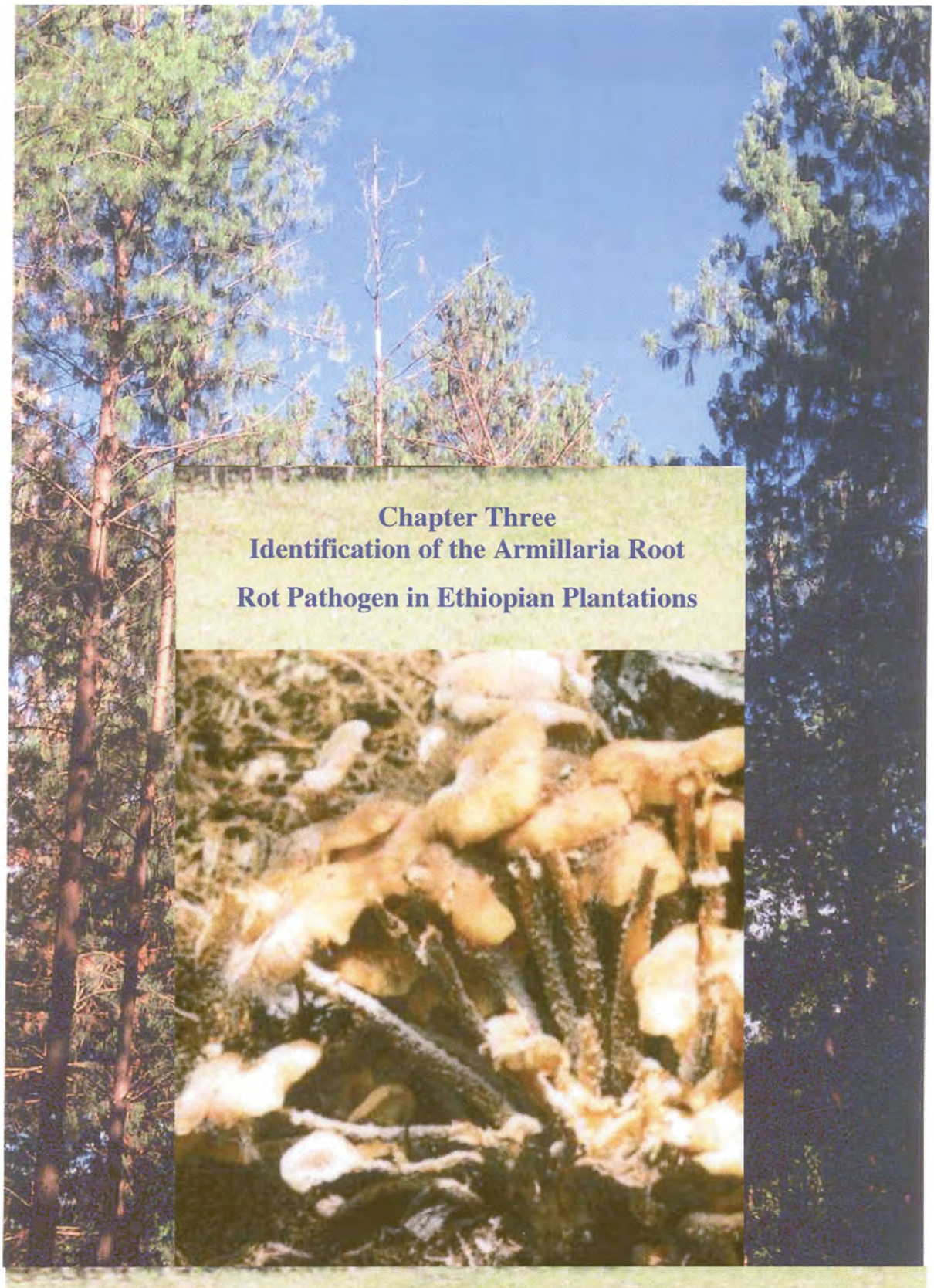

Chapter Three
Identification of the Armillaria Root Rot Pathogen in Ethiopian Plantations

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ABSTRACT

Armillaria root rot is a well-known disease on a wide range of plants, world-wide. In Ethiopia, the disease has previously been reported on *Pinus* spp., *Coffea arabica* and on various native hardwoods. The causal agent of the disease has been attributed to *Armillaria mellea*, a species now known to represent a complex of many different taxa. The aim of this study was to determine the extent of Armillaria root rot and the identity of the *Armillaria* sp. in Ethiopian plantations. As part of a plantation disease survey in 2000 and 2001, samples were collected in plantations at and around Munessa Shashemene, Wondo Genet, Jima, Mizan and Bedele, in South and South Western Ethiopia. Basidiocarps were collected and their morphology studied. Morphological identification was confirmed by sequencing the IGS-1 region of the ribosomal rRNA operon and comparing data with published sequences of *Armillaria* spp. *Armillaria* isolates were collected from *Acacia abyssinica, Pinus patula, Cederela odorata* and *Cordia alliodora* trees. Sporocarps were found on stumps of native *Juniperus exelsa*. Basidiocarp morphology suggested that the *Armillaria* sp. collected from *J. exelsa* is *A. fuscipes*. This identification was confirmed for all isolates, based on sequence data. *A. fuscipes* is known to be common in Southern Africa. Its widespread occurrence in Ethiopia suggests that it is also the major cause of Armillaria root rot in that country.

INTRODUCTION

Armillaria species cause root rot on a wide range of hosts, world-wide. These include many species such as *Eucalyptus, Pinus, Acacia* and *Cupressus* that are utilized in plantations (Wargo & Shaw 1985, Hood, Redfern & Kile 1991, Kile, McDonald & Byler 1991). *Armillaria* spp. have been regarded as primary pathogens, stress induced secondary invaders and saprophytes (Wargo & Shaw 1985, Shaw & Kile 1991). Group death, wilting and yellowing of tree tops, resin exudation, as well as the occurrence of white mycelial fans under the bark of infected trees are common symptoms of *Armillaria* infections. In many cases, rhizomorphs are also found associated with Armillaria root rot and these structures facilitate spread of *Armillaria* through the soil (Morrison, Williams & Whitney 1991).

The morphological characteristics of *Armillaria* spp. including the mycelium, rhizomorphs and basidiocarps have traditionally been the most important criteria for identification. The mycelium and the rhizomorphs of many species of these fungi, however, exhibit limited variation, restricting their use in species identification (Watling, Kile & Gregory 1982, Garraway, Hüttermann & Wargo 1991). In contrast, morphological characteristics of the basidiocarps, have provided useful taxonomic characters for species delimitation (Shaw & Kile 1991). However, the seasonal and irregular production of these structures, coupled with their scarcity, has complicated identification of *Armillaria* spp., based on morphology (Watling *et al.* 1982, Wargo & Shaw 1985, Mohammed *et al.* 1994). It is largely due to these limitations that *Armillaria mellea* (Vahl:Fr.) Kumer *sensu lato* was long considered to be a single variable species causing root rot, world-wide (Singer 1956).

In recent years, mating studies (Korhonen 1978, Ullrich & Anderson 1978, Anderson & Ullrich 1979), biochemical comparisons (Morrison *et al.* 1985, Mwangi, Lin & Hubbes 1989, Mwenje & Ride 1996) and DNA based techniques (Anderson & Stasovski 1992, Coetzee *et al.* 2000) have been used to study the biology, taxonomy, and phylogeny of *Armillaria* spp. Currently, it is known that the *Armillaria* species complex, originally

treated as *A. mellea sensu lato*, consists of at least 36 different species (Wargo & Shaw 1985, Volk & Burdsall 1995).

DNA-based characterisation provides a useful tool to identify *Armillaria* spp. The intergenic spacer region (IGS-1) of the rDNA operon is most commonly used to identify and study the relationship of *Armillaria* isolates (Anderson & Stasovski 1992, Coetzee *et al.* 2000). Restriction fragment length polymorphism (RFLP) patterns of this rDNA region are also commonly used to discriminate between *Armillaria* isolates (Harrington & Wingfield 1995).

Armillaria root rot has been reported from several countries in South, East and Western Africa. In Africa, this disease has been found associated with both cash crop plants such as coffee and tea as well as on forest plantation species including those of *Pinus*, *Eucalyptus*, *Acacia* and *Grevillea* (Mwangi *et al.* 1989, Onsando, Wargo & Waud 1997). The disease has generally been ascribed to *Armillaria mellea* (Vahl.:Fr.) P. Kumm. and *A. heimii* Pegler (Pegler 1977, Ivory 1987, Mohammed, Guillaumin & Berthelay 1989). However, recent studies conducted on *Armillaria* isolates from Africa reported the occurrence of *A. heimii*, *A. mellea sensu stricto* (Mwangi *et al.* 1989, Augustian *et al.* 1994, Guillaumin, Mohammed & Abomo-Ndongo 1994, Mohammed *et al.* 1994, Mwangi *et al.* 1994, Mwenje & Ride 1996, Abomo-Ndongo & Guillaumin 1997), *A. camerunensis* (Henn.) Volk & Burdsall [=*A. camerunensis* (Henn) = *A. mellea* (Vahl.:Fr.) P. Kumer var *camerunsis* Henn] (Singer 1986, Mohammed *et al.* 1989, Volk & Burdsall 1995), *A. mellea* (Vahl.:Fr.) P. Kumm. sub species *Africana* (Mohammed *et al.* 1994, Volk & Burdsall 1995) and *A. fuscipes* Petch (Coetzee *et al.* 2000).

In Ethiopia, damage due to Armillaria root rot has been reported from *Pinus patula* Schiede & Deppe plantations at various sites (Mengistu 1992, Dagne 1998, Alemu, Roux & Wingfield 2003). Tree death in plantations due to this disease has been estimated to be between 5-20 % (Dagne 1998). Eshetu, Teame & Girma (2000) also noted that Armillaria root rot caused minor damage in coffee (*Coffea arabica* L.) plantations. Despite this, little attention has been given to the disease. It has generally been assumed

that Armillaria root rot is caused by *A. mellea* (Mengistu 1992, Eshetu *et al.* 2000) and no detailed study has been conducted to identify the *Armillaria* spp. found in Ethiopia. However, a recent study using somatic incompatibility, isozyme comparisons and Random Amplified Polymorphic DNA (RAPD) analyses has suggested the presence of *A. mellea* on hard woods in the Kerita and Jima areas of Ethiopia (Ota, Intini & Hattori 2000).

During a survey of plantation forestry diseases in Ethiopia, conducted in 2000 and 2001, Armillaria root rot was identified as a common cause of tree mortality (Alemu *et al.* 2003). The species identity of the causal agent was, however, not known. The aim of this study was thus to identify the *Armillaria* isolates obtained from the surveys and to consider their phylogenetic relationships with other *Armillaria* species. To accomplish these objectives morphological characteristics of the basidiocarps and DNA-based comparisons including RFLP and DNA sequencing of the IGS-1 region of the rRNA operon, were used.

MATERIALS AND METHODS

Sample collection and isolation

Surveys were conducted in forestry plantations at Munessa Shashemene, Jima, Bedele, Aman/Mizan and Wondo Genet (Figure 1). Typical symptoms of Armillaria root rot were used to recognise centres of infection. Samples were collected from roots, stumps and stems of dead and dying trees. Small pieces of mycelium from the white mycelial fans, between the bark and the wood were transferred to a selective medium, containing benomyl and streptomycin (Harrington, Worall & Baker 1992). Cultures were incubated at 25 °C in the dark for three weeks. Pieces of mycelium from the tips of the cultures were then transferred to 2% MEA (2% Biolab Malt Extract, 1.5% Biolab Agar) plates to multiply them for further use. Stock cultures of all the isolates used in this study are maintained on 2% MEA slants at 5 °C in the culture collection (CMW) of the Forestry

and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1).

Basidiocarp morphology

Basidiocarps collected from stumps of felled *Juniperus exelsa* Hochest. Ex. Endl. trees were used to study their morphology. Morphological characteristics of these structures were compared with those published for other species. Characters examined included the colour of the basidiocarps and size of the pileus and stipes. Rayner's (1970) colour chart was used to determine colors.

DNA extraction

Representative isolates (CMW5837, 5844, 5846, 8967, 8969, 8971) (Table 1) from different sites and hosts were grown in liquid MY medium (2% Biolab malt extract, 0.3% Biolab yeast extract agar) in the dark at 25 °C, for approximately three weeks. Mycelium was harvested from cultures by centrifugation (8000 *g*, 30 min) and freeze dried. The dried mycelial samples were ground to a fine powder in liquid nitrogen using a mortar and pestle. DNA was extracted using a modified version of the DNA extraction method of Raeder and Broda (1985). Extraction buffer (200 mM Tris-HCl pH 8; 25 mM EDTA; 250 mM NaCl) (1000 µl) was added to about 0.5 g of powdered mycelium and incubated at 60 °C for 30 min. This was followed by a phenol:chlorophorm extraction step. Cell debris was removed by centrifugation at 13000 *g* for one hour. Further phenol:chlorophorm extractions were done on the aqueous phase until a clean interphase was obtained. Chlorophorm extractions were done to remove the traces of phenol. Sodium Acetate (3M NaAc) and absolute ethanol were added to precipitate the nucleic acids and they were collected by centrifugation at 13000 *g*. The nucleic acid pellet was washed with 70% ethanol, vacuum dried and dissolved in 50 µl sterile water. RNase A (0.01 mg/ml) (Roche) was added to the DNA and water suspensions to remove RNA and incubated overnight at 37 °C in a water bath. The resulting DNA was visualised under

UV illumination after electrophoresis on a 1% agarose gel (Promega, Madison, Wisconsin) stained with ethidium bromide.

