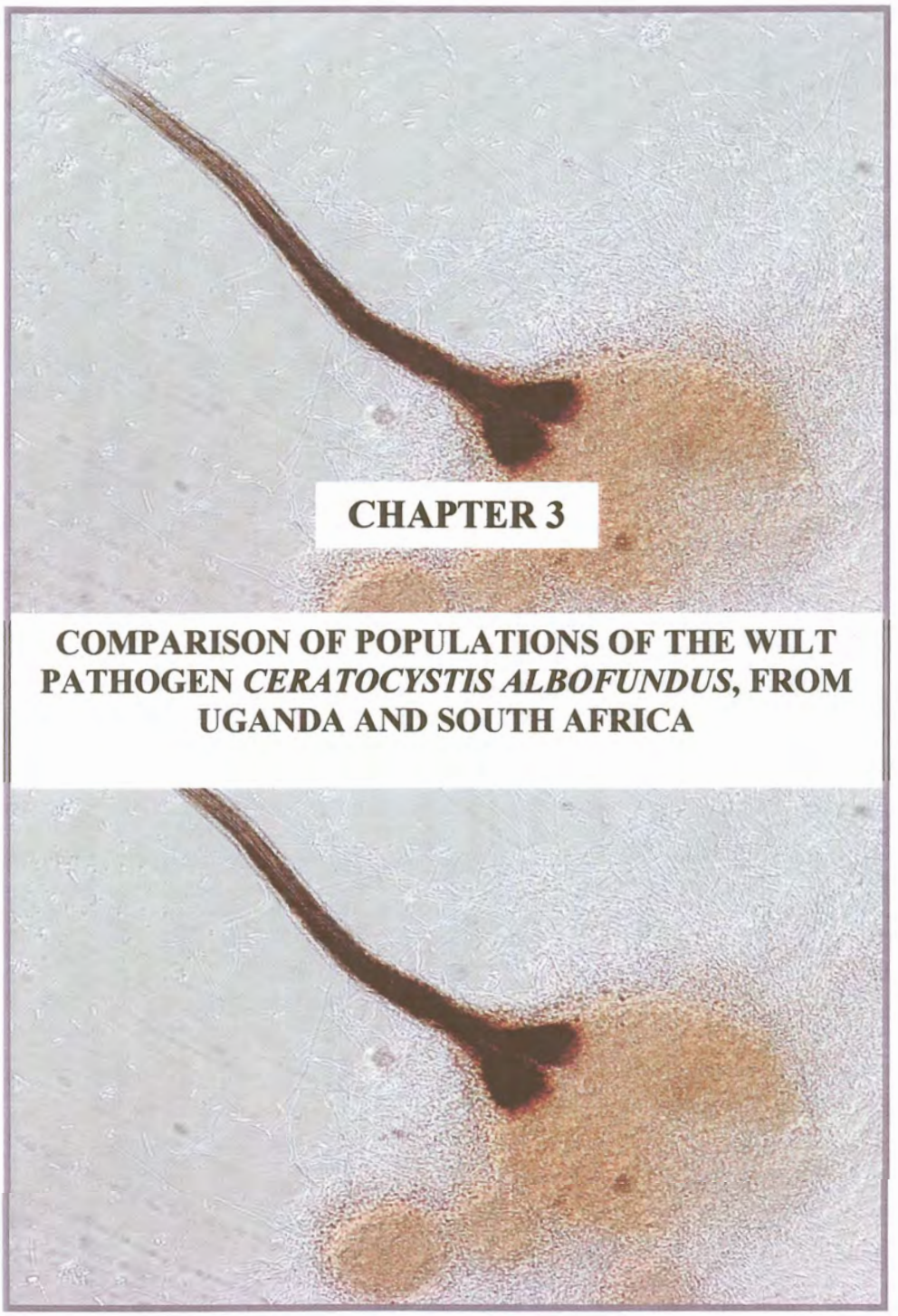


CHAPTER 3

COMPARISON OF POPULATIONS OF THE WILT PATHOGEN *CERATOCYSTIS ALBOFUNDUS*, FROM UGANDA AND SOUTH AFRICA

ABSTRACT

Ceratocystis albofundus is an important pathogen of *Acacia mearnsii* and *A. decurrens* in South Africa, where it causes stem cankers, die-back and death of these exotic trees. In 1999, *C. albofundus* was reported for the first time in South Western Uganda, where it is commonly associated with stem wounds on *A. mearnsii*, resulting from harvesting. It has previously been hypothesized that *C. albofundus* is native to South Africa. This is based on its occurrence on native *Protea* species and high gene diversity. The aim of this study was to determine the population diversity and structure of a Ugandan population of *C. albofundus* and to compare this with a South African population. Isolates were collected from 36 *A. mearnsii* stumps occurring in jungle stands in the South West of Uganda. Eight microsatellite primer pairs, previously developed for the related pathogen, *Ceratocystis fimbriata*, were used to amplify the microsatellite rich regions of the genome. Genescan analysis of the PCR amplicons showed that 7 of the loci were polymorphic and one was monomorphic, with a total of 26 alleles across all 8 loci. There were no common alleles within 2 of the 8 loci tested. Analysis of gene diversity showed that the Ugandan population is slightly more diverse than the South African population, with a gene diversity value of 0.407 compared to 0.378. Tests for gametic disequilibrium revealed clonality for both populations. Populations were highly sub-structured with very little gene flow. The high gene diversity values obtained in this study show that *C. albofundus* was probably not introduced into Uganda from South Africa and rather, that it is native to a wider area of Africa than previously assumed.

INTRODUCTION

The genus *Ceratocystis sensu stricto* Ell. & Halst. includes well-known and important canker and wilt pathogens of both forestry and agricultural crops (Kile 1993, Morris, Wingfield & De Beer 1993, Wingfield, Harrington & Solheim 1997, Roux *et al.* 2000a). Reports of *Ceratocystis* spp. causing serious disease problems on exotic trees grown intensively in plantations of the tropics and southern hemisphere have also increased in recent years. The first record of these fungi in this situation was of *C. fimbriata* Ell. & Halst., causing severe losses to *Acacia decurrens* Willd. (green wattle) in Brazil (Ribeiro *et al.* 1988). A year later, *C. albofundus* Wingfield, De Beer & Morris was recorded on *A. mearnsii* de Wild. (black wattle), *A. dealbata* Link. (silver wattle) and *A. decurrens* (green wattle) in South Africa, causing gummosis, wilting and death of trees (Morris *et al.* 1993, Wingfield *et al.* 1996, Roux & Wingfield 1997). In 1998, disease surveys in the Republic of Congo revealed that *C. fimbriata* was responsible for wilt and death of *Eucalyptus* spp. in that country (Roux *et al.* 2000b). More recently, *C. fimbriata*, has been reported as a pathogen of *Eucalyptus* spp. in Brazil (Roux *et al.* 2000a & b), Uganda (Roux & Wingfield 2001) and Uruguay (Barnes *et al.* 2002b).

Ceratocystis wilt is characterised by formation of cankers and lesions on the bark of affected trees and exudation of gum from the lesions. Internal symptoms include extensive discoloration of the sapwood. In the final stages of disease development wilting, die-back and death of the trees occurs (Morris *et al.* 1993, Wingfield *et al.* 1996, Roux, Wingfield & Dunlop 1999, Roux *et al.* 2000b), (Figure 1A & B).

Ceratocystis albofundus is distinguished from other species of *Ceratocystis* by its hyaline ascomatal bases, dark necks, and divergent ostiolar hyphae (Wingfield *et al.* 1996). In culture the fungus appears light cream colored in contrast to the green to grey appearance of other species, such as the closely related *C. fimbriata* (Wingfield *et al.* 1996). Phylogenetically, *C. albofundus* is most closely related to other *Ceratocystis* spp. with hat-shaped ascospores, such as *C. fimbriata* (Wingfield *et al.* 1996) and the newly described *C. pirilliformis* I. Barnes & M. J. Wingfield (Barnes *et al.* 2002a).

Ceratocystis spp. have a close association with insects (Kile 1993). *C. albofundus* produces a sweet aroma, which in other *Ceratocystis* spp., is known to attract insects

(De Vay, Davidson & Moller 1968, French & Stienstra 1978, Juzwik & French 1983, Kile 1993, Christen, Meza & Revah 1997). The sticky spore masses produced at the tips of the long necks of the ascomata facilitate dispersal of these spores (Hinds 1972, Juzwik & French 1983, Kile 1993).

Ceratocystis albofundus was discovered in Uganda for the first time in 1999 (Roux & Wingfield 2001). Prior to this, the fungus was known only in South Africa, where it is thought to be native (Roux *et al.* 2001, Barnes 2002). This was based partly on the fact that, apart from exotic Australian *Acacia* spp., the only other known hosts for *C. albofundus* are native South African *Protea* spp. (Gorter 1977). Molecular studies showing high levels of genetic diversity for a South African population of *C. albofundus* supported the view that the fungus is native to South Africa (Roux *et al.* 2001, Barnes 2002).

Discovery of *C. albofundus* in Uganda has raised the question as to whether it was introduced into that country from South Africa. An alternative hypothesis is that the fungus has a wide distribution in Africa, including Uganda. Our studies in Uganda have made it possible to collect a reasonably large set of isolates from this country. The aim of this study was thus to analyse the available population of *C. albofundus* in Uganda and to compare this with a population of the fungus from South Africa. This was achieved using polymorphic microsatellite DNA markers previously developed for *C. fimbriata* (Barnes *et al.* 2001).

MATERIALS AND METHODS

Isolates

Symptomatic tissue from *A. mearnsii* was collected from the Kabale district, South Western Uganda (Figure 1, Chapter 2). The area has an average temp of 17°C, which occasionally drops to 10°C at night, and the rainfall averages between 1000 – 1480mm per annum. Sampling was done randomly by collecting samples from as many trees as possible, from different woodlots. Collections were made from trees and stumps wounded during harvesting for fuelwood. Sampling involved chopping sections of symptomatic wood from the stems or branches of the trees, and wrapping these in paper bags for transportation to the laboratory and subsequent isolation of fungi.

Isolations were made on the same day as the collections. Two isolation techniques were used. Symptomatic wood pieces with streaked discoloration typical of *Ceratocystis* infections (Figure 1B) were placed in Petri dishes with moist filter paper, to induce sporulation. Wood in these moist chambers was inspected daily for the appearance of sexual fruiting structures, using a dissection microscope. In addition, small pieces of symptomatic wood were wrapped between slices of carrot and incubated for 7-14 days in plastic bags at room temperature (~ 25°C). This technique is known to be effective for isolating *Ceratocystis* spp. (Moller & De Vay 1968). After the development of sexual fruiting structures on the carrot or wood samples (Figure 1C), ascospore masses were transferred to Malt Yeast Agar (MYA) (2% malt extract, 1% yeast extract, Biolab, Midrand, Johannesburg) plates in order to obtain pure cultures (Figure 1D). All cultures, each from a different tree, are stored in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

DNA isolation

Cultures of the isolates for DNA based analyses (Table 1) were grown in sterile liquid media (2% malt extract, 1% yeast extract, Biolab, Midrand, Johannesburg) in 250 ml Erlenmyer flasks for a week at 25°C. Fungal mycelium was harvested by filtering and then freeze-dried overnight. A second set of mycelium for each isolate was stored at -20°C for later DNA isolation. Approximately 0.5 ml freeze dried mycelium for each isolate was placed in an Eppendorf tube, submerged in liquid nitrogen and ground into a fine powder using sterilized toothpicks. DNA was then extracted using the method described by Barnes *et al.* (2001).

Microsatellite Polymerase Chain Reaction (PCR) Amplification

PCR-based microsatellite DNA markers designed by Barnes *et al.* (2001) for *C. fimbriata* and used on different populations to determine their structure and diversity were used to study the Ugandan population of *C. albofundus*. The primers were first tested on a South African population collected by Roux *et al.* (2001). Eight of the eleven primers tested were effective and thus applied to the Ugandan population (Barnes 2002).

Polymerase chain reactions (PCR) were performed using 8 pairs of microsatellite primers (Table 2) following the protocol described by Barnes *et al.* (2001). The reactions were conducted in a total volume of 25 µl, comprising 2 ng DNA template (1 µl), 200 µM DNTPs (2 µl), 300 nM of each forward and reverse primer (0.75 µl) (Table 2), 5 U/µl expand high fidelity enzyme (0.1 µl) (Roche Diagnostics, Mannheim, Germany), 10 x PCR reaction buffer containing 1.5 mM $MgCl_2$ (Roche diagnostics, Mannheim, Germany) and 17.4 µl sterile distilled water. A 100 bp marker (Roche diagnostics, Mannheim, Germany) was used to estimate the DNA concentration and the size of the PCR products was determined on ethidium bromide-stained, 2% agarose gels, under UV light.

Genescan analysis

The agarose gel profiles obtained for the PCR products for all 8 microsatellite primer pairs were used to estimate DNA concentrations. Dilutions for each PCR sample were made for the genescan gel as described by Barnes *et al.* (2001). Each sample to be run on a single lane using Polyacrylamide Gel Electrophoresis (PAGE) comprised a mixture of 0.5 µl DNA (1.5 ng), 1.5 µl of loading buffer and 0.5 µl internal standard GENESCAN-TAMRA (Applied Biosystems, Warrington WA1 4SR, Great Britain). Samples were separated on a 4.25% PAGE gel, using an ABI Prism™ 377 DNA sequencer. The allele sizes for the different microsatellite regions were obtained using GeneScan 2.1 analysis software and Genotyper 2.0 (Applied Biosystems, Warrington WA1 4SR, Great Britain).

Genetic distance and population structure

The genetic distance between each isolate was calculated using allele size. A distance matrix based on total distance (D_{AD}) was obtained using the program MICROSAT (http: human.Stanford.edu/microsat). The emerging data matrix obtained was then analysed in MEGA version 2.1 (Kumar *et al.* 2001) and a dendrogram was constructed based on UPGMA (Unweighted Pair – Group Method with Arithmetic Mean). Analysis was done separately for the Ugandan population and then combined with the data obtained by Barnes (2002) for a South African population.

Genetic differentiation (G_{ST}) between the Ugandan and South African populations was calculated using the program POPGENE version 1.31 (http://www.ualberta.ca/~fyeh/fyeh). This measure determines the level of population

differentiation by comparing the gene diversities within sub-populations with that of the total population. Thus, a high G_{ST} value implies that there is low gene diversity within the sub-populations compared to the total population and *vice versa*. A value of above 0.25 indicates a very high genetic differentiation, whereas a value in the range of 0 to 0.05 indicates very low differentiation (Wright 1978). Using the G_{ST} estimates, gene flow (Nm) between the two populations was estimated using the formula $Nm = 0.5(1-G_{ST})/G_{ST}$, illustrated by McDermott & McDonald (1993) and Slatkin & Barton (1989).

Gene diversity

The gene diversity, based on allele frequencies, was calculated with the formula $H = 1 - \sum_{\kappa} \chi_{\kappa}^2$, where χ_{κ} is the frequency of the k^{th} genotype (Nei 1973). The frequency for each allele was determined by scoring each locus for the presence or absence of an allele (0 ; 1), and calculating the number of times an allele appeared in the population, divided by the sample size.

Genotypic Diversity

Multiloci genotypes were determined based on the number and frequencies of combinations of alleles at multiple loci. Genotypic diversity was calculated for both the Ugandan and South African populations using the program Multilocus v1.2 (http: www.bio.ic.ac.uk/evolve/software/multilocus) (Agapow & Burt 2000). The program is based on the probability that two individuals taken at random have different genotypes. The formula $\frac{n}{n-1}\left(1-\sum_i pi^2\right)$ where pi is the frequency of the i^{th} genotype and n is the number of individuals sampled was used. The maximum value of 1 implies that every individual is different. Thus, the program samples all possible pairs of individuals, tests whether they are different and calculates the fraction of pairs that are different.

A graph showing the genotypic diversity versus the number of loci was plotted for the Ugandan population. This was done to determine whether scoring additional loci was likely to increase the genotypic diversity or whether a plateau had been reached (Agapow & Burt 2000).

Mode of reproduction

The mode of reproduction for the Ugandan population was determined by calculating the Index of Association (I_A) (Taylor, Jacobson & Fisher 1999) and by applying the Parsimony Tree Permutation Test (PTLPT) (Burt *et al.* 1996) using the same parameters previously calculated for the South African population (Barnes 2002). The PTLPT was calculated using PAUP 4.0b3 (Phylogenetic Analysis Using Parsimony) with 500 randomisations and I_A using the program multilocus with 1000 randomisations (Agapow & Burt 2000).

RESULTS

Isolates

The carrot baiting technique was the most effective technique for retrieving isolates of *C. albofundus*. Although more than 100 samples with typical *Ceratocystis* symptoms were collected from individual trees, *C. albofundus* was obtained from only 36 trees. These all originated from an area of approximately 45 km^2 (Table 1).

Microsatellite PCR amplification

DNA was extracted for all Ugandan isolates. Successful PCR amplifications were obtained for every isolate using the 8 pairs of microsatellite primers (Table 2).

Genescan analysis

For the Ugandan population, a total of 26 alleles were obtained for the 8 loci and these ranged from 160 to 384 bp (Table 3). A total of 7 alleles were obtained for locus A7/8. Loci C17/18 and C21/22 had a total of 5 alleles each and loci A17/18, C5/6 and C23/24 had 2 alleles each. Locus A15/16 was monomorphic with an allele size of 288 bp (Table 3). The South African population (40 isolates) had a total of 24 alleles (Barnes 2002), 9 of which were shared with the Ugandan population. No alleles were shared between the two populations at loci A17/18 and C5/6 (Table 4).

