Chapter 2

Ceratocystis species on *Acacia mearnsii* and *Eucalyptus* spp. in eastern and southern Africa including six new species

(Heath *et al.* 2009, Fungal Diversity 34: 41-68)

ABSTRACT

Species of *Ceratocystis* include well-known plant pathogens causing cankers, vascular wilt and root diseases, as well as many species that are agents of sap stain of lumber. A number of *Ceratocystis* spp. have been reported from Africa, but the continent is generally poorly sampled in terms of these fungi. The aim of this study was to consider the presence of *Ceratocystis* spp. infecting wounds on plantation-grown, non-native, *Acacia mearnsii* and *Eucalyptus* spp. in Kenya, Malawi, Tanzania and South Africa. Isolates were collected from cut stumps and artificially induced wounds on the stems of trees. They were subsequently identified based on morphological characteristics and DNA sequence comparisons for the ribosomal RNA Internal Transcribed Spacer region, including the 5.8S operon as well as partial sequences of the β-tubulin and the Transcription Elongation Factor 1α genes. Analyses showed that isolates represent six previously undescribed species from Malawi, South Africa and Tanzania, while *C. moniliformis* was found for the first time in Tanzania. The undescribed *Ceratocystis* spp. are provided with the names *C. zombamontana prov. nom., C. polyconidia prov. nom., C. tanganyicensis prov. nom., C. obpyriformis prov. nom., C. oblonga prov. nom.* and *T. ceramica prov. nom.* The wilt pathogen *C. albifundus* was also commonly found on *A. mearnsii* in Tanzania and in Kenya. All the new species described in this study were pathogenic on the hosts from which they were originally isolated.

2.1. INTRODUCTION

The genus *Ceratocystis* includes some of the best-known plant pathogens in the world, responsible for a wide range of disease symptoms including stem cankers, vascular wilts and root diseases (Kile 1993). Most of these important pathogens are related to *C. fimbriata* Ell. & Halst. *sensu lato* (*s.l.*). Some have recently been provided with new names while others are recognized as unique based on phylogenetic inference (Wingfield *et al*. 1996, Barnes *et al*. 2003, Engelbrecht & Harrington 2005, Johnson *et al*. 2005, Van Wyk *et al*. 2007, Rodas *et al*. 2008). Well known tree diseases caused by *Ceratocystis* spp. include oak wilt caused by *C. fagacearum* (Bretz) Hunt (Bretz 1952, Sinclair *et al*. 1987), canker stain disease of plane trees (*Platanus* spp.) caused by *C. platani* Engelbrecht et Harrington (Engelbrecht & Harrington 2005) and wattle wilt of *Acacia mearnsii* De Wild. caused by *C. albifundus* M. J. Wingf., De Beer and M. J. Morris (Morris *et al*. 1993, Wingfield *et al*. 1996). Many species, particularly those in the *C. coerulescens* (Münch) Bakshi species complex are agents of sap stain of lumber (Münch 1907) and various species, especially in the *C. moniliformis* Hedgcock *s.l.* species complex, appear to be saprophytes (Davidson 1935, Van Wyk *et al.* 2006b).

Ceratocystis spp. residing in the *C. fimbriata s.l.* species complex require wounds for infection (DeVay *et al*. 1963, Kile 1993). These wounds can emerge from wind and hail damage, growth cracks, insect and other animal damage as well as human activities such as grafting and pruning. Insects carry the *Ceratocystis* spp., which are ecologically adapted to this mode of dissemination, to the wounds, where infection can take place (DeVay *et al*. 1963, Kile 1993). Stumps remaining from freshly harvested trees are also commonly infected by species of *Ceratocystis* (Roux *et al*. 2004). Recent studies have shown that artificially induced wounds are also commonly infected by *Ceratocystis* spp. and provide an opportunity to trap species infecting wounds from the environment (Barnes *et al*. 2003, Roux *et al*. 2004, Rodas *et al*. 2008).

Relatively little is known regarding *Ceratocystis* spp. occurring in Africa (Roux *et al.* 2005). During the course of the last two decades, there have been numerous studies

investigating these fungi on trees in the region. These have largely emerged from the discovery of a serious wilt disease of *Acacia* spp. now known to be caused by *C. albifundus* (Morris *et al*. 1993, Wingfield *et al*. 1996, Roux *et al*. 2001a,b, 2005). More recently, *C. fimbriata s.l.* was reported to result in rapid wilting and death of *Eucalyptus* spp. in the Republic of Congo (Roux *et al*. 2000) and Uganda (Roux *et al*. 2001a). This fungus has also been isolated from wounds on *Eucalyptus* trees in South Africa (Roux *et al.* 2004) but although it was pathogenic in inoculation tests, it has not been associated with disease under natural conditions. Most recently, two other species of *Ceratocystis*, *C. pirilliformis* Barnes and M. J. Wingf. and *C. moniliformis* have been recorded from *Eucalyptus* spp. in South Africa (Roux *et al*. 2004).

Population growth and globalisation is placing increasing pressure on native forests in Africa. For this reason, extensive forestry programmes, largely based on non-native species have been established. Diseases have already emerged as presenting serious constraints to the long term sustainability of forest plantations in Africa (Gibson 1964, Roux *et al*. 2005) and this is likely to be an increasing trend in the future. Concern regarding diseases has prompted surveys for important groups of tree pathogens including species of *Ceratocystis*. The aim of this study was thus to expand current knowledge relating to *Ceratocystis* spp. in Africa, particularly those occurring on wounds on non-native *A. mearnsii* and *Eucalyptus* spp. in eastern and southern Africa.

2.2. MATERIALS AND METHODS

2.2.1. Collection of isolates

Isolates were collected from *A. mearnsii* and *Eucalyptus* spp. at four localities (Piet Retief, Tzaneen, Pietermaritzburg and Lothair) in South Africa and one each in Malawi (Zomba Mountain), Tanzania (Njombe) and Kenya (Thika). Collections were made from the stumps of freshly felled *Eucalyptus* spp. and *A. mearnsii* as well as from artificially induced wounds on the stems of *Eucalyptus* trees. In the case of the stumps, samples were collected between four days and four weeks after felling, by removing pieces of wood displaying stained vascular tissue and/or the presence of fungal growth.

Stem wounds were made on *Eucalyptus* trees using the technique previously described by Barnes *et al*. (2003). Twenty trees were selected randomly at each study site and wounds were made on the stems, approximately 1.5 meters from the ground. Approximately 100 cm^2 of bark was removed from the stems to expose the cambium. A horizontal slit was made into the xylem of the wound, approximately five mm deep. Samples were collected after six weeks by removing a piece of wood and bark from the top and bottom corners of the wound site and transferred to the laboratory in brown paper bags for further study.

Wood sections were examined for the presence of fruiting structures of *Ceratocystis* spp. In addition, wood pieces displaying vascular discoloration were baited for *Ceratocystis* spp. by placing these between two carrot slices (five mm thick) and incubating them at 25°C for 7-10 days (Moller & DeVay 1968). Pieces of wood were also incubated in containers with moist tissue paper at 25°C for seven days to induce the formation of fruiting structures.

Once ascomata of *Ceratocystis* spp. were found, spore masses were lifted from their apices and transferred to 2% (w/v) malt extract agar (MEA) (Biolab, Midrand, South Africa) supplemented with streptomycin sulphate (0.001 g vol^{-1}, SIGMA, Steinheim, Germany). Plates were then incubated at approximately 25ºC under natural day/night conditions. Isolates were purified on 2% MEA and are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative isolates were also deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. In order to prepare herbarium specimens, cultures bearing fruiting structures were dried on 30% glycerol and deposited with the National Fungal Herbarium of South Africa (PREM), Pretoria.

2.2.2. Morphology and growth in culture

All isolates collected in this study were grouped based on their culture morphology on 2% MEA after five days and were then studied microscopically for further

differentiation. Representative isolates of each group were selected for further identification using DNA sequence comparisons.

For identification based on morphology, pure cultures were maintained on 2% MEA until fruiting structures formed. Fungal structures were mounted on glass slides in lactic acid and examined under a Zeiss Axioskop microscope. Images were captured using a HRc Axiocam digital camera and Axiovision 3.1 software (Carl Zeiss Ltd., Germany). Fifty measurements were made for each taxonomically relevant structure and averages and standard deviations (st. dev) were determined for each of these structures. Measurements are presented in this study as (minimum -) mean minus st. dev. - mean plus st. dev. (- maximum). Colours of cultures were defined based on the mycological colour charts of Rayner (1970).

Two test isolates of each species were selected to study growth in culture. These included one chosen to represent the holotype specimen and one of the paratypes. Growth rates of known species were not determined. Studies of growth in culture were performed by placing an agar disk (four mm diameter) overgrown with mycelium (mycelial side down) of selected five-day old isolates at the centres of 90mm Petri dishes containing 2% MEA. Petri dishes were incubated in the dark at temperatures ranging from 5°C to 35°C at 5°C intervals. Colony diameters were measured after seven days. Two measurements, perpendicular to each other, were made for each culture. Five replicates of each test strain were used at each temperature and averages of the ten measurements taken for each isolate were computed. The entire experiment was repeated once.

2.2.3. DNA isolation, PCR reactions and sequence analyses

DNA of representative *Ceratocystis* isolates (Table 1) was extracted using the method described by Van Wyk *et al*. (2006a). Three gene regions were amplified for sequencing and phylogenetic analyses. The ribosomal RNA Internal Transcribed Spacer regions (ITS) 1 and 2, and the 5.8S operon, were amplified using the primers

ITS1 and ITS4 (White *et al.* 1990). Part of the beta-tubulin (*β*-tubulin) gene was amplified with primers Bt1a and Bt1b (Glass & Donaldson 1995) and part of the Transcription Elongation Factor-1α (EF-1α) gene was amplified using the primers EF1F and EF1R (Jacobs *et al*. 2004).

Polymerase chain reaction (PCR) mixtures, for all three gene regions, consisted of 1 x Expand HF Buffer containing 1.5 mM $MgCl_2$ (Roche Diagnostics, Mannheim, Germany, supplied with the enzyme), 200 μM of each dNTP, FastStart *Taq* enzyme (2 U) (Roche Diagnostics, Mannheim, Germany), 200 ηM of the forward and reverse primers, and 2-10 ng DNA. Reactions were adjusted to a total volume of 25 μL with sterile water. The PCR programme was set for 4 min at 95°C for initial denaturation of the DNA. This was followed by 10 cycles consisting of a denaturation step at 95°C for 40s, an annealing step for 40s at 55°C and an elongation step for 45s at 70°C. Subsequently, 30 cycles consisting of 94ºC for 20s, 55ºC for 40s with a 5s extension step after each cycle, and 70ºC for 45s were performed. A final step of 10 min at 72ºC completed the programme. Amplification of the DNA for the three gene regions was confirmed under ultraviolet (UV) illumination using gel electrophoresis with 2% agarose in the presence of ethidium bromide. Amplicons were purified using 6% Sephadex G-50 columns following the manufacturer's instructions (Steinheim, Germany).

PCR amplicons were sequenced in both directions using the ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California, USA), with the same primers as those used for DNA amplification. Sequencing reactions were run on an ABI PRISM™ 3100 Autosequencer (Applied BioSystems, Foster City, California, U.S.A) and sequences were analysed using Sequence Navigator version 1.0.1 (Applied BioSystems, Foster City, California, USA). Sequences were compared with those of closely related *Ceratocystis* and *Thielaviopsis* spp. obtained from GenBank (http://www.ncbi.nlm.nih.gov), resulting in three datasets. The first set comprised three gene regions for isolates representing the *C. fimbriata s.l.* species complex, the second of the ITS gene region of *Thielaviopsis* spp. together with a

small number of *Ceratocystis* spp., and the third set was made up of three gene regions for species in the *C. moniliformis s.l.* species complex. Sequences were aligned using the web interface (http://align.bmr.kyushu-u.ac.jp/mafft/software/) of MAFFT (Katoh *et al.* 2002) and confirmed manually.

Analyses were performed using Phylogenetic Analysis Using Parsimony (PAUP) version 4.0b10* (Swofford 2002). To determine whether the sequences for the multiple gene regions could be combined into single datasets, partition homogeneity tests (Swofford 2002) were conducted. Gaps were treated as a fifth character and trees were obtained via stepwise addition of 1 000 replicates with the Mulpar option in effect. The heuristic search option based on parsimony with stepwise addition was used to obtain the phylograms. Confidence intervals using 1000 bootstrap replicates were calculated. *Ceratocystis virescens* (Davidson) Moreau was designated as the monophyletic sister out-group taxon. All sequences derived from this study were deposited in GenBank (Table 1).

Phylogenetic trees based on Bayesian probabilities using a Markov Chain Monte Carlo (MCMC) algorithm were generated using MrBayes version 3.1.1 (Ronquist & Huelsenbeck 2003). For each gene, a model of nucleotide substitution was determined using Mr Modeltest (Nylander 2004) and these were included for each gene partition in MrBayes. One million random trees were generated using the MCMC procedure with four chains and sampled every 100th generation. To avoid including trees that were sampled before convergence, tree likelihood scores were assessed to determine the number of trees that were formed before the stabilization. Trees outside the point of convergence were discarded by means of the burnin procedure in MrBayes.

2.2.4. Pathogenicity tests

The relative pathogenicity of the *Ceratocystis* spp. isolated from the various hosts was determined in inoculations performed in a greenhouse. An *E. grandis* W. Hill ex Maiden clone and *A. mearnsii* seedlings were maintained under greenhouse conditions

for two weeks to acclimatise them to these conditions, prior to inoculation. The greenhouses were subjected to natural day/night conditions (~13 hours daylight/ ~11 hours darkness) and a temperature of approximately 25ºC was maintained. Tree diameters varied from 10 to 15 mm. The two fastest growing isolates for each of the six *Ceratocystis* spp. (CMW23809, CMW23818, CMW23807, CMW23808, CMW23802, CMW23803, CMW15992, CMW15999, CMW15235, CMW15236, CMW15242, CMW15248) were selected for the inoculation tests. Twenty trees were inoculated with each test strain. In addition, ten trees were inoculated with sterile MEA plugs, to serve as controls.

Wounds were made on the tree stems using a cork borer (5 mm diam) in such a way that a disc of bark was removed to expose the cambium. Mycelial plugs of a similar size were taken from the edges of seven-day-old actively growing cultures and placed in the wounds with the mycelium facing the cambium. Wounds were sealed with laboratory film (Parafilm "M", American National Can™ Chicago, Illinois, USA) to protect the inoculated fungus and the cambium from desiccation. Lesion lengths were measured six weeks after the trees were inoculated. The experiment was repeated once and the data were pooled for analyses. The differences between variables were log10 transformed to obtain a normalized distribution. The data were then subjected to a univariate procedure using SAS (SAS Version 8.2, 2001). To determine whether the inoculated fungi were responsible for the lesion development, re-isolations were made from the lesions and the fungi identified based on morphological characteristics.

2.3. RESULTS

2.3.1. Isolates

Isolations from ascomata and mycelium on wounds or carrot baits yielded cultures that, based on morphology, clearly represented a number of different *Ceratocystis* spp. A total of 136 isolates were obtained in this study. Of these, 106 were isolated from *A. mearnsii*, 23 cultures from *Eucalyptus* spp. and seven isolates were of a *Thielaviopsis* sp. from *Eucalyptus* spp. for which no sexual fruiting bodies were found (Table 1).

Symptoms associated with fungal infection of the wounds and stumps were a discoloration of the vascular tissue in a streaked pattern, developing above and below the wounds. Vascular streaking of the wood associated with the cut stumps spread downwards into the roots.