DNA amplification

The IGS-1 region of the ribosomal RNA (rRNA) operon was amplified using the polymerase chain reaction (PCR). This region was amplified with Primers P-1 (5'-TTG CAG ACG ACT TGA ATG G- 3') (Hsiau 1996) and 5S-2B (5' CAC CGC ATC CCG TCT GAT CTG CG 3') (Coetzee *et al.* 2000). The PCR mixtures used included dNTPs (200µM of each), $MgCl_2$ (2.66mM), 10 x buffer containing $MgCl_2$ (supplied with enzyme), 0.375 µM of each primer, *Taq* polymerase (2.6 U) (Roche) and approximately 80 ng template DNA. The final reaction volume was adjusted to 50 µl with H_2O. The PCR programme consisted of an initial denaturation step at 96 °C for 2 min. This was followed by 35 cycles of annealing at 58 °C for 30 s, elongation at 72 °C for 2 min., a ramp time of 1.5 s and another denaturation at 94 °C for 30s. A final elongation step was allowed for 7 min at 72 °C. Prior to sequencing, the PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN, Germany). The fragment sizes of the PCR products were determined after electrophoresis on a 1% agarose gel stained with ethidium bromide and visualised under UV illumination. A 100 bp molecular marker was used to determine the size of the PCR fragments.

Restriction enzyme digestion

Restriction Fragment Length Polymorphism (RFLP) profiles of isolates included in this study were obtained by digesting the IGS-1 amplicons with the restriction endonuclease *Alu* I (Harrington & Wingfield 1995). IGS-1 amplicons were digested by adding 3 units of *Alu* I restriction endonuclease to 18 µl of unpurified PCR product. Digestion was allowed to occur overnight at 37 °C. DNA fragments were separated on a 3% (w/v) agarose gel (Promega, Madison, Wisconsin) stained with ethidium bromide and profiles were visualised under UV illumination. A 100 base molecular weight marker was used to determine the fragment sizes. The absolute fragment sizes were determined using the

programme Gelreader 20.5 (National Center, Supercomputing Applications, University of Illinois at Urbana-Champaign, 1991). RFLP patterns and sizes of IGS-1 amplicons for the Ethiopian *Armillaria* isolates were compared with those of *Armillaria* spp. published by Coetzee *et al.* (2000).

Cloning

It was not possible to sequence the IGS-1 amplicons directly and they were subsequently cloned to resolve this problem. Ligation of the PCR products was conducted using the PGEM® T Easy Vector System (Promega Corporation), 2X Rapid Ligation Buffer, T4 DNA Ligase, PCR products and deionized water according to the protocols outlined by the manufacturer. This reaction was incubated for one hour at room temperature. For transformation, JM109 High Efficiency Competent cells provided with the PGEM® T EASY Vector System II were used. Two µl of the ligation reactions were transferred to 1.5 ml Eppendorf tubes and 25 µl competent cell solution added to each Eppendorf tube. Isolation of recombinant plasmid DNA was accomplished using a standard plasmid miniprep procedure, using the instructions provided by the company.

DNA sequencing

Plasmid DNA was used as template to sequence the inserted IGS-1 region of the *Armillaria* samples. DNA sequences were determined using an automated (ABI PRISM™ 3100) DNA sequencer. The inserted region was sequenced in both directions using primers T7 (5'-ATT ATG CTG AGT GAT ATC CC- 3') and SP6 (5'- ATT TAG GTG ACA CTA TAG AA-3') (Promega 1999). The sequencing reactions were prepared using the Big Dye sequence system (ABI Advanced Biotechnology Institute, Perkin-Elmer) as recommended in the manufacturer's protocols.

Analysis of DNA sequence data

Sequence Navigator version 1.01 (ABI PRISM™, Perkin Elmer) was used to manually align the sequence data by inserting gaps. Analysis of the sequence data was done using PAUP* version 4.0b2 (Swofford 1998). In the sequence data analysis, indels of more than 1 base were excluded and substituted with multi-state characters and gaps treated as a 5th character. IGS-1 DNA sequences obtained in this study were aligned against the data matrix published by Coetzee *et al.* (2000) and available on Tree Base (Table 1). Phylogenetic trees generated were rooted to *A. heimii* as the monophyletic sister group to the taxa. Analyses were done using Neighbor-Joining distance analysis and the total character difference was used to generate the tree. The confidence levels of the branching points were determined by 1000 bootstrap replicates.

RESULTS

Sample collection and isolation

Symptoms of Armillaria root rot were found in plantations at Wondo Genet, Munessa Shashemene, Belete/Jima, Bedele and Aman/Mizan. Armillaria root rot was identified on 10-13 year old *P. patula*, *Acacia abyssinica* Hochest, *Cordia alliodora* (Ruiz & Pav) Oken and *Cedrela odorata* L. trees (Table 1, Figure 1). The characteristic symptoms of infection included groups of dead trees (Figure 2a), wilting and chlorosis, as well as the occurrence of white mycelial fans (Figure 2b) under the bark of diseased trees. Masses of light brown rhizomorphs were found on diseased *C. alliodora* trees, in a research plot at Aman (Figure 2c). The causal fungus was successfully isolated from symptomatic trees and grown on the selective medium. A total of 32 isolates were collected from the different hosts. In culture, the *Armillaria* isolates produced a flat whitish mycelium. Brown, cylindrical rhizomorphs were produced abundantly in cultures (Figure 2d). At the time of sample collection, the incidence of Armillaria root rot damage was most pronounced on *P. patula* at Wondo Genet.

Basidiocarp morphology

Ten basidiocarps were collected from stumps of *J. exelsa* trees, in a plantation at Wondo Genet (Figure 2e). These basidiocarps were used to partially identify the *Armillaria* sp. in this study. When the colour, the size of the stipe and the pileus of the basidiocarps were considered, the basidiocarps from Ethiopia showed close similarities with the basidiocarp morphology of *A. fuscipes* (Coetzee *et al.* 2000) and differed from those of the much smaller *A. heimii*. The pileus of the fungus had an average diameter of 45 mm and the length of the stipes varied between 60-87 mm. These measurements are more similar to those of the basidiocarps of *A. fuscipes* (Pileus = 51 mm, Stipe = 64-84 mm) than of *A. heimii* (Pileus = 30 mm, Stipe = 25-45 mm) [Figure 2e].

DNA amplification

The IGS regions of all *Armillaria* isolates from Ethiopia were successfully amplified with primers P-1 and 5S-2B. The PCR products of all *Armillaria* isolates used in this study yielded fragments of approximately 1200 base pairs (bp). This PCR fragment size is similar to that published for *A. fuscipes* (Coetzee *et al.* 2000).

Restriction enzyme digestion

Alu I restriction digestion of PCR amplicons generated identical fragment patterns for all isolates. Three distinct bands with sizes of approximately 370, 250 and 95 bp were obtained (Figure 3). Comparison of RFLP profiles of the Ethiopian *Armillaria* isolates with published profiles for *A. fuscipes* and *A. heimii* (Coetzee *et al.* 2000) showed that the RFLP patterns of *Armillaria* isolates from Ethiopia are different to both *A. fuscipes* and *A. heimii* (Table 2). Furthermore, the Ethiopian RFLP profile did not match that of any other *Armillaria* sp. for which such profiles are available.

DNA sequencing

The IGS sequence of the *Armillaria* isolates from Ethiopia, before alignment, varied between 1056 and 1100 bp. A Blast search using the IGS-1 and 5S gene sequences for these isolates against sequences in GenBank [National Centre for Biotechnology information (NCBI), US National Institute of Health Bethesad, (http:/ www.ncbi.nlm. Nih.gov/BLAST)], indicated that the DNA sequences of *Armillaria* isolates from Ethiopia most closely match with the sequences of *A. fuscipes* and *A. heimii*. Therefore, the DNA sequences of the Ethiopian *Armillaria* isolates were aligned with these two species (Coetzee *et al.* 2000). A total of 1247 characters were obtained for analysis after manual alignment.

Analysis of DNA sequence data

The *Armillaria* isolates used in this study formed two main groups in a neighbour-Joining tree (Figure 4). Sequences of *Armillaria* isolates from South Africa and La Reunion, which were previously identified as *A. fuscipes* (Coetzee *et al.* 2000) grouped together with a bootstrap support of 90%. The *Armillaria* isolates from Ethiopia resided in a separate cluster with 74% bootstrap support. The Ethiopian *Armillaria* isolates grouped separately from *A. heimii,* showing the closest affinity to *A. fuscipes,* although with some differences. The Ethiopian isolates differed from *A. fuscipes* in having several indels. Isolate CMW8971 differed from *A. fuscipes* with only 11 bp indels (of which 7 bps are deletions), while other Ethiopian isolates showed more variation. The most notable of these are isolates CMW5838 and CMW5846, which have 16 bp deletions, whereas isolates CMW5844, CMW8967 and CMW8969 have 33 bp deletions and contain one restriction site at position nine. Despite these differences, the Ethiopian isolates group with the *A. fuscipes* clade with a bootstrap of 100 % and separately from *A. heimii*.

DISCUSSION

Recently, the importance of plantation forestry diseases in Ethiopia has been afforded considerable attention. Results from this study thus, form part of the first comprehensive

plantation disease survey conducted in the country (Alemu *et al.* 2003). This study furthermore presents results of the first extensive survey of Armillaria root rot in Ethiopian forest plantations. Our data clearly show that the dominant *Armillaria* sp. causing root rot and death in plantations is *A. fuscipes*. This is the first report of *A. fuscipes* from Ethiopia and also greatly extends its host range.

Damage from Armillaria root rot has been observed in several African countries, where it has been attributed mainly to *A. mellea* and *A. heimii* (Pegler 1986, Ivory 1987). *Armillaria fuscipes* was recently reported to be common in Southern Africa (Coetzee *et al.* 2000). Outside Africa, *A. fuscipes* is known only from Sir Lanka, where it was first described and where Pegler (1986) suggested that it could have been introduced from Africa. The taxonomic status of *A. heimii* and *A. fuscipes* has, however, been confused for many years. It has thus been suggested that *A. heimii* is conspecific with *A. fuscipes* and the latter name was retained (Pegler 1986, Kile & Watling 1988, Watling, Kile & Burdsall 1991). Recent studies have shown the existence of significant variation between *A. heimii* isolates from various African countries (Augustain *et al.* 1994, Mohammed *et al.* 1994, Mwenje & Ride 1996). A DNA based study conducted on Southern African *Armillaria* isolates, thought to represent *A. heimii* showed that they are dissimilar to *A. heimii* from Zambia, Zimbabwe and Cameroon (Coetzee *et al.* 2000). In the study of Coetzee *et al.* (2000), *Armillaria* isolates from South Africa were shown to represent *A. fuscipes*, and not *A. heimii*. Similarly, *Armillaria* isolates from La Reunion, believed to represent *A. heimii* were found to be identical to the South African *Armillaria* isolates and identified as *A. fuscipes* (Coetzee *et al.* 2000). This study provided clear evidence that these two species represent distinct taxa. The results of the present study show that the Ethiopian *Armillaria* isolates represent *A. fuscipes,* although some differences were observed in RFLP and IGS sequence data.

Basidiocarp morphology has commonly been used to determine the relationships of *Armillaria* spp. (Bérubé & Dessureault 1989, Watling *et al.* 1991). The macro-morphological characters including colour and structures of the pileus, veil, annulus and stipe are reliable characters for this purpose (Bérubé & Dessureault 1989). Seasonal

availability of the basidiocarps, however, limits the use of basidiocarp morphology for species identification. In this study, very few fruiting structures were obtained and these were only from Wondo Genet. The macro-morphological characters of these basidiocarps were different from those of *A. heimii*, having larger pileus and stipes, compared to the small basidiocarps of *A. heimii* (Kile & Watling 1988). The basidiocarps from Ethiopia were very similar to those from South Africa, known to represent *A. fuscipes*. It was not possible to collect a culture linked to these basidiocarps but the proximity of the dying trees to others from which cultures and DNA sequences were obtained provides strong circumstantial evidence that the fungus is the same.

Coetzee *et al.* (2000), showed that the 5S ribosomal rRNA gene of African *A. fuscipes* and *A. heimii* isolates are in opposite orientation in comparison to other *Armillaria* spp. Because of this, primers used to amplify the IGS-1 region of non-African isolates failed to amplify the IGS-1 region of African *Armillaria* isolates (Coetzee *et al.* 2000). Primer 5S-2B was, therefore, used to amplify the IGS-1 region of African *Armillaria* spp. The IGS-1 region of the *Armillaria* isolates from Ethiopia was successfully amplified with primers P-1 and 5S-2B indicating that the 5S gene of Ethiopian *Armillaria* isolates has the same orientation as that of other African *A. fuscipes* and *A. heimii* isolates. This provides further support for our belief that the Ethiopian isolates represent *A. fuscipes*.

A recent population study on *Armillaria* spp. in Ethiopia reported that *A. mellea* is responsible for root rot on hard-wood trees in the Jima and Kerita areas (Ota *et al.* 2000). An isolate from symptomatic *P. patula* trees near Jima in our study, produced the same RFLP profile as those of other *Armillaria* isolates that we have identified as *A. fuscipes*. This suggests that the causative agent of Armillaria root rot of *P. patula* around Jima is identical to other isolates included in our study and that it also represents *A. fuscipes*. The results of Ota *et al.* (2000) and this study, thus suggest that more than one *Armillaria* spp. might be involved in causing Armillaria root rot in Ethiopia. This emphasises the importance of conducting further and more comprehensive studies on the diversity, distribution, and host range of Armillaria root rot in Ethiopia.

RFLP patterns of all Ethiopian *Armillaria* isolates differed from those of *A. fuscipes* and all other *Armillaria* spp. This difference in RFLP pattern was supported by DNA sequence data, which showed the deletion of indels within one of the restriction sites. Although the Ethiopian isolates grouped closely to *A. fuscipes,* they formed a separate sub-clade. This suggests that the *Armillaria* samples from Ethiopia could represent a distinct species, closely related to *A. fuscipes*. Macro- and micro-morphological comparison of the basidiocarps will be essential to understand the significance of this variation.