Genetic distance and population structure

The dendrogram obtained by UPGMA from the distance matrix, revealed that the Ugandan population is structured, although there are no significant sub-groups within the population (Figure 2). Isolates from different plantations/farms/plots had similar genotypes and thus were evenly distributed within the population. With the combined Ugandan and South African populations, isolates grouped according to geographical

origin. Three distinct groups (Figure 3) were thus obtained with South African isolates dominant in Group I and Ugandan isolates dominant in group III. Isolate CMW 4074 from Uganda occurred within the South African group comprised of 33 isolates, whereas isolate CMW 4107 from South Africa occurred within the Ugandan group, comprised of 27 isolates. Group II (Figure 3) included a mixture of isolates from the two countries, with 8 from Uganda and 6 from South Africa (Figure 3). Some of the isolates grouped separately from the three major main groups, residing in separate minor groups (Figure 3).

The measure of genetic differentiation revealed a high differentiation between the Ugandan and South African populations. A G_{ST} value of 0.3116 (Table 5) was obtained. Estimation of gene flow revealed a Nm value of 1.1043, indicating very low gene flow between the two populations.

Gene diversity

A diversity value of 0.407 was obtained for the Ugandan population, compared to a value of 0.378 for the South African population (Table 4 and 6). Ugandan locus C17/18, with 5 alleles had the highest gene diversity value of 0.6188 (Table 4). In the South African population, locus A7/8, with 9 alleles had the highest diversity (0.751) (Barnes 2002).

Genotypic diversity

A maximum genotypic value of 0.963, equivalent to 96% was obtained for the Ugandan population. For the South African population, a value of 0.977, equivalent to 98% was obtained, slightly higher than the Ugandan population.

A p-value of < 0.001 was obtained for the graph plotted for genotypic diversity versus the number of loci. A plateau had been reached indicating that additional sampling would not have changed the genotypic diversity obtained for the Ugandan population (Figure 4).

Mode of reproduction

For the Ugandan population, the observed index of association (I_A) was 0.46 ($P<0.001$), slightly beyond the normal distribution for a recombining population

(Figure 5). The test rejects the null hypothesis of recombination, and suggests that reproduction is predominantly clonal.

DISCUSSION

Results of this study have shown that *C. albofundus* in Uganda has a high genetic diversity and this reflects a fungus that is most likely native to the country. Previously, it was thought that the fungus was native only to South Africa (Roux *et al.* 2001), and one hypothesis on which the present study was based, was that *C. albofundus* had been introduced into Uganda. This is clearly not so and the fungus appears to have a much wider distribution in Africa, than previously assumed.

The higher gene diversity for the Ugandan population in contrast to that in South Africa, might give an impression that the fungus has been present in Uganda for an extended period of time. This would have allowed sufficient time for mutational events to occur. Populations that have been in an area for a long period of time are expected to have more alleles than recently introduced populations (McDonald 1997, McDonald & Linde 2002). The increase in allele number could also be due to genetic drift, which enhances the frequencies of new alleles to recognisable standards (McDonald 1997, McDonald & Linde 2002). Thus, a high level of gene diversity is expected at the centre of origin (McDonald & McDermott 1993, McDonald 1997, Hoegger *et al.* 2000). The high gene diversity revealed within both the South African and Ugandan populations gives an impression that the fungus is native to the African continent and not necessarily only to South Africa as previously hypothesized.

The occurrence of *C. albofundus* on native *Protea* species in South Africa (Gorter 1977) supports the view that the fungus is well established in that country, living on native species and causing disease on exotic trees such as *A. mearnsii* (Morris *et al.* 1993, Wingfield *et al.* 1996). Research pertaining to the occurrence of *C. albofundus* on native species, especially *Proteas* in Uganda and South Africa is necessary to give greater insight into the possible origin of the fungus.

Results of this study have shown that populations of *C. albofundus* in Uganda and South Africa are clonal. This could imply that sexual reproduction within the populations is dictated by the MAT 2 gene, which promotes selfing, resulting in clonal progeny (De Beer 1994, Witthunn *et al.* 2000). Our findings further support previous studies done on related *Ceratocystis* species, which have indicated that although members in the genus tend to produce sexual structures profusely, the offspring produced by these species are very similar, varying only in their mating types (Harrington & McNew 1997). The high gene diversities combined with the clonal reproductive mode of the two populations further, suggests that the fungus is well established in Africa.

The high level of genotypic diversity estimated for the Ugandan population (96%), similar to that of the South African population (98%), contradicts the indication of clonality revealed by measures of gametic disequilibrim. Measures of genotypic diversity provide information of the proportion of the population that results from sexual reproduction (McDonald 1997, McDonald & Linde 2002). In this context, recombination resulting in new genotypes is not expected due to the clonal nature of the populations. The higher genotypic diversities obtained in this study could be attributed to mutations. Because the fungus is haploid, mutation occurring at any locus will most likely change the genotypes. The high genotypic diversities, further supports the view that *C. albofundus* has been in Africa for a long time.

Analysis of genetic distance for isolates in the Ugandan population, showed a high structure within the Ugandan population, although no pure subgroups were formed. This could be due to the small geographic area from which the population was sampled. For the combined population (Ugandan and South African), sub-structuring was revealed. The fact that recently introduced pathogen populations are expected to have little genetic differentiation (Leung, Nelson & Leach 1993), implies that the Ugandan and South African populations have been present in their respective countries for an extended period of time. This view is supported by the fact that very few alleles are shared between the two populations. The high levels of genetic differentiation between these populations might imply that sufficient time has passed to allow for selection of different alleles in different locations, and genetic drift, which changes allele frequencies randomly (Leung *et al.* 1993).

The low levels of gene flow between the Ugandan and South African populations of *C. albofundus* is consistent with the fact that the two countries are very distant from each other. Populations from countries between Uganda and South Africa might provide a link between populations or make tracing the movement of the pathogen possible. The results we have obtained in this study are, therefore, insufficient to further elucidate the origin of *C. albofundus*. To fully explain the origin of this pathogen, attempts will need to be made to obtain populations from other African countries and from alternative, preferably native, hosts of *C. albofundus*.

REFERENCES

Agapow, P-M. & Burt, A. (2000) Multilocus 1.2 [http://www.bio.ic.ac.uk/evolve/software/multilocus].

Barnes, I., Gaur, A., Burgess, T., Roux, J., Wingfield, B. D. & Wingfield, M. J. (2001) Microsatellite markers reflect intra-specific relationships between isolates of the vascular wilt pathogen, *Ceratocystis fimbriata*. *Molecular Plant Pathology* **2**: 319-325.

Barnes, I., Roux, J., Dudzinski, M. J., Old, K. M., Wingfield, B. D. & Wingfield, M. J. (2002a) *Ceratocystis pirilliformis*, a new species from *Eucalyptus nitens* in Australia. *Mycologia* (in Press).

Barnes, I., Roux, J., Wingfield, B. D., Neill, M. O. & Wingfield, M. J. (2002b) *Ceratocystis fimbriata* infecting *Eucalyptus grandis* in Uruguay. *Australasian Plant Pathology* (in Press).

Barnes, I. (2002) Taxonomy, phylogeny and population biology of *Ceratocystis* species with particular reference to *Ceratocystis fimbriata*. M. Sc. thesis, University of Pretoria, Pretoria, South Africa.

Burt, A., Carter, D. A., Koenig, G. L., White, T. J. & Taylor, J. W. (1996) Molecular markers reveal cryptic sex in the human pathogen *Coccidioides immitis*. *Proceedings of the National Academy of Science U.S.A.* **93**: 770-773.

Christen, P., Meza, J. C. & Revah, S. (1997) Fruity aroma production in solid state fermentation by *Ceratocystis fimbriata*: influence of the substrate type and presence of precursors. *Mycological Research* **101**: 911-919.

De Beer, C. (1994) *Ceratocystis fimbriata* with special reference to its occurrence as a pathogen of *Acacia mearnsii* in South Africa. M. Sc. thesis, University of the Orange Free State, Bloemfontein, South Africa.

De Vay, J. E., Davidson, R. W. & Moller, W. J. (1968) New species of *Ceratocystis* associated with bark injuries on deciduous fruit trees. *Mycologia* **60**: 635-641.

French, D. W. & Stienstra, W. C. (1978) Oak wilt. *Extension Folder 310*, Agricultural Extension Service, University of Minnesota, U.S.A.

Gorter, G. J. A. (1977) Index of plant pathogens and the diseases they cause in cultivated plants in South Africa. Department of Agricultural Technical Services, South Africa. *Science Bulletin* **392**.

Harrington, T. C. & McNew, D. L. (1997) Self-fertility and unidirectional mating type switching in *Ceratocystis coerulescens*, a filamentous ascomycete. *Current Genetics* **32**: 52-59.

Hinds, T. E. (1972) Insect transmission of *Ceratocystis* species associated with aspen cankers. *Phytopathology* **62**: 221-225.

Hoegger, P. J., Rigling, D., Holdenrieder, O. & Heiniger, U. (2000) Genetic structure of newly established populations of *Cryphonectria parasitica*. *Mycological Research* **104**: 1108-1116.

Juzwik, J. & French, D. W. (1983) *Ceratocystis fagacearum* and *C. piceae* on the surfaces of free-flying and fungus mat inhabiting nitidulids. *Phytopathology* **73**: 11164-1168.

Kile, G. A. (1993) Plant diseases caused by species of *Ceratocystis* sensu strico and *Chalara*. In *Ceratocystis* and *Ophiostoma*. Taxonomy, Ecology and Pathogenicity. (M. J. Wingfield, K. A. Seifert & J. F. Webber, eds): 173–284. APS Press, St. Paul, Minnesota.

Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001) Molecular Evolutionary Genetics Analysis software, Arizona State University, Tempe, Arizona, USA.

Leung, H., Nelson, R. J. & Leach J. E. (1993) Population structure of plant pathogenic fungi and bacteria. *Advances in Plant Pathology* **10**: 157-205.

McDermott, J. M. & McDonald, B. A. (1993) Gene flow in plant pathosystems. *Annual Review of Phytopathology* **31**: 353-373.

McDonald, B. A. (1997) The population genetics of fungi. Tools and techniques. *Phytopathology* **87**: 448-453.

McDonald, B. A. & McDermott, J. M. (1993) Population genetics of plant pathogenic fungi. *Bioscience* **43**: 311-319.

McDonald, B. A. & Linde, C. (2002) The population genetics of plant pathogens and breeding strategies for durable resistance. *Euphytica* **124**: 163-180.

Moller, W. J. & De Vay, J. E. (1968) Carrot as a species – selective isolation medium for *Ceratocystis fimbriata*. *Phyptopathology* **58**: 123-124.

Morris, M. J., Wingfield, M. J. & De Beer, C. (1993) Gummosis and wilt of *Acacia mearnsii* in South Africa caused by *Ceratocystis fimbriata*. *Plant Pathology* **42**: 814-817.

Nei, M. (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Science USA* **70**: 3321–3323.

Slatkin, M. & Barton, N. H. (1989) A comparison of three indirect methods for estimating average levels of gene flow. *Evolution* **43**: 1349-1368.

Ribeiro, I. J. A., Fumikoito, M., Filho, O. P. & De Castro, J. L. (1988) Gomose da *Acacia negra* causada por *Ceratocystis fimbriata* Ell. & Halst. *Bragantia Campinas* **47**: 71-74.

Roux, J. & Wingfield, M. J. (1997) Survey and virulence of fungi occurring on diseased *Acacia mearnsii* in South Africa. *Forest Ecology and Management* **99**: 327-336.

Roux, J., Wingfield, M. J. & Dunlop, R. (1999) Susceptibility of elite *Acacia mearnsii* families to Ceratocystis wilt in South Africa. *Journal of Forestry Research* **4**: 187-190.

Roux, J., Wingfield, M. J., Coutinho, T. A., Bouillett, J. P. & Leigh, P. (2000a) Diseases of plantation *Eucalyptus* in the Republic of Congo. *South African Journal of Science* **96**: 454-456.

Roux, J., Wingfield, M. J., Wingfield, B. D., Bouillett, J. P. & Alfenas, A. C. (2000b) A serious new disease of *Eucalyptus* caused by *Ceratocystis fimbriata* in Central Africa. *Forest Pathology* **30**: 175-184.

Roux, J., Harrington, T. C., Steimel, J. P. & Wingfield, M. J. (2001) Genetic variation in the wattle wilt pathogen *Ceratocystis albofundus*. *Mycoscience* **42**: 327-332.

Roux, J. & Wingfield, M. J. (2001) First report of Ceratocystis wilt of *Acacia mearnsii* in Uganda. *Plant Disease* **85**: 1029.

Taylor, J. W., Jacobson, D. J. & Fisher, M. C. (1999) The evolution of Asexual Fungi: Reproduction, Speciation and Classification. *Annual Review of Phytopathology* **37**: 197-246.

Wingfield, M. J., De Beer, C., Visser, C. D. & Wingfield, B. D. (1996) A new *Ceratocystis* species defined using morphological and ribosomal DNA comparisons. *Systematics and Applied Microbiology* **19**: 191-202.

Wingfield, M. J., Harrington, T. C. & Solheim, H. (1997) Two species in the *Ceratocystis coerulescens* complex from conifers in western North America. *Canadian Journal of Botany* **75**: 827-834.

Witthunn, R. C., Harrington. T. C., Wingfield, B. D., Steimel, J. P. & Wingfield, M. J. (2000) Deletion of the *MAT-2* mating type gene during uni-directional mating type switching in *Ceratocystis*. *Current Genetics* **38**: 48-52.

Wright, S. (1978) Variability within and among natural populations. Vol. 4. The University of Chicago Press, Chicago.

Table 1. Isolates of *C. albofundus* collected from diseased *Acacia mearnsii* in Uganda and used in this study.

[A]**Isolates**	**Area collected**
CMW 4074	Kagalama
CMW 4998	Kakarome
CMW 5329 -5364	Mafuga
CMW 7111-7112	Kakarome
CMW 7113-7114	Kachwekano
CMW 7115	Mafuga
CMW 7116	Karungu
CMW 7153-7162	Mafuga
CMW 7268	"
CMW 9173	Murutunga
CMW 9174	Kachwekano
CMW 9175-9176	Murutunga
CMW 9177	Kachwekano
CMW 9178	Mafuga
CMW 9179	Murutunga
CMW 9180	"
CMW 9181	Kakarome
CMW 9182-9184	Karungu
CMW 9375-9376	"
CMW 9377	Kachwekano

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

Table 2. Microsatellite primer sequences used for Polymerase Chain Reaction amplifications[A].

Primers	Forward primer sequence	Reverse primer sequence
AG7/8	CGA GAC AGC AAC ACA AGC CC	GGG GCG GTG GTG CAA TTG TC
AG15/16	CTT GAC CGA CCT GCC GAT TG	GGA TAG CAG CGA CAA GGA CC
AG17/18	GTC GGT GGT GGA GAC GGT C	CGG CCC TGC CAA CGG ATG
CF5/6	GAC CAA AGA TGG TGG CGA GC	CAT GGG CAT GCC TAG CCT TG
CF15/16.2	CGT TTG CAA GGC AAG GCA GC	CAG GGA CTA GGG TCT GCC AG
CF17/18	GAA ACC GAG AGT CAT CGT CC	CGA GCC AAG ACG TTC ATT GAA G
CF21/22	GCG TTG AAA GAT GTG GCG TG	GCA CTA CGA GAA TAG AAT GCA G
CF23/24	CAT GAT CGA CAA GGG CGC TG	CAG GGA ATT CCC GAT GGC AG

[A]Information obtained from Barnes *et al.* 2001.

Table 3. Genescan results of eight microsatellite markers indicating allele sizes and genotypes obtained for each isolate in the Ugandan population.

CMW No.	A7/8							A15/16	A17/18		C5/6		C15/16.2				C17/18			C21/22					C23/24		Microsatellite	Genotype
	296	299	308	319	320	331	332	288	304	305	382	384	260	261	283	284	290	291	292	250	251	254	283	284	160	168	Profile	
4074	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0	1	51212442	A
4998	1	0	0	0	0	0	0	1	1	0	0	1	0	1	0	1	0	0	0	0	0	0	1	0	1	0	11122241	B
7111	1	0	0	0	0	0	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	0	0	1	1	0	11212251	C
7112	0	0	0	0	1	0	0	1	1	0	1	0	1	0	0	0	1	0	0	0	0	0	0	1	0	1	51111352	D
7113	0	0	0	0	1	0	0	1	1	0	1	0	0	1	0	1	0	0	0	0	0	1	0	0	1	0	51112231	E
7114	0	0	0	0	1	0	0	1	0	1	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	1	51222442	F
7116	0	0	1	0	0	0	0	1	1	0	1	0	1	0	0	0	0	1	0	0	0	0	1	0	0	1	31111442	G
7153	0	0	0	1	0	0	0	1	1	0	1	0	1	0	0	1	0	0	0	1	0	0	0	0	1	0	41111211	H
7154	0	0	0	0	1	0	0	1	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	1	0	51112251	I
7155	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	0	1	0	1	51212452	J
7156	0	0	0	0	1	0	0	1	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	1	0	51112251	I
7157	0	0	0	0	0	1	0	1	1	0	1	0	1	0	0	0	0	1	0	1	0	0	0	0	0	1	61111412	K
7115	0	0	0	1	0	0	0	1	1	0	1	0	1	0	0	1	0	0	0	0	0	0	1	0	1	0	41111241	L
7158	0	0	0	1	0	0	0	1	1	0	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0	41112241	M
7159	0	0	0	1	0	0	0	1	0	1	1	0	1	0	0	0	0	1	0	0	0	0	1	0	0	1	41211442	N
7160	0	0	0	1	0	0	0	1	1	0	1	0	0	1	0	0	0	1	0	0	1	0	0	0	0	1	41112422	O
7161	0	0	1	0	0	0	0	1	1	0	1	0	0	1	0	0	0	1	0	0	0	0	0	1	0	1	31112452	P
7162	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	0	1	0	1	51212452	J
7268	0	1	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	0	1	21121442	Q
5329	0	0	0	0	1	0	0	1	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	1	1	0	51211151	R
5364	0	0	0	0	1	0	0	1	0	1	0	1	1	0	0	0	1	0	0	0	0	0	1	0	0	1	51221342	S
9178	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0	51212241	T
9181	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0	51212241	T
9173	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0	51212241	T
9180	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0	51212441	U
9376	0	1	0	0	0	0	0	1	0	1	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	1	21222442	V
9184	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0	51212241	T
9183	0	0	0	0	1	0	0	1	0	1	1	0	0	1	1	0	0	0	0	0	0	0	1	0	1	0	51212141	W
9176	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	1	0	1	0	51212441	U
9175	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0	1	51212442	A
9177	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	0	1	0	0	1	51212242	X
9375	0	0	0	0	0	0	1	1	0	1	1	0	0	1	0	0	0	0	1	0	0	0	1	0	1	0	71212541	Y
9174	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0	1	51212442	A
9182	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0	51212241	T
9377	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0	1	51212442	A
9179	0	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	1	0	0	0	0	1	0	0	1	51212442	A

Table 4. Comparison between the Ugandan and South African populations of *C. albofundus*. **A)** Number of polymorphic alleles present for each locus within the Ugandan and South African populations of *C. albofundus* and number of alleles shared between each population. **B)** Index of association, reproductive mode, gene diversity and maximum genotypic diversity of both the Ugandan and South African populations of *C. albofundus*.