2.3.2. Morphology and growth in culture

The *Ceratocystis* spp. obtained in this study could be separated into eight distinct groups based on culture morphology. Selected isolates representing these groups (designated Group A to Group H) were chosen for further study and others have been preserved (Table 1). Group A isolates were from *A. mearnsii* in Tanzania (CMW15773, CMW15781), South Africa (CMW23825, CMW23838) and Kenya (CMW 24685, CMW 24686) and produced light-coloured mycelium bearing ascomata with light coloured bases, similar to those of *C. albifundus*. Group B isolates (CMW15235, CMW15236, CMW15251) from *E. grandis* in Malawi, produced light brown to greyish-white colonies (17''''i) and perithecia with black ascomatal necks. Group C isolates (CMW23806, CMW23807, CMW23808) were obtained from *A. mearnsii* from South Africa and had greyish-white colonies (21''k) and perithecia with black ascomatal necks similar to those of *C. pirilliformis.* Group D isolates (CMW15991, CMW15992, CMW15999) were from *A. mearnsii* in Tanzania and produced dark grey to greenish colonies (25''m) similar to those of *C. fimbriata s.l.* This fungus produced abundant chlamydospores not typically found in *C. fimbriata s.s.* Isolates (CMW23809, CMW23818, CMW23819) residing in Group E were from *A. mearnsii* in South Africa and produced dark brown to greenish-brown colonies (19''i) and perithecia with black ascomatal necks and had an extremely strong alkaloid odour. Group F isolates (CMW23802, CMW23803, CMW23804) were from *A. mearnsii* in South Africa and produced white-coloured colonies that turned light brown with age and were similar to those of *C. savannae* Kamgan Nkuekam and Jol. Roux. These isolates, however, produced nodules on the hyphae, a characteristic not found in *C. savannae* (Kamgan *et al*. 2008). Group G isolates were from *Eucalyptus* spp. in Tanzania (CMW22284, CMW22289) and South Africa (CMW17587, CMW17960). These isolates were characterized by hyaline to grey to black mycelium, similar to that found in *C.*

moniliformis. Group H isolates (CMW15245, CMW15246, CMW15248) represented a *Thielaviopsis* sp., from a *Eucalyptus* sp. in Malawi without any production of sexual structures and were characterized by light to dark brown mycelium (21"b).

No growth rate studies were performed for Groups A and G as these represent known species. The optimal growth for isolates (CMW15236, CMW15235) representing Group B was 20-25°C. No growth was observed at 5°C and 35°C. At 20 and 25°C an average of 41mm of growth was observed after 14 days. The optimal growth for Group C (CMW23808, CMW23807) isolates was 25°C and they did not grow at 5°C or at 35°C. At 25°C, an average of 52mm growth was observed after seven days. Group D isolates (CMW15992, CMW15999) grew optimally at 20°C but did not grow at 5°C or at 35°C and reached an average of 27mm of growth after seven days. Group E isolates (CMW23809, CMW23818) grew optimally at 20-25°C with minimal growth at 5°C and 35°C and produced an average growth of 81mm in seven days. Isolates in Group E produced an average growth of 72mm at 20°C and 81mm at 25°C after seven days. Group F isolates (CMW23803, CMW23802) grew optimally at 25°C. Growth was also observed at 5°C and at 35°C. Cultures reached an average of 83mm of growth after seven days. Group H isolates (CMW15245, CMW 15248) produced optimal growth at 25°C reaching an average of 90mm of growth after four days. No growth was observed at 5°C, 10°C or at 35°C.

2.3.3. Phylogenetic analyses

DNA sequencing yielded amplicons of ~500bp for both the ITS and *β*-tubulin gene regions and amplicons of ~750bp for the EF1-α gene region. Partition homogeneity tests showed that data from the three gene regions could be combined for both the *C. fimbriata s.l.* and *C. moniliformis s.l.* datasets. The *C. fimbriata s.l.* data set had a P value of 0.001 and the *C. moniliformis s.l.* data set a P value of 0.16 for the partition homogeneity tests.

One most parsimonious tree was obtained for the combined data set of the *C. fimbriata s.l.* group (Figure 1). This tree had a length of 1597 base pairs. There were 1915 characters, with 1135 of these characters being constant, 37 characters being parsimony-uninformative and 743 characters being parsimony-informative, with Consistency Index (CI) = 0.7276, Retention Index (RI) = 0.9003 and Rescaled Consistency (RC) = 0.6551. In the phylogenetic tree, *C. fimbriata s.s.*, *C. platani* Engelbrecht and Harrington, *C. cacaofunesta* (Walter) Engelbrecht and Harrington, *C. pirilliformis, C. polychroma* M. van Wyk and M.J. Wingf., *C. albifundus*, *C. caryae* J.A. Johnson and Harrington, *C. smalleyi* J.A. Johnson and Harrington, *C. variospora* (Davids.) C. Moreau, *C. populicola* J.A. Johnson and Harrington, *C. tsitsikammensis* Kamgan Nkuekam and Jol. Roux and *C. atrox* M. van Wyk and M.J. Wingf. all represented distinct clades, supported by high bootstrap values. The isolates collected in this study formed four separate and distinct clades. Groups emerging from the phylogenetic analyses were consistent with the groups defined based on their morphology.

The isolates obtained from *A. mearnsii* from Tanzania (Group A, Group D) grouped in two clades. Group D isolates (CMW15991, CMW15992, CMW15999) resided in a distinct clade (Clade 1), close to *C. tsitsikammensis* with strong bootstrap support (100%). Group A isolates (CMW24806, CMW24861) resided in the *C. albifundus* clade (Clade 2), with 100% bootstrap support. Isolates (CMW24685, CMW24686) from *A. mearnsii* from Kenya, treated as Group A (CMW24685, CMW24686), also grouped in the *C. albifundus* clade (Clade 2). Isolates from *A. mearnsii* in South Africa resided in three clades (Group A, Group C, Group E). The first group of isolates, Group A (CMW23825, CMW23838) grouped within the *C. albifundus* clade (Clade 2). The other two groups of isolates (CMW23806, CMW23807, CMW23808) designated Group C (Clade 3) and Group E (CMW23809, CMW23818, CMW23819) (Clade 4) resided in two distinct clades with strong (100%) bootstrap support, close to the *C. pirilliformis* clade. Isolates (CMW15235, CMW15236, CMW15251) obtained from *E. grandis* from Malawi, designated Group B, also formed a distinct clade, separate from any other isolates and with strong (100%) bootstrap support, close to *C. pirilliformis* (Clade 5). Posterior probability values calculated for the branch nodes supported the bootstrap

values for all these clades and suggested that four previously undescribed species in the *C. fimbriata s.l.* group were collected during this study (Figure 1).

Three most parsimonious trees were obtained for the data set including the *Thielaviopsis* spp. (Figure 2). In the phylogenetic tree, *C. bhutanensis* M. van Wyk, M.J. Wingf. and T. Kirisits, *C. omanensis* Al-Subhi, M.J. Wingf, M. van Wyk and Deadman, *T. ovoidea* (Nag Raj et Kend.) Paulin, Harrington et McNew, *T. populi* (Veldeman ex Kiffer et Delan) Paulin, Harrington et McNew, *T. basicola* (Baker et Br.) Ferr., *C. polonica* (Siem.) C. Moreau and *C. resenifera* Harrington and M.J. Wingf. all represented distinct clades, supported by high bootstrap values. *Thielaviopsis* isolates collected from *Eucalyptus* in Tanzania (Group H) formed a separate, well supported clade (Figure 2). This tree had a length of 265 steps, the total number of characters were 468, with 303 of these being constant and 165 parsimony-informative, with CI = 0.8868, RI = 0.9690 and RC = 0.8593. In the phylogenetic tree, the *Thielaviopsis* isolates obtained in this study grouped most closely to *C. bhutanensis* and *C. omanensis*, separate from other known *Thielaviopsis* spp. and with strong bootstrap support (Figure 2). These isolates were then further compared to all *Ceratocystis* spp. in the *C. moniliformis s.l.* group using all three gene regions, confirming their unique nature, as well as their affinity to *C. bhutanensis* and *C. omanensis* (Figure 3).

Two most parsimonious trees were obtained for the combined data set of the *C. moniliformis s.l.* group, of which one is presented (Figure 3). This tree had a length of 578 steps, the total number of characters were 1273, with 844 constant, three characters parsimony-uninformative and 426 parsimony-informative characters, with CI = 0.9135, RI = 0.9595 and RC = 0.8765. In the phylogenetic tree, *C. savannae*, *C. omanensis*, *C. bhutanensis, C. moniliformis*, *C. tribiliformis* M. van Wyk and M.J. Wingf. and *C. moniliformopsis* Z.Q. Yuan and C. Mohammed all resided in distinct clades, supported by high bootstrap values. Clades emerging from the phylogenetic analyses were consistent with those (Group G, Group F, Group H) that emerged based on morphology for the African isolates. Isolates (CMW23802, CMW23803, CMW23804) obtained from *A. mearnsii* from South Africa (Group G) grouped in a distinct clade (Clade 6)

with strong (100%) bootstrap support, closest to *C. savannae*. Isolates in Group F obtained from *Eucalyptus* in Malawi (CMW15242, CMW15245, CMW15248) grouped in a distinct clade (Clade 7) with strong (100%) bootstrap support, most closely related to the isolates obtained from *A. mearnsii* from South Africa (Clade 6) and *C. savannae*. Isolates in Group H obtained from *Eucalyptus* spp. in Tanzania (CMW22284, CMW22289) and South Africa (CMW17587, CMW17960) grouped together within the *C. moniliformis* clade (Clade 8). Posterior probability values calculated for the branch nodes supported the bootstrap values (Figure 3).

Taxonomy

Comparison of DNA sequence data for the African isolates resulted in distinct phylogenetic lineages, consistent with the groups that emerged from morphological comparisons. These thus showed that five previously undescribed *Ceratocystis* spp. and one new *Thielaviopsis* sp. were amongst the isolates collected in this study (Table 2, Table 3). These fungi are consequently described as new taxa.

Ceratocystis zombamontana **R.N. Heath & Jol. Roux. sp. nov.** **(Fig. 4)**

(MB511245)

Etymology: The name refers to the Zomba mountain in Malawi where this fungus was first isolated.

Coloniae badiae. *Colla ascomatum* atrobrunnea vel nigra, apicem versus pallescentia (264-) 315 – 442 (-535) µm longa, apicibus (13-) 156 – 21 (-23) µm latis. *Ascosporae* lateraliter visae cucullatae vel pileiformes (4-) 5 – 6 (-6) X (3-) 3 – 4 (-5) µm, e supra visae doliiformes (5-) 6 – 7 (-8) X (5-) 6 – 7 (-8) µm. *Anamorpha Thielaviopsis* cum conidiophoris phialidicis in mycelio singulis, hyalinis. Conidiophorae primariae ampulliformes. Conidia primaria cylindrica baciliformia; secondaria doliiformia. *Chlamydosporae* absunt.

Colonies hair brown (17''''i), reverse hair brown (17''''i). *Mycelium* mostly superficial and smooth, sparse tawny olive (19''i) aerial mycelium. *Optimal temperature* for growth 20-25°C, minimal growth at 5°C and no growth at 35°C. Slow growing, reaching

41mm in 14 days at 20 and at 25°C. *Ascomatal bases* dark brown to black, subglobose to ovoid, (130-) 152 – 196 (-222) µm long, (106-) 132 – 181 (-208) µm wide. Ornamentation absent. *Ascomatal necks* dark brown to black at base becoming lighter brown to hyaline towards apices, (264-) 315 – 443 (-535) µm long, (24-) 30 – 38 (-42) µm wide at bases of necks, (13-) 16 – 21 (-23) µm wide at tips of necks. *Ostiolar hyphae* divergent, hyaline, (16-) 20 – 25 (-27) µm long. *Asci* not observed. *Ascospores* accumulating in round, white to yellow (yellow-buff 19d) masses at the apices of the ascomatal necks, embedded in sheath tissue, aseptate, hyaline, cucullate (hat-shaped) in side view, (4-) 5 – 6 (-6) µm long, (3-) 3 – 4 (-5) µm wide without sheath, doliiform in top view, (5-) 6 – 7 (-8) µm long, (5-) 6 – 7 (-8) µm wide including sheath.

Thielaviopsis anamorph: *Conidiophores* occurring singly on mycelium, conidiophores phialidic, hyaline, primary conidiophores flask shaped (55-) 69 – 101 (-119) µm long, (4-) 5 – 7 (-8) µm wide at bases, (3-) 3 – 5 (-5) µm wide at tips. Secondary conidiophores flaring, (72-) 74 – 117 (-128) µm long, (4-) 4 – 6 (-7) µm wide at bases, (4-) 4 – 4 (-6) µm wide at tips. Phialidic *conidium* development through ring wall building, *conidia* of two types, formed singly or in chains, primary conidia cylindrical, baciliform, (15-) 16 – 21 (-26) µm long, (3-) 3 – 4 (-6) µm wide, secondary conidia barrel-shaped, (5-) 6 – 7 (-9) µm long, (3-) 3 – 4 (-4) µm wide. *Chlamydospores* absent.
Specimens examined: Malawi, Zomba Mountain (S 15° 21.269 E 035° 18.163). Isolated from wounds on *Eucalyptus grandis*. Collected: R.N. Heath and J. Roux, 2004. **Holotype**, PREM59804, **culture ex-type** CMW15236 = CBS122296.

Additional specimens: Malawi, Zomba Mountain (S 15° 21.269 E 035° 18.163). Isolated from wounds on *Eucalyptus grandis*. Colected: R.N. Heath and J. Roux, 2004. **Paratype**, PREM59805, **culture ex-type** CMW15235 = CBS122297; CMW15251 = CBS122298 = PREM59806; CMW15242 = PREM59807.

***Ceratocystis polyconidia* R.N. Heath & Jol. Roux. sp. nov. (Fig. 5)**

(MB511246)

Etymology: The name refers to the abundance of primary conidia produced.

Coloniae isabellinae. *Colla ascomatum* atrobrunnea vel nigra, apicem versus pallescentia vel hyalina (326-) 589 – 429 (-694) µm longa, apicibus (11-) 13 – 19 (-23) µm latis. *Ascosporae* lateraliter visae cucullatae vel pileiformes (3-) 3.1 – 4.1 (-5) X (3.5-) 4.3 – 5.3 (-5.8) µm, e supra visae doliiformes. *Anamorpha Thielaviopsis* cum conidiophoris phialidicis in mycelio singulis, hyalinis. Conidiophorae primariae ampulliformes. Conidiophorae secondariae expansae. Conidia primaria cylindrica baciliformia; secondaria doliiformia. *Chlamydosporae* terminales, singulae, parietibus crassis, subglobosae, brunneae.

Colonies isabella (19''i), reverse isabella (19''i). *Mycelium* mostly superficial, smooth, segmented, sparse hazel (17''i) aerial mycelium. *Optimal temperature* for growth 20-25°C. Minimal growth at 5°C and 35°C. Fast growing, reaching 81mm in 7 days at 25°C. *Ascomatal bases* dark brown to black, globose, (121-) 153 – 221 (-269) µm long, (133-) 153 – 223 (-277) µm wide. No ornamentation. *Ascomatal necks* dark brown to black at bases becoming lighter brown to hyaline towards apices, (326-) 389 – 429 (-694) µm long, (20-) 26 – 34 (-39) µm wide at bases of necks, (11-) 13 – 19 (-23) µm wide at tips of necks. *Ostiolar hyphae* divergent, hyaline, (36-) 39 – 47 (-57) µm long. *Asci* not observed. *Ascospores* accumulating in round, white to yellow (yellow-buff 19d) masses at the apices of the ascomatal necks, cucullate in side view, (3-) 3 – 4 (-5) µm long, (4-) 4 – 5 (-6) µm wide without sheath and (4-) 4 – 5 (-6) µm wide with sheath, doliiform in top view.

Thielaviopsis anamorph: Primary and secondary *conidiophores* occurring singly on mycelium, conidiophores phialidic, hyaline, primary conidiophores flask shaped, (72-) 74 – 117 (-128) µm long, (4-) 4 – 6 (-7) µm wide at bases, (6-) 6 – 8 (-8) at the widest point, (4-) 4 – 4 (-6) µm wide at tips. Secondary conidiophores flaring at apices, (38-) 52 – 87 (-105) µm long, (4-) 5 – 7 (-7) µm wide at bases, (5-) 6 – 8 (-9) µm wide at tips. Phialidic *conidium* development through ring wall building, *conidia* of two types, formed singly or in chains, primary conidia cylindrical, baciliform, (14-) 18 – 25 (-28) µm long, (4-) 4 – 6 (-6) µm wide, secondary conidia barrel-shaped, (8-) 9 – 11 (-13) µm long, (5-) 6 – 8 (-8) µm wide. *Chlamydospores* terminal, single, thick walled, sub-globose, argus brown (13m), (9-) 9 – 11 (-13) µm long, (8) 11 – 14 (-16) µm wide.