Results of this study have shown that *Armillaria* root rot not only affects *P. patula,* but that it also kills *Co. alliodora* and *C. odorata* trees planted in research plots at Aman, near Mizan. The fungus was also found on *A. abyssinica* and *J. excelsa,* species native to Ethiopia and growing in the *Pinus* plantations at Bedele and Wondo Genet. Most plantations in Ethiopia are made up of exotic species and these are planted on sites previously occupied by indigenous hardwoods. This suggests that stumps of the native hardwoods could be sources of the initial inoculum needed to infect exotic species. Planting of *Pinus* spp. in these areas should be avoided. The occurrence of the same *Armillaria* sp. on these different tree species implies that this pathogen could be damaging to a wider range of trees in the country. In order to better understand the distribution, diversity and host range of *Armillaria* spp. as well as to investigate its importance in other plantation areas, this study should be extended to other parts of Ethiopia.

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Table 1. *Armillaria* isolates used in this study

Identity	Culture number [a]	Host	Origin	Collector	Accession No.
A. fuscipes [b]	CMW5838	*Pinus patula*	Wondo Genet, Ethiopia	Alemu Gezahgne & Roux, J.	AY172029
A. fuscipes [b]	CMW5844	*P. patula*	Wondo Genet, Ethiopia	Alemu Gezahgne & Roux, J.	AY172032
A. fuscipes [b]	CMW5846	*P. patula*	Wondo Genet, Ethiopia	Alemu Gezahgne & Roux, J.	AY172030
A. fuscipes [b]	CMW8967	*Cordia alliodora*	Aman/Mizan, Ethiopia	Alemu Gezahgne & Roux, J.	AY172031
A. fuscipes [b]	CMW8969	*Acacia abyssinica*	Bedele, Ethiopia	Alemu Gezahgne & Roux, J.	AY172034
A. fuscipes [b]	CMW8971	*P. patula*	Belete/Jima, Ethiopia	Alemu Gezahgne & Roux, J.	AY172033
A. fuscipes [c]	CMW2717	*P. elliottii*	Sabie, South Africa	Wingfield, M. J.	AF204821
A. fuscipes [c]	CMW2740	*P. patula*	Entabeni, South Africa	Wingfield, M. J.	AF204822
A. fuscipes [c]	CMW3167	*P. elliottii*	Sabie, South Africa	Ivory, M.	AF204823
A. heimii [c]	CMW3152	Unknown	Western Province, Cameroon	Watling, R.	AF204826
A. heimii [c]	CMW3164	*Pelargonium asperum*	Saint-Denis, La Reunion	Fabergue, C.	AF204824
A. heimii [c]	CMW3173	*Tectona grandis*	Dola Hill, Zambia	Ivory, M.	AF204825
A. heimii [c]	CMW3955	*Acacia xanthophloea*	Harare, Zimbabwe	Wingfield, M. J. & Coetzee, M. P. A.	AF204827

[a] CMW numbers refer to the culture collection numbers of the Tree Pathology Co-operative Programme (TPCP), FABI, University of Pretoria. Pretoria, South Africa.
[b] Isolates sequenced in this study.
[c] Sequence of *Armillaria*, in FABI database, identical to those submitted to GenBank (Coetzee *et al.* 2000).

Table 2. Comparison of RFLP sizes of *Armillaria* isolates

Armillaria from Ethiopia	*A. fuscipes*[a]	*A. heimii*[a]	*A. mellea*[b]
370	365	530	215
250	245	220	175
95	135	175	150

[a] Data obtained from Coetzee *et al.* 2000

[b] Data obtained from Coetzee *et al.* 2001

Figure 1. Map of Ethiopia showing the plantation areas where surveys were conducted.

Figure 2. Symptoms and signs of *Armillaria* infection. (A) Group death of infected trees in a *Pinus patula* stand in Wondo Genet, Ethiopia, (B) White mycelial fan, (C) Rhizomorphs found between bark and wood on *Cordia alliodora*, (D) Culture of *Armillaria* growing on MEA, (E) Basidiocarps of *Armillaria* on a *Juniperus exelsa* stump.

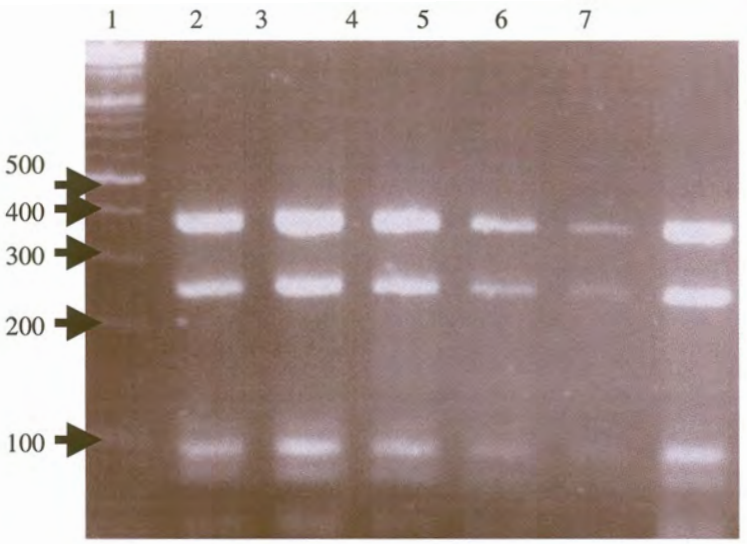

Figure 3. *Alu I* restriction fragment patterns of *Armillaria* isolates from Ethiopia on 3% agarose gel stained with ethidium bromide. Lane 1=Molecular marker, 2=CMW 5844, 3=CMW 5846, 4=CMW 5838, 5=CMW 8969, 6=CMW 8967 and 7=CMW 8971.

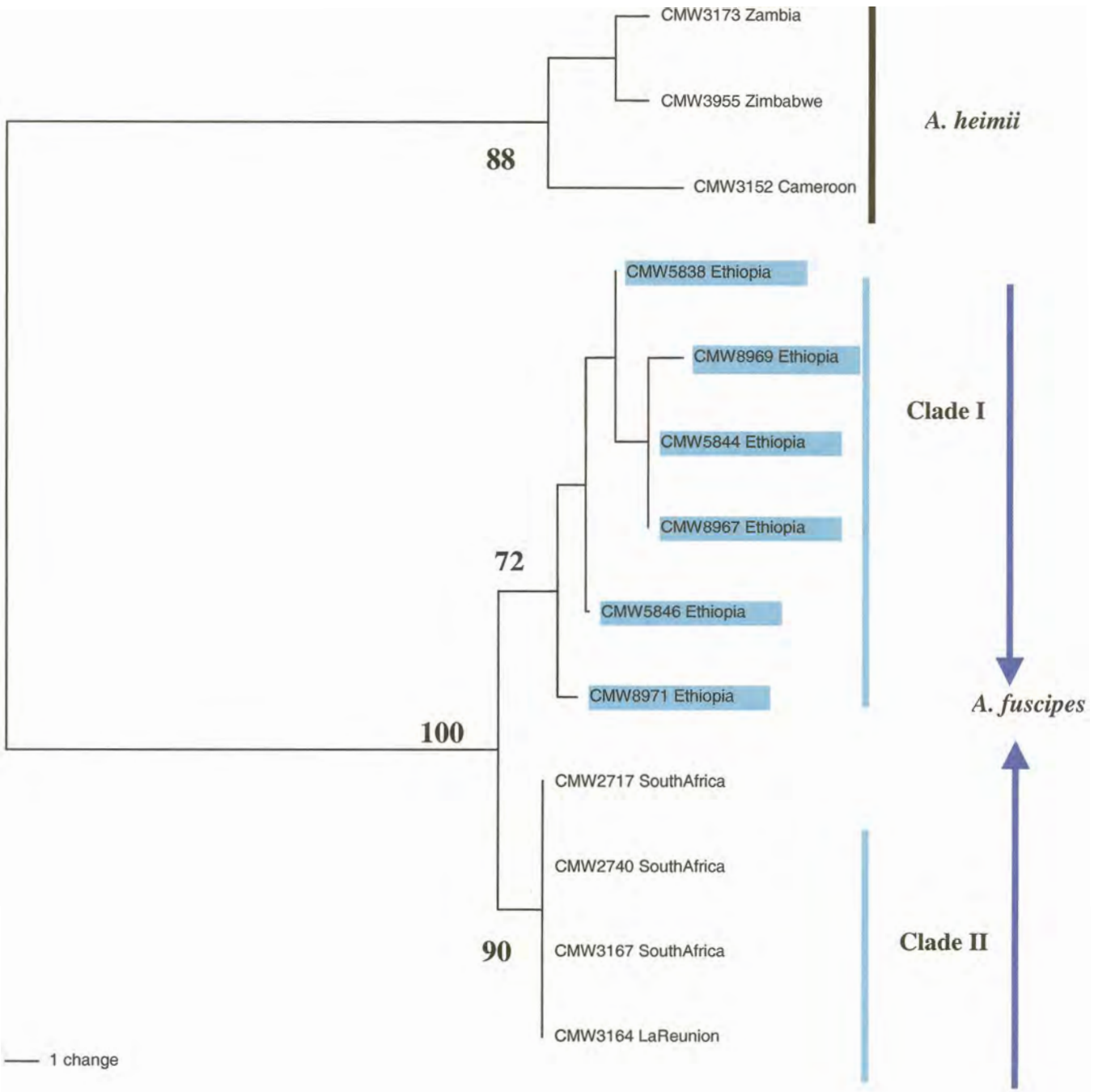

Figure 4. Phylogram generated from Neighbor Joining analysis of the IGS-1 sequence data used in this study. Bootstrap values are shown on the branches.

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                          150        160        170        180        190        200        210
CMW3173_ZAMBIA        AACAGCATGT TTAATGGA-- ---------- --AGCCTATT GTGTATAATA TTGGTATATA CGGTGTACGG
CMW3152_CAMEROON      .......... ........-- ---------- --.......G .......... .......... ..........
CMW3955_ZIMBABWE      .......... ........-- ---------- --........ .......... .......... ..........
CMW5837_ETHIOPIA      .......... .G......GG GGTATGGATC CA...G.... ..-------- -----..... .......T..
CMW5846_ETHIOPIA      .......... .G......GG GGTATGGATC CA...G.... ..-------- -----..... .......T..
CMW8968_ETHIOPIA      .......... .G......GG GGTATGGATC CA...G.... ..-------- -----..... .......T..
CMW5844_ETHIOPIA      .......... .G......GG GGTATGGATC CA...G.... ..-------- -----..... .......T..
CMW8967_ETHIOPIA      .......... .G......GG GGTATGGATC CA...G.... ..-------- -----..... .......T..
CMW8971_ETHIOPIA      .......... .G......GG GGTATGGATC CA...G.... ..-------- -----..... .......T..
CMW2717_SOUTH_AFRICA  .......... .G......GG GGTATGGATC CA...G.... ..-------- -----..... .......T..
CMW2740_SOUTH_AFRICA  .......... .G......GG GGTATGGATC CA...G.... ..-------- -----..... .......T..
CMW3167_SOUTH_AFRICA  .......... .G......GG GGTATGGATC CA...G.... ..-------- -----..... .......T..
CMW3164_LA_REUNION    .......... .G......GG GGTATGGATC CA...G.... ..-------- -----..... .......T..

                          220        230        240        250        260        270        280
CMW3173_ZAMBIA        AGTACGGGTA TACAGAAGAG -----TATAC AGTACAGTAG ACAGTATATA TATATATA-- --TTATAT-A
CMW3152_CAMEROON      .......... .......... -----..... .......... .......... ........-- --.......-.
CMW3955_ZIMBABWE      .......... .......... -----..... .......... .......... ........-- --.......-.
CMW5837_ETHIOPIA      ...G...... .......... AAGAG..... ...G.....C .......... ........TA TA....G.A.
CMW5846_ETHIOPIA      ...G...... .......... AAGAG..... ...G.....C .......... ........TA --....G.A.
CMW8968_ETHIOPIA      ...G...... .......... AAGAG..... ...G.....C .......... ........TA TA....G.A.
CMW5844_ETHIOPIA      ...G...... .......... AAGAG..... ...G.....C .......... ........TA TA....G.A.
CMW8967_ETHIOPIA      ...G...... .......... AAGAG..... ...G.....C .......... ........TA TA....G.A.
CMW8971_ETHIOPIA      ...G...... .......... AAGAG..... ...G.....C .......... ........TA TA....G.A.
CMW2717_SOUTH_AFRICA  ...G...... .......... AAGAG..... ...G.....C .......... ........TA --....G.A.
CMW2740_SOUTH_AFRICA  ...G...... .......... AAGAG..... ...G.....C .......... ........TA --....G.A.
CMW3167_SOUTH_AFRICA  ...G...... .......... AAGAG..... ...G.....C .......... ........TA --....G.A.
CMW3164_LA_REUNION    ...G...... .......... AAGAG..... ...G.....C .......... ........TA --....G.A.
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                                290        300        310        320        330        340        350
CMW3173_ZAMBIA          TCTAT--GAC TTGGACTTGG ACTTGTACTT GGACTTGGAT CTTGGATCAC AATGCAAGTA AGGTAGTAGG
CMW3152_CAMEROON        .....--... .C........ .......... .......... .......... .......... ..........
CMW3955_ZIMBABWE        .....--... .......... .......... .......... .......... .......... ..........
CMW5837_ETHIOPIA        ....CAT... .......... .......... .......... .....G---- ---------- ---.T.A.AT
CMW5846_ETHIOPIA        ....CAT... .......... .......... .......... .....G---- ---------- ---.T.A.AT
CMW8968_ETHIOPIA        ....CAT... .......... .......... .......... .....G---- ---------- ---.T.A.AT
CMW5844_ETHIOPIA        ....CAT... .......... .......... .......... .....G---- ---------- ---.T.A.AT
CMW8967_ETHIOPIA        ....CAT... .......... .......... .......... .....G---- ---------- ---.T.A.AT
CMW8971_ETHIOPIA        ....CAT... .......... .......... .......... .....G---- ---------- ---.T.A.AT
CMW2717_SOUTH_AFRICA    ....CAT... .......... .......... .......... .....G..-- --------.T G...T.A.AT
CMW2740_SOUTH_AFRICA    ....CAT... .......... .......... .......... .....G..-- --------.T G...T.A.AT
CMW3167_SOUTH_AFRICA    ....CAT... .......... .......... .......... .....G..-- --------.T G...T.A.AT
CMW3164_LA_REUNION      ....CAT... .......... .......... .......... .....G..-- --------.T G...T.A.AT