A

Loci	A7/8	A15/16	A17/18	C5/6	C15/16.2	C17/18	C21/22	C23/24	Total Number of alleles
Uganda	7	1	2	2	2	5	5	2	26
S. Africa	9	2	2	2	1	3	3	2	24
No. of alleles shared	2	1	-	-	1	2	2	1	9

B

	Uganda	South Africa[B]
Sample size	36	40
Index of Association (observed value)	0.46	0.992
- **Minimum value**	-0.227	-0.246
- **Maximum value**	0.414	0.379
- **p-values[A]**	<0.001	< 0.001
Gene diversity	0.4072	0.3778
Maximum genotypic diversity	96%	98%
Reproductive mode	Clonal	Clonal

[A] p-Values reveal the relevance of linkage disequilibria. P-values greater than 0.05 are not significant, whereas, p-values less than 0.01 are highly significant.

[B] Information obtained from Barnes (2002).

Table 5. Results of Genetic differentiation (Gst) and geneflow (Nm) obtained for the combined Ugandan and South African populations.

Locus	**Gst**	**Nm[A]**
A7/8	0.2060	1.9267
A15/16	0.2121	1.8571
A17/18	0.3985	0.7546
C5/6	0.7137	0.2006
C15/16.2	0.6000	0.3333
C17/18	0.1031	4.3481
C21/22	0.0612	7.6638
C23/24	0.2360	1.6190
Mean	**0.3116**	**1.1047**
St. Dev	0.0281	0.0363

[A]Nm is the estimate of gene flow from G_{ST}. E.g., $Nm = 0.5(1 - G_{ST})/ G_{ST}$. A G_{ST} of 0-0.05 indicates very low differentiation whereas a value of 0.25 indicates high differentiation.

Table 6. Summary of genetic variation statistics for the Ugandan population for all 8 loci.

Locus	Sample Size	na*[A]	h*[B]
A7/8	36	7.00	0.5617
A15/16	36	1.00	0.0000
A17/18	36	2.00	0.4614
CF5/6	36	2.00	0.2392
C15/16.2	36	2.00	0.3750
C17/18	36	5.00	0.6188
C21/22	36	5.00	0.5015
C23/24	36	2.00	0.5000
Mean	36	3.25	**0.4072**

[A] Observed number of alleles.

[B] Nei's gene diversity.

Figure 1. Cultural characteristics and disease symptoms caused by *C. albofundus*. **A)** Wilt and death of *A. mearnsii* trees after infection by *C. albofundus*. **B)** Typical discoloration of the wood caused by *C. albofundus* infection. **C)** Light colored bases of ascomata and dark necks characteristic of *C. albofundus*. **D)** Creamy, whitish mycelium produced by *C. albofundus* grown on 2 % Malt Extract Agar.

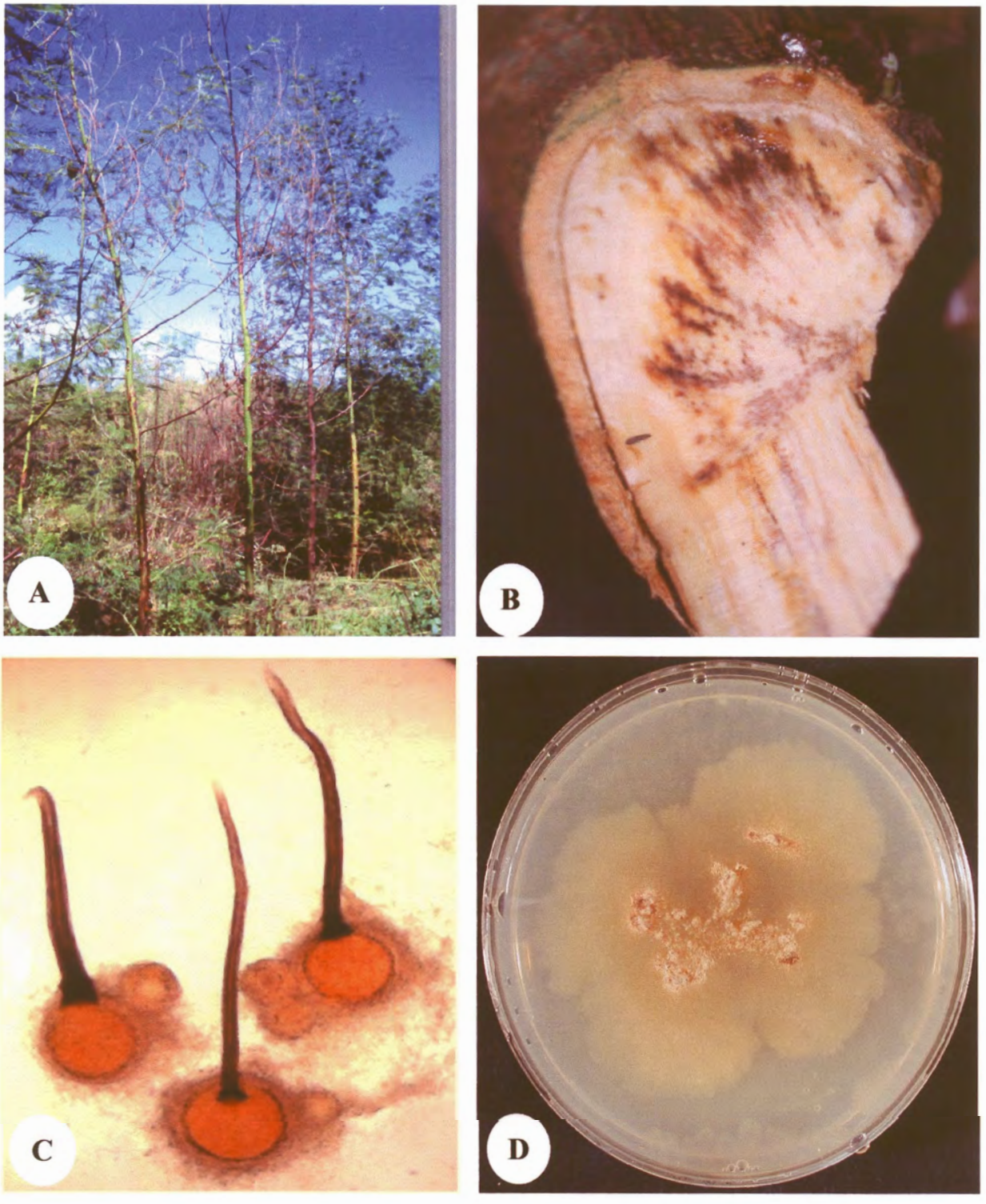

A
B
C
D

Figure 2. UPGMA dendrogram of the Ugandan population derived from genetic distances based on total nucleotide length calculated using total distance D_{AD}.

Ugandan population of *C. albofundus*

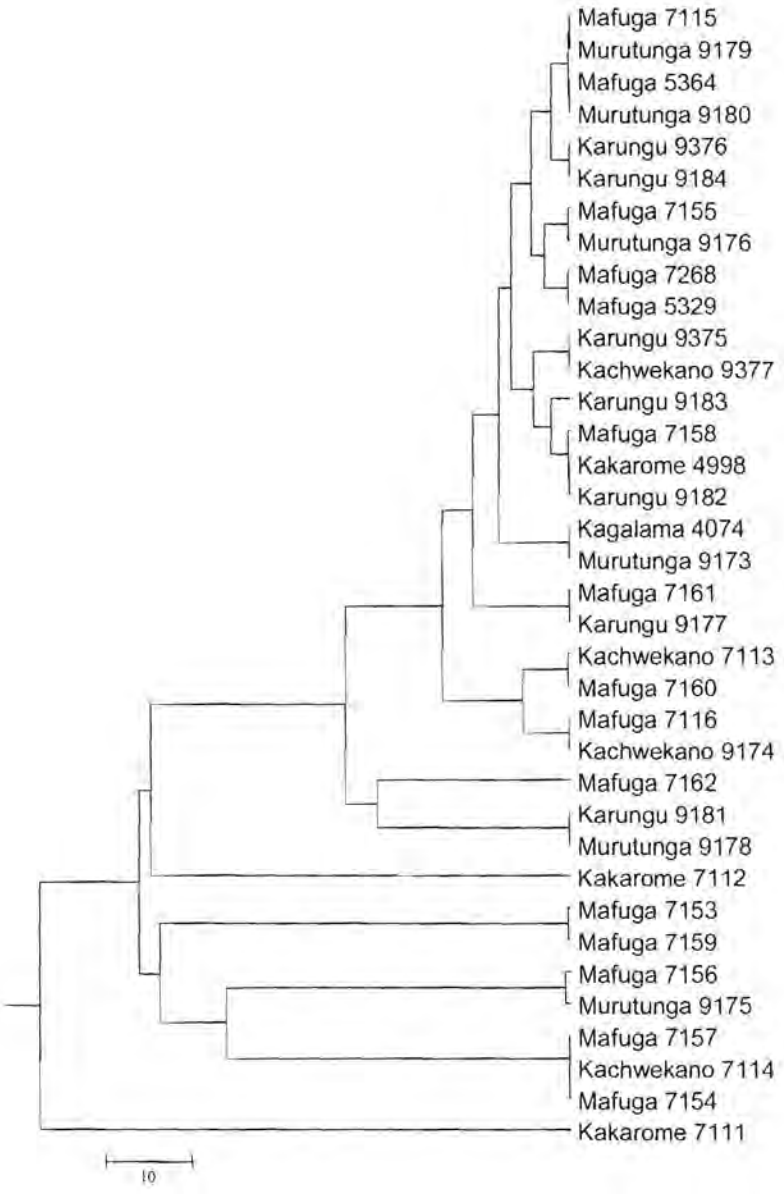


Figure 3. UPGMA dendrogram obtained using genetic distances for combined populations of Uganda and South Africa. Distances were calculated using D_{AD} based on total distance. A high differentiation within the populations was revealed with a G_{ST} value of 0.3116.

Ugandan and South African populations of *C. albofundus*

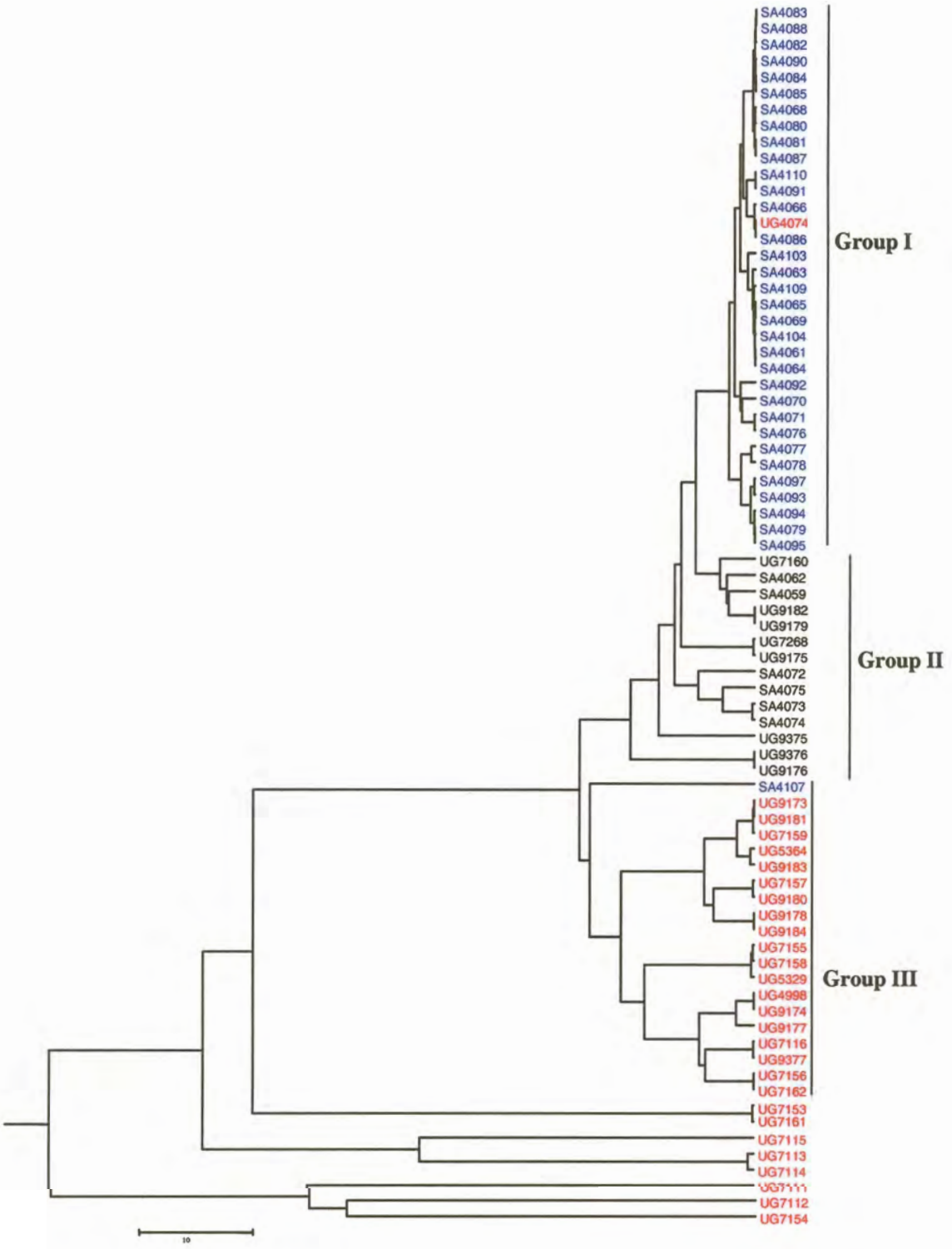


Figure 4. Curve of genotypic diversity against number of loci ($p<0.001$) for Ugandan population with 1000 sampling events.