Specimens examined: South Africa, Piet Retief (S 26° 58' 68.5", E 030° 54' 28.3"). Isolated from cut stumps of *Acacia mearnsii*. Collected: R.N. Heath, 2006. **Holotype**, PREM59788, **culture ex-type** CMW23809 = CBS122289.

Additional specimens: South Africa, Piet Retief (S 26° 58' 68.5", E 030° 54' 28.3"). Isolated from cut stumps of *Acacia mearnsii*. Collected: R.N. Heath, 2006. **Paratype**, PREM59789, **culture ex-type** CMW23818 = CBS122290; CMW23819 = CBS122821 = PREM59790; CMW23817 = PREM59791, CMW23810 = PREM59863.

***Ceratocystis tanganyicensis* R.N. Heath & Jol. Roux sp. nov. (Fig. 6)**

(MB511247)

Etymology: The name refers to the famous lake Tanganyika in Tanzania, not far from where this fungus was first isolated.

Coloniae eburneo-atrovirides. *Colla ascomatum* atrobrunnea vel nigra, apicem versus pallescentia vel hyalina (302-) 366 – 484 (-558) µm longa, apicibus (13-) 14 – 18 (-21) µm latis. *Ascosporae* lateraliter visae cucullatae vel pileiformes (3-) 3 –5 (-6) X (4-) 4 – 6 (-8) µm vagina exclusa, e supra visae doliiformes. *Anamorpha Thielaviopsis* cum conidiophoris phialidicis. Conidiophorae primariae ampulliformes. Conidiophorae secondariae expansae. Conidia primaria cylindrica baciliformia; secondaria doliiformia. *Chlamydosporae* in hyphis singulae, globosae.

Colonies dark ivory green (25"m), reverse ivory green (25"m). *Mycelium* mostly superficial and smooth, sparse tawny olive (19"i) aerial mycelium. *Optimal temperature* for growth 20°C. No growth at 5°C or at 35°C. Slow growing, reaching 27mm in 7 days at 20°C. *Ascomatal bases* dark brown to black, subglobose, (127-) 149 – 190 (-216) µm long, (119-) 138 – 177 (-205) µm wide. No ornamentation. *Ascomatal necks* dark brown to black at base becoming lighter brown to hyaline towards apices, (302-) 366 – 484 (-558) µm long, (19-) 24 – 32 (-37) µm wide at bases of necks, (13-) 14 – 18 (-21) µm wide at tips of necks. *Ostiolar hyphae* divergent, hyaline, (17-) 39 – 47 (-47) µm long. *Asci* not observed. *Ascospores* accumulating in round, white to yellow (yellow-buff 19d) masses at the apices of the ascomatal necks, cucullate in side

view, (3-) 3 – 5 (-6) µm long, (4-) 4 – 6 (-8) µm wide without sheath and (5-) 6 – 7 (-8) µm wide with sheath, doliiform in top view.

Thielaviopsis anamorph: Primary and secondary *conidiophores* occurring singly on mycelium, conidiophores phialidic, hyaline, primary conidiophores flask shaped, (49-) 60 – 116 (-179) µm long, (4-) 5 –7 (-8) µm wide at bases, (4-) 6 – 8 (-9) at the widest point, (3-) 4 – 5 (-8) µm wide at tips. Secondary conidiophores flaring at the apices, (43-) 55 – 85 (-98) µm long, (4-) 5 – 7 (-8) µm wide at bases, (5-) 6 – 8 (-9) µm wide at tips. Phialidic *conidium* development through ring wall building, *conidia* of two types, formed singly or in chains, primary conidia cylindrical, bacilliform, (12-) 14 – 19 (-24) µm long, (3-) 4 – 5 (-5) µm wide, secondary conidia barrel-shaped, (3-) 3 – 9 (-13) µm long, (6-) 7 –10 (-12) µm wide. *Chlamydospores* developing singly on hyphae, argus brown (13m), globose, (10-) 10 – 13 (-14) µm long, (3-) 10 – 12 (-13) µm wide.

Specimens examined: Tanzania, Njombe area (S 09° 16.366 E 034° 38.765). Isolated from cut-stumps of *Acacia mearnsii*. Collected R.N. Heath and J. Roux, 2004. **Holotype**, PREM59800, **culture ex-type** CMW15992 = CBS122293.

Additional specimens: Tanzania, Njombe area (S 09° 16.366 E 034° 38.765). Isolated from cut-stumps of *Acacia mearnsii*. Collected R.N. Heath and J. Roux, 2004. **Paratype**, PREM59801, **culture ex-type** CMW15999 = CBS122294; CMW15991 = CBS122295 = PREM59802; CMW15993 = PREM59803, CMW15988 = PREM59864.

***Ceratocystis obpyriformis* R.N. Heath & Jol. Roux sp. nov. (Fig. 7)**

(MB511248)

Etymology: The name refers to the distinctly obpyriform shape of the ascomatal bases.

Coloniae olivaceae. *Colla ascomatum* atrobrunnea vel nigra, apicem versus pallescentia (477-) 569 – 675 (-708) µm longa, apicibus (13-) 16 – 21 (-26) µm latis. *Ascosporae* lateraliter visae cucullatae vel pileiformes (3-) 3 – 4 (-6) µm longa, apicibus (3-) 4 – 5 (-7) µm latis vagina exclusa, e supra visae doliiformes. *Anamorpha Thielaviopsis* cum conidiophoris phialidicis in mycelio singulis, hyalinis. Conidiophorae primariae ampulliformes. Conidiophorae secondariae expansae. Conidia primaria cylindrica baciliformia; secondaria doliiformia. *Chlamydosporae* absunt.

Colonies olivaceous (21"k), reverse olivaceous (21"k). *Mycelium* mostly superficial and smooth, sparse white aerial mycelium. *Optimal temperature* for growth at 25°C. No growth at 5°C or at 35°C. Fast growing, reaching 52mm in 7 days at 25°C. *Ascomatal bases* dark brown to black, obpyriform, (152-) 177 - 217 (-233) µm long, (149-) 166 – 206 (-228) µm wide. No ornamentations. *Ascomatal necks* dark brown to black at base becoming lighter brown to hyaline towards apices, (477-) 569 – 675 (-708) µm long , (26-) 28 – 36 (-45) µm wide at bases of necks, (13-) 16 – 21 (-26) µm wide at tips of necks. *Ostiolar hyphae* divergent, hyaline, (34-) 37 – 47 (-54) µm long. *Asci* not observed. *Ascospores* accumulating in round, white to yellow (yellow-buff 19d) masses at the apices of the ascomatal necks, embedded in sheath, aseptate, hyaline, cucullate in side view, (3-) 3 – 4 (-6) µm long, (3-) 4 – 5 (-7) µm wide without sheath and (4-) 5 – 8 (-8) µm wide with sheath, doliiform in top view.

Thielaviopsis anamorph: Primary and secondary *conidiophores* occurring singly on mycelium, conidiophores phialidic, hyaline, primary conidiophores flask shaped, (57-) 75 – 124 (-157) µm long, (4-) 5 – 6 (-7) µm wide at bases, (3-) 6 – 8 (-8) µm at the widest point, (3-) 4 – 5 (-5) µm wide at tips. Secondary conidiophores flaring at apices, (54-) 56 – 74 (-83) µm long, (4-) 4 – 6 (-7) µm wide at bases, (5-) 6 –8 (-9) µm wide at tips. Phialidic *conidium* development through ring wall building, *conidia* of two types, formed singly or in chains, primary conidia cylindrical, bacilliform, (14-) 15 – 20 (-24) µm long, (3-) 3 – 5 (-6) µm wide, secondary conidia barrel-shaped, (8-) 10 – 12 (-13) µm long, (5-) 6 – 8 (-9) µm wide. *Chlamydospores* absent.

Specimens examined: South Africa, Piet Retief (S 26° 58' 68.5", E 030° 54' 28.3"). Isolated from cut stumps of *Acacia mearnsii*. Collected: R.N. Heath, 2006. **Holotype**, PREM59796, **culture ex-type** CMW23808 = CBS122511.

Additional specimens: South Africa, Piet Retief (S 26° 58' 68.5", E 030° 54' 28.3"). Isolated from cut stumps of *Acacia mearnsii*. Collected: R.N. Heath, 2006. **Paratype**, PREM59797, **culture ex-type** CMW23807 = CBS122608; CMW23806 = CBS12609 = PREM59798; CMW27862 = PREM59799.

***Ceratocystis oblonga* R.N. Heath & Jol. Roux sp. nov.** **(Fig. 8)**

(MB511249)

Etymology: The name refers to the oblong shape of the secondary conidia.

Coloniae iuvenes albae, fuscantes. *Colla ascomatum* atrobrunnea vel nigra, apicem versus pallescentia (405-) 5025 – 721 (-881) µm longa, apicibus (12-) 13 – 18 (-23) µm latis. *Ascosporae* lateraliter visae cucullatae vel pileiformes (3-) 3 – 4 (-4) X (4-) 5 – 6 (-6) µm vagina exclusa, e supra visae doliiformes. *Anamorpha Thielaviopsis* cum conidiophoris phialidicis in mycelio singulis, hyalinis. Conidiophorae secondariae expansae. Conidia primaria cylindrica baciliformia; secondaria oblonga, apicibus truncatis. *Chlamydosporae* absunt.

Colonies white when young, becoming deep colonial buff (21''b), reverse grayish sepia (15''''i). *Mycelium* superficial producing aerial mycelia. *Hyphae* granular. *Optimal temperature* for growth at 20-25°C. Minimal growth at 5°C and 35°C. Fast growing, reaching 83mm in 7 days at 25°C. *Ascomatal bases* dark brown to black, obpyriform, (149-) 206 – 329 (-372) µm long, (130-) 180 – 254 (-315) µm wide with conical spines, (8-) 11 – 16 (-19) µm long. *Ascomatal necks* dark brown to black at base becoming lighter brown to hyaline towards apices, (405-) 502 – 721 (-881) µm long, (30-) 46 – 69 (-76) µm wide at bases of necks, (12-) 13 – 18 (-23) µm wide at tips of necks with disciform bases. *Ostiolar hyphae* divergent, hyaline, (22-) 22 – 27 (-31) µm long. *Asci* not observed. *Ascospores* accumulating in round, white to yellow (yellow-buff 19d) masses at the apices of the ascomatal necks, embedded in sheath, aseptate, hyaline, cucullate (hat-shaped) in side view, (3-) 3 – 4 (-4) µm long, (4-) 5 – 6 (-6) µm wide without sheath and (6-) 7 – 8 (-8) µm with sheath, doliiform in top view.

Thielaviopsis anamorph: Primary and secondary *conidiophores* occurring singly on mycelium, conidiophores phialidic, hyaline, primary conidiophores flask shaped, (19-) 21 – 35 (-41) µm long, (2-) 3 – 4 (-4) µm wide at bases, (2-) 3 – 4 (-5) µm at the widest point, (2-) 2 – 3 (-3) µm wide at tips. Secondary conidiophores flaring at apices, (23-) 29 – 50 (-59) µm long, (3-) 3 – 4 (-5) µm wide at bases, (4-) 4 – 6 (-6) µm wide at tips. Phialidic *conidium* development through ring wall building, *conidia* of two types, formed singly or in chains, primary conidia cylindrical, bacilliform, (12-) 14 – 19 (-23) µm long, (3-) 3 – 5 (-5) µm wide, secondary conidia oblong, apices truncate, (5-) 6 – 7 (-9) µm long, (3-) 4 – 5 (-6) µm wide. *Chlamydospores* absent.

Specimens examined: South Africa, Piet Retief (S 26° 58' 68.5", E 030° 54' 28.3"). Isolated from cut stump of *Acacia mearnsii*. Collected: R.N. Heath, 2006. **Holotype**, PREM59792, culture **ex-type** CMW23803 = CBS122291.

Additional specimens: South Africa, Piet Retief (S 26° 58' 68.5", E 030° 54' 28.3"). Isolated from cut stump of *Acacia mearnsii*. Collected: R.N. Heath, 2006. **Paratype**, PREM59793, **culture ex-type** CMW23802 = CBS122820; CMW23804 = CBS122292 = PREM59794; CMW23805 = PREM59795.

Thielaviopsis ceramica **R.N. Heath & Jol. Roux. sp. nov. (Fig. 9)**

(MB511250)

Etymology: The name originates from the historic name of the geographical region (Zomba, Malawi) where the fungus was found. Historically, the name Zomba originated from the fact that early settlers used the area to produce clay pots (called Zoomba omba), where after the region was called Zomba. "*ceramica*" means clay in Greek.

Coloniae iuvenes albae, cum aetate atrobubalinae. Mycelium granulare. Conidiophorae phialidicae in mycelio singulae, hyalinae. *Conidiophorae* primariae ampulliformes (20-) 22 – 30 (-36) µm longae, basi (2-) 3 – 4 (-5) µm, apicibus (2-) 2 – 2 (-3) µm latae. *Conidiophorae* secondariae expansae (23-) 33 – 46 (-53) µm longae, basi (1-) 2 –3 (-3) µm, apicibus (2-) 2 – 3 (-3) µm. *Conidia* primaria cylindrica baciliformia; secondaria doliiformia. *Chlamydosporae* absunt.

Colonies white when young, becoming deep colonial buff (21''b). *Mycelium* superficial producing aerial mycelia, granular. *Optimal temperature* for growth at 25°C. No growth at 5, 10 or at 35°C. Fast growing, reaching 90mm in four days at 25°C. *Conidiophores* occurring singly on mycelium, conidiophores phialidic, hyaline, primary conidiophores flask shaped, (20-) 22 – 30 (-36) µm long, (2-) 3 – 4 (-5) µm wide at bases, (3-) 3 – 4 (-5) µm wide at middle, (2-) 2 – 2 (-3) µm wide at tips. Secondary conidiophores flaring at apices, (25-) 35 – 46 (-53) µm long, (1-) 2 – 3 (-3) µm wide at bases, (2-) 2 – 3 (-3) µm wide at tips. Phialidic *conidium* development through ring wall building, *conidia* of two types, formed singly or in chains, primary conidia cylindrical,

bacilliform, (4-) 6 – 8 (-10) µm long, (1-) 2 (-3) µm wide, secondary conidia barrel-shaped, (2-) 2 – 3 (-4) µm long, (3-) 4 – 6 (-8) µm wide. *Chlamydospores* absent.
Specimens examined: Malawi, Zomba Mountain (S 15° 21.269 E 035° 18.163). Isolated from wounds on *Eucalyptus grandis*. Collected: R.N. Heath and J. Roux, 2004. **Holotpe**, PREM59808, **culture ex-type** CMW15245 = CBS122299, CMW15251

Additional specimens: Malawi, Zomba (S 15° 21.269 E 035° 18.163). Isolated from wounds on *Eucalyptus grandis*. Colected: R.N. Heath and J. Roux, 2004. **Paratype**: PREM59809, **culture ex-type** CMW15248 = CBS122300; CMW15246 = CBS122624 = PREM59810; CMW15238 = PREM59811, CMW15249 = PREM59865.

2.3.4. Pathogenicity tests

Greenhouse inoculations on *A. mearnsii* trees with *Ceratocystis* isolates collected in this study resulted in distinct lesions, whereas the control inoculations produced no lesions (Figure 10). Lesions associated with all isolates differed significantly from the controls ($P<0.0001$). Although *C. polyconidia* (CMW23809, CMW23818) produced the longest lesions, and *C. obpyriformis* (CMW23807, CMW23808) produced the shortest lesions, there were no statistical differences between the lesion lengths produced by the test isolates. After six weeks, the wounds of the control inoculations had begun to recover and to produce callus tissue. All test organisms were consistently re-isolated from the lesions after six weeks. Both replicates of the experiments produced similar results.

Greenhouse inoculations on the *E. grandis* (ZG14) trees with all *Ceratocystis* and *Thielaviopsis* isolates resulted in distinct lesions, whereas the control inoculations produced no lesions (Figure 11). *Ceratocystis zombamontana* (CMW15235, CMW15236) produced significantly larger lesions than *T. ceramica* (CMW15248, CMW 15245) or the control ($P<0.0001$). However, *T. ceramica* also produced significantly larger lesions than the control inoculations ($P<0.0001$). Both the test organisms were consistently isolated from the lesions. Both replicates of the experiments produced the same results.