                                360        370        380        390        400        410        420
CMW3173_ZAMBIA          CAATGCAACG CAAGGCTAGT AGACAACGCA AGGCAATGCA AGGATAGTAG ACAATGCAAG GCAATGCAAG
CMW3152_CAMEROON        .......... .......... .A..G..... .......... .......... .......... .......---
CMW3955_ZIMBABWE        .......... .......... .......... .......... .......... .......... ..........
CMW5837_ETHIOPIA        ..-------- ---------- ------.CA. ..A....... ....A----- ---------- ----------
CMW5846_ETHIOPIA        ..-------- ---------- ------.CA. ..A....... ....A----- ---------- ----------
CMW8968_ETHIOPIA        ..-------- ---------- ------.CA. ..A....... ....A----- ---------- ----------
CMW5844_ETHIOPIA        ..-------- ---------- ------.CA. ..A....... ....A----- ---------- ----------
CMW8967_ETHIOPIA        ..-------- ---------- ------.CA. ..A....... ....A----- ---------- ----------
CMW8971_ETHIOPIA        ..-------- ---------- ------.CA. ..A....... ....A----- ---------- ----------
CMW2717_SOUTH_AFRICA    ..-------- ---------- ------.CA. ..A....... ....A----- ---------- ----------
CMW2740_SOUTH_AFRICA    ..-------- ---------- ------.CA. ..A....... ....A----- ---------- ----------
CMW3167_SOUTH_AFRICA    ..-------- ---------- ------.CA. ..A....... ....A----- ---------- ----------
CMW3164_LA_REUNION      ..-------- ---------- ------.CA. ..A....... ....A----- ---------- ----------
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                          430        440        450        460        470        480        490
CMW3173_ZAMBIA        GCTAGTAGAC AACGCAACGC AATGCAA-GG CTAGTAGACA ACGCAAGGC- -AAGTAAGCT AGCAGGCAGA
CMW3152_CAMEROON      ---------- ---------- -----..-.. .......... .........- -......... ..........
CMW3955_ZIMBABWE      .......... .......... .......-.. .......... .........- G......... ..........
CMW5837_ETHIOPIA      .......... ..T....G.- -----..-.- .......... .GA.G.C--- -......... .C.......T
CMW5846_ETHIOPIA      .......... ..T....G.- -----..-.- .......... .GA.G.C--- -......... .C.......T
CMW8968_ETHIOPIA      .......... ..T....G.- -----..-.- .......... .GA.G.C--- -......... .C.G......
CMW5844_ETHIOPIA      .......... ..T....G.- -----..-.- .......... .GA.G.C--- -......... .C........
CMW8967_ETHIOPIA      .......... ..T....G.- -----..-.- .......... .GA.G.C--- -......... .C........
CMW8971_ETHIOPIA      .......... ..T....G.- -----..-.- .......... .GA.G.C--- -......... .C........
CMW2717_SOUTH_AFRICA  ---------- ---------- --------.- .......... .GA.G.C--- -......... .C........
CMW2740_SOUTH_AFRICA  ---------- ---------- --------.- ..T....... .GA.G.C--- -......... .C........
CMW3167_SOUTH_AFRICA  ---------- ---------- --------.- ..T....... .GA.G.C--- -......... .C........
CMW3164_LA_REUNION    ---------- ---------- --------.- ..N....... .GA.G.C--- -......... .C........

                          500        510        520        530        540        550        560
CMW3173_ZAMBIA        CTTGTGAG-- TTGAGAGCTT GTACGCATGT CTTAGTTGGT GTGCA----- ---------- ----------
CMW3152_CAMEROON      ........-- .......... .......... .......... .....----- ---------- ----------
CMW3955_ZIMBABWE      ........-- .......... .......... .......... .....----- ---------- ----------
CMW5837_ETHIOPIA      ....A...-- -------... .......... T........C .C...TAGAG TCTTTGGACT TGGGACTTGG
CMW5846_ETHIOPIA      ....A...-- -------... .......... .........C .C...TAGAG TCTTTGGACT TGGGACTTGG
CMW8968_ETHIOPIA      ........-- ---------- ---------- ----.....C .C...TAGAG TCTTTGGACT TGGGACTTGG
CMW5844_ETHIOPIA      ........-- ---------- ---------- ----.....C .C...TAGAG TCTTTGGACT TGGGACTTGG
CMW8967_ETHIOPIA      ........-- ---------- ---------- ----.....C .C...TAGAG TCTTTGGACT TGGGACTTGG
CMW8971_ETHIOPIA      ........TC .......... .......... .........C .C...TAGAG TCTTTGGACT TGGGACTTGG
CMW2717_SOUTH_AFRICA  ........TC .......... ........C. .........C .C...TAGAG TCTTTGGACT TGGGACTTGG
CMW2740_SOUTH_AFRICA  ........TC .......... ........C. .........C .C...TAGAG TCTTTGGACT TGGGACTTGG
CMW3167_SOUTH_AFRICA  ........TC .......... ........C. .........C .C...TAGAG TCTTTGGACT TGGGACTTGG
CMW3164_LA_REUNION    ........TC .......... ........C. .........C .C...TAGAG TCTTTGGACT TGGGACTTGG
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                          570        580        590        600        610        620        630
CMW3173_ZAMBIA            ---------- --TTGCGGAC TTGG------ ---------G CATTGA-GGG CTTGTATGCA -CGCA--CCT
CMW3152_CAMEROON          ---------- --........ ....------ ---------. ......-... .......... T...--...
CMW3955_ZIMBABWE          ---------- --........ ....------ ---------. ......-... .......... -...--...
CMW5837_ETHIOPIA          ACAGCCAACG GA........ ....ACAGAA TTGCAAGCT. ......-.C. ..C...C... T-...TG...
CMW5846_ETHIOPIA          ACAGCCAACG GA........ ....ACAGAA TTGCAAGCT. ......-.C. ..C...C... T-...TG...
CMW8968_ETHIOPIA          ACAGCCAACG GA........ ....ACAGAA TTGCAAGCT. ......-.C. ..C...C... T-...TG...
CMW5844_ETHIOPIA          ACAGCCAACG GA........ ....ACAGAA TTGCAAGCT. ......-.C. ..C...C... T-...TG...
CMW8967_ETHIOPIA          ACAGCCAACG GA........ ....ACAGAA TTGCAAGCT. ......-.C. ..C...C... T-...TG...
CMW8971_ETHIOPIA          ACAGCCAACG GA........ ....ACAGAA TTGCAAGCT. ......-.C. ..C...C... T-...TG...
CMW2717_SOUTH_AFRICA      ACACCCAATG GA........ ....ACAGAA TTGCAAGCT. ......-.C. ..C...C... T-...TG...
CMW2740_SOUTH_AFRICA      ACACCCAATG GA........ ....ACAGAA TTGCAAGCT. ......-.C. ..C...C... T-...TG...
CMW3167_SOUTH_AFRICA      ACACCCAATG GA........ ....ACAGAA TTGCAAGCT. ......A.C. ..C...C... T-...TG...
CMW3164_LA_REUNION        ACACCCAATG GA........ ....ACAGAA TTGCAAGCT. ......-.C. ..C...C... T-...TG...

                          640        650        660        670        680        690        700
CMW3173_ZAMBIA            TAACGGACTT GGACATTGAG GTGTATGCAC G---CTT--- ---------- -GGACATTGA G---------
CMW3152_CAMEROON          ..-....... .......... .......... .GACA..GAG GTGTATGCAC -......... .---------
CMW3955_ZIMBABWE          ..-....... .......... .......... .CAC...ACG GACTT----- -......... .---------
CMW5837_ETHIOPIA          ..CTT.T... .C.-----.. C..C..C..T .--A...GCC CTCAAGCAAA T.--....C. ATGCGTCGAC
CMW5846_ETHIOPIA          ..CTT.T... .C.-----.. C..C..C..T .--A...GCC CTCAAGCAAA T.--....C. ATGCGTCGAC
CMW8968_ETHIOPIA          ..CTT.T... .C.-----.. C..C..C..T .--A...GCC CTCAAGCAAA T.--....C. ATGCGTCGAC
CMW5844_ETHIOPIA          ..CTT.T... .C.-----.. C..C..C..T .--A...GCC CTCAAGCAAA T.--....C. ATGCGTCGAC
CMW8967_ETHIOPIA          ..CTT.T... .C.-----.. C..C..C..T .--A...GCC CTCAAGCAAA T.--....C. ATGCGTCGAC
CMW8971_ETHIOPIA          ..CTT.T... .C.-----.. C..C..C..T .--A...GCC CTCAAGCAAA T.--....C. ATGCGTCGAC
CMW2717_SOUTH_AFRICA      ..CTT.T... .C.-----.. C..C..C..T .--A...GCC CTCAAGCAAA T.--..... ATGCGTCGAC
CMW2740_SOUTH_AFRICA      ..CTT.T... .C.-----.. C..C..C..T .--A...GCC CTCAAGCAAA T.--..... ATGCGTCGAC
CMW3167_SOUTH_AFRICA      ..CTT.T... .C.-----.. C..C..C..T .--A...GCC CTCAAGCAAA T.--..... ATGCGTCGAC
CMW3164_LA_REUNION        ..CTT.T... .C.-----.. C..C..C..T .--A...GCC CTCAAGCAAA T.--..... ATGCGTCGAC
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                                710        720        730        740        750        760        770
CMW3173_ZAMBIA          ---------- GTGT------ ----ATGCA- ---------- ---------- ---CGGACAT TGAGGTGTAT
CMW3152_CAMEROON        ---------- ....------ ----.....- ---------- ---------- ---....... ..........
CMW3955_ZIMBABWE        ---------- ....------ ----.....- ---------- ---------- ---....... ..........
CMW5837_ETHIOPIA        TTGCAAGCTA ....TGCGCA TATT.....T GTCTTACTTG CATTTCGCTA GTTA.C.... ...CT..C.A
CMW5846_ETHIOPIA        TTGCAAGCTA ....TGCGCA TATT.....T GTCTTACTTG CATTTCGCTA GTTA.C.... ...CT..C.A
CMW8968_ETHIOPIA        TTGCAAGCTA ....TGCGCA TATT.....T GTCTTACTTG CATTTCGCTA GTTA.C.... ...CT..C.A
CMW5844_ETHIOPIA        TTGCAAGCTA ....TGCGCA TATT.....T GTCTTACTTG CATTTCGCTA GTTA.C.... ...CT..C.A
CMW8967_ETHIOPIA        TTGCAGGCTA ....TGCGCA TATT.....T GTCTTACTTG CATTTCGCTA GTTA.C.... ...CT..C.A
CMW8971_ETHIOPIA        TTGCAAGCTA ....TGCGCA TATT.....T GTCTTACTTG CATTTCGCTA GTTA.C.... ...CT..C.A
CMW2717_SOUTH_AFRICA    TTGCAAGCTA ....TGCGCA TATT.....T GTCTTACTTG CATTTCGCTA GTTA.C.... ...CT..C.A
CMW2740_SOUTH_AFRICA    TTGCAAGCTA ....TGCGCA TATT.....T GTCTTACTTG CATTTCGCTA GTTA.C.... ...CT..C.A
CMW3167_SOUTH_AFRICA    TTGCAAGCTA ....TGCGCA TATT.....T GTCTTACTTG CATTTCGCTA GTTA.C.... ...CT..C.A
CMW3164_LA_REUNION      TTGCAAGCTA ....TGCGCA TATT.....T GTCTTACTTG CATTTCGCTA GTTA.C.... ...CT..C.A

                                780        790        800        810        820        830        840
CMW3173_ZAMBIA          GCACGCACCT TACG------ -----GAC-- ---------- ---------- ---------- ----------
CMW3152_CAMEROON        .......... ....------ -----...-- ---------- ---------- ---------- ----------
CMW3955_ZIMBABWE        .......... ....------ -----...-- ---------- ---------- ---------- ----------
CMW5837_ETHIOPIA        ..---..AG. ....CTAGTT AGTTA...AA GCTTGGTTTG ACTTTGGCAA ATGCGTTCAC TTGCAAGCTT
CMW5846_ETHIOPIA        ..---..AG. ....CTAGTT AGTTA...AA GCTTGGTTTG ACTTTGGCAA ATGCGTTCAC TTGCAAGCTT
CMW8968_ETHIOPIA        ..---..AG. ....CTAGTT AGTTA...AA GCTTGGTTTG ACTTTGGCAA ATGCGTTCAC TTGCAAGCTT
CMW5844_ETHIOPIA        ..---..AG. ....CTAGTT AGTTA...AA GCTTGGTTTG ACTTTGGCAA ATGCGTTCAC TTGCAAGCTT
CMW8967_ETHIOPIA        ..---..AG. ....CTAGTT AGTTA...AA GCTTGGTTTG ACTTTGGCAA ATGCGTTCAC TTGCAAGCTT
CMW8971_ETHIOPIA        ..---..AG. ....CTAGTT AGTTA...AA GCTTGGTTTG ACTTTGGCAA ATGCGTTCAC TTGCAAGCTT
CMW2717_SOUTH_AFRICA    ..---..AG. ....CTAGTT AGTTA...AA CCTTGGTTTG ACTTTGGCAA ATGCGTTCAC TTGCAAGCTT
CMW2740_SOUTH_AFRICA    ..---..AG. ....CTAGTT AGTTA...AA CCTTGGTTTG ACTTTGGCAA ATGCGTTCAC TTGCAAGCTT
CMW3167_SOUTH_AFRICA    ..---..AG. ....CTAGTT AGTTA...AA CCTTGGTTTG ACTTTGGCAA ATGCGTTCAC TTGCAAGCTT
CMW3164_LA_REUNION      ..---..AG. ....CTAGTT AGTTA...AA CCTTGGTTTG ACTTTGGCAA ATGCGTTCAC TTGCAAGCTT
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                          850        860        870        880        890        900        910
CMW3173_ZAMBIA        --TTGGACAT TGAGGGCTTG TA-------- ---------- ---------- -------CGC ACGCACCTTA
CMW3152_CAMEROON      --........ .......... ..-------- ---------- ---------- -------... ..........
CMW3955_ZIMBABWE      --........ .......... ..-------- ---------- ---------- -------... ..........
CMW5837_ETHIOPIA      AG......TA ...TTT.G.. C.TTGAAATA CAAGTCAACA TGCTAGCTAG CACTTCAT.A .T.G.A...G
CMW5846_ETHIOPIA      AG......TA ...TTT.G.. C.TTGAAATA CAAGTCAACA TGCTAGCTAG CACTTCA..A .T.G.A...G
CMW8968_ETHIOPIA      AG......TA ...TTT.G.. C.TTGAAATA CAAGTCAACA TGCTAGCTAG CACTTCA..A .T.G.A...G
CMW5844_ETHIOPIA      AG......TA ...TTT.G.. C.TTGAAATA CAAGTCAACA TGCTAGCTAG CACTTCA..A .T.G.A...G
CMW8967_ETHIOPIA      AG......TA ...TTT.G.. C.TTGAAATA CAAGTCAACA TGCTAGCTAG CACTTCA..A .T.G.A...G
CMW8971_ETHIOPIA      AG......TA ...TTT.G.. C.TTGAAATA CAAGTCAACA TGCTAGCTAG CACTTCA..A .T.G.A...G
CMW2717_SOUTH_AFRICA  AG......TA ...TTT.G.. C.TTGAAATA CAAGTCAACA TGCTAGCTAG CACTTCA..A .T.G.A...G
CMW2740_SOUTH_AFRICA  AG......TA ...TTT.G.. C.TTGAAATA CAAGTCAACA TGCTAGCTAG CACTTCA..A .T.G.A...G
CMW3167_SOUTH_AFRICA  AG......TA ...TTT.G.. C.TTGAAATA CAAGTCAACA TGCTAGCTAG CACTTCA..A .T.G.A...G
CMW3164_LA_REUNION    AG......TA ...TTT.G.. C.TTGAAATA CAAGTCAACA TGCTAGCTAG CACTTCA..A .T.G.A...G