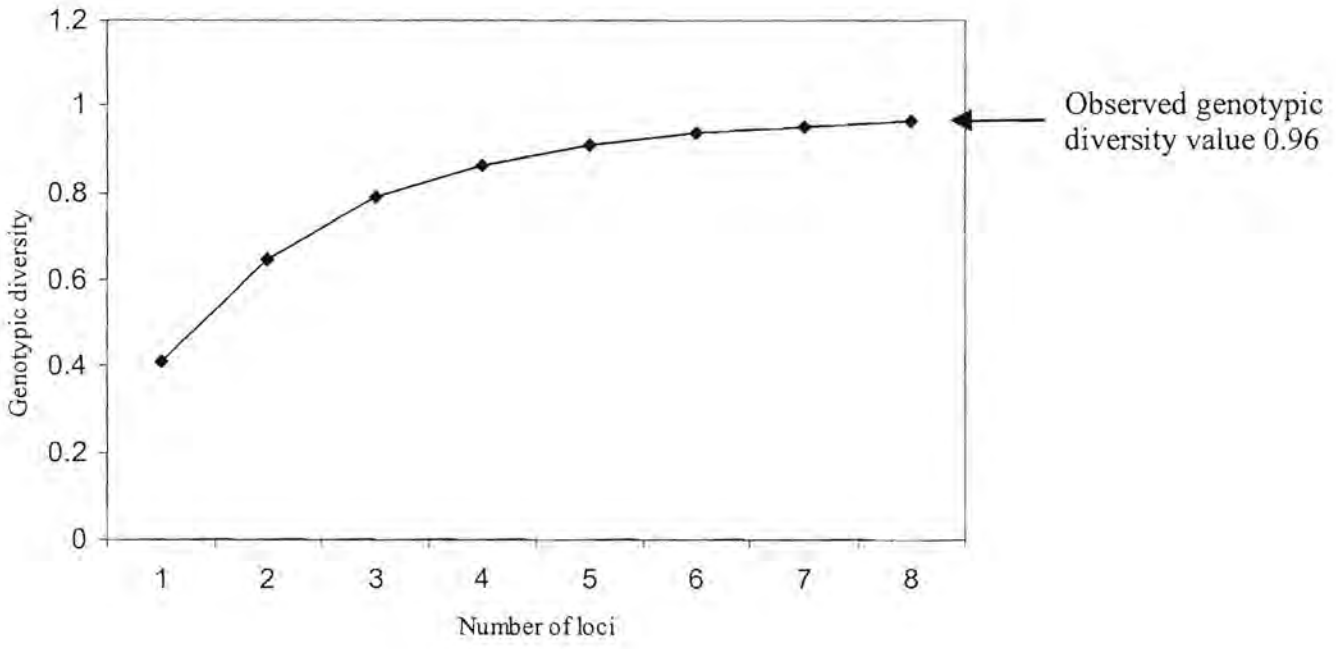
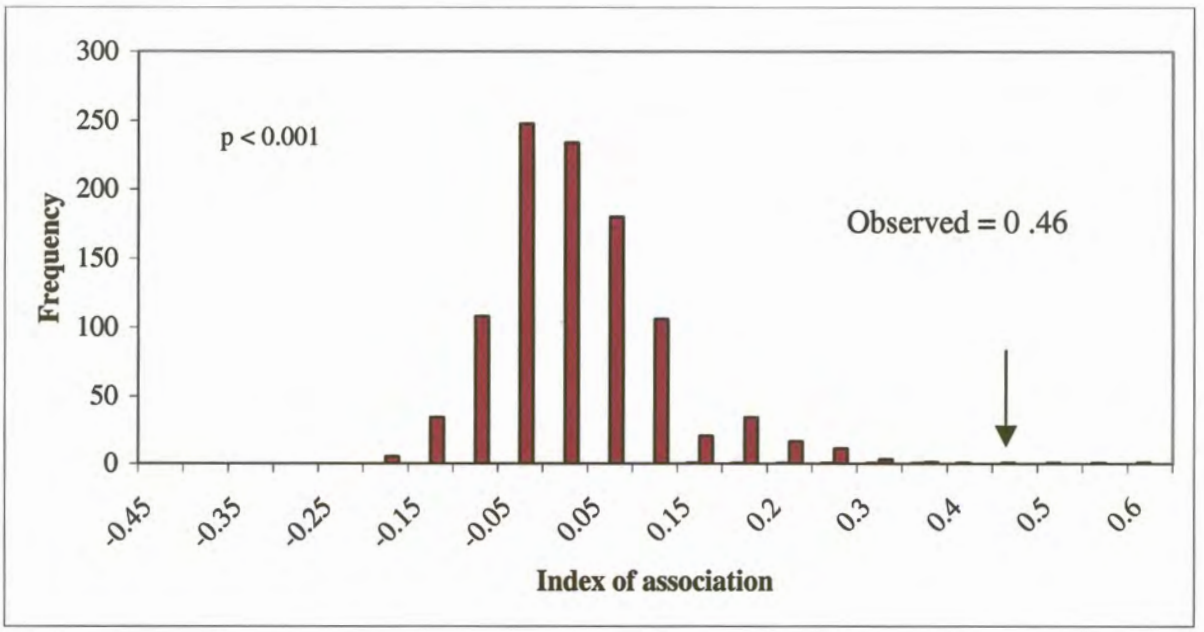
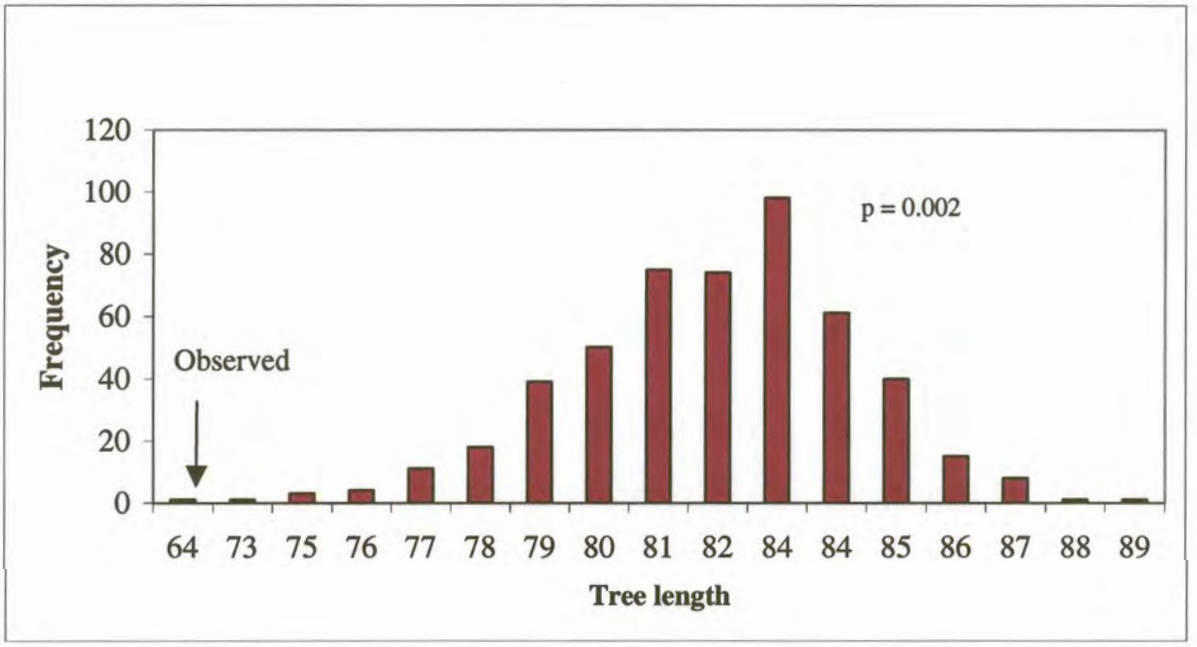

1.2
1
0.8
0.6
0.4
0.2
0
Genotypic diversity
1
2
3
4
5
6
7
8
Number of loci
Observed genotypic diversity value 0.96

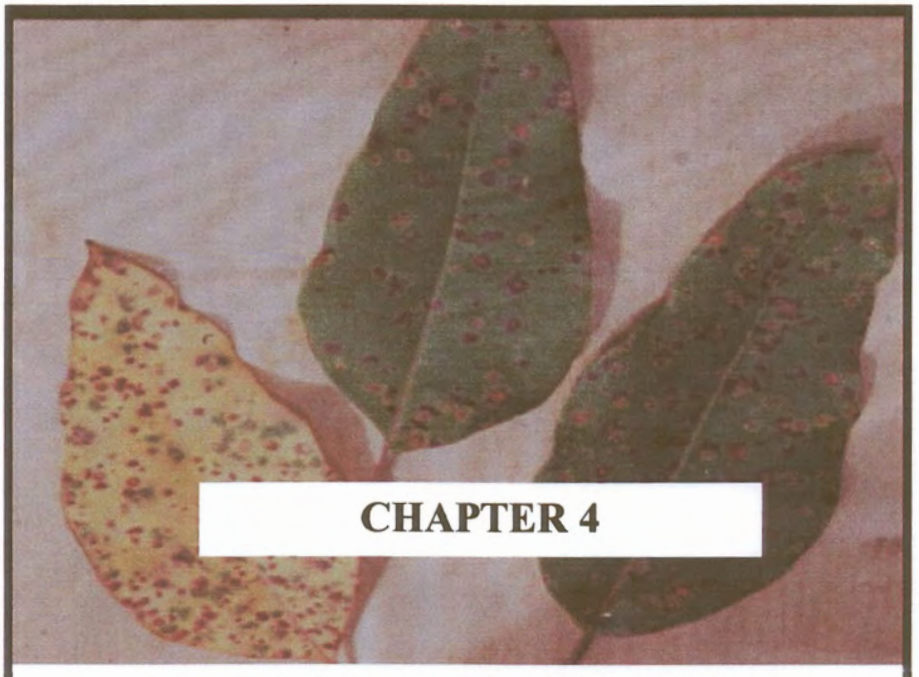
Figure 5. Histograms representing frequency distribution of pairwise distances among multiloci genotypes. **A)** Histogram representing the observed index of association for the Ugandan population, falling slightly beyond the distribution for randomised data sets ($P<0.001$, $I_A = 0.46$). **B)** Parsimony Tree Length Permutation Tests (PTLPT) based on 500 randomisation events. The tree length for the randomised data set is longer than that of the observed data. Both distributions indicate clonal reproduction in the Ugandan population of *C. albofundus*.

A


300
250
200
150
100
50
0
Frequency
p < 0.001
Observed = 0 .46
-0.45
-0.35
-0.25
-0.15
-0.05
0.05
0.15
0.2
0.3
0.4
0.5
0.6
Index of association

B


120
100
80
60
40
20
0
Frequency
p = 0.002
Observed
64
73
75
76
77
78
79
80
81
82
84
84
85
86
87
88
89
Tree length



CHAPTER 4

BACTERIAL BLIGHT OF *EUCALYPTUS* SPECIES IN UGANDA CAUSED BY *PANTOEA ANANATIS*.

Submitted as: Grace Nakabonge, Teresa Coutinho, Jolanda Roux & Michael Wingfield (2003) Bacterial blight of *Eucalyptus* species in Uganda. *Journal of Phytopathology.*

ABSTRACT

Dependency on exotic plantations for supplies of wood and wood products has increased significantly in the tropics and Southern Hemisphere. In Uganda, *Eucalyptus* species are amongst the most widely grown plantation trees, covering 40% of afforested areas. Diseases, however, reduce their growth rate, survival and the quality of products emerging from them. Most *Eucalyptus* diseases are caused by fungi, but bacterial pathogens are increasing in importance. During a disease survey in 2001, a leaf spot and blight disease was observed in Southern Uganda. Diseased trees showed leaf spots, lesions along the main leaf veins, premature abscission of the leaves, die-back and death. Isolations made from wilted shoots and leaves with water soaked lesions, consistently yielded bacterial isolates. The bacteria were characterized using Gram stain, Hugh-Leifson oxidation fermentation, oxidase and catalase tests as well as 16S rRNA gene sequences. The bacterium was identified as *Pantoea ananatis*, which is known to cause similar symptoms on *Eucalyptus* spp. in South Africa. This is the first record of bacterial blight caused by *P. ananatis* outside South Africa. The disease primarily damages young trees and it is thus of great concern in terms of plantation establishment. It will now be necessary to establish trials to select bacterial blight-tolerant planting stock.

INTRODUCTION

In Uganda, trees contribute to over 90% of the total energy consumed (Ruyooka 1999, Anonymous 2000a). *Eucalyptus* species, especially *Eucalyptus grandis* W. Hill, make up approximately 40% of afforested areas in the country (Ruyooka 1999). These plantations are owned by private companies, individual farmers as well as the Uganda Forest Department (Anonymous 2000a, Anonymous 2000b). The trees from these plantations are important for structural poles, transmission poles, fuelwood and sawn timber (Ruyooka 1999, Anonymous 2000a).

Diseases pose a significant threat to exotic plantation forestry. In Uganda, diseases such as Botryosphaeria canker and Ceratocystis wilt of *Eucalyptus* have already been recognised as serious threats to the emerging and important plantation forestry industry (Roux *et al.* 2001). This is similar to the situation in other countries, such as South Africa, where new diseases continue to be discovered and increase the overall costs of forestry. Despite intensive research, diseases such as Mycosphaerella leaf blotch caused by *Mycosphaerella* spp., Cryphonectria canker caused by *Cryphonectria cubensis* (Bruner) Hodges and Coniothyrium canker caused by *Coniothyrium zuluense* Wingfield, Crous & Coutinho continue to threaten exotic plantation forestry, especially in the Southern Hemisphere (Conradie, Swart & Wingfield 1990, Crous & Wingfield 1996, Wingfield, Crous & Coutinho 1997). Despite the importance of diseases in plantation forestry, little is known regarding the situation in Uganda.

Most serious diseases of exotic plantation trees are caused by fungal pathogens. However, in recent years a number of bacterial diseases have had a serious negative impact on *Eucalyptus* plantations. Bacterial wilt caused by *Ralstonia solanacearum* (synonyms *Pseudomonas solanacearum* and *Burkholderia solanacearum*) Yabuuchi *et al.* has, for example, caused damage to plantations in many parts of the world (Dianese 1986, Wu & Liang 1988, Akiew & Trevorrow 1994, Coutinho *et al.* 2000, Roux *et al.* 2000). Crown gall caused by *Agrobacterium tumefaciens* (Smith & Townsend 1907) Conn 1942 and shoot blight caused by *Xanthomonas campestris* (Pammel 1895) Dowson 1939 pv. *eucalypti* (Truman 1974) Dye 1978, are less common and are considered to be of minor importance (Wardlaw, Kile & Dianese 2000). The most recently described bacterial disease of *Eucalyptus* spp. is bacterial

blight caused by *Pantoea ananatis* Serrano (synonyms *Erwinia ananas* and *Erwinia uredovora*). It is currently only known from South Africa (Coutinho *et al.* 2002). Very little is known regarding this newly discovered pathogen, but its damage to young trees at establishment is of considerable concern (Coutinho *et al.* 2002).

During surveys of *Eucalyptus* diseases in Uganda, a leaf blight disease was commonly observed on young trees, in several areas of the country. The aim of this study was to identify the causal agent of this leaf disease. Use was made of both biochemical and molecular techniques to identify the pathogen and to compare it with isolates from South Africa.

MATERIALS AND METHODS

Disease incidence and isolations

In February 2001, a survey of *Eucalyptus* plantations was conducted in the Southern parts of Uganda to identify the distribution and cause of diseases of these trees. A leaf disease was identified on young trees with symptoms similar to those of bacterial blight in South Africa (Coutinho *et al.* 2002). Samples were collected from leaves of diseased *E. grandis* trees in Bweyogerere, Entebbe (central Uganda) and Tororo (eastern Uganda) (Figure 1, Chapter 2).

Isolations were made from wilted shoots and leaves with water soaked lesions after surface disinfestation of the tissue by dipping it in 70% ethanol and placing small leaf pieces on a drop of sterile water on a microscope slide. These samples were viewed under a phase contrast microscope (Nikon SMZ645) for bacterial exudates. Excised lesions from diseased leaves were macerated in 1ml of sterile distilled water using a mortar and pestle and the resulting macerate was streaked on to nutrient agar [3.0 g beef extract (Difco), 5.0 g peptone (Difco) and 15 g agar in 1000 ml H_2O]. Pure cultures were obtained from single bacterial colonies appearing from these primary isolations. Bacterial cultures were preserved using the Microbank™ Bacterial Preservation System according to the manufacturer's protocols (http://www.pro-lab.com/Microbank.html) (Prolab diagnostics, Canada). All isolates are stored at –70°C in the bacterial culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Physiological and Biochemical tests

Gram staining was performed using the Hucker's modification Gram stain protocol (http://biology.clc.uc.edu/Fankhauser/Labs/Microbiology/Gram_Stain/Gram_stain.htm). Hugh Leifson, oxidative and catalase tests were also conducted once pure bacterial cultures were obtained (Hugh & Leifson 1953, Richard 1994, Schaad, Jones & Chun 2001).

Molecular characterisation

DNA Extraction

Bacterial isolates were grown overnight in 5 ml nutrient broth (2.0 g Yeast extract, 5.0 g Peptone, 5.0 g NaCl in 1000 ml distilled water) (Biolab Diagnostics, Midrand, Johannesburg) and a pellet was obtained by centrifugation. Nucleic acids were extracted and purified as described by Hauben *et al.* (1998). DNA pellets were dried and suspended in 50 µl H_20. The concentration of nucleic acid extracts was estimated on a 2% agarose gel containing ethidium bromide, exposed to UV light.

Amplification of the 16S rRNA gene.

The polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene. The reaction was done in a volume of 25 µl comprising of a 2 ng DNA template, 800 µM dNTPs, 300 nM of universal forward primer (W3) (5'- AGA GTT TGA TCC TGG CTC AG- 3') and reverse primer (W4) (5' - AAG GAG GTG ATC CAG CCG CA -3') (Coutinho *et al.* 2002), 5U/µl Taq polymerase (Roche Diagnostics, Mannheim, Germany) and sterile distilled water (17.4 µl). The reaction consisted of an initial denaturation step at 94°C for 2 minutes. This was followed by 30 amplification cycles consisting of 1 min at 92°C and 30 seconds of annealing at 56°C. The PCR products were visualised under UV light on a 2% agarose gel containing Ethidium bromide, to determine the presence or absence of bands. The PCR products were purified using the High pure PCR product purification kit according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany).

The purified PCR products were ligated overnight into pGEM-T Easy vector at 10°C. The total volume was 10 µl, consisting of 2X–rapid buffer, T4 DNA ligase (5 µl), pGEM®-T Easy vector (50 ng) (1 µl), PCR product (3 µl), and T4 DNA ligase (1 µl) (Promega, Madison, USA). The ligation products were transformed by mixing 2 µl of

ligation product with 25 µl of competent *Escherichia coli* JM109 (Promega) in sterile Eppendorf tubes. These were kept on ice for 20 minutes, heat shocked for 45 seconds in a water bath (42°C), transferred to test tubes containing 475 µl Luria–Bertani (LB) broth (10 g Bacto®-Tryptone, 5 g Bacto®-Yeast Extract, 5 g NaCl) and incubated for 2 hrs at 37°C with shaking (~150 rpm). 150 µl of each transformation culture was incubated overnight at 37°C on LB medium [10 g Bacto®-Tryptone, 5 g Bacto®-Yeast Extract, 5 g NaCl, 80 µg X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside), 100 µg ampicillin]. The positive clones (without blue colour) were transferred to LB broth containing 100 µg ampicillin and incubated overnight at 37°C with shaking (~150 rpm). Plasmid DNA was recovered by alkaline lysis as described by Sambrook, Fritsch & Maniatis (1989). To release the insert (16S rRNA gene) and to determine its size, plasmid DNA was digested with *EcoR*1, run on an ethidium bromide stained 2% gel and visualized under UV illumination.