2.4. DISCUSSION

This study focused on identifying *Ceratocystis* spp. from two non-native plantation tree species in southern and eastern Africa, led to the discovery of six previously undescribed *Ceratocystis* spp. In addition, *C. albifundus* and *C. moniliformis s.s.* were commonly encountered. Identification of the species arose from a combination of morphological characteristics and comparisons of DNA sequence data, the latter of which were important in recognizing new species for fungi that are morphologically similar.

Two of the previously undescribed species encountered in this study are related to *C. moniliformis* and reside in a group that we refer to as the *C. moniliformis s.l.* species complex. Fungi in the *C. moniliformis s.l.* group can easily be distinguished from other *Ceratocystis* spp. based on the presence of conical spines on their ascomatal bases (Hedgcock 1906, Hunt 1956, Upadhyay 1981). This group also produces disc-like structures at the bases of the ascomatal necks (Bakshi 1951, Hunt 1956). The two species described in this study have been provided with the names *C. oblonga* and *T. ceramica*.

Ceratocystis oblonga grouped close to *C. savannae* within the *C. moniliformis s.l.* species complex, but in a discrete clade with strong bootstrap support. It can also be distinguished from *C. savannae* based on colony colour. In this regard, *C. oblonga* produces white colonies when young, turning colonial buff with age, whereas *C. savannae* produces smokey gray cultures (Kamgan *et al*. 2008). *Ceratocystis oblonga* also has hyphae that have a granular appearance, whereas *C. savannae* has smooth-walled hyphae. Furthermore, *C. savannae* does not produce secondary phialides, but these structures are common in *C. oblonga*. *Ceratocystis oblonga* produces significantly smaller primary and secondary conidiophores than those of *C. savannae* and it produces secondary conidia that are oblong with truncate apices, which separates it from all other *Ceratocystis* spp. Another clear distinction between *C. oblonga* and *C. savannae* is found in the shapes of their ascomatal bases. *Ceratocystis oblonga*

produces obpyriform ascomatal bases in contrast to the globose ascomatal bases produced by *C. savannae*. *Ceratocystis oblonga* also differs from *C. savannae* and most other species in the *C. moniliformis s.l.* group, other than *C. bhutanensis* (Van Wyk *et al*. 2004, Kamgan *et al*. 2008), in the fact that it is able to grow at 5°C, producing colonies of up to 5mm diameter after four days of growth. Together with *C. bhutanensis*, it is one of two species in this group adapted to growth at low temperatures.

Thielaviopsis ceramica is phylogenetically most closely related to *C. bhutanensis*. These species are, however, fungi with vastly different ecologies with one occurring on wounds on *Eucalyptus* spp. in Africa and the other associated with conifer infesting bark beetles in Bhutan (Van Wyk *et al*. 2004). The two species can further be distinguished based on various morphological characteristics. *Thielaviopsis ceramica* produces colonial buff-coloured colonies whereas those of *C. bhutanensis* are cream-buff to dark olive to black in colour. *Thielaviopsis ceramica* also produces slightly longer (4-6 µm) barrel shaped conidia than that of *C. bhutanensis* (3-5 µm). As no teleomorph structures could be induced for *T. ceramica* no other morphological comparisons were possible.

Ceratocystis moniliformis was isolated from *Eucalyptus* stumps in South Africa and Tanzania. This fungus has previously been reported from artificially induced wounds on *Eucalyptus* spp. in South Africa (Roux *et al.* 2004) but this is the first confirmed report of its occurrence elsewhere in Africa. Isolation of *C. moniliformis* was not surprising as it is a fungus with a broad global distribution and host range (Davidson 1935, Bakshi 1951, Hunt 1956, Van Wyk *et al*. 2006b). It is not known to be a pathogen of *Eucalyptus* spp. or other tree species.

Five of the species collected in this study reside in the *C. fimbriata s.l.* species complex. Of these, four were of previously undescribed species which we have provided with the names *C. tanganyicensis*, *C. zombamontana*, *C. polyconidia* and *C. obpyriformis*. Fungi in the *C. fimbriata s.l.* species complex are generally known as virulent pathogens and they include species such as *C. fimbriata s.s.*, *C. platani, C. cacaofunesta* and *C.*

albifundus. Although species in this group are aggressive pathogens, they are not as fast growing as species in the *C. moniliformis s.l.* species complex and in this respect, they can generally be distinguished from the later group based on culture morphology.

Ceratocystis zombamontana is most closely related to *C. pirilliformis* within the *C. fimbriata s.l.* species complex, but it resides in a distinct phylogenetic clade. This species produces hair brown cultures in contrast to the pale olivaceous grey cultures found in *C. pirilliformis*. *Ceratocystis zombamontana* has shorter ascomatal necks that are wider at the tip compared to that of *C. pirilliformis* and it has significantly larger ascospores than those of the former species. Furthermore, *C. zombamontana* produces flask-shaped primary phialides compared to the cylindrical to lageniform primary phialides of *C. pirilliformis*. Another distinct morphological difference between these two phylogeneticaly closely related species is that *C. pirilliformis* produces clamydospores (Barnes *et al*. 2003), whereas these structures have not been found in *C. zombamontana*.

Ceratocystis tanganyicensis, isolated from *A. mearnsii,* resides in a large clade, with *C. tsitsikammensis,* but forming a distinct clade with strong bootstrap support. Although this species is phylogenetically closest to *C. tsitsikammensis*, it can clearly be distinguished from that species based on culture morphology. In this regard, *C. tanganyicensis* produces ivory green cultures, whereas those of *C. tsitsikammensis* are a greenish olivaceous colour. *Ceratocystis tanganyicensis* also grows optimally at 20°C while *C. tsitsikammensis* grows optimally at 25°C. *Ceratocystis tanganyicensis* can be distinguished from *C. tsitsikammensis* based on the sub-globose ascomatal bases in *C. tanganyicensis* compared to the globose ascomatal bases of *C. tsitsikammensis*. Furthermore, *C. tanganyicensis* produces longer (39-47µm) ostiolar hyphae than *C. tsitsikammensis* (23-38 µm) (Kamgan *et al*. 2008).

Ceratocystis polyconidia and *C. obpyriformis*, from *A. mearnsii* in South Africa reside in distinct clades, however, both grouped closely with *C. pirilliformis* and *C. tanganyicensis* in phylogenetic analyses. *Ceratocystis polyconidia* could be

distinguished from *C. pirilliformis* based on culture morphology as it produces dark ivory grey cultures compared to those of *C. pirilliformis* which are pale olivaceous grey. *Ceratocystis polyconidia* can be distinguished from *C. obpyriformis* and *C. pirilliformis* by its globose ascomatal bases, compared to the pyriform ascomatal bases in the latter species and the obpyriform ascomatal bases in *C. obpyriformis*. *Ceratocystis obpyriformis* can further be distinguished from *C. pirilliformis* and *C. polyconidia* as these species produce chlamydospores (Barnes *et al*. 2003) whereas these structures are absent in *C. obpyriformis*.

Ceratocystis albifundus was isolated from *A. mearnsii* stumps in three of the countries in which collections were made. Identification of this species can easily be achieved using morphology, as it is the only species in the group with light coloured ascomatal bases. Isolation of *C. albifundus* from *A. mearnsii* from Tanzania, Kenya and South Africa was expected as this species has previously been reported from these countries causing wilting and death of trees (Roux & Wingfield 1997, Roux *et al.* 2005). In South Africa it is considered the most important pathogen of *A. mearnsii* and an important factor in plantation health (Roux & Wingfield 1997).

Pathogenicity tests showed that all the species described in this study can give rise to lesions on the host plants from which they were isolated. Under field conditions all the species were isolated from wounds and although inoculation trials gave rise to lesions, it is unknown whether they are able to cause disease in nature. As a number of species in the *C. fimbriata s.l.* group are known to be pathogens, it is possible to speculate that isolates obtained in this study, and residing in the *C. fimbriata s.l.* species complex, could be important pathogens. In contrast, based on knowledge of the isolates residing in the *C. moniliformis s.l.* complex, these are probably not pathogenic in nature, but predominantly saprophytic.

This study presents the most comprehensive consideration of wound-infecting *Ceratocystis* spp. on non-native plantation grown tree species in Southern and Eastern Africa. Recently it has been estimated that there are approximately 171 500 fungal

species in South Africa (Crous *et al.* 2006). The fact that six new fungal species were found in a limited study in three countries emphasizes the distinct lack of knowledge regarding microfungi in Africa. This is even more so if one considers that this study reports three previously undescribed species for South Africa, a country in which research on the health of plantation forestry trees has a significant history.

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Table 1. *Ceratocystis* isolates obtained and used in this study.

Species	Culture nr.	Host	Geographical origin	Collector(s)	GenBank Accession no. ITS. BT, EF1α
C. albifundus	[b] CMW4068	*Acacia mearnsii*	South Africa	J. Roux	DQ520638, EF070429, EF070400
"	[b] CMW5329	"	Uganda	J. Roux	AF 388947, DQ371649, EF070401
"	[a] CMW23823, CMW23824	"	South Africa	R.N. Heath	
"	[a b] CMW23825	"	"	"	EU245010, EU244982, EU244942
"	[a] CMW23826-CMW23837	"	"	"	
"	[a b] CMW23838	"	"	"	EU245009, EU244981, EU244941
"	[a b] CMW24860	"	Tanzania	R.N. Heath & J. Roux	EU245011, EU244983, EU244943
"	[a b] CMW24861	"	"	"	EU245012, EU244984, EU244944
	[a] CMW24862-CMW24887	"	"	"	
"	[a b] CMW24685	"	Kenya	"	EU245013, EU244985, EU244945
"	[a b] CMW24686	"	"	"	EU245014, EU244986, EU244946
"	[a] CMW27876, CMW27877	"	"	"	
C. atrox	[b] CMW19383	*Eucalyptus grandis*	Australia	M.J. Wingfield	EF070414, EF070430, EF070402
"	[b] CMW19385	"	"	"	EF070415, EF070431, EF070403
C. bhutanensis	[b] CMW8217, CBS114289	*Ips schmutzenhoferi*	Bhutan	M.J. Wingfield, T. Kirisits, D.B. Chhetri	AY528957, AY528962, AY528952
C. bhutanensis	[b] CMW8242, CBS112907	"	Bhutan	"	AY528956, AY528961, AY528951
C. cacaofunesta	[b] CMW15051, CBS152.62	*Theobroma cacao*	Costa Rica	A.J. Hansen	DQ520636, EF070427, EF070398
C. cacaofunesta	[b] CMW14809, CBS115169	"	Ecuador	C. Suarez	DQ520637, EF070428, EF070399
C. caraye	[b] CMW14793, CBS114716	*Carya cordiformis*	USA	J. Johnson	EF070424, EF070439, EF070412
C. caraye	[b] CMW14808, CBS115168	*C. ovata*	"	"	EF070423, EF070440, EF070411
C. fimbriata	[b] CMW15049, CBS141.37	*Ipomaea batatas*	"	C.F. Andrus	DQ520629, EF070442, EF070394
C. fimbriata	[b] CMW1547	*I. batatas*	Papua New Guinea	E.C.H. McKenzie	AF264904, EF070443, EF070395
C. moniliformis	[a b] CMW22284	*E. grandis*	Tanzania	J. Roux & R.N. Heath	EU245015, EU244987, EU244947

Species	Culture nr.	Host	Geographical origin	Collector(s)	GenBank Accession no. ITS. BT, EF1α
C. moniliformis	[a b] CMW22289	*E. grandis*	Tanzania	J. Roux & R.N. Heath	EU245016, EU244988, EU244948
"	[a] CMW27864 – CMW27875	"	"	"	
"	[a] CMW17584	*Eucalyptus* sp.	South Africa	G. Kamgan Nkuekam	
"	[a b] CMW17587	"	"	"	EU245017, EU244989, EU244949
"	[a b] CMW17690	"	"	"	EU245018, EU244990, EU244950
"	[a] CMW27863	"	"	"	
"	[b] CMW9590	"	"	J. Roux	AY528985, AY528996, AY529006
"	[b] CMW4114	"	Ecuador	M. J. Wingfield	AY528986, AY528997, AY529007
C. moniliformopsis	[b] CMW10215, CBS115793	*E. oblique*	Australia	Z.Q. Yuan	AY528999, AY528988, AY529009
"	[b] CMW9986, CBS109441	"	"	"	AY528987, AY528998, AY529008
C. oblonga	[a b] CMW23802	*A. mearnsii*	South Africa	R.N. Heath	EU245020, EU244992, EU244952
"	[a b] CMW23803	"	"	"	EU245019, EU244991, EU244951
"	[a b] CMW23804	"	"	"	EU245021, EU244993, EU244953
"	[a] CMW23805	"	"	"	
C. obpyriformis	[a b] CMW23806	*A. mearnsii*	South Africa	"	EU245005, EU244977, EU244937
"	[a b] CMW23807	"	"	"	EU245004, EU244976, EU244936
"	[a b] CMW23808	"	"	"	EU245003, EU244975, EU244935
"	[a] CMW27862	"	"	"	
C. omanensis	[b] CMW11046, CBS118112	*Mangifera indica*	Oman	A. O. Al-Adawi	DQ074739, DQ074729, DQ074734
"	[b] CMW11048, CBS115780	"	"	"	DQ074742, DQ074732, DQ074737
C. pirilliformis	[b] CMW6569	*Eucalyptus nitens*	Australia	M.J. Wingfield	AF427104, DQ371652, AY528982
"	[b] CMW6579	"	"	"	AF427105, DQ371653, AY528983
C. platani	[b] CMW14802, CBS115162	*Platanus occidentalis*	USA	T.C. Harrington	DQ520630, EF070425, EF070396
C. platani	[b] CMW23918	"	Greece	M.J. Wingfield	EU426554, EU426555, EU426556
C. polonica	[b] CMW5026	n/a	n/a	n/a	AY233907

Species	Culture nr.	Host	Geographical origin	Collector(s)	GenBank Accession no. ITS. BT, EF1α
C. polonica	[b] CMW1165	n/a	n/a	n/a	AY233906
C. polychroma	[b] CMW11424, CBS115778	*Syzygium aromaticum*	Indonesia	M.J. Wingfield	AY528970, AY528966, AY528978
"	[b] CMW11436, CBS115777	"	"	"	AY528971, AY528967, AY528979
C. polyconidia	[a b] CMW23809	*A. mearnsii*	South Africa	R.N. Heath	EU245006, EU244978, EU244938
"	[a] CMW23810-CMW23817	"	"	"	
"	[a b] CMW23818	"	"	"	EU245007, EU244979, EU244939
"	[a b] CMW23819	"	"	"	EU245008, EU244980, EU244940
"	[a] CMW23820-CMW23822	"	"	"	
C. populicola	[b] CMW14789, CBS119.78	*Populus* sp.	Poland	J. Gremmen	EF070418, EF070434, EF070406
"	[b] CMW14819, CBS114725	"	USA	T. Hinds	EF070419, EF070435, EF070407
C. resinifera	[b] CMW20931, CBS100202	*Picea* sp.	Norway	H. Solheim	U75616
"	[b] CMW26371, CBS100204	"	USA	T. Hinds	U75618
C. savannae	[b] CMW17300	*Acacia nigrescens*	South Africa	G. Kamgan Nkuekam	EF408551, EF408565, EF408572
"	[b] CMW17279	*Combretum zeyheri*	"	"	EF408552, EF408566, EF408573
C. smalleyi	[b] CMW14800, CBS114724	*Carya cordiformis*	USA	G. Smalley	EF070420, EF070436, EF070408
C. tanganyicensis	[a] CMW15988-CMW15990	*A. mearnsii*	Tanzania	R.N. Heath & J. Roux	
"	[a b] CMW15991	"	"	"	EU244997, EU244969, EU244929
"	[a b] CMW15992	"	"	"	EU244999, EU244971, EU244931
"	[a] CMW15993-CMW15998	"	"	"	
"	[a b] CMW15999	"	"	"	EU244998, EU244970, EU244939
C. tanganyicensis	[a] CMW16000-CMW16007	*A. mearnsii*	Tanzania	R.N. Heath & J. Roux	
"	[a] CMW27878-CMW27892	"	"	"	
C. tribiliformis	[b] CMW13013	*Pinus merkusii*	Indonesia	M.J. Wingfield	AY528993, AY529003, AY529014
"	[b] CMW13015	"	"	"	AY528994, AY529004, AY529015
C. tsitsikammensis	[b] CMW14276	*Rapanea melanophloeos*	South Africa	G. Kamgan Nkuekam	EF408555, EF408569, EF408576