                          920        930        940        950        960        970        980
CMW3173_ZAMBIA        CTTTGTTGGC GCAA------ ---------- --------AA AT-AAAGACT TGCAAGCTAA GCTTGATTGG
CMW3152_CAMEROON      .......... ....------ ---------- --------.. ..-....... .......... ..........
CMW3955_ZIMBABWE      .......... ....------ ---------- --------.. ..-....... .......... ..........
CMW5837_ETHIOPIA      G...A.A.-- -...GTATGC CACCTATAGC CAAGTACG.. .GC.TT.... .......... ....CG....
CMW5846_ETHIOPIA      G...A.A.-- -...GTATGC CACCTATAGC CAAGTACG.. .GC.TT.... .......... ....CG....
CMW8968_ETHIOPIA      G...A.A.-- -...GTATGC CACCTATAGC CAAGTANG.. .GC.TT.... .......... ....CG....
CMW5844_ETHIOPIA      G...A.A.-- -...GTATGC CACCTATAGC CAAGTACG.. .GC.TT.... .......... ....CG....
CMW8967_ETHIOPIA      G...A.A.-- -...GTATGC CACCTATAGC CAAGTACG.. .GC.TT.... .......... ....CG....
CMW8971_ETHIOPIA      G...A.A.-- -...GTATGC CACCTATAGC CAAGTACG.. .GC.TT.... .......... ....CG....
CMW2717_SOUTH_AFRICA  G...A.A.-- -...GTATGC CACCTATAGC CAAGTACG.. .GC.TT.... .......... ....CG....
CMW2740_SOUTH_AFRICA  G...A.A.-- -...GTATGC CACCTATAGC CAAGTACG.. .GC.TT.... .......... ....CG....
CMW3167_SOUTH_AFRICA  G...A.A.-- -...GTATGC CACCTATAGC CAAGTACG.. .GC.TT.... .......... ....CG....
CMW3164_LA_REUNION    G...A.A.-- -...GTATGC CACCTATAGC CAAGTACG.. .GC.TT.... .......... ....CG....
```

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                          990        1010       1020       1030       1040       1050       1060
CMW3173_ZAMBIA        ACT------- ---------- ----GGAGT- ---------- ---------- ---------- --------CA
CMW3152_CAMEROON      ...------- ---------- ----.....- ---------- ---------- ---------- --------..
CMW3955_ZIMBABWE      ...------- ---------- ----.....- ---------- ---------- ---------- --------..
CMW5837_ETHIOPIA      .T.CTCTATT AGTTACATCT ACTT...C.A TGGCTGACAC GCAAAAAGCA AAGGGGG--A CTTGTTGG..
CMW5846_ETHIOPIA      .T.CTCTATT AGTTACATCT ACTT...C.A TGGCTGACAC GCAAAAAGCA AAGGGGG--A CTTGTTGG..
CMW8968_ETHIOPIA      .T.CTCTATT AGTTACATCT ACTT...C.A TGGCTGACAC GCAAAAAGCA AAGGGGGGGA CTTGTTGG..
CMW5844_ETHIOPIA      .T.CTCTATT AGTTACATCT ACTT...C.A TGGCTGACAC GCAAAAAGCA AAGGGGG--A CTTGTTGG..
CMW8967_ETHIOPIA      .T.CTCTATT AGTTACATCT ACTT...C.A TGGCTGACAC GCAAAAAGCA AAGGGGG--A CTTGTTGG..
CMW8971_ETHIOPIA      .T.CTCTATT AGTTACATCT ACTT...C.A TGGCTGACAC GCAAAAAGCA AAGGGGG--A CTTGTTGG..
CMW2717_SOUTH_AFRICA  .T.CTCTATT AGTTACATCT ACTT...C.A TGGCTGACAG GCAAAAAGCA AAGGGGG--A CTTGTTGG..
CMW2740_SOUTH_AFRICA  .T.CTCTATT AGTTACATCT ACTT...C.A TGGCTGACAG GCAAAAAGCA AAGGGGG--A CTTGTTGG..
CMW3167_SOUTH_AFRICA  .T.CTCTATT AGTTACATCT ACTT...C.A TGGCTGACAG GCAAAAAGCA AAGGGGG--A CTTGTTGG..
CMW3164_LA_REUNION    .T.CTCTATT AGTTACATCT ACTT...C.A TGGCTGACAG GCAAAAAGCA AAGGGGG--A CTTGTTGG..

                          1060       1070       1080       1090       1100       1110       1120
CMW3173_ZAMBIA        GACTTGA--- ---TATTCGT ---------- ---------- ---------- ---ACTTAAT GCTATCTTGC
CMW3152_CAMEROON      .......--- ---.......  ---------- ---------- ---------- ---.......  ..........
CMW3955_ZIMBABWE      .......--- ---.......  ---------- ---------- ---------- ---.......  ..........
CMW5837_ETHIOPIA      ..A....ACT TTT.C..... TTACAGCGTG CGCCGTGCGC CGTGCTGGGT CAG....... ..C..G..--
CMW5846_ETHIOPIA      ..A....ACT TTT.C..... TTACAGCGTG CGCCGTGCGC CGTGCTGGGT CAG....... ..C..G..--
CMW8968_ETHIOPIA      ..A....ACT TTT.C..... TTACAGCGTG CGCCGTGCGC CGTGCTGGGT CAG....... ..C..G..--
CMW5844_ETHIOPIA      ..A....ACT TTT.C..... TTACAGCGTG CGCCGTGCGC CGTGCTGGGT CAG....... ..C..G..--
CMW8967_ETHIOPIA      ..A....ACT TTT.C..... TTACAGCGTG CGCCGTGCGC CGTGCTGGGT CAG....... ..C..G..--
CMW8971_ETHIOPIA      ..A....ACT TTT.C..... TTACAGCGTG CGCCGTGCGC CGTGCTGGGT CAG....... ..C..G..--
CMW2717_SOUTH_AFRICA  ..A....ACT TTT.C..... TTACAGCGTG CACCGTGTGC CGTGCTGGGT CAG....... ..C..G..--
CMW2740_SOUTH_AFRICA  ..A....ACT TTT.C..... TTACAGCGTG CACCGTGTGC CGTGCTGGGT CAG....... ..C..G..--
CMW3167_SOUTH_AFRICA  ..A....ACT TTT.C..... TTACAGCGTG CACCGTGTGC CGTGCTGGGT CAG....... ..C..G..--
CMW3164_LA_REUNION    ..A....ACT TTT.C..... TTACAGCGTG CACCGTGTGC CGTGCTGGGT CAG....... ..C..G..--
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                          11130       1140       1150       1160       1170       1180       1190
CMW3173_ZAMBIA          TATCTTACTA TCTT------ --ACTATCAA AAACCACAGC ACCCAGGATT GCCCACGTGG TCC-CCCACC
CMW3152_CAMEROON        .......... ....------ --........ .......... .......... C..G-..... ..GG......
CMW3955_ZIMBABWE        .......... ....ACTATC TT........ .......... .......... C..G-..... ...-......
CMW5837_ETHIOPIA        ---------- ---------- --........ .......... .......... C..GCG-... ...-......
CMW5846_ETHIOPIA        ---------- ---------- --T....... .......... .......... C..GCG-... ...-......
CMW8968_ETHIOPIA        ---------- ---------- --........ .......... .......... C..GCG-... ...-......
CMW5844_ETHIOPIA        ---------- ---------- --........ .......... .......... C..GCG-... ...-...C..
CMW8967_ETHIOPIA        ---------- ---------- --........ .......... .......... C..GCG-... ...-......
CMW8971_ETHIOPIA        ---------- ---------- --........ .......... .......... C..GCG-... ...-......
CMW2717_SOUTH_AFRICA    ---------- ---------- --........ .......... .......... C..GCA-... ...-......
CMW2740_SOUTH_AFRICA    ---------- ---------- --........ .......... .......... C..GCA-... ...-......
CMW3167_SOUTH_AFRICA    ---------- ---------- --........ .......... .......... C..GCA-... ...-......
CMW3164_LA_REUNION      ---------- ---------- --........ .......... .......... C..GCA-... ...-......

                          1200       1210       1220       1230       1240    1247
CMW3173_ZAMBIA          GTGGTACTAA CTAGGCGGCA CTTTGNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNN
CMW3152_CAMEROON        .......... ...A...... ..NNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNN
CMW3955_ZIMBABWE        .......... ..G....... .....G-TTA ACTGCGCAGA TNNNNNNNNN NNNNNNN
CMW5837_ETHIOPIA        .......... ..NNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNN
CMW5846_ETHIOPIA        .........G ..G....... .....-ATTA ACTGCGCAGA TCAGACGGGA TGCGGNN
CMW8968_ETHIOPIA        .......... ..G....... .....-ATTA ACTGCGCAGA TCAGACGGNN NNNNNNN
CMW5844_ETHIOPIA        .......... ..G....... .....-ATTA ACTGCGCAGA TCAGACGGGA TGCGGNN
CMW8967_ETHIOPIA        .......... ..G....... .....-ATTA ACTGCGCAGA TCAGACGGGA TGCGGNN
CMW8971_ETHIOPIA        .......... ..G....... .....-ATTA ACTGCGCAGA TCAGACGGNN NNNNNNN
CMW2717_SOUTH_AFRICA    .......... ..G....... .....-ATTA ACTGCGCAGA TCAGACGGGA TGCGGTG
CMW2740_SOUTH_AFRICA    .......... ..G....... .....GATTA ACTGCGCAGA TCAGACGGGA TGCGGTG
CMW3167_SOUTH_AFRICA    .......... ..G....... .....GATTA ACTGCGCAGA TCAGACGGGA TGCGGTG
CMW3164_LA_REUNION      .......... ..G....... .....-ATTA ACTGCGCAGA TCAGACGGGA TGCGGTG
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Chapter Four

Charaterisation of the Coniothyrium Stem Canker Pathogen on *Eucalyptus camaldulensis* in Ethiopia

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. The aim of this study was to identify the causal agent of the disease in Ethiopia. This was achieved by sequencing the ITS region of the rRNA operon and the β-tubulin gene 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. Based on these data, the *Coniothyrium* isolates from Ethiopia and Uganda grouped together in a separate clade, but closely related to *C. zuluense* from South Africa, Mexico and Thailand. This study represents the first definitive report of *C. zuluense* and the disease caused by it in Ethiopia and Uganda. In Ethiopia, Coniothyrium canker is causing considerable losses in yield and quality of timber and it impacts negatively on the lives of the subsistence farmers. Research will thus be required to minimize these losses.

INTRODUCTION

Eucalyptus species, which originate from Australia and nearby islands, have been introduced and planted in many tropical and subtropical countries. Estimates indicate that plantations of *Eucalyptus* spp. cover approximately 10 million hectares of land, world-wide (Eldridge *et al.* 1997). These plantations provide furniture, timber, 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, the planting of exotic trees commenced with the introduction of *Eucalyptus* spp. in the 1890's. *Eucalyptus globulus* Labill, *E. camaldulensis* Dehn., *E. saligna* Sm., *E. grandis* Hill ex Maid and *E. citriodora* Hook are the most common species planted in Ethiopia. *E. camaldulensis* is widely planted, usually at lower elevation and warmer localities, while *E. globulus* is commonly planted in cooler areas. Plantations of *Eucalyptus* constitute the major proportion of exotic plantation species and cover about 100 000 ha of land. These *Eucalyptus* plantations provide wood for energy, construction material, transmission poles and fencing material (Pohjonen & Pukkala 1990, Persson 1995).

Eucalyptus spp. have showed great promise in most areas where they have been planted as exotics. However, diseases pose a serious threat to these economically important plantation species. A number of important diseases have been recorded on different *Eucalyptus* species and clones. These diseases infect stems, roots and leaves. Cryphonectria canker caused by *Cryphonectria cubensis* (Bruner) Hodges (Hodges, Alfenas & Ferreria 1986, Wingfield, Swart & Abear 1989, Conradie, Swart & Wingfield 1990), canker and die-back caused by *Botryosphaeria* spp. (Smith, Kemp & Wingfield 1994), vascular wilt of *Eucalyptus* caused by *Ceratocystis fimbriata* Ell. & Halst. (Roux *et al.* 2000), pink disease caused by *Erythricium salmonicolor* (Berk. & Broome) Burds. (Sharma, Mohanan & Florence 1984, Roux *et al.* 2001, Alemu, Roux & Wingfield 2002) and Leaf blotch caused by *Mycosphaerella* spp. (Park & Keane 1982, Crous 1998) are examples of diseases in commercial *Eucalyptus* plantations. Recently, a serious stem canker disease caused by *Coniothyrium zuluense* Wingfield, Crous & Coutinho has also been described causing losses to *Eucalyptus* trees in various countries (Wingfield, Crous & Coutinho 1996, Roux, Wingfield & Cibrián 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* S. T. Blake 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) and Mexico (Roux *et al.* 2002).

During a disease survey of plantation forestry species in Western and South Western Ethiopia, several pathogens were identified (Alemu, Roux & Wingfield 2003). Symptoms of stem canker similar to those of Coniothyrium canker were observed on *E. camaldulensis* trees at a number of these localities (Alemu *et al.* 2003). *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.* An additional objective was to determine the phylogenetic relationship between the fungus occurring in Ethiopia and isolates from other parts of the world. To achieve this DNA sequences of the ITS regions of the rRNA operon and β-tubulin gene were used.

MATERIALS AND METHODS

Sample collection and isolation