DNA sequencing and analysis

The 16S rRNA gene was sequenced from the undigested plasmid DNA. The reaction was done in a total volume of 10 µl consisting of 5x dilution buffer (1 µl), 4.5 µl H_2O, 2 µl DNA (50ng PCR product), 10x reaction mix (2 µl), ~ 2 pmol/µl of one of either reverse or forward universal primers, T7 and Sp6 (pGEM®-T Easy vector systems, Promega, Madison, USA) or one of the internal primers 0T1 (5' - GAA GAA GGC CTT CGG GTT G -3') and 0T2 (5'- CAC GAC ACG AGC TGA CGA C - 3') (Coutinho *et al.* 2002). The DNA was precipitated by addition of 2µl sodium acetate (3 M) and 50 µl of 100% ethanol. The mixture was agitated using a vortex mixer and centrifuged for 30 min (10000 rpm). The pellet was then washed with 70% ethanol and vacuum dried for 5 minutes. The PCR products were sequenced in both directions using the Big Dye Cycle Sequencing kit (Applied Biosystems, Foster City, California) on an ABI Prism™ 3100 DNA sequencer.

The 16S rRNA gene sequences were analyzed and edited using the Sequence Navigator programme (Applied Biosystems). The Blast program (National Center for Biotechnology Information, United States National Institutes of Health, Bethesda, MD) was used for homology searches using GenBank/EMBL databases (http://www.ncbi.nlm.nih.gov/BLAST/). An assortment of 16S rRNA gene sequences was obtained using blast searches. These were aligned with sequences obtained from

the Ugandan bacterial isolates using the program Clustal X (Thompson, Higgins & Gibson 1994).

Phylogenetic analysis

Phylogenetic analysis of aligned sequences was achieved by using PAUP 4.0b3 (Phylogenetic Analysis Using Parsimony) (Swofford 1998). Heuristic searches with maximum parsimony, using tree bisection-reconstruction and simple stepwise addition was used to produce phylogenetic trees. Gaps were treated as missing data. Support for branching was determined by performing 1000 bootstrap replicates (Felsenstein 1985). The tree was rooted to the outgroup taxon *Klebsiella pneumoniae* (Schroeter) Trevisan., which was treated as a monophyletic sister group to the other taxa (Coutinho *et al.* 2002).

RESULTS

Disease incidence and isolations

The disease was detected in all the areas surveyed (Table 1) on two to four-year-old *E. grandis* trees. Most severe damage was, however, observed in the Entebbe area (Wakiso district). Symptoms included tip die-back and leaf spots on young leaves. Leaf spots first appeared as water soaked lesions, which finally became corky. Infections appeared to begin from the leaf petioles spreading into the main veins and subsequently to adjacent tissue. In some cases, the infected trees appeared bush-like due to the formation of numerous new growth leader shoots (Figure 1).

Numerous rod shaped bacteria were observed streaming from leaf tissues macerated in sterile distilled water, on microscope slides. A total of 20 isolates, each obtained from different symptomatic trees, were used in physiological and biochemical tests. Four of these isolates (BCC0107, BCC0109, BCC0105, BCC0160) were subsequently selected for DNA-based characterisation (Table 2).

Physiological and biochemical tests

Isolates formed yellow-pigmented round colonies on nutrient agar. All cells were Gram negative, straight rods varying between 0.45 to 0.6 µm in width and 1.0 to 2.0 µm in length. All were catalase positive, oxidase negative, exhibiting a fermentative metabolism (Table 3). The physiological and biochemical characteristics confirmed

that the bacterial isolates belong to the family *Enterobacteriaceae* (*Erwinia – Pantoea* group).

Molecular characterization

PCR amplification and DNA Sequence analysis

The 16S rRNA gene was successfully amplified and all Ugandan isolates produced a ~1500bp fragment (Figure 2). The fragments were successfully cloned and sequenced. A blast search revealed that the Ugandan isolates were most similar to *Pantoea ananatis* (Z96081). The sequences obtained for isolates from Uganda were aligned with other related *Enterobacteriaceae* in the genus *Erwinia* and *Pantoea* obtained from Genebank (http://www.ncbi.nlm.gov). Manual alignment of these sequences resulted in a total of 1337 base pairs for final analysis (Figure 3).

Phylogenetic analysis of the aligned sequences resulted in 7 most parsimonious trees after 8686 rearrangements. A consistency index (CI) of 0.905 and retention index (RI) of 0.839 was obtained. The trees had the same topologies, differing only in the internal position of isolates. The total tree length obtained was 142. Of the 1337 characters, 1257 were constant, 51 parsimony uninformative and 29 parsimony informative. The most parsimonious tree had the same topology as the consensus tree and clearly showed that the Ugandan isolates group together with *P. ananatis* isolated from *Eucalyptus* in South Africa and with the type species isolated from pineapples (*P. ananatis*, Z96081). Isolates of *P. ananatis* (U80209 and U8019), which are hyperparasites on uredia of *Puccinia graminis* Pers.: Pers also grouped in this clade, separate from closely related species such as *P. agglomerans* (Kwon *et al.* 1997) (Figure 4).

DISCUSSION

In this study, we have established that the bacterium associated with bacterial blight and die-back on young *E. grandis* trees in Uganda is *Pantoea ananatis*. Given the association of this bacterium with a similar disease in South Africa (Coutinho *et al.* 2002), we believe that *P. ananatis* is the cause of the disease in Uganda. This represents the first report of *P. ananatis* associated with leaf and shoot blight of *Eucalyptus* spp., outside South Africa. This disease appears to be widespread in

Eucalyptus growing areas of Uganda and it is clearly an important constraint to tree establishment.

Using physiological and biochemical tests, we were able to identify Ugandan bacterial isolates only as belonging to the family *Enterobacteriaceae*. By sequencing the 16S rRNA gene, we were able to align the sequences from Ugandan isolates with those of other *Pantoea* species and related *Erwinia* species. DNA sequence data clearly showed that the Ugandan isolates are *P. ananatis*, making this the second report of bacterial blight of *Eucalyptus* caused by *P. ananatis* in the world.

The genus *Pantoea* contains a number of pathogenic speceis, including *P. stewartii* (Smith 1898) and *P. ananatis*. Before its establishment in 1989, these pathogens resided in *Erwinia* (Mergaert, Verdonck & Kersters 1993). *P. ananatis* Serrano (Synonyms: *Erwinia uredovora*, *Erwinia ananas*) was first described in 1928, where it was responsible for fruitlet rot on pineapples (Serrano 1928). Since then, there have been various reports of its occurrence on other crops. These include leaf blotch on sorghum (Azad, Holmes & Cooksey 2000), cantaloupe post harvest diseases (Bruton *et al.* 1991), honeydew melon rot (Wells *et al.* 1987) and bulb decay of onions (Gitaitis & Gay 1997). In 1998, it was reported as the cause of bacterial blight of *Eucalyptus* clones (Coutinho *et al.* 2002). The association of *P. ananatis* with disease is enigmatic, as it is commonly known as an epiphyte on the fungal smut pathogen *Ustilago maydis* and on the panicles of barley, buckwheat and rice (Watanabe, Kawakita & Sato 1996, Paccola-Meirelles *et al.* 2001).

In Uganda, bacterial blight has been reported in *Eucalyptus* plantation areas, previously occupied by natural forests. The plantations are also in close proximity to agricultural crops such as cassava, maize, beans and sweet potatoes. At the time of the survey in which this disease was discovered, damage was not observed on other crops. It is possible, however, that this bacterium originated on other crops which might act as alternative hosts. The possibility of seed transmission can also not be ruled out, since some farmers might have purchased *Eucalyptus* seed from suppliers in South Africa.

Since this disease report originates from a limited survey, more detailed surveys will be required to establish the relationships between *P. ananatis*, *Eucalyptus* trees,

agricultural crops and weeds growing in proximity. In this way a method of developing future recommendations for commercial activities, in the absence of disease losses might be developed.

The occurrence of bacterial blight on young *Eucalyptus* species may pose a serious problem to the Ugandan forestry industry. The disease leads to the stunting of trees and eventual death. The extent of losses in Uganda has not been quantified and detailed studies are needed in this regard. However, during the current survey, the levels of damage appeared to be heavy. In South Africa, for example, susceptible clones had to be removed from breeding programmes, as infection by *P. ananatis* resulted in severe stunting and malformation of trees, especially in nurseries (Coutinho *et al.* 2002).

Long-term control through the selection and development of clones resistant to bacterial blight shows the most potential for reducing the impact of bacterial blight of *Eucalyptus* spp. Matching trees to sites might also be considered, since trees planted on inappropriate sites are subject to severe stress, thus enhancing their chance of being affected by diseases. In cases where seedlings are used for propagation, care should also be taken to ensure that pathogen-free seeds are planted. This is especially since *P. ananatis* has been shown to be seed borne and seed-transmitted in onions and Sudangrass (Azad *et al.* 2000, Walcott *et al.* 2002). These strategies will ensure reduced losses due to bacterial blight and other diseases in Uganda.

REFERENCES

Akiew, E. & Trevorrow, P. R. (1994) Management of bacterial wilt. In *Bacterial wilt. A disease and its causative agent, Pseudomonas solanacearum*. (A. C. Hayward & G. L. Hartman, eds): 179-198. CAB International, Wallingford, Oxford, UK.

Anonymous (2000a) *National Biomass study, output for the period 1996-2000*. Uganda Forest Department, Ministry of Water, Lands and Environment, Kampala, Uganda. [http://www.ubos.org/abstractpdf/forestry.pdf.htm]

Anonymous (2000b) *The Uganda Forestry Policy*. Ministry of Water Lands and Environment, Kampala, Uganda.

Azad, H. R., Holmes, G. J. & Cooksey, D. A. (2000) A new leaf blotch disease of Sudangrass caused by *Pantoea ananas* and *Pantoea stewartii*. *Plant Disease* **84**: 973-979.

Bruton, B. D., Well, J. M., Lester, G. E. & Patterson, C. L. (1991) Pathogenicity and characterization of *Erwinia ananas* causing a post harvest disease of cantaloup fruit. *Plant Disease* **75**: 180-183.

Conradie, E., Swart, W. J. & Wingfield, M. J. (1990) Cryphonectria canker of *Eucalyptus*, an important disease in plantation forestry in South Africa. *South African Forestry Journal* **152**: 43-49.

Coutinho, T. A., Wingfield, M. J., Roux, J., Riedel, K-H. & Terblanche, J. (2000) First report of bacterial wilt caused by *Ralstonia solanacearum* on eucalypts in South Africa. *Forest Pathology* **30**: 205-210.

Coutinho, T. A., Preisig, O., Mergaert, J., Cnockaert, M. C., Riedel, K-H., Swings, J. & Wingfield, M. J. (2002) Bacterial blight and dieback of *Eucalyptus* species, hybrids and clones in South Africa. *Plant Disease* **86**: 20-25.

Crous, P. W. & Wingfield, M. J. (1996) Species of *Mycosphaerella* and their anamorphs associated with leaf blotch disease of *Eucalyptus* in South Africa. *Mycologia* **8**: 44-458.

Dianese, J. C. (1986) Problemas patológicas des florestas plantadas do vale do Rio Jari. *Fitopatologia Brasileira* **11**: 274-275.

Felsenstein, J. (1985) Confidence intervals on phylogenetics: an approach using bootstrap. *Evolution* **39**: 783-791.

Gitaitis, R. D. & Gay, J. D. (1997) The first report of a leaf blight, seed, stalk rot, and bulb decay of onion by *Pantoea ananas*. *Plant Disease* **81**: 1096.

Hauben, L., Moore, E. R. B., Vauterin, L., Steenackers, M., Mergaert, J., Verdonck, L. & Swings, J. (1998) Phylogenetic position of phytopathogens within the *Enterobacteriaceae*. *Systematics of Applied Microbiology* **21**: 384–397.

Hugh, R. & Leifson, E. (1953) The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram-negative bacteria. *Journal of Bacteriology* **66**: 24-26.

Kwon, S. W., Go, S-J., Kang, H-W., Ryu, J-C. & Jo, J-K. (1997) Phylogenetic analysis of *Erwinia* species based on 16S rRNA gene sequences. *International Journal of Systematic Bacteriology* **47**: 1061-1067.

Mergaert, J., Verdonck, L. & Kersters, K. (1993) Transfer of *Erwinia ananas* (synonym, *Erwinia uredovora*) and *Erwinia stewartii* to the Genus *Pantoea* emend. as *Pantoea ananas* (Serrano, 1928) comb. nov and *Pantoea stewartii* (Smith, 1898) comb. nov., respectively, and description of *Pantoea stewartii* subsp. indologenes subsp. Nov. *International Journal of Systematic Bacteriology* **43**: 162-173.

Paccola-Meirelles, L. D., Ferreira, A. S., Meirelles, W. F., Marriel, I. E. & Casela, C. R. (2001) Detection of a bacterium associated with leaf spot disease of maize in Brazil. *Journal of Phytopathology* **149**: 275-279.

Richard, C. (1994) Genus VI. *Enterobacter*. In *Bergey's manual of Systematic Bacteriology* (N. R. Krieg and J. G. Holt, eds): 465-469. The Williams & Wilkins Co., Baltimore.

Roux, J., Coutinho, T. A., Wingfield, M. J. & Bouillett, J. P. (2000) Diseases of plantation *Eucalyptus* in the Republic of Congo. *South African Journal of Science* **96**: 454-456.

Roux, J., Coutinho, T. A., Mujuni, Byabashaija & Wingfield, M. J. (2001) Diseases associated with plantation *Eucalyptus* in Uganda. *South African Journal of Science* **97**: 16-18.

Ruyooka, D. B. A. (1999) *Sowing the Eucalyptus seed a solution to household incomes*. Faculty of Forestry and Nature Conservation, Makerere University, Uganda.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schaad, N. W., Jones, J. B. & Chun, W. (2001) *Laboratory guide for identification of Plant pathogenic bacteria*, 3rd edn. The American Phytopathological Society, St Paul, Minnesota.

Serrano, F. B. (1928) Bacterial fruitless brown rot of pineapples in the Philippines. *Philippine Journal of Science* **36**: 271-305.

Swofford, D. L. (1998) PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4.0 Beta version. (Sinauer Associates): 128. Sunderland, Massachusetts.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673-4680.

Walcott, R. R., Gitaitis, R. D., Castro, A. C., Sanders, F. H. Jr. & Diaz-Perez, J. C. (2002) Natural infestation of onion seed by *Pantoea ananatis*, causal agent of soft rot. *Plant Disease* **86**: 106-111.

Wardlaw, T. J., Kile, G. A. & Dianese, J. C. (2000) Diseases of Eucalypts associated with viruses, phytoplasmas, bacteria and nematodes. In *Diseases and pathogens of eucalypts* (P. J. Keane, G. A. Kile, F. D. Podger & B. N. Brown, eds): 339-349. CSIRO publishing, Collingwood, Australia.

Watanabe, K., Kawakita, H. & Sato, M. (1996) Epiphytic bacterium, *Erwinia ananas*, commonly isolated from rice plants and brown planthoppers (*Nilaparvata lugens*) in hopperburn patches. *Applied Entomology and Zoology* **31**: 459-462.

Wells, J. M., Sheng., W-S., Ceponis, M. J. & Chen, T. A. (1987) Isolation and characterization of strains of *Erwinia ananas* from honeydew melons. *Phytopathology* **77**: 511-514.

Wingfield, M. J., Crous, P. W., & Coutinho, T. A. (1997) A serious canker disease of *Eucalyptus* in South Africa caused by a new species of *Coniothyrium*. *Mycopathologia* **136**: 139-145.

Wu, Q. P. & Liang, Z. C. (1988) Identification and pathogenic tests of the causal organism of the bacterial wilt of *Eucalyptus*. *Journal of South China Agricultural University* **9**: 59-67.

Table 1. Weather conditions and altitude of areas sampled[A].

Sampling area	Average temperature (°C)	Average rainfall (mm/yr^{-1})	Altitude (m)
Entebbe (Central Uganda)	21.5	1538	1155
Bweyogerere (Central Uganda)	21.5	1538	1155
Tororo (Eastern Uganda)	22.5	1427	1170
Kabale (Western Uganda)	15.3	1180	2241

[A]Information obtained from Uganda Meteorological Department, Entebbe.

Table 2. List of bacterial isolates used in this study.

Species	Host	Culture number	Origin	Accession No.
P. ananatis[a]	*E. grandis*	BCC0107	Entebbe, Uganda	AY173020
"	"	BCC0109	"	AY173023
"	"	BCC0105	"	AY173021
"	"	BCC0160	"	AY173022
"	*Eucalyptus* clones	LMG 20106	"	AF364844
"	"	LMG 20103	South Africa	AF364847
"	"	LMG 20104	South Africa	AF364846
"	"	LMG 20105	"	AF364845
"	*Ananas comosus*	LMG 2665	Brazil	Z96081
"	*Puccinia graminis f. sp. tritici*			U80209
"	*Puccinia graminis f. sp. tritici*			U8019
P. agglomerans				AJ251466
P. agglomerans				U80183
Klebsiella pneumoniae				Y17656

[a] Ugandan isolates characterized in this study

- Isolates designated by BCC are maintained in the bacterial culture collection at the Forestry and Agricultural Biotechnology Institute (FABI), Faculty of Biological and Agricultural Sciences, University of Pretoria, Pretoria, South Africa.
- Isolates designated by LMG are deposited in the culture collection at the Universiteit Gent, K. L. Ledegranckstraat 35, B-9000 Gent, Belgium.

Table 3. Characteristics of *P. ananatis* strains isolated from diseased *Eucalyptus* species from Uganda.

Characteristic	**Result**
Cell dimensions	0.45 to 0.6 x 1.0 to 2.0 µm
Colony characteristics	Entire, round
Pigmentation	Yellow
Mucoid growth	Yes
Cell morphology	Rods & motile
Gram stain	Negative
Hugh Leifson test	Fermentative
Oxidase test	Negative
Catalase	Positive

Figure 1. Symptoms associated with bacterial blight and dieback. **A)** Leaf spots, which appear water soaked in the initial stages and eventually become corky. **B)** Manifestation of lesions along the main leaf vein. The arrow shows bacteria exuding out of the leaf. **C)** Bushy appearance of an infected tree due to formation of numerous new shoots. **D)** Stunted *Eucalyptus* tree and death of main shoot.