Species	Culture nr.	Host	Geographical origin	Collector(s)	GenBank Accession no. ITS. BT, EF1α
C. tsitsikammensis	[b] CMW14278	*Rapanea melanophloeos*	South Africa	G. Kamgan Nkuekam	EF408556, EF408570, EF408577
C. variospora	[b] CMW20935, CBS 114715	*Quercus alba*	USA	J. Johnson	EF070421, EF070437, EF070409
"	[b] CMW20936, CBS 114714	*Q. robur*	"	"	EF070422, EF070438, EF070410
C. virescens	[b] CMW11164	*Fagus americanum*	"	D. Houston	DQ520639, EF070441, EF070413
"	[b] CMW3276	*Quercus* sp.	USA	T. Hinds	AY528984, AY528990, AY5289991
C. zombamontana	[a b] CMW15251	*Eucalyptus* spp.	Malawi	R.N. Heath & J. Roux	EU245001, EU244973, EU244933
"	[a b] CMW15235	"	"	R.N. Heath & J. Roux	EU245002, EU244974, EU244934
"	[a b] CMW15236	"	"	R.N. Heath & J. Roux	EU245000, EU244972, EU244932
"	[a] CMW15242	"	"	R.N. Heath & J. Roux	
T. basicola	[b] C1602	n/a	n/a	n/a	AF275490
"	[b] C1373	n/a	n/a	n/a	AF275482
T. ceramica	[a b] CMW15245	*Eucalyptus* sp.	Malawi	R.N. Heath & J. Roux	EU245022, EU244994, EU244926
	[a] CMW15237	"	"	"	
"	[a] CMW15238	"	"	"	
	[a] CMW15240, CMW15241	"	"	"	
"	[a b] CMW15246	"	"	"	EU245023, EU244995, EU244927
"	[a b] CMW15248	"	"	"	EU2450024, EU244996, EU244928
	[a] CMW15249	"	"	"	
T. ceramica	[a] CMW17139-CMW17141	*Eucalyptus* sp.	Malawi	R.N. Heath & J. Roux	
"	[a] CMW24169-CMW24172	"	"	"	
T. ovoidea	[b] C1375	n/a	n/a	n/a	AF275483
"	[b] C1376	n/a	n/a	n/a	AF275484
T. populi	[b] CBS484.71	*Populus* sp.	n/a	n/a	AF275479
"	[b] CBS486.71	"	n/a	n/a	AF275480

[a] Isolates obtained in this study
[b] Isolates used in phylogenetic analysis in this study

Table 2. Morphological differences between species described in this study and closely related species in the *C. fimbriata sensu lato* group.

Character	*C. pirilliformis*	*C. zombamontana*	*C. polyconidia*	*C. tanganyicensis*	*C. obpyriformis*	*C. tsitsikammensis*
ASCOMATA						
Base						
Shape	Pyriform to Globose	Obpyriform	Globose	Subglobose	Obpyriform	Globose
Colour	Black	Dark brown to black	Dark brown to black	Dark brown to black	Dark brown to black	Black
Diameter	115-187(-206) µm	(106-)132-181(-208) µm	(133-)153-223(-277) µm	(119-)138-177(-205) µm	(149-)166-206(-228) µm	(124-)143-175(-186) µm
Ornamentation	None	None	None	None	None	None
Neck						
Colour	Black	Dark brown to black	Dark brown to black	Dark brown to black	Dark brown to black	Black
Collar	None	None	None	None	Collar	None
Length	372-683(-778)µm	(264-)315-443(-535) µm	(326-)429-589(694) µm	(302-)365-484(-558) µm	(477)569-675(-708) µm	(217-)321-425(-465) µm
Width (tip)	12-21 (-25) µm	(13-)16-21(-23) µm	(11-)13-19(-23) µm	(13-)14-18(-21) µm	(13-)16-21(-26) µm	n/a
Width (base)	19-33(-40)µm	(24-)30-38(-42) µm	(20-)26-34(-39) µm	(19-)24-32(-37) µm	(26-)28-36(-45) µm	(31-)32-47(62) µm
Ostiolar hyphae						
Shape	Convergent	Divergent	Divergent	Divergent	Divergent	Divergent
Length	n/a	(16-)20-25(-27) µm	(36-)39-47(-57) µm	(17-)39-47(-47) µm	(34.-)39-47(-54) µm	(23-)28-38(-42) µm
Ascospores						
Colour	Hyaline	Hyaline	Hyaline	Hyaline	Hyaline	Hyaline
Shape (side view)	Hat-shaped	Hat-shaped	Hat-shaped	Hat-shaped	Hat-shaped	Hat-shaped
Length	3-5(-7)µm	(4-)5-6(-6) µm	(3-)3-4(-5) µm	(3-)3-5(-6) µm	(3-)3-4(-6) µm	(4-)5-(-7) µm
Width	2-4(-5)µm	(3-)3-4(-5) µm	(4-)4-5(-6) µm	(4-)4-6(-8) µm	(3-)4-5 (-7) µm	(2-)3-4(-5) µm
Aggregation	n/a	Yellow-buff	Yellow-buff	Yellow-buff	Yellow-buff	n/a

Table 3. Morphological differences between species described in this study and closely related species in the *C. moniliformis sensu lato* group.

Character	***C. oblonga***	***C. savannae***	***C. moniliformis*[a]**
ASCOMATA			
Base			
Shape	Obpyriform	Globose	Globose
Colour	Dark brown to black	Dark brown	Brown / black
Diameter	(13-)180-254(-315) µm	(155-)178-217(-248) µm	90-180
Ornamentation	(8-)11-16(-19) µm	(1-)3-8(-13) µm	12-16 x 6 µm
Neck			
Colour	Dark brown to black	Dark brown	Na
Collar	Disc shaped	Disc shaped	Na
Length	(405-)502-721(-881) µm	(359-)455-703(-775) µm	Na
Width (tip)	(12-)13-18(-23) µm	(13-)12-21(-24) µm	Na
Width (base)	(30-)46-69(-76) µm	(37-)48-59(-62) µm	Na
Ostiolar hyphae			
Shape	Divergent	Divergent	Divergent
Length	(22-)23-27(31) µm	(17-)25-40(-46) µm	12-18 µm
Ascospores			
Colour	Hyaline	Hyaline	Hyaline
Shape (side view)	Hat-shaped	Hat-shaped	Oval, one side flat
Length	(3)-3-4(-4) µm	(5-)5-5 (-6) µm	4-5 µm
Width	(6-)7-8(-8) µm	(2-)3-3(-4) µm	3-4 µm
Aggregation	Yellow-buff	Straw yellow	Na

[a]Hedgcock 1906

Figure 1. Phylogenetic tree based on the combined ITS, *β*-tubulin and EF1-α gene regions of the *Ceratocystis fimbriata sensu lato* group of isolates. The phylogram was obtained using the heuristic search option based on parsimony. Bootstrap values are indicated above the branches with posterior probability values in brackets. *Ceratocystis virescens* represents the out-group taxon. Clades 1-5 (Bold) indicate isolates identified in this study.

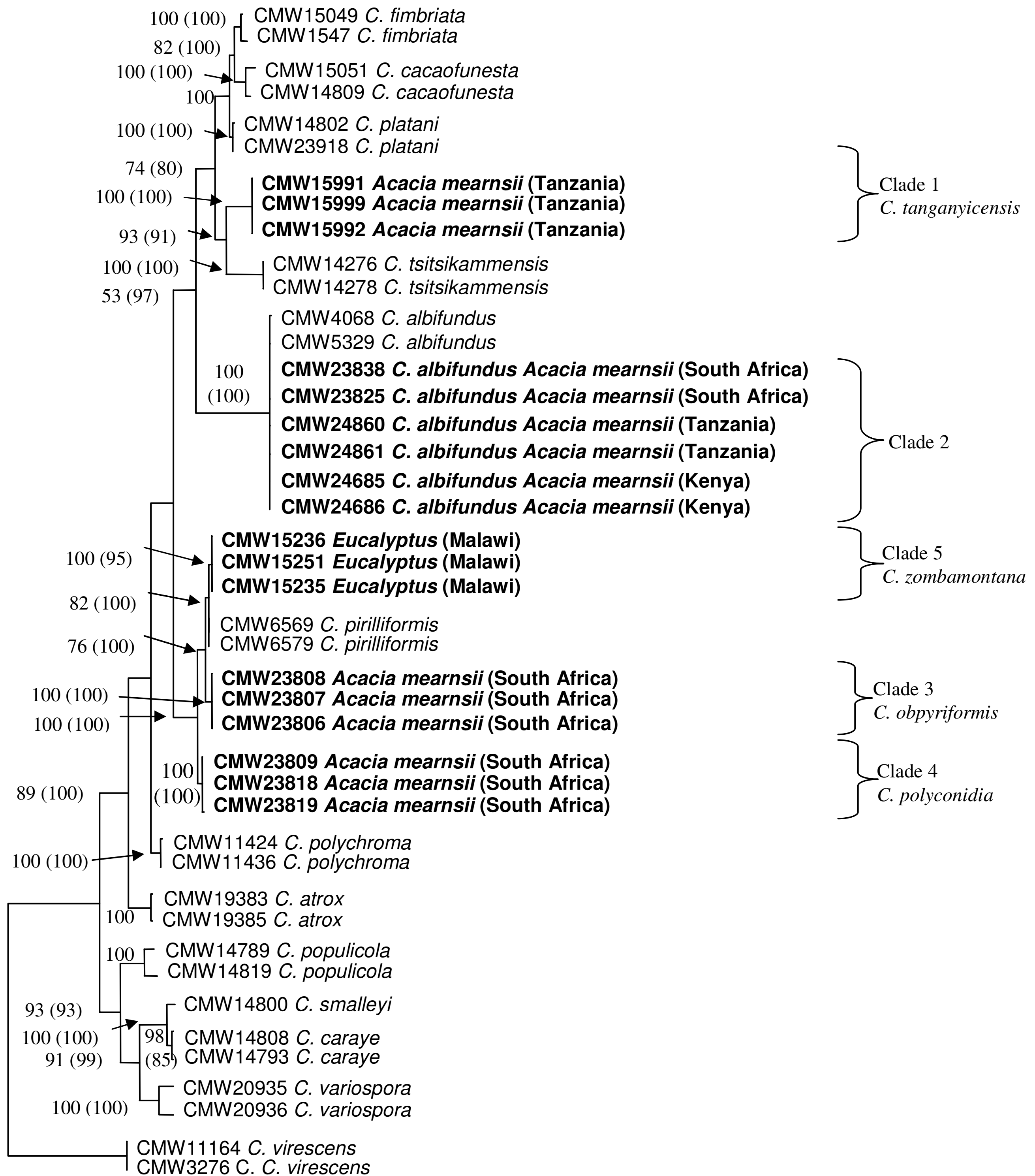

100 (100)
CMW15049 C. fimbriata
CMW1547 C. fimbriata
82 (100)
100 (100)
CMW15051 C. cacaofunesta
CMW14809 C. cacaofunesta
100
100 (100)
CMW14802 C. platani
CMW23918 C. platani
74 (80)
100 (100)
CMW15991 Acacia mearnsii (Tanzania)
CMW15999 Acacia mearnsii (Tanzania)
CMW15992 Acacia mearnsii (Tanzania)
Clade 1
C. tanganyicensis
93 (91)
100 (100)
CMW14276 C. tsitsikammensis
CMW14278 C. tsitsikammensis
53 (97)
CMW4068 C. albifundus
CMW5329 C. albifundus
100
(100)
CMW23838 C. albifundus Acacia mearnsii (South Africa)
CMW23825 C. albifundus Acacia mearnsii (South Africa)
CMW24860 C. albifundus Acacia mearnsii (Tanzania)
CMW24861 C. albifundus Acacia mearnsii (Tanzania)
CMW24685 C. albifundus Acacia mearnsii (Kenya)
CMW24686 C. albifundus Acacia mearnsii (Kenya)
Clade 2
100 (95)
CMW15236 Eucalyptus (Malawi)
CMW15251 Eucalyptus (Malawi)
CMW15235 Eucalyptus (Malawi)
Clade 5
C. zombamontana
82 (100)
CMW6569 C. pirilliformis
CMW6579 C. pirilliformis
76 (100)
100 (100)
CMW23808 Acacia mearnsii (South Africa)
CMW23807 Acacia mearnsii (South Africa)
CMW23806 Acacia mearnsii (South Africa)
100 (100)
Clade 3
C. obpyriformis
100
(100)
CMW23809 Acacia mearnsii (South Africa)
CMW23818 Acacia mearnsii (South Africa)
CMW23819 Acacia mearnsii (South Africa)
Clade 4
C. polyconidia
89 (100)
100 (100)
CMW11424 C. polychroma
CMW11436 C. polychroma
CMW19383 C. atrox
100
CMW19385 C. atrox
100
CMW14789 C. populicola
CMW14819 C. populicola
CMW14800 C. smalleyi
93 (93)
100 (100)
98
CMW14808 C. caraye
91 (99)
(85)
CMW14793 C. caraye
CMW20935 C. variospora
100 (100)
CMW20936 C. variospora
CMW11164 C. virescens
CMW3276 C. C. virescens
- 10 changes

Figure 2. Phylogenetic tree based on the ITS gene region of *Thielaviopsis* spp. and *Ceratocystis* spp. The phylogram was obtained using the heuristic search option based on parsimony. Bootstrap values are indicated above the branches with posterior probability values in brackets. *Ceratocystis virescens* represents the out-group taxon.

CMW8242 *C. bhutanensis*
CMW8217 *C. bhutanensis*
64 (53)
CMW15246 *Eucalyptus* (Malawi)
(74)
CMW15245 *Eucalyptus* (Malawi)
T. ceramica
100 (100)
CMW15248 *Eucalyptus* (Malawi)
CMW11046 *C. omanensis*
CMW11048 *C. omanensis*
84 (99)
100 (98)
C1375 *T. ovoidea*
C1376 *T. ovoidea*
100 (56)
CBS484.71 *T. populi*
89 (94)
86 (64)
CBS486.71 *T. populi*
100
(100)
C1606 *T. basicola*
100 (100)
C1373 *T. basicola*
CMW5026 *C. polonica*
100 (100)
CMW1165 *C. polonica*
64 (64)
C. resinifera
93 (83)
C resinifera
CMW11164 *C. virescens*
CMW3276 *C. virescens*
5 changes

Figure 3. Phylogenetic tree based on the combined ITS, *β*-tubulin and EF1-α gene regions of the *Ceratocystis moniliformis sensu lato* group. The phylogram was obtained using the heuristic search option based on parsimony. Bootstrap values are indicated above the branches with posterior probability values in brackets. *Ceratocystis virescens* represents the out-group taxon. Clades 6-8 (Bold) indicate isolates identified in this study.

100 (100)
CMW17300 *C. savannae*
CMW17297 *C. savannae*
100 (100)
CMW23803 *Acacia mearnsii* (South Africa)
CMW23802 *Acacia mearnsii* (South Africa)
100 (99)
CMW23804 *Acacia mearnsii* (South Africa)
Clade 7 *C. oblonga*
CMW15246 *Eucalyptus* (Malawi)
CMW15245 *Eucalyptus* (Malawi)
100 (99)
CMW15248 *Eucalyptus* (Malawi)
Clade 8 *T. ceramica*
97 (84)
93 (100)
CMW8242 *C. bhutanensis*
CMW8217 *C. bhutanensis*
100 (100)
CMW11048 *C. omanensis*
CMW11046 *C. omanensis*
94 (81)
CMW22289 *C. moniliformis* ***Eucalyptus***
CMW22284 *C. moniliformis* ***Eucalyptus* (Tanzania)**
CMW17587 *C. moniliformis* ***Eucalyptus* (South**
100 (100)
CMW9590 *C. moniliformis*
CMW17960 *C. moniliformis* ***Eucalyptus***
CMW4114 *C. moniliformis*
100 (94)
Clade 6
CMW13015 *C. tribiliformis*
CMW13013 *C. tribiliformis*
100 (100)
100
CMW10215 *C. moniliformopsis*
CMW9986 *C. moniliformopsis*
100 (100)
CMW3276 *C. virescens*
CMW11164 *C. virescens*
— 10 changes

Figure 4. Morphological characteristics of ***Ceratocystis zombamontana***: A. Obpyriform ascomata. Scale bar = 25µm B. Divergent ostiolar hyphae. Scale bar = 5µm C. Hat-shaped ascospores. Scale bar = 2.5µm D. Flask shaped primary phialide Scale bar = 10µm E. Secondary phialides producing barrel-shaped conidia. Scale bar = 10µm F. Primary conidia, cylindrical (above) and secondary conidium, barrel-shaped (below) Scale bar = 10µm.