Samples were collected from infected *E. camaldulensis* trees planted in Southern and South Western Ethiopia (Figure 1). Disease symptoms were used to select infected trees for sampling. 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 plates containing malt extract agar (MEA, 20 g Biolab Malt Extract; 15 g Biolab Agar), spread on

the agar surface with sterilised water and incubated at 25 °C for two weeks. Stock cultures of all isolates were maintained on 2% MEA slants at 5 °C. *Coniothyrium* cultures collected from Ethiopia are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA extraction

Total genomic DNA was extracted from isolates (Table 1) grown in liquid MY medium (2% Malt Extract, 0.3% Yeast Extract Agar) for two 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. A modified version of the method of Raeder and Broda (1985) was used to extract DNA from the mycelium. Approximately 0.5 g of powdered mycelium was placed in 1.5 µl Eppindorf (Epps.) tubes and 1000 µl extraction buffer (100 mM Tris-HCl, pH 8; 50 mM EDTA; 500 mM NaCl; 5 g CTAB) was added to each tube. These suspensions were then incubated in a 60 °C water bath for 2 hours, and frequently mixed by inverting the tubes. Phenol (500 µl) was added and the solution was mixed using a vortex mixer. Thereafter, 300µl chloroform was added and mixed. The cell debris were removed by centrifugation at 12500 g, for 60 min at 4 °C. The upper aqueous layer of this mixture was transferred to new tubes, whereafter a further phenol:chlorophorm extraction was carried out by adding 200 µl phenol and 200 µl chloroform. This mixture was centrifuged at 12500 g for 5 min at 4 °C and the upper aqueous layer transferred to a fresh tube. To remove the excess phenol it was washed with 400 µl chloroform and centrifuged at 12500 g for 5 min at 4 °C. This step was repeated until a clear interface was obtained. Next, 0.1 volume of 3M NaAc (pH 5.5) and two volumes of absolute ethanol were added and the mixture was centrifuged for 30 min at 4 °C to precipitate the nucleic acid. The liquid phase was discarded and the precipitated nucleic acid was washed with 70% ethanol and centrifuged for 5 min at 4 °C to obtain a DNA pellet.

The DNA pellets were vacuum dried to remove excess ethanol and resuspended in 50 µl water. RNase A (1mg/ml) (Roche Diagnostics, South Africa) was added to the DNA solution to remove the contaminating RNA and incubated at 37 °C in a water bath over night. The presence of DNA in the samples was detected by using agarose gel electrophoresis in a 1% agarose gel, stained with ethidium bromide and visualised under UV light.

PCR amplification

The internal transcribed spacer (ITS) regions of the ribosomal RNA 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) to amplify the ITS 1, ITS 2 and 5.8S genes of the ribosomal RNA operon (White *et al.* 1990). The PCR reaction mixture contained DNA polymerase (*Taq*, 2.5 U/µl, Roche Diagnostics, South Africa), 2.5 mM dNTP's, 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 water to make up a final volume of 50 µl. Denaturation was performed at 96 °C for 1 min. This was followed by 35 cycles of primer annealing at 55 °C for 30 s, chain elongation was undertaken at 72 °C for 1 min and denaturation was conducted at 92 °C for 1 min. Final chain elongation was carried out at 72 °C for 5 min.

The β-tubulin gene was partially amplified using the forward primer Bt2a (5' GGT AAC CAA ATC GGT GCT GCT TTC 3') and the reverse primer Bt2b (5' ACC CTC AGT GTA G TG ACC CTT GGC 3') (Glass & Donaldson 1995). The PCR reaction mix included DNA polymerase (*Taq*, 2.5U/µl), 0.2 mM dNTP's, 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, 1 µl of DNA and 37 µl sterilised water to make up a final volume of 50 µl. Amplification was conducted using the following PCR reaction conditions: an initial denaturation at 94 °C for 1 min, which was followed by 40 cycles at 94 °C for 1 min, primer annealing at 51 °C for 30 s, chain elongation at 72 °C for 1 min and an additional chain elongation step at 72 °C for another 1 min. All PCR products were detected using agarose gel electrophoresis on 1% agarose gels stained with ethidium bromide under UV illumination.

DNA sequencing

The PCR products of both the ITS regions and the β-tubulin gene were purified using the High Pure PCR Product Purification Kit (QUIAGEN, 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 protocol, on an ABI PRISM™ 3100 DNA Autosequencer (Perkin-Elmer). Primers ITS 1 and ITS 4 were used for sequencing the ITS regions and for the β-tubulin gene,

primers Bt2a and Bt2b were used. The sequences for the Ethiopian isolates were compared with ITS DNA sequences obtained from GenBank [National Centre for Biotechnology Information (NCBI), US 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 sequence data of *Coniothyrium* spp. and *Mycosphaerella* spp. were included in the study and the ITS and β-tubulin data analyzed.

Sequence analysis

The ITS and β-tubulin gene sequences were aligned manually 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). *Mycosphaerella molleriana* (Thumb.) Lindau. and *M. nubilosa* (Cooke) Hansf. were used as an outgroup for the combined data set. A Partition homogeneity test was used to check the combinability of the ITS and β-tubilin sequence data sets (Farris *et al.* 1995, Huelsenbeck, Bull & Cunningham 1996).

Pathogenicity test

An inoculation study was conducted on an 18-month-old *E. grandis* clone (ZG 14) (approximately 1 cm diameter) in the green house at a temperature of ~25 °C. Prior to inoculation, plants acclimatised to greenhouse conditions for one week. Seven of the *Coniothyrium* isolates from Ethiopia were randomly selected for the inoculation study (Table 1). Cultures were grown on MEA for two weeks before inoculation. A 9 mm cork borer was used to remove the bark and expose the cambium. Mycelial plugs, of equal size, overgrown with the test cultures, were placed into each wound with the mycelium facing the wood. All the wounds were covered with parafilm (Pechiney Plastic Packaging, Chicago) to prevent contamination and desiccation. Each isolate was inoculated on 10 trees and an additional ten trees were inoculated with sterile MEA to serve as controls.

After six 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 statistically compare lesion development associated with the isolates and the control.

RESULTS

Sample collection and Isolation

Symptoms of Coniothyrium stem canker were observed in several *E. camaldulensis* growing localities in South and South Western Ethiopia. These areas were between Woliso and Jima and between Wolkite and Sodo (Figure 1). *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 (Figure 2a-2d) were evident on most infected trees. Initially, small discrete lesions developed on young green bark (Figure 2a, 2d). When these lesions coalesced, large necrotic lesions developed on the stems, branches and twigs (Figure 2b). Kino pockets were observed in the wood associated with the bark lesions on infected trees (Figure 2c).

After one day in moisture chambers, pycnidial structures, 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 had similar growth and colour in culture.

PCR amplification and analysis of sequence data

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. Amplification of the partial β-tubulin gene yielded fragments of approximately 400 bp.

The ITS regions and 5.8S gene were sequenced and after alignment yielded a total of 551 characters of which 495 characters were constant, 40 variable characters were parsimony uninformative and 16 characters were parsimony informative. A total of 485 characters were obtained when the β-tubulin gene was aligned. Of these, 397 characters were constant, 67 were parsimony uninformative and 21 characters were parsimony informative.

Comparison of the ITS and 5.8S gene sequences to sequences available in the NCBI data base revealed that the sequences of the samples from Ethiopia are most similar to that of *C. zuluense* (98%) followed by *Mycosphaerella vespa* (Carnegie & Keane) and *M. molleriana* (96% homology) and *M. nubilosa* (94% homology). Analysis of the ITS sequence data, using sequences obtained from Genbank and the data set from Van Zyl *et al.* (2002) produced 1 tree. The tree had a CI = 0.976 and RI = 0.944 (Figure 3), and showed that the *Coniothyrium* isolates from Ethiopia and Uganda grouped together in the larger *C. zuluense* clade (83% bootstrap). Two distinct sub-clades, were however, apparent. Isolates from South Africa, Thailand and Mexico grouped in one clade (97% bootstrap) and isolates from Ethiopia and Uganda grouped in another (80% bootstrap). *C. zuluense* isolates grouped more closely with *M. molleriana* and *M. nubilosa*, than with other species of *Coniothyrium*, including *C. ovatum* Swart and *C. fuckelii* Sacc.

A partition homogeneity test showed that the ITS and β-tubulin sequences could be combined (P value = 1). The combined sequences had a total of 956 characters of which 796 characters were constant, 116 variable characters were parsimony uninformative and 44 characters were parsimony informative. Analysis of the combined data sets generated 1 tree (Figure 4). The tree generated from the combined data set had a consistency index (CI) of 0. 969 and retention index (RI) of 0.942. Ethiopian and Uganda isolates grouped together with *C. zuluense* (100% bootstrap). Two sub-clades were, however, produced within *C. zuluense* (Figure 4). Isolates from South Africa, Thailand and Mexico grouped together in clade I with a 96% bootstrap support. This clade represents *C. zuluense*. Clade II contained the *Coniothyrium* isolates from Ethiopia and Uganda with a 100% confidence limit. The *Coniothyrium* isolates grouped separately from any of the *Mycosphaerella* isolates.

Pathogenicity test

Small lesions developed on *E. grandis* trees inoculated with Ethiopian *Coniothyrium* isolates (Figure 2f). Lesion lengths differed statistically from those of the control ($P<0.0001$) (R-square = 0.48). No variation was observed in lesion development between the *C. zuluense* isolates used in the inoculation study (Table 3, Figure 5).

DISCUSSION

Coniothyrium stem canker, caused by *C. zuluense* is considered to be one of the most important new threats to plantation grown *Eucalyptus* species. Until recently, this disease was known only from South Africa (Wingfield *et al.* 1996), Thailand (Van Zyl *et al.* 2002) and Mexico (Roux *et al.* 2002). 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), this study provides the first clear evidence for its occurrence in the country and 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 based 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). The disease is restricted to specific areas in 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*. In South Africa Coniothyrium stem canker is only a problem in warmer sub-tropical areas (Wingfield *et al.* 1996) while the only other reports of this disease is from tropical areas such as Thailand (Van Zyl *et al.* 2002) and Mexico (Roux *et al.* 2002).

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. vespa*, *M. molleriana* and *M. nubilosa*. This is particularly interesting as other *Coniothyrium* spp. such as *C. ovatum* and *C. fuckelii* were 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 choose *Mycosphaerella* spp. 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.