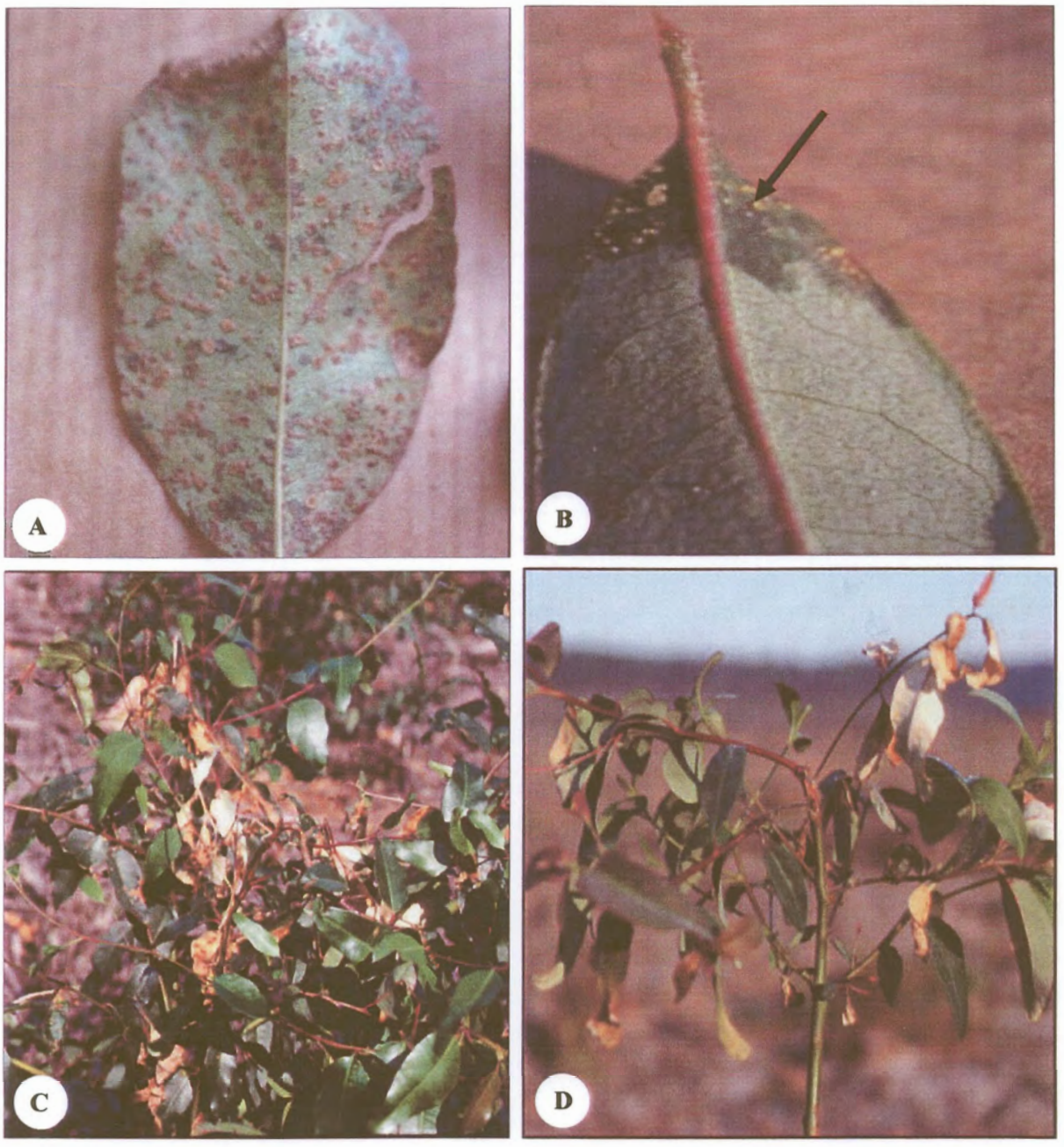


Figure 2. Gel profile (on a 2% agarose gel) obtained after amplifying the 16S rRNA gene. A fragment size of approximately 1500 was obtained.

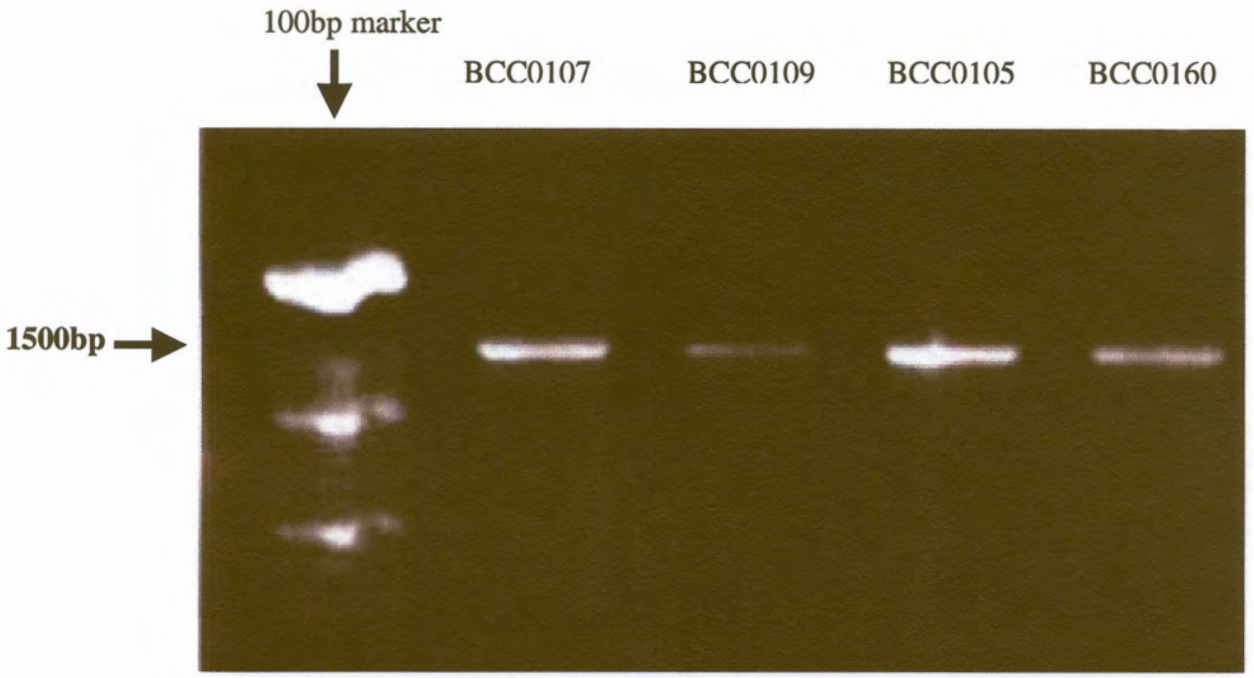


Figure 3. DNA sequence data for the partial 16S rRNA gene of Ugandan isolates, aligned against sequences of *Pantoea* species obtained from gene bank.
(- represents gaps, N represents missing data, . represents identical bases)

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                                    10         20         30         40         50
P.stewartii     (U80208)    GGGTGACGAG TGGCGGACGG GTGAGTAATG TCTGGGAAAC TGCCCGATGG
P.agglomerans   (U80183)    .......... .......... .......... ......G.T. ........A.
P.agglomerans   (AJ251466)  .......... .......... .......... ......G.T. ........A.
P.ananatis      (AF364844)  .......... .......... .......... ......G.T. ........A.
P.ananatis      (AF364845)  .......... .......... .......... ......G.T. ........A.
P.ananatis      (AF364847)  .......... .......... .......... ......G.T. ........A.
P.ananatis      (AF364846)  .......... .......... .......... ......G.T. ........A.
P.ananatis      (U80209)    .T........ .......... .......... ......G.T. ........A.
P.ananatis      (U8019)     .......... .......... .......... ......G.T. ........A.
P.ananatis      (Z96081)    .......... .......... .......... ......G.T. ........A.
P.ananatis      (AY173020)  .......... .......... .......... ......G.T. ........A.
P.ananatis      (AY173021)  .......... .......... .......... ......G.T. ........A.
P.ananatis      (AY173022)  .......... .......... .......... ......G.T. ........A.
P.ananatis      (AY173023)  .......... .......... .......... ......G.T. ........A.
K. pneumoniae   (Y17656)    .......... C......... .......... .......... ....T.....

                                    60         70         80         90        100
P.stewartii     (U80208)    AGGGGGATAA CTACTGGAAA CGGTAGCTAA TACCGCATAA CGTCGCAAGA
P.agglomerans   (U80183)    .......... .C........ ....G..... .......... ..........
P.agglomerans   (AJ251466)  .......... .C........ ....G..... .......... ..........
P.ananatis      (AF364844)  .......... .C........ ....G..... .......... ..........
P.ananatis      (AF364845)  .......... .C........ ....G..... .......... ..........
P.ananatis      (AF364847)  .......... .C........ ....G..... .......... ..........
P.ananatis      (AF364846)  .......... .C........ ....G..... .......... ..........
P.ananatis      (U80209)    .......... .C........ ....G..... .......... ..........
P.ananatis      (U8019)     .......... .C........ ....G..... .......... ..........
P.ananatis      (Z96081)    .......... .C........ ....G..... .......... ..........
P.ananatis      (AY173020)  .......... .C........ ....G..... .......... ..........
P.ananatis      (AY173021)  .......... .C........ ....G..... .......... ..........
P.ananatis      (AY173022)  .......... .C........ ....G..... .......... ..........
P.ananatis      (AY173023)  .......... .C........ ....G..... .......... ..........
K. pneumoniae   (Y17656)    .......... .......... .......... .......... ..........
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                                  110        120        130        140        150
P.stewartii      (U80208)    CCAAAGTGGG GGACCTCCGG GCCTCACACC ATCGGATGTG CCCAGATGGG
P.agglomerans    (U80183)    ......A... ......T... .....T...T ........AA ..........
P.agglomerans    (AJ251466)  ......A... ......T... .....T...T ........AA ..........
P.ananatis       (AF364844)  ......A... ......T... .....T...T ........AA ..........
P.ananatis       (AF364845)  ......A... ......T... .....T...T ........AA ..........
P.ananatis       (AF364847)  ......A... ......T... .....T...T ........AA ..........
P.ananatis       (AF364846)  ......A... ......T... .....T...T ........AA ..........
P.ananatis       (U80209)    ......A... ......T... .....T...T ........AA ..........
P.ananatis       (U8019)     ......A... ......T... .....T...T ........AA ..........
P.ananatis       (Z96081)    ......A... ......T... .....T...T ........AA ..........
P.ananatis       (AY173020)  ......A... ......T... .....T...T ........AA ..........
P.ananatis       (AY173021)  ......A... ......T... .....T...T ........AA ..........
P.ananatis       (AY173022)  ......A... ......T... .....T...T ........AA ..........
P.ananatis       (AY173023)  ......A... ......T... .....T...T ........AA ..........
K. pneumoniae    (Y17656)    .......... ......T... ......TG.. ...A...... ..........

                                  160        170        180        190        200
P.stewartii      (U80208)    ATTAGCTAGT AGGCGGGGTA ACGGCCCACC TAGGCGACGA TCCCTAGCTG
P.agglomerans    (U80183)    .......... .......... .T........ .......... ..........
P.agglomerans    (AJ251466)  .......... .......... .T........ .......... ..........
P.ananatis       (AF364844)  .......... .......... .......... .......... ..........
P.ananatis       (AF364845)  .......... .......... .......... .......... ..........
P.ananatis       (AF364847)  .......... .......... .......... .......... ..........
P.ananatis       (AF364846)  .......... .......... .......... .......... ..........
P.ananatis       (U80209)    .......... .......... .......... .......... ..........
P.ananatis       (U8019)     .......... .......... .......... .......... ..........
P.ananatis       (Z96081)    .......... .......... .......... .......... ..........
P.ananatis       (AY173020)  .......... .......... .......... .......... ..........
P.ananatis       (AY173021)  .......... .......... .......... .......... ..........
P.ananatis       (AY173022)  .......... .......... .......... .......... ..........
P.ananatis       (AY173023)  .......... .......... .......... .......... ..........
K. pneumoniae    (Y17656)    .......... ...T...... .....T.... .......... ..........
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                                    210        220        230        240        250
P.stewartii      (U80208)     GTCTGAGAGG ATGACCAGCC ACACTGGAAC TGAGACACGG TCCAGACTCC
P.agglomerans    (U80183)     .......G.. .......... .......... .......... .........-
P.agglomerans    (AJ251466)   .......... .......... .......... .......... ..........
P.ananatis       (AF364844)   .......... .......... .......... .......... ..........
P.ananatis       (AF364845)   .......... .......... .......... .......... ..........
P.ananatis       (AF364847)   .......... .......... .......... .......... ..........
P.ananatis       (AF364846)   .......... .......... .......... .......... ..........
P.ananatis       (U80209)     .......... .......... .......... .......... ..........
P.ananatis       (U8019)      .......... .......... .......... .......... ..........
P.ananatis       (Z96081)     .......... .......... .......... .......... ..........
P.ananatis       (AY173020)   .......... .......... .......... .......... ..........
P.ananatis       (AY173021)   .......... .......... .......... .......... ..........
P.ananatis       (AY173022)   .......... .......... .......... .......... ..........
P.ananatis       (AY173023)   .......... .......... .......... .......... ..........
K. pneumoniae    (Y17656)     .......... .......... .......... .......... ..........

                                    260        270        280        290        300
P.stewartii      (U80208)     TACGGGAGGC AGCAGTGGGG AATATTGCAC AATGGGCGCA AGCCTGATGC
P.agglomerans    (U80183)     .......... .......... .......... .......... ..........
P.agglomerans    (AJ251466)   .......... .......... .......... .......... ..........
P.ananatis       (AF364844)   .......... .......... .......... .......... ..........
P.ananatis       (AF364845)   .......... .......... .......... .......... ..........
P.ananatis       (AF364847)   .G........ .......... .......... .......... ..........
P.ananatis       (AF364846)   .......... .......... .......... .......... ..........
P.ananatis       (U80209)     .......... .......... .......... .......... ..........
P.ananatis       (U8019)      .......... .......... .......... .......... ..........
P.ananatis       (Z96081)     .......... .......... .......... .......... ..........
P.ananatis       (AY173020)   .......... .......... .......... .......... ..........
P.ananatis       (AY173021)   .......... .......... .......... .......... ..........
P.ananatis       (AY173022)   .......... .......... .......... .......... ..........
P.ananatis       (AY173023)   .......... .......... .......... .......... ..........
K. pneumoniae    (Y17656)     .......... .......... .......... .......... ..........
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                                  310        320        330        340        350
P.stewartii       (U80208)    AGCCATGCCG CGTGTATGAA GAAGGCCTTC GGGTTGTAAA GTACTTTCAG
P.agglomerans     (U80183)    .......... .......... .......... .......... ..........
P.agglomerans     (AJ251466)  .......... .......... .......... .......... ..........
P.ananatis        (AF364844)  .......... .......... .......... .......... ..........
P.ananatis        (AF364845)  .......... .......... .......... .......... ..........
P.ananatis        (AF364847)  .......... .......... .......... .......... ..........
P.ananatis        (AF364846)  .......... .......... .......... .......... ..........
P.ananatis        (U80209)    .......... .......... .......... .......... ..........
P.ananatis        (U8019)     .......... .......... .......... .......... ..........
P.ananatis        (Z96081)    .......... .......... .......... .......... ..........
P.ananatis        (AY173020)  .......... .......... .......... .......... ..........
P.ananatis        (AY173021)  .......... .......... .......... .......... ..........
P.ananatis        (AY173022)  .......... .......... .......... .......... ..........
P.ananatis        (AY173023)  .......... .......... .......... .......... ..........
K. pneumoniae     (Y17656)    .......... .....G.... .......... .......... .C........

                                  360        370        380        390        400
P.stewartii       (U80208)    C-GGGGAGGA AGGTGGT--G AGGTTAATAA CCTC-ATCAA TTGACATTAC
P.agglomerans     (U80183)    .-........ ...C.AC--. -......... ..CT-G..G. .....G....
P.agglomerans     (AJ251466)  .-........ ...C.A.--. G......... ...T-...G. .....G....
P.ananatis        (AF364844)  .-........ ...C.A.--. T......... ..G.-..TG. .....G....
P.ananatis        (AF364845)  .-........ ...C.A.--. T......... ..G.-G..G. .....G....
P.ananatis        (AF364847)  .-........ ...C.A.--. T......... ..G.-..TG. .....G....
P.ananatis        (AF364846)  .-........ ...C.A.-GT -......... ..G.-G.TG. .....G....
P.ananatis        (U80209)    .-........ ...C.A.--. T......... ..G.-..TG. .....G....
P.ananatis        (U8019)     .-........ ...C.A.--. T......... ..G.-..TG. .....G....
P.ananatis        (Z96081)    .-........ ...C.A.--. T......... ..G.-G..G. .....G....
P.ananatis        (AY173020)  .-........ ...C.A.--. T......... ..G.-G..G. .....G....
P.ananatis        (AY173021)  .-........ ...C.A.--. T......... ..G.-G..G. .....G....
P.ananatis        (AY173022)  .-........ ...C.A.--. T......... ..G.-G..G. .....G....
P.ananatis        (AY173022)  .-........ ...C.A.--. T......... ..G.-G..G. .....G....
K. pneumoniae     (Y17656)    .-........ ...C.A.--. ..............-...G. .....G....
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                                  410        420        430        440        450
P.stewartii      (U80208)    CCG-CAGAAG AAGCACCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG
P.agglomerans    (U80183)    ...-...... .......... .......... .......... ..........
P.agglomerans    (AJ251466)  ...-...... ....-..... .......... .......... ..........
P.ananatis       (AF364844)  ...-...... .......... .......... .......... ..........
P.ananatis       (AF364845)  ...-...... .......... .......... .......... ..........
P.ananatis       (AF364847)  ...-...... .......... .......... .......... ..........
P.ananatis       (AF364846)  ...-...... .......... .......... .......... ..........
P.ananatis       (U80209)    ...-...... .......... .......... .......... ..........
P.ananatis       (U8019)     ...-...... .......... .......... .......... ..........
P.ananatis       (Z96081)    ...-...... .......... .......... .......... ..........
P.ananatis       (AY173020)  ...-...... .......... .......... .......... ..........
P.ananatis       (AY173021)  ...-...... .......... .......... .......... ..........
P.ananatis       (AY173022)  ...-...... .......... .......... .......... ..........
P.ananatis       (AY173023)  ...-...... .......... .......... .......... ..........
K. pneumoniae    (Y17656)    ...-...... .......... .......... .......... ..........