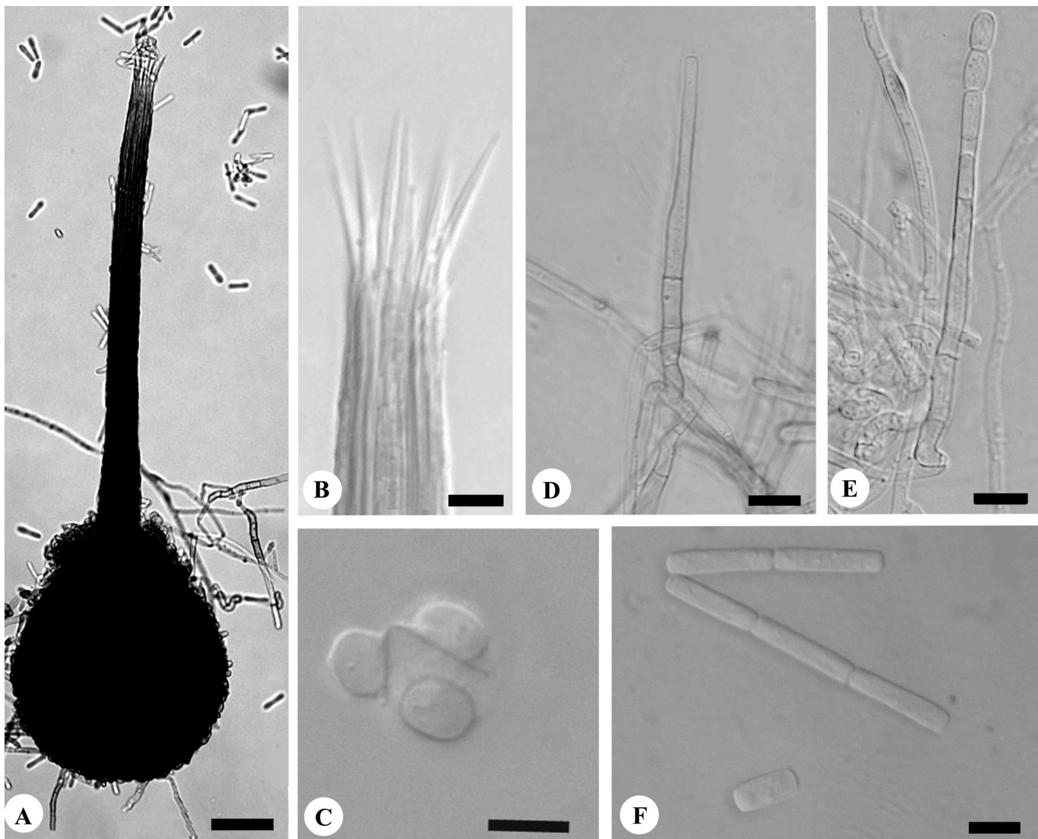

A
B
C
D
E
F

Figure 5. Morphological characteristics of ***Ceratocystis polyconidia***: A. Globose ascomatal base. Scale bar = 100µm B. Divergent ostiolar hyphae with emerging hat-shaped ascospores. Scale bar = 10µm. C. Hat-shaped ascospore. Scale bar = 2.5µm. D. Segmented hyphae. Scale bar = 10µm. E. Flask-shaped primary phialide with emerging cylindrical conidia. Scale bar = 20µm. F. Subglobose chlamydospores. Scale bar = 10µm. G. Secondary phialide producing barrel-shaped conidia in chains. Scale bar = 20µm. H. Barrel shaped conidia in chains. Scale bar = 10µm. I. Cylindrical conidia in chains. Scale bar = 5µm.

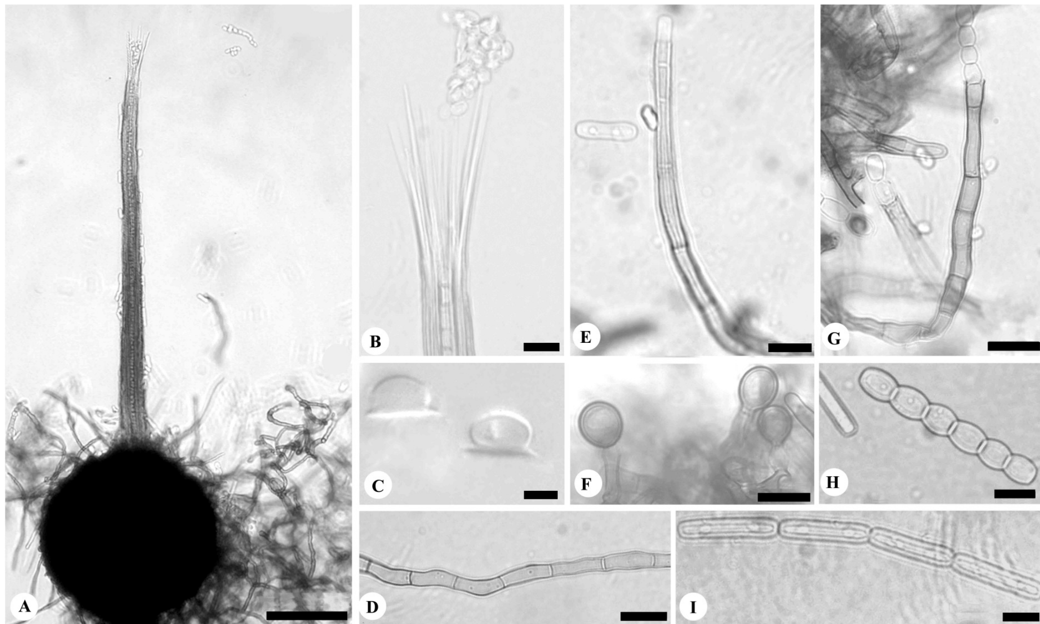

A
B
C
D
E
F
G
H
I

Figure 6. Morphological characteristics of ***Ceratocystis tanganyicensis***: A. Subglobose ascomatal base. Scale bar = 100µm. B. Divergent ostiolar hyphae. Scale bar = 10 µm. C. Hat-shaped ascospores. Scale bar = 5µm. D. Flask shaped primary phialide producing cylindrical conidia. Scale bar = 20µm. E. Cylindrical conidia. Scale bar = 10µm. F. Secondary phialide producing barrel shaped conidia. Scale bar = 5µm. G. Barrel shaped conidia. Scale bar = 5µm. H. Globose chlamydospores produced singly or in chains. Scale bar = 10µm.

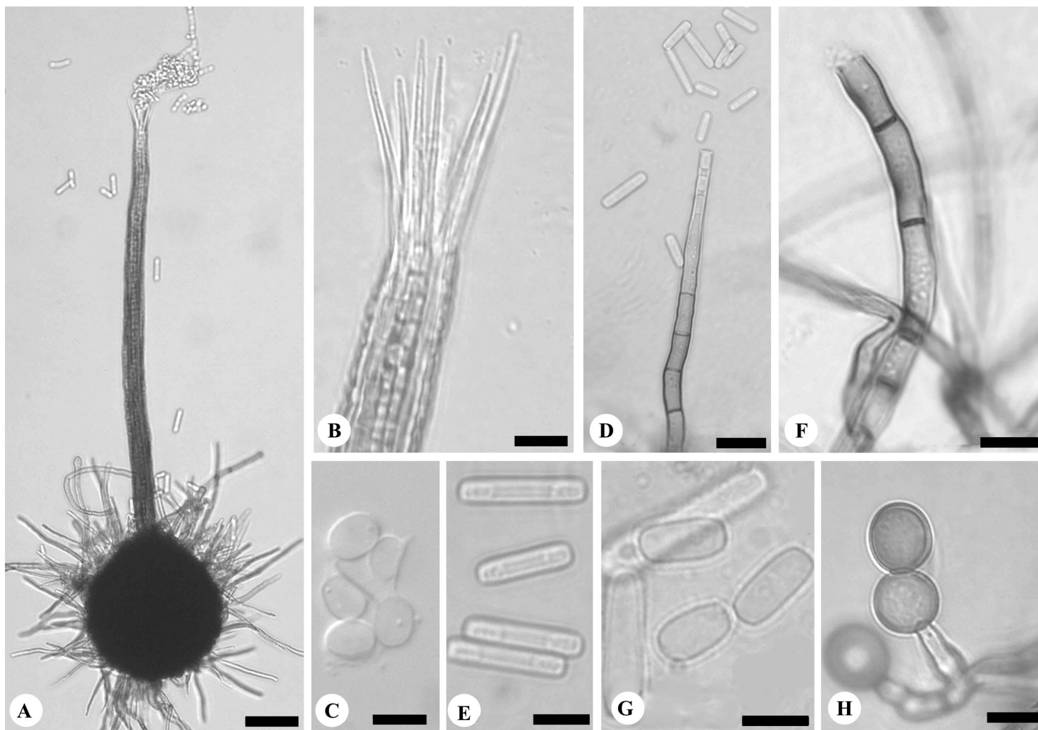

A
B
C
D
E
F
G
H

Figure 7. Morphological characteristics of ***Ceratocystis obpyriformis*** A. Obpyriform ascomatal base. Scale bar = 100µm. B. Divergent ostiolar hyphae with emerging hat-shaped ascospores. Scale bar = 20µm. C. Hat-shaped ascospores. Scale bar = 5µm. D. Primary, flasked shaped phialide producing cylindrical conidia. Scale bar = 50µm. E. Cylindrical conidia. Scale bar = 5µm. F. Secondary phialides. Scale bar = 20µm. G. Barrel shaped conidia in chains. Scale bar = 20µm.

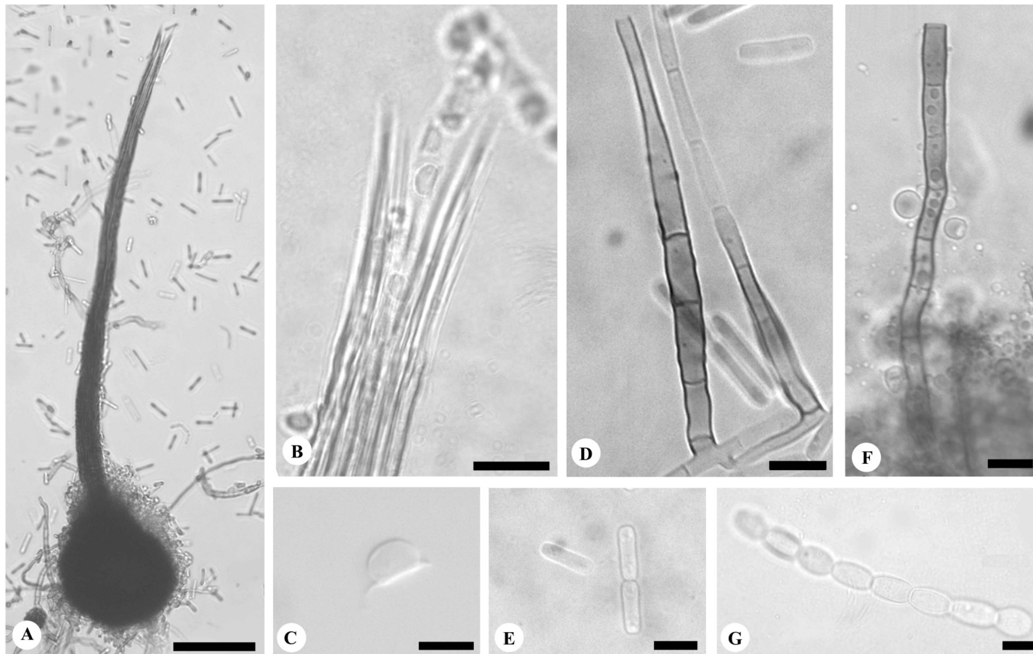
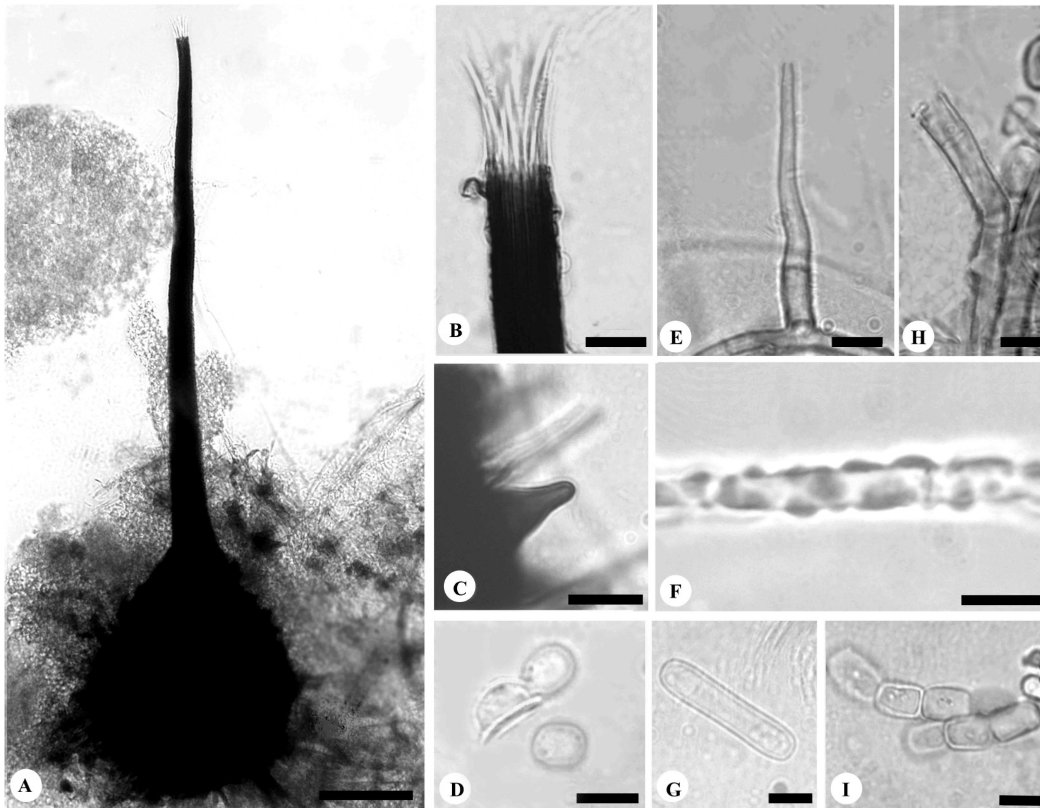

A
B
C
D
E
F
G

Figure 8. Morphological characteristics of ***Ceratocystis oblonga***: A. Obpyriform ascomatal base. Scale bar = 100µm. B. Divergent ostiolar hyphae. Scale bar = 20µm. C. Ascomatal base with short conical spines. Scale bar = 5µm. D. Hat-shaped ascospores. Scale bar = 5µm. E. Flask shaped primary phialide producing cylindrical conidia. Scale bar = 10µm. F. Hyphae with rough walls. Scale bar = 5µm. G. Cylindrical conidia. Scale bar = 10µm. H. Secondary phialide producing barrel-shaped conidia. Scale bar = 10µm. I. Oblong-shaped conidia. Scale bar = 5µm.



A
B
C
D
E
F
G
H
I

Figure 9. Morphological characteristics of ***Thielaviopsis ceramica***: A. Primary phialide Scale bar = 5µm. B. Flask shaped secondary phialide. Scale bar = 5µm. C. Primary cylindrical conidia. Scale bar = 5µm. D. Secondary barrel-shaped conidia. Scale bar = 2.5µm. E. Hypha with granulated wall. Scale bar = 5µm.