Results of our combined sequence data set separated the *C. zuluense* isolates into two distinct groups. One of these groups mainly constituted authentic *C. zuluense* isolates from South Africa, Thailand and Mexico. The Ethiopian isolates and one isolate from Uganda were identical and resided in a separate clade. These data might suggest that *C. zuluense* represents a species complex, and this deserves further scrutiny.

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. During an extensive survey of *Eucalyptus* diseases in Western and Southern Ethiopia (Alemu *et al.* 2003), Coniothyrium stem canker was not observed on *E. grandis*, or any other species 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. The disease is wide spread in *E. camaldulensis* growing areas between Wolkite and Sodo as well as 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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Table 1. List of fungal isolates used in this study

Isolate No.	Origin	Species	Host	Collector	Accession No.	
					ITS	B-tubilin
CMW11220, CMW7399	South Africa	*Coniothyrium zuluense*	*E. grandis*	L.M. van Zyl	AF376823	AY244383
CMW11221, CMW7459	South Africa	,,	,,	,,	AF376816	AY244384
CMW11225	Ethiopia	,,	,,	Alemu Gezahgne & J. Roux	AY244415	AY244390
CMW11226	Ethiopia	,,	,,	,,	AY244413	AY244391
CMW11227	Ethiopia	,,	,,	,,	AY244414	AY244392
CMW11228	Uganda	,,	*Eucalyptus* spp.	J. Roux	AY244416	AY244389
CMW11230	Mexico	,,	,,	M. J. Wingfield & J. Roux	AF385610	AY244385
CMW11231	Mexico	,,	,,	,,	AF385611	AY244386
CMW5232	Thailand	,,	,,	M. J. Wingfield & van Zyl	AF376828	AY244387
CMW5234	Thailand	,,	,,	,,	AF376825	AY244388
CMW3032	South Africa	*Mycosphaerella nubilosa*	*E. bicostata*	P.W. Crous	-	AY244393
CMW8575	Chile	*M. molleriana*	*E. globulus*	R. Ahumada	-	AY244394

[a] CMW numbers refer to the culture collection numbers of the Tree Pathology Co-operative Programme (TPCP), FABI, University of Pretoria, South Africa.

Table 2. Results of inoculation of an *E. grandis* clone with Ethiopian *Coniothyrium* isolates

Isolates	Mean Lesion Length (mm)	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
CMW11238	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

Each mean lesion length is the average of 10 measurements.

R-Square =0.48.

Mean values with the same letters did not differ significantly at P = 0.05.

Figure 1. Map of Ethiopia showing the plantation areas where surveys were conducted.

Figure 2. Symptoms of Coniothyrium stem canker on *E. camaldulensis*. (A) Discrete lesions on stem, (B) stem malformation and discoloration, (C) Kino pockets in *E. camaludensis* wood, (D) Development of necrotic lesions on branches, (E) lesions produced on ZG14 after artificial inoculation with *C. zuluense*. (1) control (2) *C. zuluense*.

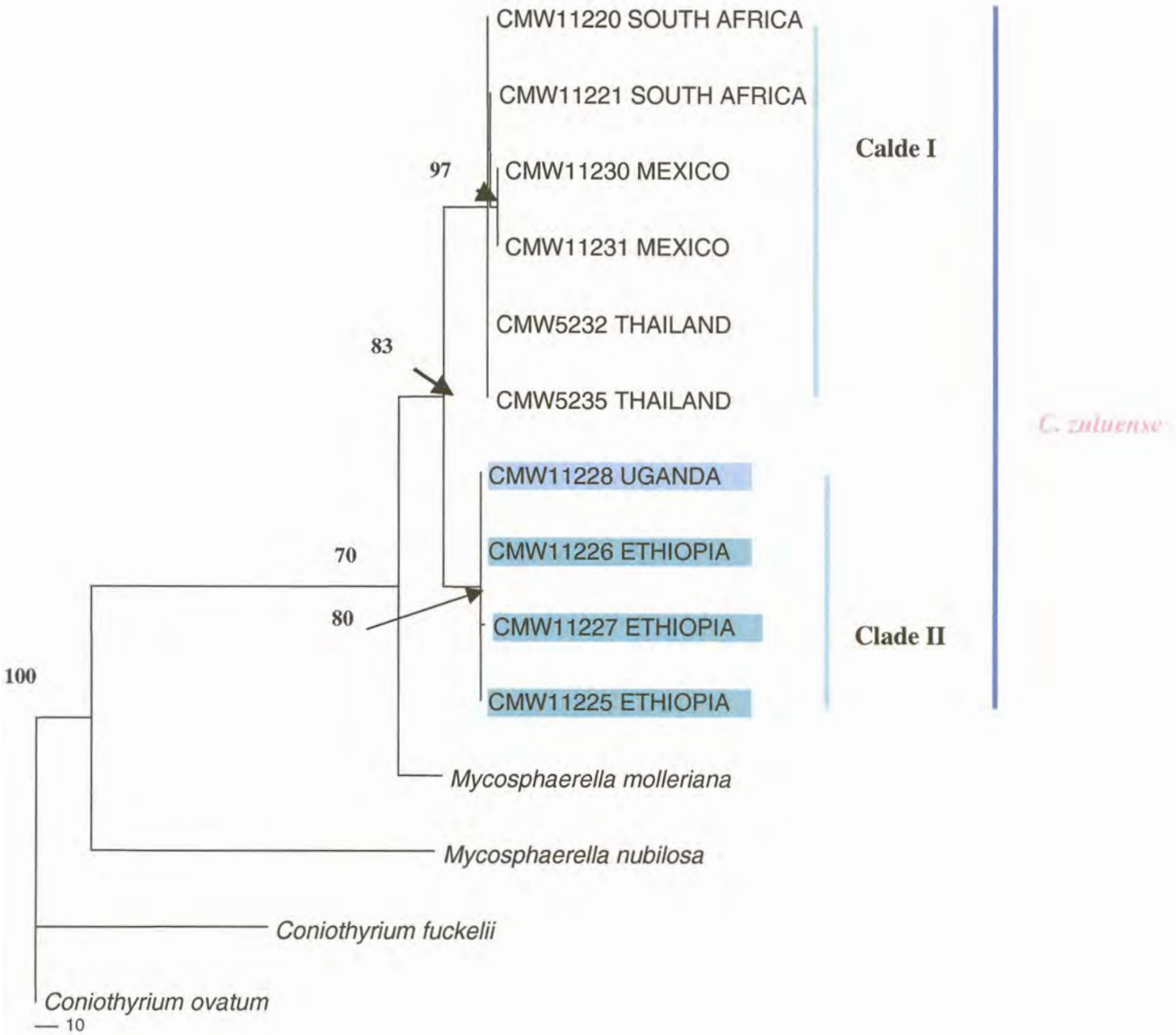

Figure 3. Phylogenetic tree of the ITS sequence data of *Coniothyrium* spp. and *Mycosphaerella* spp. CI=0.976 and RI=0.944. Bootstrap values are shown above the branches.

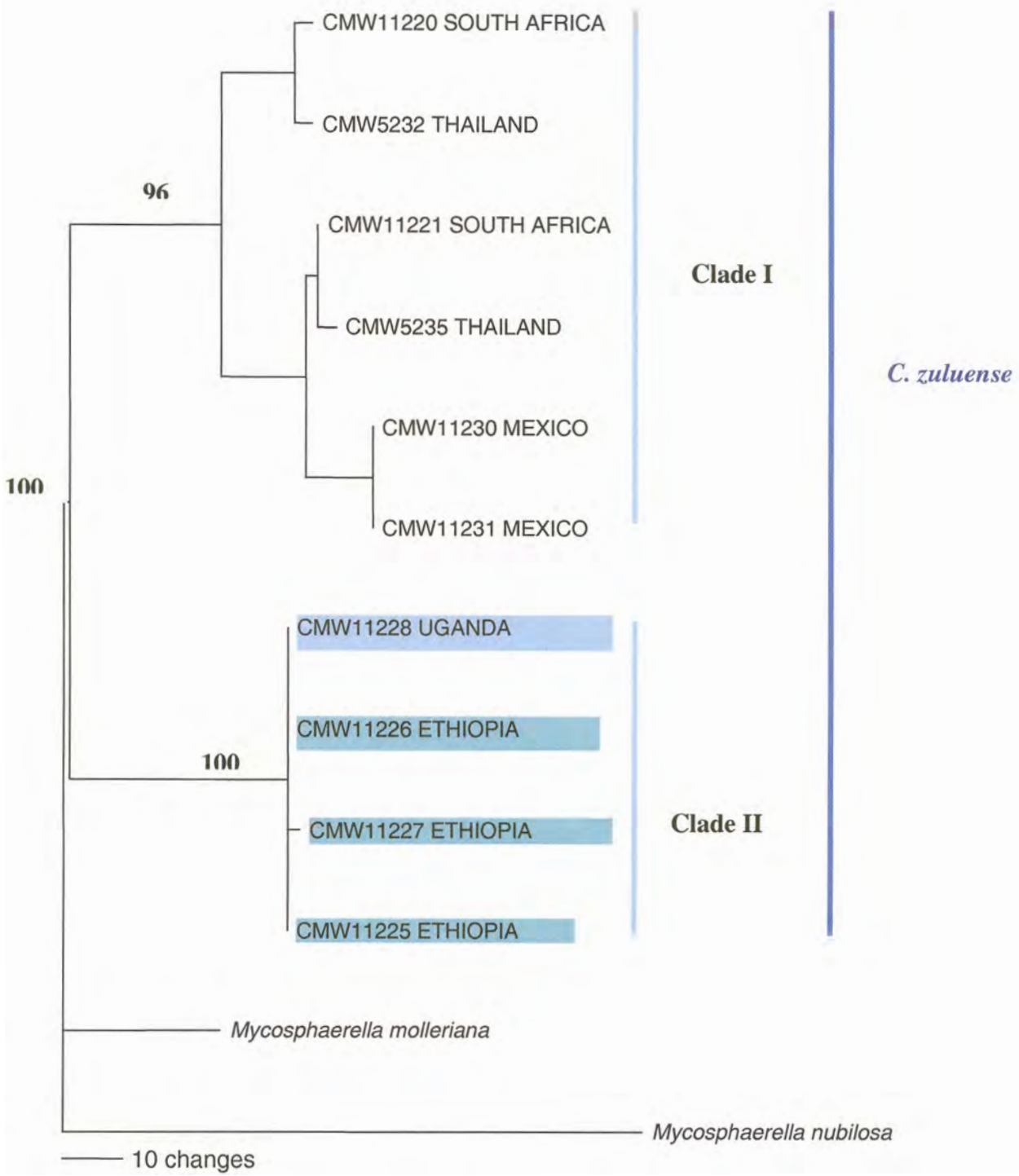

Figure 4. Phylogenetic tree of *Coniothyrium* spp. generated from the combined ITS and β-tubulin sequences. CI = 0.969 and RI = 0.942. Bootstrap values are shown at each branch.

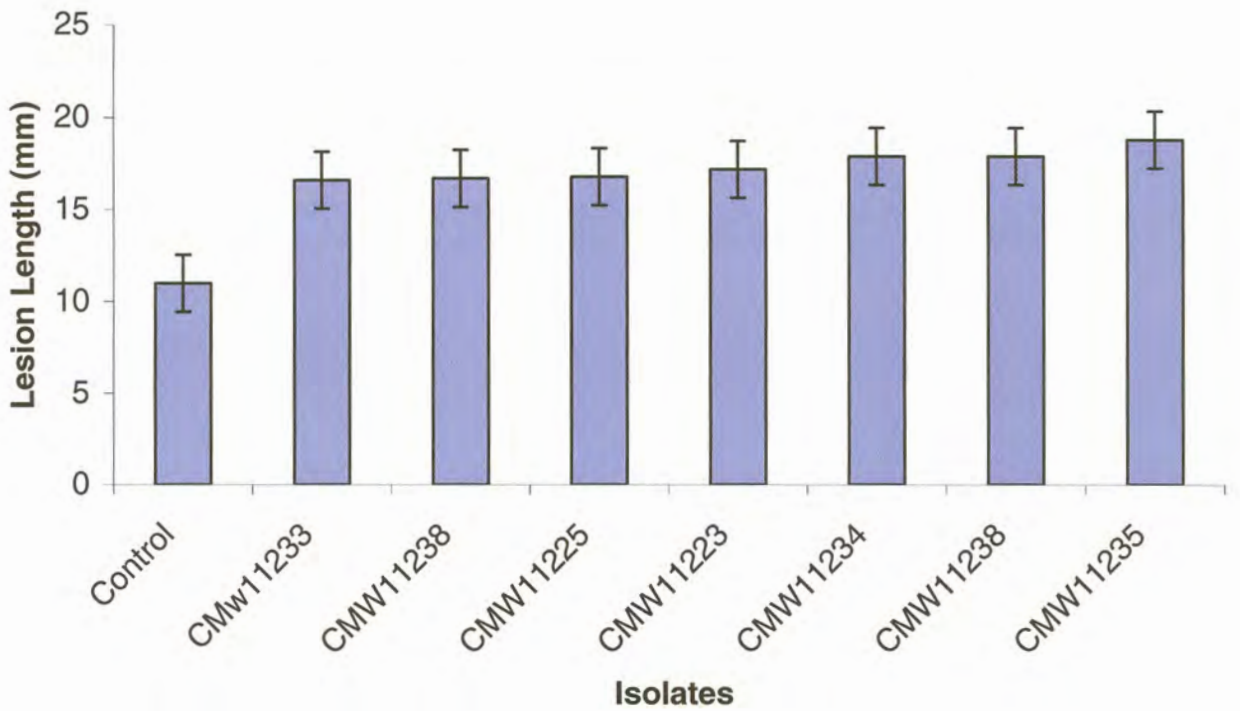

Figure 5. Means and confidence limits of lesion lengths of *Coniothyrium* isolates from the greenhouse inoculation trial.

Figure 7. Aligned sequences of the ITS and β-Tubulun genes of isolates used in this study. (-)= Gaps, (.)= homologous nucleotides (N)= Unknown bases.

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                                10         20         30         40         50         60         70         80
CMW11220_South_Africa           TCCGTAGGTG GAACCTGCGG AGGGATCATT ACTGAGTGAG GGCGCAAGCC CGACCTCC-A ACCCCATGTT TTCCAACCAT
CMW11221_South_Africa           .......... .......... .......... .......... .......... ........-. .......... ...T......
CMW11230_Mexico                 .......... .......... .......... .......... .......... ........-. .......... ...T......
CMW11231_Mexico                 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN N......... ...T......
CMW5232_Thailand                .......... .......... .......... .......... .......... ........-. .......... ..........
CMW5235_Thailand                .......... .......... .......... .......... .......... ........-. .......... ..........
CMW11228_Uganda                 .......... .......... .......... ......C... .....G.... ........-. .......... ...-......
CMW11226_Ethiopia               .......... .......... .......... ......C... .....G.... ........-. .......... ...-......
CMW11227_Ethiopia               NNNNNNNNNN NNN....... .......... ......C... .....G.... ........-. .......... ...-......
CMW11225_Ethiopia               .......... .......... .......... ......C... .....G.... ........-. .......... ...-......
Mycosphaerella_molleriana       .......... .......... .......... .......... .......... ........-. ....-..... .C.A.....C
Mycosphaerella_nubilosa         .......... -......... .......... ..C....... ....GC.... ........T. C...-..... .C...-...C

                                90         100        110        120        130        140        150        160
CMW11220_South_Africa           GTTGCCTCGG GGGCGACCCG GCCATCGCGC CGGGGCCCCC GGTGGACCCC TCCAACTCTG CATCTTTGCG TCTGAGTCAC
CMW11221_South_Africa           .......... .......... .......... .......... .......... .......... .......... ..........
CMW11230_Mexico                 .......... .......... .......... .......... .......... .......... .......... ..........
CMW11231_Mexico                 .......... .......... .......... .......... .......... .......... .......... ..........
CMW5232_Thailand                .......... .......... .......... .......... .......... .......... .......... ..........
CMW5235_Thailand                .......... .......... .......... .......... .......... .......... .......... ..........
CMW11228_Uganda                 .......... .......... ....C..... .......... ..C....... ..-....... .....C.... ..........
CMW11226_Ethiopia               .......... .......... ....C..... .......... ..C....... ..-....... .....C.... ..........
CMW11227_Ethiopia               .........TC .......... ....C..... .......... ..C....... ..-....... .....C.... ..........
CMW11225_Ethiopia               .......... .......... ....C..... .......... ..C....... ..-....... .....C.... ..........
Mycosphaerella_molleriana       .......... .......... ...GC..... .......... .........- ..-....... .....C.... ..........
Mycosphaerella_nubilosa         .......... .......... ...CC..... ........T. .CA.A..... ..-...GGCT GGATC.GTGC GTG....A.T
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                                170        180        190        200        210        220        230        240
CMW11220_South_Africa           AAAATAAAAT CAATCAAAAC TTTCAACAAC GGATCTCTTG GTTCTGGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT
CMW11221_South_Africa           .......... .......... .......... .......... .......... .......... .......... ..........
CMW11230_Mexico                 .......... .......... .......... .......... .......... .......... .......... ..........
CMW11231_Mexico                 .......... .......... .......... .......... .......... .......... .......... ..........
CMW5232_Thailand                .......... .......... .......... .......... .......... .......... .......... ..........
CMW5235_Thailand                .......... .......... .......... .......... .......... .......... .......... ..........
CMW11228_Uganda                 .......... .......... .......... .......... .......... .......... .......... ..........
CMW11226_Ethiopia               .......... .......... .......... .......... .......... .......... .......... ..........
CMW11227_Ethiopia               .......... .......... .......... .......... .......... .......... .......... ..........
CMW11225_Ethiopia               .......... .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_molleriana       .....C..-. .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_nubilosa         .C..CC..-. ....T..... .......... .......... .......... .......... .......... ..........

                                250        260        270        280        290        300        310        320
CMW11220_South_Africa           AATGTGAATT GCAGAATTCA GTGAATCATC GAATCTTTGA ACGCACATTG CGCCCTCTGG TATTCCGGAG GGCATGCCTG
CMW11221_South_Africa           .......... .......... .......... .......... .......... .......... .......... ..........
CMW11230_Mexico                 .......... .......... .......... ...C...... .......... .......... .......... ..........
CMW11231_Mexico                 .......... .......... .......... ...C...... .......... .......... .......... ..........
CMW5232_Thailand                .......... .......... .......... .......... .......... .......... .......... ..........
CMW5235_Thailand                .......... .......... .......... .......... .......... .......... .......... ..........
CMW11228_Uganda                 .......... .......... .......... .......... .......... .......... .......... ..........
CMW11226_Ethiopia               .......... .......... .......... .......... .......... .......... .......... ..........
CMW11227_Ethiopia               .......... .......... .......... .......... .......... .......... .......... ..........
CMW11225_Ethiopia               .......... .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_molleriana       .......... .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_nubilosa         .......... .......... .......... .......... .......... .......... .......... ..........
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                                330        340        350        360        370        380        390        400
CMW11220_South_Africa           TTCGAGCGTC ATTACACCAC TCCAGCCTCG CTGGGTATTG GGCGCCGCGG CCTCCGCGCG CCTT-AATGT CTCCGGCCGA
CMW11221_South_Africa           .......... .......... .......... .......... .......... .......... ....-..... ..........
CMW11230_Mexico                 .......... .......... .......... .......... .......... .......... ....-..... ..........
CMW11231_Mexico                 .......... .......... .......... .......... .......... .......... ....-..... ..........
CMW5232_Thailand                .......... .......... .........? .......... .......... .......... ....-..... ..........
CMW5235_Thailand                .......... .......... .......... .......... .......... .......... ....-..... ..........
CMW11228_Uganda                 .......... .......... .......... .......... .......... .......... ....-..... ..........
CMW11226_Ethiopia               .......... .......... .......... .......... .......... .......... ....-..... ..........
CMW11227_Ethiopia               .......... .......... .......... .......... .......... .......... ....-..... ..........
CMW11225_Ethiopia               .......... .......... .......... .......... .......... .......... ....-..... ..........