                                  460        470        480        490        500
P.stewartii      (U80208)    GAGGGTGCAA GCGTTAATCG GAATTACTGG GCGTAAAGCG CACGCAGGCG
P.agglomerans    (U80183)    .......... .......... .......... .......... ..........
P.agglomerans    (AJ251466)  .......... .......... .......... .......... ..........
P.ananatis       (AF364844)  .......... .......... .......... .......... ..........
P.ananatis       (AF364845)  .......... .......... .......... .......... ..........
P.ananatis       (AF364847)  .......... .......... .......... .......... ..........
P.ananatis       (AF364846)  .......... .......... .......... .......... ..........
P.ananatis       (U80209)    .......... .......... .......... .......... ..........
P.ananatis       (U8019)     .......... .......... .......... .......... ..........
P.ananatis       (Z96081)    .......... .......... .......... .......... ..........
P.ananatis       (AY173020)  .......... .......... .......... .......... ..........
P.ananatis       (AY173021)  .......... .......... .......... .......... ..........
P.ananatis       (AY173022)  .......... .......... .......... .......... ..........
P.ananatis       (AY173023)  .......... .......... .......... .......... ..........
K. pneumoniae    (Y17656)    .......... .......... .......... .......... ..........
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                                  510        520        530        540        550
P.stewartii      (U80208)    GTCTGTTAAG TCAGATGTGA AATCCCCGGG CTTAACCTGG GAACTGCATT
P.agglomerans    (U80183)    .......... .......... .......... .......... ..........
P.agglomerans    (AJ251466)  .......... .......... .......... .......... ..........
P.ananatis       (AF364844)  .......... .......... .......... .......... ..........
P.ananatis       (AF364845)  .......... .......... .......... .......... ..........
P.ananatis       (AF364847)  .......... .......... .......... .......... ..........
P.ananatis       (AF364846)  .......... .......... .......... .......... ..........
P.ananatis       (U80209)    .......... .......... .......... .......... ..........
P.ananatis       (U8019)     .......... .......... .......... .......... ..........
P.ananatis       (Z96081)    .......... .......... .......... .......... ..........
P.ananatis       (AY173020)  .......... .......... .......... .......... ..........
P.ananatis       (AY173021)  .......... .......... .......... .......... ..........
P.ananatis       (AY173022)  .......... .......... .......... .......... ..........
P.ananatis       (AY173023)  .......... .......... .......... .......... ..........
K. pneumoniae    (Y17656)    ......C... ..G..... .......... ..C..... ..........

                                  560        570        580        590        600
P.stewartii      (U80208)    TGAAACTGGC AGGCTTGAGT CTCGTAGAGG GGGGTAGAAT TCCAGGTGTA
P.agglomerans    (U80183)    .......... .......... ..T..... .......... ..........
P.agglomerans    (AJ251466)  .......... .......... ..T..... .......... ..........
P.ananatis       (AF364844)  .......... .......... .......... .......... ..........
P.ananatis       (AF364845)  .......... .......... .......... .......... ..........
P.ananatis       (AF364847)  .......... .......... .......... .......... ..........
P.ananatis       (AF364846)  .......... .......... .......... .......... ..........
P.ananatis       (U80209)    .......... .......... .......... .......... ..........
P.ananatis       (U8019)     .......... .......... .......... .......... ..........
P.ananatis       (Z96081)    .......... .......... .......... .......... ..........
P.ananatis       (AY173020)  .......... .......... .......... .......... ..........
P.ananatis       (AY173021)  .......... .......... .......... .......... ..........
P.ananatis       (AY173022)  .......... .......... .......... .......... ..........
P.ananatis       (AY173023)  .......... .......... .......... .......... ..........
K. pneumoniae    (Y17656)    C......... .....A.... ..T..... .......... ..........
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                                    610        620        630        640        650
P.stewartii     (U80208)        GCGGTGAAAT GCGTAGAGAT CTGGAGGAAT ACCGGTGGCG AAGGCGGTCC
P.agglomerans   (U80183)        .......... .......... .......... .......... .......C..
P.agglomerans   (AJ251466)       .......... .......... .......... .......... .......C..
P.ananatis      (AF364844)       .......... .......... .......... .......... .......C..
P.ananatis      (AF364845)       .......... .......... .......... .......... ..........
P.ananatis      (AF364847)       .......... .......... .......... .......... ..........
P.ananatis      (AF364846)       .......... .......... .......... .......... ..........
P.ananatis      (U80209)         .......... .......... .......... .......... .......C..
P.ananatis      (U8019)          .......... .......... .......... .......... .......C..
P.ananatis      (Z96081)         .......... .......... .......... .......... .......C..
P.ananatis      (AY173020)       .......... .......... .......... .......... .......C..
P.ananatis      (AY173021)       .......... .......... .......... .......... .......C..
P.ananatis      (AY173022)       .......... .......... .......... .......... .......C..
P.ananatis      (AY173023)       .......... .......... .......... .......... .......C..
K. pneumoniae   (Y17656)         .......... .......... .......... .......... .......C..

                                    660        670        680        690        700
P.stewartii     (U80208)        CCTGGACGAA GACTGACGCT CAGGTGCGAA AGCGTGGGGA GCAAACAGGA
P.agglomerans   (U80183)        .......A.. .......... .......... .......... ..........
P.agglomerans   (AJ251466)       .......A.. .......... .......... .......... ..........
P.ananatis      (AF364844)       .......... .......... .......... .......... ..........
P.ananatis      (AF364845)       .......... .......... .......... .......... ..........
P.ananatis      (AF364847)       .......... .......... .......... .......... ..........
P.ananatis      (AF364846)       .......... .......... .......... .......... ..........
P.ananatis      (U80209)         .......... .......... .......... .......... ..........
P.ananatis      (U8019)          .......... .......... .......... .......... ..........
P.ananatis      (Z96081)         .......... .......... .......... .......... ..........
P.ananatis      (AY173020)       .......A.. .......... .......... .......... ..........
P.ananatis      (AY173021)       .......A.. .......... .......... .......... ..........
P.ananatis      (AY173022)       .......A.. .......... .......... .......... ..........
P.ananatis      (AY173023)       .......A.. .......... .......... .......... ..........
K. pneumoniae   (Y17656)         .......A.. .......... .......... .......... ..........
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                                    710        720        730        740        750
P.stewartii     (U80208)    TTAGATACCC TGGTAGTCCA CGCCGTAAAC GATGTCGACT TGGAGGTTGT
P.agglomerans   (U80183)    .......... .......... .......... .......... ..........
P.agglomerans   (AJ251466)  .......... .......... .......... .......... ..........
P.ananatis      (AF364844)  .......... .......... .......... .......... ..........
P.ananatis      (AF364845)  .......... .......... .......... .......... ..........
P.ananatis      (AF364847)  .......... .......... .......... .......... ..........
P.ananatis      (AF364846)  .......... .......... .......... .......... ..........
P.ananatis      (U80209)    .......... .......... .......... .......... ..........
P.ananatis      (U8019)     .......... .......... .......... .......... ..........
P.ananatis      (Z96081)    .......... .......... .......... .......... ..........
P.ananatis      (AY173020)  .......... .......... .......... .......... ..........
P.ananatis      (AY173021)  .......... .......... .......... .......... ..........
P.ananatis      (AY173022)  .......... .......... .......... .......... ..........
P.ananatis      (AY173023)  .......... .......... .......... .......... ..........
K. pneumoniae   (Y17656)   .......... .......... .......... ........T. ..........

                                    760        770        780        790        800
P.stewartii     (U80208)    TCCCTTGAGG AGTGGCTTCC GGAGCTAACG CGTTAAGTCG ACCGCCTGGG
P.agglomerans   (U80183)    .......... .......... .......... .......... ..........
P.agglomerans   (AJ251466)  .......... .......... .......... .......... ..........
P.ananatis      (AF364844)  .......... .......... .......... .......... ..........
P.ananatis      (AF364845)  .......... .......... .......... .......... ..........
P.ananatis      (AF364847)  .......... .......... .......... .......... ..........
P.ananatis      (AF364846)  .......... .......... .......... .......... ..........
P.ananatis      (U80209)    .......... .......... .......... .......... ..........
P.ananatis      (U8019)     .......... .......... .......... .......... ..........
P.ananatis      (Z96081)    .......... .......... .......... .......... ..........
P.ananatis      (AY173020)  .......... .......... .......... .......... ..........
P.ananatis      (AY173021)  .......... .......... .......... .......... ..........
P.ananatis      (AY173022)  .......... .......... .......... .......... ..........
P.ananatis      (AY173023)  .......... .......... .......... .......... ..........
K. pneumoniae   (Y17656)   G......... C......... .......... ......A... ..........
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                                  810        820        830        840        850
P.stewartii     (U80208)     GAGTACGGCC GCAAGGTTAA AACTCAAATG AATTGACGGG GGCCCGCACA
P.agglomerans   (U80183)     .......... .......... .......... .......... ..........
P.agglomerans   (AJ251466)   .......... .......... .......... .......... ..........
P.ananatis      (AF364844)   .......... .......... .......... .......... ..........
P.ananatis      (AF364845)   .......... .......... .......... .......... ..........
P.ananatis      (AF364847)   .......... .......... .......... .......... ..........
P.ananatis      (AF364846)   .......... .......... .......... .......... ..........
P.ananatis      (U80209)     .......... .......... .......... .......... ..........
P.ananatis      (U8019)      .......... .......... .......... .......... ..........
P.ananatis      (Z96081)     .......... .......... .......... .......... ..........
P.ananatis      (AY173020)   .......... .......... .......... .......... ..........
P.ananatis      (AY173021)   .......... .......... .......... .......... ..........
P.ananatis      (AY173022)   .......... .......... .......... .......... ..........
P.ananatis      (AY173023)   .......... .......... .......... .......... ..........
K. pneumoniae   (Y17656)     .......... .......... .......... .......... ..........

                                  860        870        880        890        900
P.stewartii     (U80208)     AGCGGTGGAG CATGTGGTTT AATTCGATGC AACGCGAAGA ACCTTACCTA
P.agglomerans   (U80183)     .......... .......... .......... .......... ..........
P.agglomerans   (AJ251466)   .......... .......... .......... .......... ..........
P.ananatis      (AF364844)   .......... .......... .......... .......... ..........
P.ananatis      (AF364845)   .......... .......... .......... .......... ..........
P.ananatis      (AF364847)   .......... .......... .......... .......... ..........
P.ananatis      (AF364846)   .......... .......... .......... .......... ..........
P.ananatis      (U80209)     .......... ...A...... .......... .......... ..........
P.ananatis      (U8019)      .......... ...A...... .......... .......... ..........
P.ananatis      (Z96081)     .......... .......... .......... .......... ..........
P.ananatis      (AY173020)   .......... .......... .......... .......... ..........
P.ananatis      (AY173021)   .......... .......... .......... .......... ..........
P.ananatis      (AY173022)   .......... .......... .......... .......... ..........
P.ananatis      (AY173023)   .......... .......... .......... .......... ..........
K. pneumoniae   (Y17656)     .......... .......... .......... .......... .........G
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                                      910        920        930        940        950
P.stewartii     (U80208)      CTCTTGACAT CCAGCGAACT TGGC----AG AGATGCCTTG GTGCCTTC-G
P.agglomerans   (U80183)      .......... ...CG...T. ....----.. .........A ........-.
P.agglomerans   (AJ251466)    .......... ...-G...T. ....----.. .........A ........-.
P.ananatis      (AF364844)    .......... ....A..... .A..----.. ......T... ........-.
P.ananatis      (AF364845)    .......... ....A..... .A..----.. ......T... ........-.
P.ananatis      (AF364847)    .......... ....A..... .A..----.. ......T... ........-.
P.ananatis      (AF364846)    .......... ....A..... .A..----.. ......T... ........-.
P.ananatis      (U80209)      .......... ....A..... .A..----.. ......T... ........-.
P.ananatis      (U8019)       .......... ....A..... .A..----.. ......T... ........-.
P.ananatis      (Z96081)      .......... ....A..... ....----.. ......T... ........-.
P.ananatis      (AY173020)    .......... ....A..... .A..----.. ......T... ........-.
P.ananatis      (AY173021)    .......... ....A..... .A..----.. ......T... ........-.
P.ananatis      (AY173022)    .......... ....A..... .A..----.. ......T... ........-.
P.ananatis      (AY173023)    .......... ....A..... .A..----.. ......T... ........-C
K. pneumoniae   (Y17656)      G......... ...CA..... .TC.----.. .....GA... ........-.

                                      960        970        980        990       1000
P.stewartii     (U80208)      GGAACGCTGA GACAGGTGCT GCATGGCTGT CGTCAGCTCG TGTTGTGAAA
P.agglomerans   (U80183)      ..G..CG... .......... .......... .......... ..........
P.agglomerans   (AJ251466)    .....CG... .......... .......... .......... ..........
P.ananatis      (AF364844)    .....T.... .......... .......... .......... ..........
P.ananatis      (AF364845)    ...G.T.... .......... .......... .......... ..........
P.ananatis      (AF364847)    .....T.... .......... .......... .......... ..........
P.ananatis      (AF364846)    .....T.... .......... .......... .......... ..........
P.ananatis      (U80209)      .....T.... .......... .......... .......... ..........
P.ananatis      (U8019)       .....T.... .......... .......... .......... ..........
P.ananatis      (Z96081)      ...G.C.... .......... .......... .......... ..........
P.ananatis      (AY173020)    .....C.... .......... .......... .......... ..........
P.ananatis      (AY173021)    .....C.... .......... .......... .......... ..........
P.ananatis      (AY173022)    .....C.... .......... .......... .......... ..........
P.ananatis      (AY173023)    .....C.... .......... .......... .......... ..........
K. pneumoniae   (Y17656)      .....TG... .......... .......... .......... ..........
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                                  1010       1020       1030       1040       1050
P.stewartii       (U80208)    TGTTGGGTTA AGTCCCGCAA CGAGCGCAAC CCTTATCCTT TGTTGCCAGC
P.agglomerans     (U80183)    .......... .......... .......... .......... ..........
P.agglomerans     (AJ251466)  .......... .......... .......... .......... ..........
P.ananatis        (AF364844)  .......... .......... .......... .......... ..........
P.ananatis        (AF364845)  .......... .......... .......... .......... ..........
P.ananatis        (AF364847)  .......... .......... .......... .......... ..........
P.ananatis        (AF364846)  .......... .......... .......... .......... ..........
P.ananatis        (U80209)    .......... .......... .......... .......... ..........
P.ananatis        (U8019)     .......... .......... .......... .......... ..........
P.ananatis        (Z96081)    .......... .......... .......... .......... ..........
P.ananatis        (AY173020)  .......... .......... .......... .......... ..........
P.ananatis        (AY173021)  .......... .......... .......... .......... ..........
P.ananatis        (AY173022)  .......... .......... .......... .......... ..........
P.ananatis        (AY173023)  .......... .......... .......... .......... ..........
K. pneumoniae     (Y17656)    .......... .......... .......... .......... ..........

                                  1060       1070       1080       1090       1100
P.stewartii       (U80208)    GATTCGGTCG GGAACTCAAA GGAGACTGCC GGTGATAAAC CGGAGGAAGG
P.agglomerans     (U80183)    .......... .......... .......... .......... ..........
P.agglomerans     (AJ251466)  .......... .......... .......... .......... ..........
P.ananatis        (AF364844)  .......... .......... .......... .......... ..........
P.ananatis        (AF364845)  .......... .......... .......... .......... ..........
P.ananatis        (AF364847)  .......... .......... .......... .......... ..........
P.ananatis        (AF364846)  .......... .......... .......... .......... ..........
P.ananatis        (U80209)    .......... .......... .......... .......... ..........
P.ananatis        (U8019)     .......... .......... .......... .......... ..........
P.ananatis        (Z96081)    .......... .......... .......... .......... ..........
P.ananatis        (AY173020)  .......... .......... .......... .......... ..........
P.ananatis        (AY173021)  .......... .......... .......... .......... ..........
P.ananatis        (AY173022)  .......... .......... .......... .......... ..........
P.ananatis        (AY173023)  .......... .......... .......... .......... ..........
K. pneumoniae     (Y17656)    .G..A..C.. .......... .......... A......... T.........
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                                1110       1120       1130       1140       1150
P.stewartii      (U80208)   TGGGGATGAC GTCAAGTCAT CATGGCCCTT ACGAGTAGGG CTACACACGT
P.agglomerans    (U80183)   .......... .......... .......... .......... ..........
P.agglomerans    (AJ251466) .......... .......... .......... .......... ..........
P.ananatis       (AF364844) .......... .......... .......... .......... ..........
P.ananatis       (AF364845) .......... .......... .......... .......... ..........
P.ananatis       (AF364847) .......... .......... .......... .......... ..........
P.ananatis       (AF364846) .......... .......... .......... .......... ..........
P.ananatis       (U80209)   .......... .......... .......... .......... ..........
P.ananatis       (U8019)    .......... .......... .......... .......... ..........
P.ananatis       (Z96081)   .......... .......... .......... .......... ..........
P.ananatis       (AY173020) .......... .......... .......... .......... ..........
P.ananatis       (AY173021) .......... .......... .......... .......... ..........
P.ananatis       (AY173022) .......... .......... .......... .......... ..........
P.ananatis       (AY173023) .......... .......... .......... .......... ..........
K. pneumoniae    (Y17656)   .......... .......... .......... ....CC.... ..........