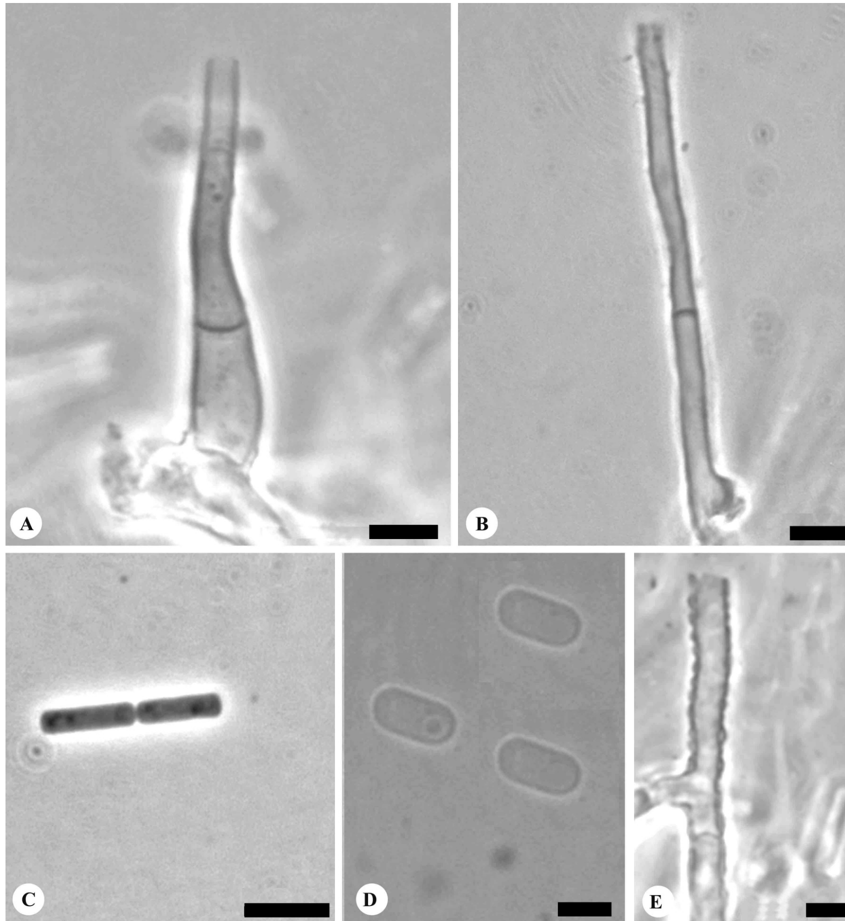

A
B
C
D
E

Figure 10. Bar chart indicating the average lesion length (in millimetres) resulting from inoculation trials with *Ceratocystis oblonga* (CMW23802, CMW23803), *C. obpyriformis* (CMW23807, CMW23808), *Ceratocystis polyconidia* (CMW23809, CMW23818), and *C. tanganyicensis* (CMW15992, CMW15999) onto *Acacia mearnsii.* Different letters above the bars indicate treatments that are statistically different based on a 95 % confidence limit.

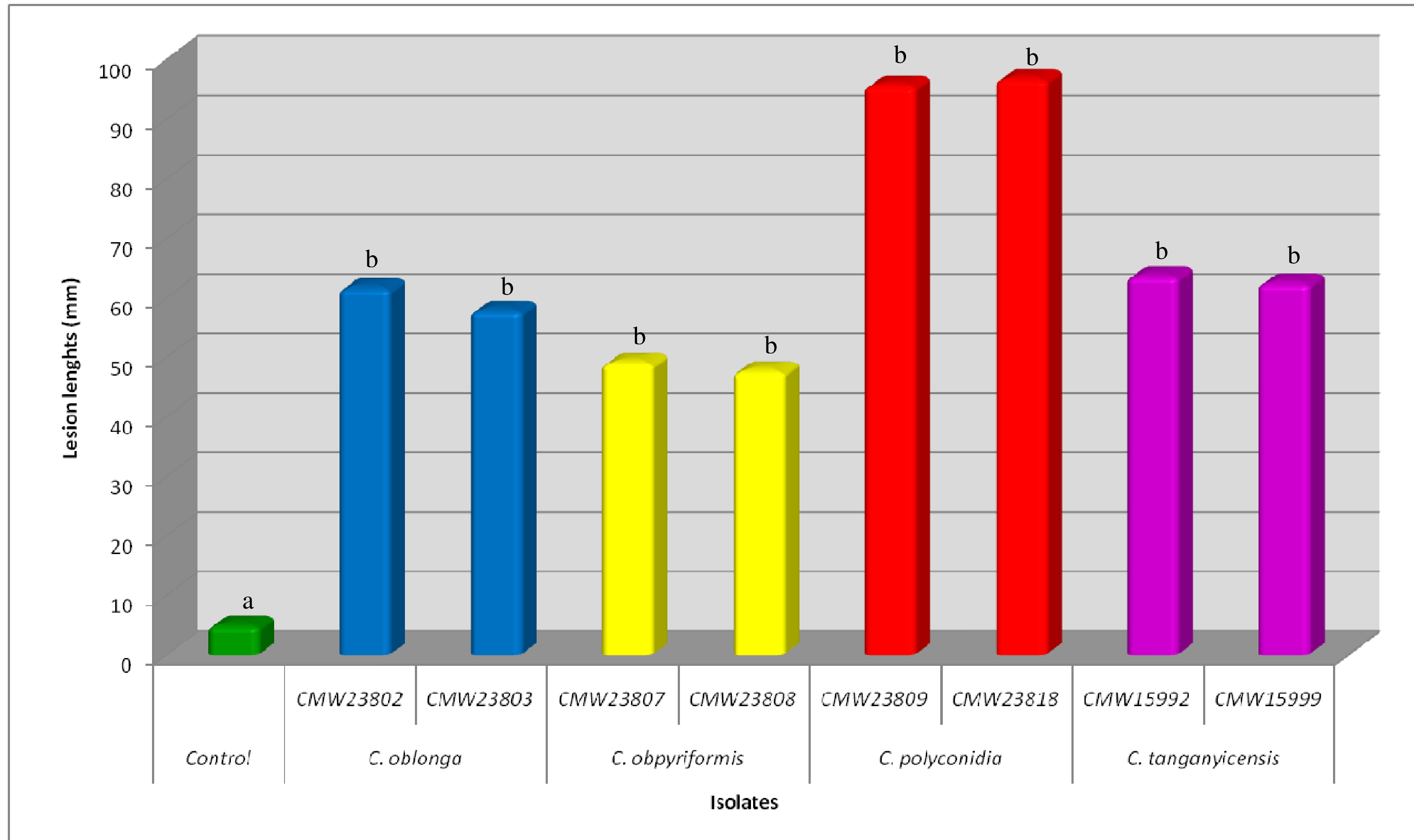
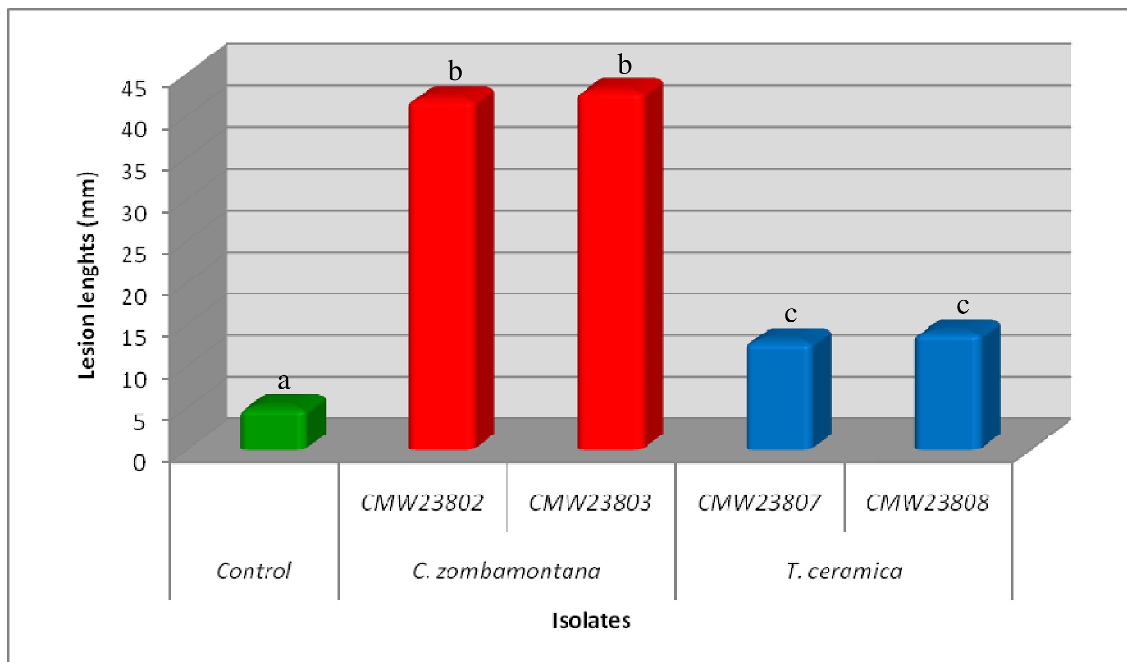

Lesion lenghts (mm)
100
90
80
70
60
50
40
30
20
10
0
a
b
b
b
b
b
b
b
b
CMW23802
CMW23803
CMW23807
CMW23808
CMW23809
CMW23818
CMW15992
CMW15999
Control
C. oblonga
C. obpyriformis
C. polyconidia
C. tanganyicensis
Isolates

Figure 11. Bar chart indicating the average lesion length (in millimetres) resulting from inoculation trials with *C. zombamontana* (CMW15235, CMW15236) and *T. ceramica* (CMW15245, CMW15248) onto *Eucalyptus grandis* (ZG 14). Different letters above the bars indicate treatments that are statistically different based on a 95 % confidence limit.


45
40
35
30
25
20
15
10
5
0
Lesion lenghts (mm)
a
b
b
c
c
CMW23802
CMW23803
CMW23807
CMW23808
Control
C. zombamontana
T. ceramica
Isolates

CHAPTER 3

POPULATION ANALYSES OF *CERATOCYSTIS ALBIFUNDUS* IN SOUTHERN AND EASTERN AFRICA SUGGEST A PROGRESSIVELY SOUTHERLY HOST COLONISATION ROUTE

ABSTRACT

Ceratocystis albifundus causes wilt and death of non-native *Acacia mearnsii* trees in Southern and Eastern Africa, resulting in considerable economic losses to forestry industries. The fungus is believed to be native to the African continent based on its host and geographic ranges, as well as high level of genotypic diversity on *A. mearnsii* in Uganda and South Africa. Surveys conducted between 2005 and 2007, yielded several populations of *C. albifundus* from various hosts and geographic areas in Southern and Eastern Africa. These isolates provided a first opportunity to consider the diversity of the pathogen from native African trees and to gain further insight into the possible origin and movement of *C. albifundus*. For this purpose polymorphic markers, previously applied to populations of the pathogen, were used. The presence or absence of alleles in populations was analysed using standard population genetic methods. The highest percentage of unique alleles (52%) was observed in the populations from native hosts and those from the northern range of the samples collected (48%). Maximum percentage of genotypic diversity ranged from 30.7% to 100%. The highest percentage of genotypic diversity exhibited by a population was for the northern range of isolates collected. The results suggest that *C. albifundus* is native to regions north of South Africa and that it has more recently become established in the country. These results further support the hypothesis that *C. albifundus* has undergone a host jump from native African trees to plantation grown *A. mearnsii*.

3.1. INTRODUCTION

Ceratocystis albifundus is the causal agent of Ceratocystis wilt of non-native *A. mearnsii* trees in South Africa (Morris *et al*. 1993) and Eastern Africa (Roux *et al*. 2001, 2005). The disease is characterized by the formation of cankers and lesions on the bark of infected trees that also exude copious amounts of gum. Internally, the pathogen causes extensive discolouration of the sapwood and in the final stages of disease development, wilting, die-back and mortality of trees are observed (Roux *et al*. 1999). The wilt disease caused by *C. albifundus* has resulted in significant losses in Southern and Eastern African *A. mearnsii* plantations (Roux *et al*. 2001a, Roux *et al*. 2005) and it poses a significant risk to the future sustainability of forestry operations that depend on these trees.

In recent years, *C. albifundus* has been reported from a number of native tree genera in South Africa (Roux *et al*. 2007), Zambia and Malawi (Roux *et al*. 2004). The first report of *C. albifundus* from a native host was when it was recorded as *C. fimbriata* on *Protea gigantea* (Gorter 1977, Wingfield *et al*. 1996). Later the fungus was reported from eight other native host tree genera in South Africa (Roux *et al*. 2007). On these native hosts, the fungus was found on wounds resulting from strong winds and there was no associated disease or tree death found (Roux *et al*. 2007).

Ceratocystis albifundus has been found only on the African continent and is thought to be native to Africa. Population biology studies on isolates from *A. mearnsii* in South Africa and Uganda have shown a high genetic diversity for the fungus in these two regions (Roux *et al*. 2001b, Barnes *et al.* 2005). Roux *et al*. (2001b) determined the nuclear and mitochondrial gene diversity of this pathogen using a microsatellite marker and mitochondrial restriction enzyme profiles. In that study, gene diversity for *C. albifundus* was high in comparison to other native *Ceratocystis* spp. (Roux *et al*. 2001b). In a study by Barnes *et al*. (2005), isolates of *C. albifundus* from *A. mearnsii* in South Africa and Uganda were compared using polymorphic microsatellite DNA markers to determine the genetic diversity, population structure and gene flow between the two populations. The gene and genotypic diversity was

high in both populations, but they were genetically isolated and gene flow was low. It was, therefore, suggested that the ancestral population had yet to be discovered (Barnes *et al*. 2005).

The report of *C. albifundus* from several native host genera in South Africa (Roux *et al*. 2007), Zambia and Malawi (Roux *et al*. 2004) provided a first opportunity to compare populations from native hosts to those on non-native *A. mearnsii* trees. These populations, together with recently collected populations from non-native *A. mearnsii* trees in Tanzania, makes available the most extensive collection of *C. albifundus* isolates to date for Southern and Eastern Africa. The aim of this study was to consider the population diversity of *C. albifundus* isolates from native and non-native hosts from southern and eastern Africa, using polymorphic microsatellite DNA markers. Previously collected populations of the pathogen from Uganda, Zambia and South Africa were included and additional isolates were collected from native hosts, non-native *A. mearnsii*, as well as from insects in South Africa and non-native *A. mearnsii* in Tanzania.

3.2. MATERIALS AND METHODS

3.2.1. Isolates

Isolates of *C. albifundus* included in this study originated from previous collections housed in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), and from collections made specifically for this investigation. Isolates sourced from previous studies (Barnes *et al*. 2005, Roux *et al*. 2004, Roux *et al*. 2007) originated from six geographical areas (Figure 1). These included three regions of South Africa (Gauteng Province, Eastern Cape Province, Mpumalanga Province, KwaZulu-Natal), one from Uganda (Kabale region) and Zambia (Kitwe Province). Samples included those from native African hosts (South Africa, Zambia) and those from non-native *A. mearnsii* trees (South Africa, Uganda). Samples from native hosts were obtained from wounds resulting from bark harvesting operations (Roux *et al*. 2004, Roux *et al*. 2007). Those collected from *A. mearnsii*

were obtained from stumps of recently felled trees or from diseased and dying trees (Roux *et al*. 2001).

To increase the population size of *C. albifundus,* collections were made from native and non-native hosts in South Africa and from non-native hosts in Tanzania (Njombe) (Figure 1). Collections were made from cut stumps of non-native *A. mearnsii*, between four days and four weeks after felling, by removing pieces of wood and bark displaying stained vascular tissue or the presence of fungal growth. Isolates from native tree species were collected in South Africa at the Leeuwfontein Collaborative Nature Reserve (Gauteng Province) and southern parts of the Kruger National Park (Limpopo Province). Isolates collected in the Kruger National Park were from natural wounds created by elephants, while isolates from Leeuwfontein Collaborative Nature Reserve were collected from natural wounds, artificially induced wounds and from insects that visit wounds.