Mycosphaerella_molleriana       .......... .......... ...G...... .......... .......... .......... ...CG..-.. ..........
Mycosphaerella_nubilosa         .......... ...T...... .......C.. ..T....... .......... .......... ...C-..... ..........

                                410        420        330        440        450        460        470        480
CMW11220_South_Africa           GCCGACCGTC TCCAAGCGTT GTGGCACAAC TGTTTCGCTT TCGGG-ACCG GTCCGGCGAC GCGCCGTTAA ACCCTTTCAC
CMW11221_South_Africa           .......... .......... .......... .......... .....-.... .......... .......... ..........
CMW11230_Mexico                 .......... .......... .......... .......... .....-.... .......... .......... ..........
CMW11231_Mexico                 .......... .......... .......... .......... .....-.... .......... .......... ..........
CMW5232_Thailand                .......... .......... .......... .......... .....-.... .......... .......... ..........
CMW5235_Thailand                .......... .......... .......... .......... .....-.... .......... .......... ..........
CMW11228_Uganda                 .......... ..T....... .......... .......... .....-.... ...T...... .......... ..........
CMW11226_Ethiopia               .......... ..T....... .......... .......... .....-.... ...T...... .......... ..........
CMW11227_Ethiopia               .......... ..T....... .......... .......... .....-.... ...T...... .......... ..........
CMW11225_Ethiopia               .......... ..T....... .......... .......... .....-.... ...T...... .......... ..........
Mycosphaerella_molleriana       .......... .......... .......... .......... .....-.... ...T....G. .......... ..........
Mycosphaerella_nubilosa         .......... ..TC...... .......T.. .........G A....G.... ...T....-. .......... ..........
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                                490        500        510        520        530        540        550        560
CMW11220_South_Africa           CAAAGGTTGA CCTCGGATCA GGTAGGGATA CCCGCTGAAC TTAAGCATAT CAATTAAAGC GGAGGATGGT AACCAAA---
CMW11221_South_Africa           .......... .......... .......... .......... .......... ..-....-.. .......... .......---
CMW11230_Mexico                 .......... .......... .......... .......... .......... .????????? ??????.... .......---
CMW11231_Mexico                 .......... .......... .......... .......... .......... ..T.A.-GCG .AG.A..... .......---
CMW5232_Thailand                .......... .......... .......... .......... .......... ....-..-.. .......... .......---
CMW5235_Thailand                .......... .......... .......... .......... .......... ....-..-.. .......... .......---
CMW11228_Uganda                 .......... .......... .......... .......... .......... ..-....-.. .......... .......---
CMW11226_Ethiopia               .......... .......... .......... .......... .......... ..-....... .......... .......A--
CMW11227_Ethiopia               .......... .......... .......... .......... .......... ..-....... .......... .......---
CMW11225_Ethiopia               .......... .......... .......... .......... .......... ....-..-.. .......... .......---
Mycosphaerella_molleriana       -......... .......... .......... ?????????? ?????????? ?????????? ??????.... .......A--
Mycosphaerella_nubilosa         .......... .......... .......... ?????????? ?????????? ?????????? ?????????. .......AA-

                                570        580        590        600        610        620        630        640
CMW11220_South_Africa           TCGGTGCTGC TTTCTGGCAG AACATCTCCG GCGAGCACGG CCTCGACGGC TCCGGCGTGT AGGTCTAGCA GGAGTGGGAT
CMW11221_South_Africa           .......... .......... .......... .......... .........T .......... .......... ..........
CMW11230_Mexico                 .......... .......... .......... .......?.. .......... .......... .........G ..........
CMW11231_Mexico                 .......... .......... .......... .......... .......... .......... .........G ..........
CMW5232_Thailand                .......... .......... .......... .......... .......... .......... .......... ..........
CMW5235_Thailand                .......... .......... .......... .......... .........T .......... .......... ..........
CMW11228_Uganda                 .......... .......... .......... .......... ......T... .......... ......GA.. ...A..A..G
CMW11226_Ethiopia               .......... .......... .......... .......... ......T... .......... ......GA.. ...A..A..G
CMW11227_Ethiopia               .......... .......... .......... .......... ......T... .......... ......GA.. ...A..A..G
CMW11225_Ethiopia               .......... .......... .......... .......... ......T... .......... ......GA.. ...A..A..G
Mycosphaerella_molleriana       .......... .......... .......... .......T.. .......... ..T....... .T..G..... ATGC...C.G
Mycosphaerella_nubilosa         .......... .......... .C........ ....A..T.. .........T G......... GA..G..A.. ACGCGAAAGA
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                                650        660        670        680        690        700        710        720
CMW11220_South_Africa           CGAAGGAGAA GAGGATACTG ACGCGAGGCA GGTACAATGG CACGTCTGAC CTCCAGCTCG AGCGCATGAA CGTGTACTTC
CMW11221_South_Africa           ....T..... .......... .......... .......... .......... .......... .......... ..........
CMW11230_Mexico                 ...G...... .......... .......... .......... .......... .......... .......... ..........
CMW11231_Mexico                 ...G...... .......... .......... .......... .......... .......... .......... ..........
CMW5232_Thailand                .......... .......... .......... .......... .......... .......... .......... ..........
CMW5235_Thailand                ....T..... .......... .......... .......... .......... .......... .......... ..........
CMW11228_Uganda                 ..G.A...G. .......... .......... .......... .......... .......... .......... ..........
CMW11226_Ethiopia               ..G.A...G. .......... .......... .......... .......... .......... .......... ..........
CMW11227_Ethiopia               ..G.A...G. .......... .......... .......... .......... .......... .......... ..........
CMW11225_Ethiopia               ..G.A...G. .......... .......... .......... .......... .......... .......... ..........
Mycosphaerella_molleriana       T.G....... .C...C.... .......A.. .......... .........T .......... .......... ..........
Mycosphaerella_nubilosa         GCCT.AG... CGC..C.... .TAT.GT... .......... .......... .......... .......... ...C......

                                730        740        750        760        770        780        790        800
CMW11220_South_Africa           AACGAGGTAT GGCCTGAGGC AGCAACTATC -TCCAATCCA CACAC----- --TAACGCGA TACGCAGGCA TCCGGCAACA
CMW11221_South_Africa           .......... .....T.... .......... C.T...-... .....----- --......... .......... ..........
CMW11230_Mexico                 .......... .....T.... .......... C.T...-... .....----- --......... .......... ..........
CMW11231_Mexico                 .......... .....T.... .......... C.T...-... .....----- --......... .......... ..........
CMW5232_Thailand                .......... .......... .......... -......... .....----- --......... .......... ..........
CMW5235_Thailand                .......... .....T.... .......... G.T..C-... .....----- --......... .......... ..........
CMW11228_Uganda                 .......... .....T.... .......... C.T...-... .....----- --...T.... C......... ..........
CMW11226_Ethiopia               .......... .....T.... .......... C.T...-... .....----- --...T.... C......... ..........
CMW11227_Ethiopia               .......... .....T.... .......... C.T...-... .....----- --...T.... C......... ..........
CMW11225_Ethiopia               .......... .....T.... .......... C.T...-... .....----- --...T.... C......... ..........
Mycosphaerella_molleriana       .......... .....C.... ...C.T.... CCT.T.-.A. ..ACA---CC AC.G..CGC. A..AT..... ..T.......
Mycosphaerella_nubilosa         ........GC .A.ACCGCT. TTTCCA.... AGG.T.-TGG ..GTGAGGAT AC.G..T..C A..A.....G ..........
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                                 810        820        830        840        850        860        870        880
CMW11220_South_Africa        AGTATGTCCC GCGTGCCGTC CTCGTCGACT TGGAGCCGGG CACCATGGAC GCTGTCCGCG CTGGTCCGTT CGGTCAGCTC
CMW11221_South_Africa        .......... .......... ..G....... .......... .......... .......... .C........ ..........
CMW11230_Mexico              .......... .......... ..G....... .......... .......... .......... .C........ ..........
CMW11231_Mexico              .......... .......... ..G....... .......... .......... .......... .C........ ..........
CMW5232_Thailand             .......... .......... .......... .......... .......... .......... .......... ..........
CMW5235_Thailand             .......... .......... ..G....... .......... ...T...... ......-... .C........ ..........
CMW11228_Uganda              .......... .......... .......... .......... .......... .......... .C.....C.. ..........
CMW11226_Ethiopia            .......... .......... .......... .......... .......... .......... .C.....C.. ..........
CMW11227_Ethiopia            .......... .......... .......... .......... .......... .......... .C.....C.. ..........
CMW11225_Ethiopia            .......... .......... .......... .......... .......... .......... .C.....C.. ..........
Mycosphaerella_molleriana    .......... .......... .......... .......... T......... ..C....... .......T.. ..........
Mycosphaerella_nubilosa      .......... A......... ........T. .......A.. T......... ..C.....T. .-..C..A.. ...A......

                                 890        900        910        920        930        940        950      958
CMW11220_South_Africa        TTCCGCCCGG ACAACTTCGT CTTCGGTCAG TCGGGTGCTG GCAACAACTG GGCCAAGGGT CACTAC-ACT GAGGGTA
CMW11221_South_Africa        .......... .......... .......... .......... .......... .......... ......-... .......
CMW11230_Mexico              .......... .......... .......... .......... .......... .......... ......-... .......
CMW11231_Mexico              .......... .......... .......... .......... .......... .......... ..-...-... .......
CMW5232_Thailand             .......... .......... .......... .......... .......... .......... ......C... .......
CMW5235_Thailand             ...-...... .......... .......... .......... .......... .......... ......-... .......
CMW11228_Uganda              .......... .......... ......C... .......... .......... .......... ......-... .......
CMW11226_Ethiopia            .......... .......... ......C... .......... .......... .......... .-....C... .......
CMW11227_Ethiopia            .......... .......... ......C... .......... .......... .......... ......-... .......
CMW11225_Ethiopia            .......... .......... ......C... .......... .......... .......... ......-... .......
Mycosphaerella_molleriana    .......... .......... ......C... ..C....... .......... .......... .....NNNNN NNNNNNN
Mycosphaerella_nubilosa      ........A. .T........ ......C... .......... .A........ .......... .-...AC... .......
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