                                1160       1170       1180       1190       1200
P.stewartii      (U80208)   GCTACAATGG CGCATA-CAA AGAGAAGCGA CCTCGCGAGA GCAAGCGGAC
P.agglomerans    (U80183)   .......... ......-... ........A. .......... ..........
P.agglomerans    (AJ251466) .......... ......-... .......... .......... ..........
P.ananatis       (AF364844) .......... ......-... .......... .......... ..........
P.ananatis       (AF364845) .......... ......-... .......... .......... ..........
P.ananatis       (AF364847) .......... ......-... .......... .......... ..........
P.ananatis       (AF364846) .......... ......-... .......... .......... ..........
P.ananatis       (U80209)   .......... ......-... .......... .......... ..........
P.ananatis       (U8019)    .......... ......-... .......... .......... ..........
P.ananatis       (Z96081)   .......... ......-... .......... .......... ..........
P.ananatis       (AY173020) .......... ......-... .......... .......... ..........
P.ananatis       (AY173021) .......... ......-... .......... .......... ..........
P.ananatis       (AY173022) .......... ......-... .......... .......... ..........
P.ananatis       (AY173023) .......... ......-... .......... .......... ..........
K. pneumoniae    (Y17656)   .......... .AT...-... .......... .......... ..........
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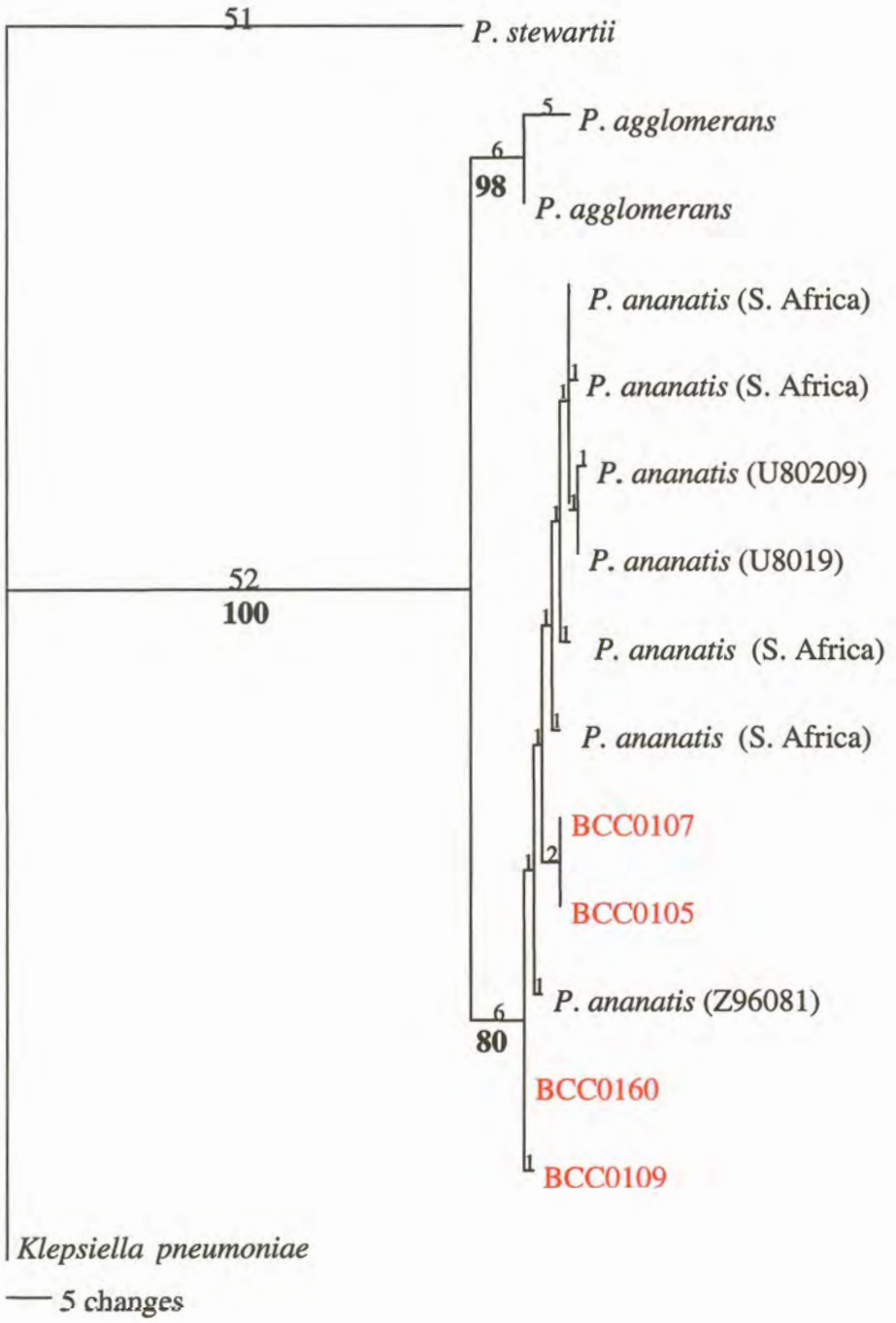
                                1210       1220       1230       1240       1250
P.stewartii       (U80208)      CTCATAAAGT GCGTCGTAGT CCGGATCGGA GTCTGCAACT CGACTCCGTG
P.agglomerans     (U80183)      ....C..... .......... .......... .......... ..........
P.agglomerans     (AJ251466)    ....C..... .......... .......... .......... ..........
P.ananatis        (AF364844)    .......... .......... .......... .......... ..........
P.ananatis        (AF364845)    .......... .......... .......... .......... ..........
P.ananatis        (AF364847)    .......... .......... .......... .......... ..........
P.ananatis        (AF364846)    .......... .......... .......... .......... ..........
P.ananatis        (U80209)      .......... .......... .......... .......... ..........
P.ananatis        (U8019)       .......... .......... .......... .......... ..........
P.ananatis        (Z96081)      .......... .......... .......... .......... ..........
P.ananatis        (AY173020)    ....C..... .......... .......... .......... ..........
P.ananatis        (AY173021)    ....C..... .......... .......... .......... ..........
P.ananatis        (AY173022)    .......... .......... .......... .......... ..........
P.ananatis        (AY173023)    .......... .......... .......... .......... ..........
K. pneumoniae     (Y17656)      .......... AT........ ......T... .......... .......A..

                                1260       1270       1280       1290       1300
P.stewartii       (U80208)      AAGTCGGAAT CGCTA-GTAA TCGTGGATCA GAATGCCACG GTGAATACGT
P.agglomerans     (U80183)      .......... .....-.... .......... .......... ..........
P.agglomerans     (AJ251466)    .......... .....-.... .......... .......... ..........
P.ananatis        (AF364844)    .......... .....-.... .......... .......... ..........
P.ananatis        (AF364845)    .......... .....-.... .......... .......... ..........
P.ananatis        (AF364847)    .......... .....-.... .......... .......... ..........
P.ananatis        (AF364846)    .......... .....-.... .......... .......... ..........
P.ananatis        (U80209)      .......... .....-.... .......... .......... ..........
P.ananatis        (U8019)       .......... .....-.... .......... .......... ..........
P.ananatis        (Z96081)      .......... .....-.... .......... .......... ..........
P.ananatis        (AY173020)    .......... .....-.... .......... .......... ..........
P.ananatis        (AY173021)    .......... .....-.... .......... .......... ..........
P.ananatis        (AY173022)    .......... .....-.... .......... .......... ..........
P.ananatis        (AY173023)    .......... .....-.... .......... .......... ..........
K. pneumoniae     (Y17656)      .......... .....-.... ....A..... ......T... ..........
```

		1310	1320	1330	1340
P.stewartii	(U80208)	TCCCGGGCCT	TGTACACACC	GCCCGTCACA	CCATGGG
P.agglomerans	(U80183)	....	....	....	....
P.agglomerans	(AJ251466)	....	....	....	....
P.ananatis	(AF364844)	....	....	....	....
P.ananatis	(AF364845)	....	....	....	....
P.ananatis	(AF364847)	....	....	....	....
P.ananatis	(AF364846)	....	....	....	....
P.ananatis	(U80209)	....	....	....	....
P.ananatis	(U8019)	....	....	....	....
P.ananatis	(Z96081)	....	....	....	....
P.ananatis	(AY173020)	....	....	....	....
P.ananatis	(AY173021)	....	....	....	....
P.ananatis	(AY173022)	....	....	....	....
P.ananatis	(AY173023)	....	....	....	....
K. pneumoniae	(Y17656)	....	....	....	....

Figure 4. One of the 7 most parsimonious phylogenetic trees obtained after aligning the 16S rRNA gene for *P. ananatis*. *P. stewartii*, *P. agglomerans* and Ugandan isolates (in red). *Klepsiella pneumoniae* is the out-group taxon, rooted at internal nodes as a monophyletic sister group to ingroup. Total length of the tree = 142, CI = 0.905, RI = 0.839. Bootstrap values are typed in bold and appear below the branches.


51
P. stewartii
5
P. agglomerans
6
98
P. agglomerans
P. ananatis (S. Africa)
1
P. ananatis (S. Africa)
1
P. ananatis (U80209)
1
P. ananatis (U8019)
52
100
P. ananatis (S. Africa)
1
P. ananatis (S. Africa)
BCC0107
2
BCC0105
1
P. ananatis (Z96081)
6
80
BCC0160
1
BCC0109
Klepsiella pneumoniae
5 changes

SUMMARY

Plantation forestry is important to Uganda as it represents a future resource of timber for fuel and structural purposes. Diseases, however, pose a serious threat to the productivity and sustainability of this emerging industry. A number of serious diseases have already been reported from surveys recently conducted in Uganda. The aim of the present study was to expand on the current knowledge of some of the diseases and to gain additional information regarding their causal agents.

The first chapter of this thesis reviewed the literature pertaining to diseases of plantation trees in Uganda. The observation is made that diseases were recognised as problematic, as early as the 1950's and trials were conducted at that time to select disease resistant planting stock. Due to political unrest, these initiatives ceased and a reliance on natural forests for the supply of wood and wood products persisted. Over exploitation of natural forests has now resulted in awareness that this resource is threatened and plantation forestry is widely recognised as the only alternative for the supply of wood and wood products. The Uganda Forestry Department is now actively promoting the establishment of plantations to reduce destruction of natural forests. This initiative has included surveys to identify the diseases affecting forest plantations in Uganda. These surveys have shown that diseases pose a serious threat to the establishment of plantation forests in the country.

Botryosphaeria canker is the most common disease of plantation *Eucalyptus* in Uganda. In chapter two of this thesis, I have shown that *B. parva*, *L. theobromae* and an undescribed species are associated with Botryosphaeria canker of *Eucalyptus* spp. in Uganda. Pathogenicity trials revealed statistically significant differences in virulence between different species, with *L. theobromae* being the least pathogenic and the undescribed species the most pathogenic. Further investigations are now needed to evaluate the pathogenicity of these fungi in the field and to consider their biology and relative importance.

Ceratocystis albofundus is an important pathogen of *Acacia mearnsii* and *A. decurrens* in South Africa. In 1999, *C. albofundus* was reported for the first time in south western Uganda. The results obtained in the current study indicate that the Ugandan population has high gene diversity similar to that of a South African

population. The results further show that the fungus reproduces clonally in both countries, with very little gene flow occurring between them. The high gene diversity values obtained in this study indicate that *C. albofundus* is native to Africa and not only South Africa, as previously hypothesized. The high gene diversity of *C. albofundus* revealed within the two populations gives a clear indication that management of Ceratocystis wilt should focus on breeding for resistance. Selection of resistant varieties is in progress in South Africa and in future, it will be useful in management of the disease.

Most *Eucalyptus* diseases are caused by fungi, but bacterial pathogens are increasing in importance. In the fourth chapter of this thesis, a bacterium known as *Pantoea ananatis* was identified as the causal agent of blight and dieback on *Eucalyptus* spp. in Uganda. This is the first report of the disease outside South Africa. The disease primarily damages young trees and it is thus of great concern in terms of plantation establishment. It will now be necessary to establish trials to select bacterial blight-tolerant planting stock. The bacterium has also been reported to infect agricultural crops which is especially important in the Ugandan situation, where trees are grown in close proximity to agricultural crops.

The results obtained in this thesis will help to manage diseases affecting plantation forest species in Uganda. The study will hopefully also provide farmers and foresters with an elevated understanding of the importance of diseases in plantation forestry. This should impact strongly on the capacity of Ugandans to deal with diseases, not only those caused by the pathogens identified in the study, but also other diseases, particularly of forest and fruit crops.

OPSOMMING

Plantasiebosbou is belangrik in Uganda, omdat dit 'n toekomstige bron van hout vir energie en strukturele behoeftes bied. Siektes is egter 'n ernstige bedreiging vir die produktiwiteit en volhoubaarheid van hierdie opkomende industrie. 'n Aantal ernstige siektes is alreeds aangemeld tydens vorige opnames wat in Uganda gedoen is. Die doelwit van die huidige studie is om die kennis oor sommige van hierdie siektes te verbreed en om verdere inligting oor siekteveroorsakende agente te verkry.

Die eerste hoofstuk van hierdie verhandeling gee 'n oorsig van die literatuur i.v.m. die siektes van plantasiespesies in Uganda. Dit word uitgewys dat siektes as problematies beskou is van so vroeg as die 1950's en dat proewe gedoen is om siekteweerstandbiedende plantmateriaal te selekteer. As gevolg van politieke oproer is hierdie inisiatiewe gestaak en het die land afhanklik gebly van natuurlike woude vir die verskaffing van hout en houtprodukte. Huidiglik is daar 'n bewuswording dat oorbenutting die voortbestaan van natuurlike woude bedrieg, en dat plantasiebosbou die enigste alternatief bied vir die verskaffing van hout en houtprodukte. Die Uganda Bosbou Departement promofeer nou aktief die vestiging van plantasies om die vernietiging van natuurlike woude te verminder. Hierdie inisiatiewe sluit in pogings om die siektes wat bosbouplantasies in Uganda beïnvloed, te identifiseer. Vroër het ondersoeke het getoon dat siektes 'n ernstige bedreiging inhou vir die vestiging van plantasiebosbou in die land.

Botryosphaeria kanker is die mees algemene siekte van *Eucalyptus* spp. in Uganda. In hoofstuk twee van die verhandeling, wys ek dat *B. parva*, *L. theobromae* en 'n onbekende *Botryosphaeria* sp. geassosieer word met Botryosphaeria kanker van *Eucalyptus* in Uganda. Patogenisiteitstoetse het gewys dat daar statisties betekenisvolle verskille in die virulensie van die genoemde spesies is. *Lasiodiplodia theobromae* was die mins virulente spesie, terwyl die onbekende spesie die mees virulent was. Verdere ondersoek is nodig om die virulensie van hierdie swamme in die veld te toets en om hulle biologie en relatiewe belang te bepaal.

Ceratocystis albofundus is 'n belangrike patogeen van *Acacia mearnsii* en *A. decurrens* in Suid-Afrika. In 1999, is *C. albofundus* die eerste keer geidentifiseer in die suid weste van Uganda. Die resultate in die huidige studie wys daarop dat die

Ugandese populasie hoë geendiversiteit het, wat vergelykbaar is met die Suid Afrikaanse populasie van die swam. Die resultate wys verder dat die swam klonaal voortplant in beide bg. lande, met baie min genetiese vloei tussen hulle. Die hoë geendiversiteits-waardes wat in hierdie studie verkry is, wys dat *C. albofundus* inheems is aan Afrika en nie Suid-Afrika soos 'n vroeëre hipotese voorstel nie. Die hoë geendiversiteit van *C. albofundus* in die twee populasies, wys daarop dat daar gefokus moet word op die kweek van weerstandbiedende variëteite as 'n beheerstrategie. Die seleksie van weerstandbiedende variëteite is reeds aan die gang in Suid-Afrika en sal baie bruikbaar wees vir beheer van die siekte in die toekoms.

Meeste siektes op *Eucalyptys* word veroorsaak deur swamme, maar bakteriese patogene word al meer belangrik. In die vierde hoofstuk van die verhandeling word die bakterium, *Pantoea ananatis,* geïdentifiseer as die oorsaak van skroeisiekte en terugsterwing van *Eucalyptus* spesies in Uganda. Dit is die eerste aanmelding van die siekte buite Suid-Afrika. Die siekte affekteer hoofsaaklik jong boompies en is daarom 'n bron van ernstige kommer vir die vestiging van plantasies. Dit is nou nodig om proewe op te stel om plantmateriaal te selekteer wat weerstanbiedend is teen bakteriese skroeisiekte. Die bakterium infekteer ook ander landbou-gewasse. Laasgenoemde is belangrik in Uganda, aangesien bome dikwels naby aan ander landbou gewasse geplant word.

Die resultate van hierdie verhandeling sal help met die beheer van siektes van bosbouplantasies in Uganda. Die studie sal hopelik ook dien as hulpmiddel om land- en bosbouers 'n beter begrip te gee van die belang van siektes in bosbouplantasies. Dit sal ook 'n wesenlike impak hê op die kapasiteit van die mense van Uganda om plantasiesiektes te hanteer, nie net die wat veroorsaak word deur die patogene wat in hierdie verhandeling geïdentifiseer is nie, maar ook ander siektes in die bosbou- en vrugtebedrywe.