Wood and bark sections collected from *A. mearnsii* as well as native tree species were examined for the presence of fruiting structures of *Ceratocystis* spp. Where present, isolations were made from these fruiting structures directly onto 2% (w/v) malt extract agar (MEA) (Biolab, Midrand, South Africa) supplemented with streptomycin sulphate (0.001g/litre, SIGMA, Steinheim, Germany). In addition, wood pieces displaying vascular discoloration were also baited for *Ceratocystis* spp. by placing these between two slices of carrots (5mm thick) and incubating them at 25°C for 7-10 days following the method described by Moller & DeVay (1968). Pieces of wood were also incubated in moist containers at 25°C for seven days to induce the formation of fruiting structures. Once ascomata of *Ceratocystis* spp. were found, spore masses were lifted from their apices and transferred to 2% (w/v) MEA supplemented with streptomycin sulphate (0.001 g/litre). Isolates were identified as *C. albifundus* based on their light coloured ascomatal bases, as this is the only *Ceratocystis* sp. that has this distinguishing characteristic (Wingfield *et al.* 1996). Plates were then incubated at approximately 25ºC under natural day/night conditions. Isolates were purified on 2% (w/v) MEA and are maintained in the culture collection

(CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

3.2.2. DNA extraction, PCR amplification and allele size determination

Genomic DNA of the *C. albifundus* isolates was extracted using the method described by Van Wyk *et al*. (2006). Polymerase chain reactions (PCR) were performed using an Eppendorf Mastercycler PCR machine (Eppendorf AG, Germany). Twenty four fluorescently labelled primer pairs developed for *C. fimbriata* Ell. & Hast. were used for PCR reactions (Barnes *et al*. 2001, Steimel *et al*. 2004). Of these, seven primer pairs that successfully produced PCR products were used for the study. The PCR reaction mixture and conditions used were those described previously (Barnes *et al*. 2001, Steimel *et al.* 2004). Amplified DNA was visualised on 2% (w/v) agarose gels stained with ethidium bromide and viewed under ultra violet light.

Allele sizes of amplified PCR products were determined by electrophoresis on an ABI PRISM™ 3100 Automated DNA sequencer (Applied Biosystems, Foster City, USA) and compared against a GENESCAN–500 LIZ (Applied Biosystems, Warrington, UK) internal size standard. Allele sizes were analysed with GENESCAN and GENEMAPPER software (Applied Biosystems, Foster City, USA). Allele size data from GENESCAN and GENEMAPPER were summarised in spreadsheets and the number of alleles per locus and the total number of haplotypes across all the populations were determined.

3.2.3. Genotypic diversity

The frequency of alleles at each locus was calculated for each geographical region by dividing the number of times each allele occurred by the total number of isolates. Each isolate was assigned a haplotype code according to the observed allele sizes across the microsatellite loci. Genotypic diversity (G_{ST}) was calculated using the equation of Stoddard & Taylor (1988). To compare genotypic diversities between collections of different numbers of isolates, the maximum percentage of genotypic diversity ($\hat{G}$) was calculated (Chen *et al*. 1994). Statistical confirmation that

sufficient numbers of isolates and markers were available for each collection was obtained by plotting genotypic diversity against number of loci sampled with 1000 re-samplings using the computer software program Multilocus v1.3b (Agapow & Burt 2001).

3.3. RESULTS

3.3.1. Isolates

A total of 190 isolates were included in this study (Table 1). Each isolate used for the analyses originated from a different tree or insect. For the analyses, populations were defined based on host and geographical area. The first two populations were defined based on whether the isolates had been obtained from native tree species and insects in native areas (hereafter referred to as the "Native" population) versus isolates from *A. mearnsii* trees (hereafter referred to as the "Non-native" population). The "Native" population consisted of 102 isolates whereas the "Non-native" population consisted of 88 isolates. Furthermore, populations were defined based on geographical origin with isolates obtained from Zambia, Uganda and Tanzania grouped into a "Northern" population (78 isolates) versus isolates obtained from South Africa grouped into a "Southern" population (112 isolates). These "Northern" and "Southern" populations were further subdivided into isolates obtained from native hosts or non-native hosts. A total of eight populations designated as described above were then subjected to analyses (Table 2).

3.3.2. Determination of allele size

A total of 89 alleles were observed across the eight loci detected by the seven markers (one marker produced two loci) used for all the populations, collectively resulting in an average of 10 alleles per locus. The "Native" population exhibited the greatest number of alleles, namely 69, within which 36 (52%) unique alleles were identified (Table 2). This was the population with the highest number of unique alleles. The lowest number of alleles was obtained for the Southern population of non-native trees (33), with only four unique alleles (Table 2).

3.3.3. Genotypic diversity

A total of 447 different haplotypes were observed across all the populations (Table 2). The number of haplotypes per population ranged between 17 and 81. The populations from non-native hosts exhibited the highest number of haplotypes (81). Of these, 444 (99%) haplotypes were unique to the population. The populations obtained from native hosts from the "Northern" population exhibited the lowest number of haplotypes (17) with all (100%) being unique to the population.

The maximum percentage of genotypic diversity ($\hat{G}$) between the various populations ranged between 30.7% and 100% (Table 2). The population from native hosts from the "Southern" population had the lowest $\hat{G}$ of 30.7%, while the population from native hosts from the "Northern" population exhibited the highest $\hat{G}$ value of 100%. The genotypic diversity plotted against the number of loci provided a saturated matrix thereby indicating that all of the populations defined in the study had been sufficiently sampled.

3.4. DISCUSSION

This study presents the first analyses of the population structure of the wilt pathogen *C. albifundus,* collected from non-native as well as native hosts from Southern and Eastern Africa. Previous studies were performed only on isolates collected from *A. mearnsii* from South Africa and Uganda (Barnes *et al*. 2005, Roux *et al*. 2001) and concluded that *C. albifundus* is native to Africa. The limited number of populations, as well as the unavailability of isolates from native hosts, made it difficult for the authors to identify a founder population and resulted in many unanswered questions.

If *C. albifundus* were to have originated on native hosts in Africa, it would be expected that the genotypic diversity would be highest in populations obtained from these hosts (Tsutsui *et al*. 2000). Our results do not fully support this expectation. The population obtained from native African species (southern and northern combined) had a low $\hat{G}$ value (34.9%) compared to the population from non-native

hosts (73.3%). However, when the populations are evaluated individually, a different picture emerges. The population obtained from native hosts from the "Northern" population, exhibited the maximum $\hat{G}$ value (100%), compared to that of the "Southern" native hosts with only 30.7%. Furthermore, the "Northern" population showed nearly double the amount of genotypic diversity (72.3%) compared to the "Southern" population (36.8%). This suggests that the fungus most likely originated from hosts north of South Africa and that it has spread in a southerly direction.

The difference in $\hat{G}$ value between populations obtained from native hosts could be due to the different sampling strategy used to collect the "Northern" and "Southern" populations. The population from native hosts from the "Northern" population was collected from numerous tree species from two sites situated close to each other (~50ha, ~50km), in contrast to the population obtained from native hosts from the "Southern" population. The last-mentioned population was collected in two relatively small geographical areas situated a long distance from each other, with one area situated in the Kruger National park (~100 ha) (Figure 1D) and the other obtained from three collection sites in the Leeuwfontein Collaborative Nature Reserve (~50ha) (Figure 1E). However, the statistical analysis that is used to confirm that sufficient numbers of isolates and markers have been used to sample a population (plotting genotypic diversity against number of loci sampled) reached matrix saturation. This suggests that both populations were fully sampled and that the different sampling strategies cannot be used as an explanation of the differences between the northern and southern populations.

An alternative explanation for the differences between the "Southern" and "Northern" populations isolated from trees native to each region is that *C. albifundus* originated in an area north of South Africa and that it was introduced into the country only recently. This notion also explains the difference in diversity between the *C. albifundus* isolates from non-native tree species in the northern collections, compared to the South African collections, where the "Southern" collections are less diverse than the "Northern" collections. The isolates obtained from non-native species in the north are also less diverse than the isolates obtained from native species in the north. This would be expected if the pathogen had moved from native species to plantation species. In support of this hypothesis, the isolates from the non-native species in the

north have fewer unique alleles than those observed in the corresponding native population.

Our results show that it is not possible to simply compare isolates of *C. albifundus* from native hosts with isolates from non-native hosts throughout the geographic regions from which they were collected. Each region should rather be evaluated on its own for the current set of populations. The data suggest that *C. albifundus* originated in a region to the north of South Africa and that it has progressively followed a southerly route of host colonisation. One explanation for the movement of *C. albifundus* in a southerly direction could be due to climatic changes that have occurred in a southerly direction (Barrett *et al*. 1992). The end of the previous ice age and aridification brought about by the closure of the Indonesian seaway 3-4 million years ago (Cane & Molnar 2001) and this resulted in a gradual increase in temperatures over the southern hemisphere. This warming of the continent in a southerly direction has resulted in the expansion of the habitat of possible hosts of the fungus. To the best of our knowledge, this is the first study to demonstrate the possible correlation between the habitat expansion of plants on the African continent and the spread of a fungus. The data also suggest that *C. albifundus* has made a number of host jumps in the recent past. The first (based on fairly high diversity values) was probably onto non-native species north of southern Africa. These host jumps commonly occur in tree pathogens, although they are poorly understood (Slippers *et al*. 2005). It appears that the pathogen has subsequently been moved into Southern Africa. It is not possible to determine whether this has occurred as the result of movement of non-native or native tree species into this region, but both options are equally likely.

The number of unique alleles exhibited by each population of *C. albifundus* corresponded with the genetic diversity obtained for that population. The "Northern" population exhibited more unique alleles (48%) than the "Southern" populations (41%). Within the "Northern" population, isolates from native hosts (44%) exhibited a much higher percentage of unique alleles than those from non-native hosts (14%). A similar trend was observed within the "Southern" population with the isolates from native trees exhibiting a higher percentage of unique alleles (36%) than those from non-native hosts (12%). These results again indicate that *C. albifundus* most probably

originated north of southern Africa and subsequently followed a progressively southerly host colonisation route.

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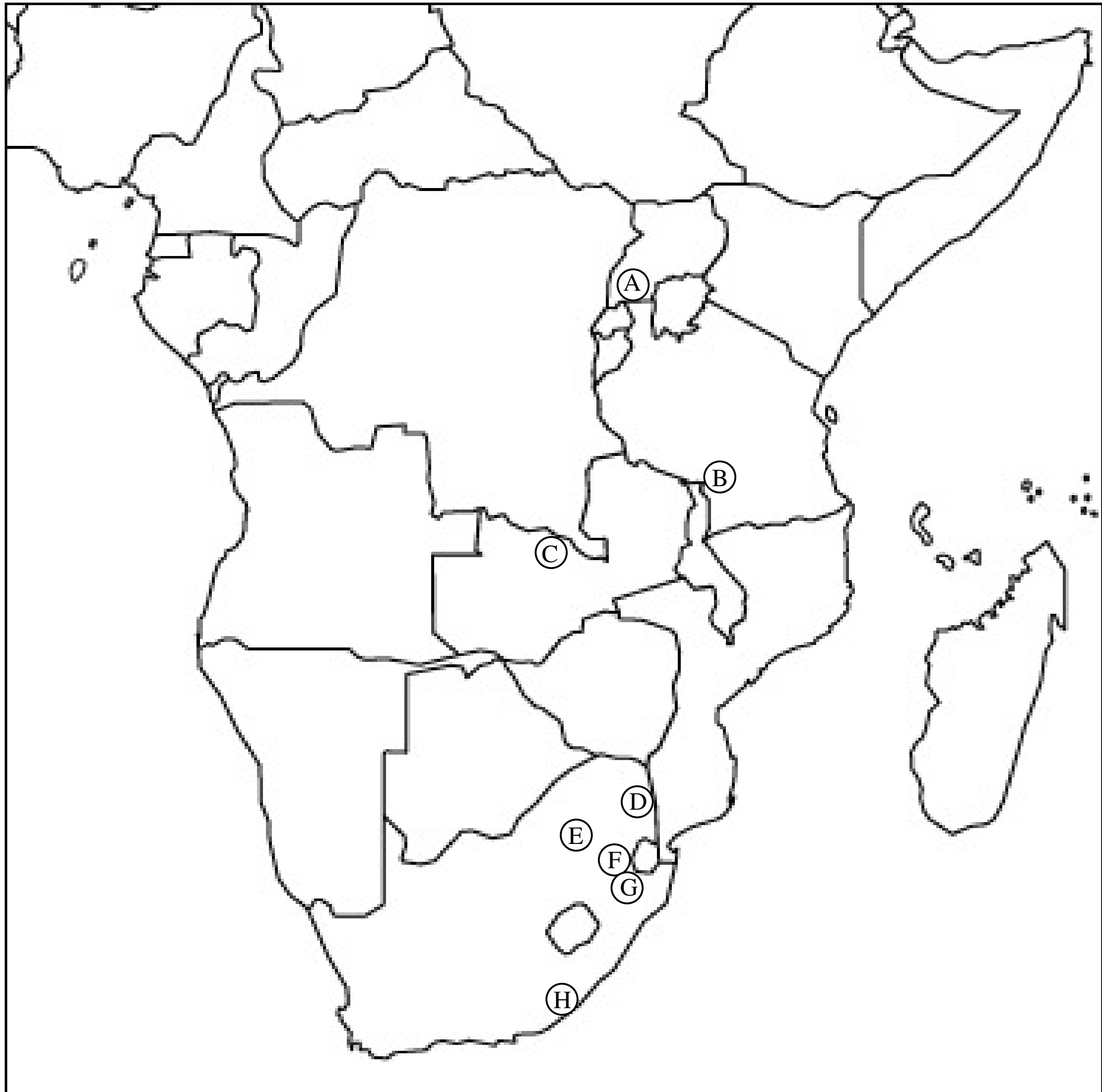
Table 1. List of isolates used in this study.

Culture number	Host	Geographical origin	Collector (s)
CMW17620-CMW17623	Native	Kruger National Park, RSA	R.N. Heath & J. Roux
CMW21146-CMW21154	"	"	"
CMW23840-CMW23848	"	"	"
CMW14157-CMW14163	"	Leeuwfontein Nature Reserve, RSA	"
CMW14165, 14166	"	"	"
CMW14168-CMW14173	"	"	"
CMW14605	"	"	"
CMW17270-CMW17288	"	"	"
CMW17307-CMW17312	"	"	"
CMW17628-CMW17631	"	"	"
CMW17633-CMW17639	"	"	"
CMW17773-CMW17775	"	"	"
CMW21469-CMW21475	"	"	"
CMW21661-CMW21695	"	"	"
CMW22292, 22293	"	"	"
CMW22300-CMW22302	"	"	"
CMW13979,13988	"	Kitwe Province, Zambia	J. Roux
CMW14607-CMW14610	"	"	"
CMW14612-CMW14617	"	"	"
CMW14619, 14620	"	"	"
CMW4076, 4079, 4084	Non-native	KwaZulu-Natal Province, RSA	"
CMW4097	"	Eastern Cape Province, RSA	T.C. Harrington
CMW23823- CMW23839	"	Mpumalanga Province, RSA	R.N. Heath
CMW23849-CMW23853	"	"	"
CMW4074, 4998, 5329	"	Kabale region, Uganda	J. Roux & G. Nakabonge
CMW7111-CMW7114	"	"	"
CMW7153-CMW7156	"	"	"
CMW7160-CMW7162	"	"	"
CMW7208	"	"	"
CMW9173-CMW9175	"	"	"
CMW9177-CMW9180	"	"	"
CMW9182, 9184	"	"	"
CMW9375-CMW9277	"	"	"
CMW15997-CMW15999	"	Njombe, Tanzania	R.N. Heath & J. Roux
CMW16003-CMW16006	"	"	"
CMW24860-CMW24887	"	"	"

Table 2. Summary of values obtained during statistical analyses of polymorphic DNA markers.

Population	No. of isolates	No. of alleles	Unique alleles	No. of haplotypes	Unique haplotypes	$\hat{G}$ -value
Native	102	69	36	77	77	34.9%
Non-native	88	46	13	81	80	73.3%
Northern	78	60	29	71	70	72.3%
Southern	112	53	22	75	74	36.8%
Northern native	17	43	19	17	17	100%
Northern non-native	60	37	5	52	52	66.7%
Southern native	84	38	14	48	48	30.7%
Southern non-native	28	33	4	26	26	93.3%

Figure 1. Map indicating the geographical areas from which *C. albifundus* isolates were obtained for this study. (A) Uganda (Kabale region), (B) Tanzania (Njombe), (C) Zambia (Kitwe, Copperbelt Province), (D) Kruger National Park (South Africa), (E) Leeuwfontein Collaborative Nature Reserve (South Africa), (F) Mpumalanga Province (South Africa), (G) KwaZulu-Natal Province (South Africa), (H) Eastern Cape Province (South Africa).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