

Occurrence of the wattle wilt pathogen, *Ceratocystis albifundus* on native South African trees

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Summary

Ceratocystis albifundus causes the disease known as wattle wilt of non-native *Acacia meurnsii* trees in South Africa, Uganda and Kenya. Infection results in rapid wilt and death of susceptible trees and stem cankers on more tolerant trees. It has been suggested that *C. albifundus* is indigenous to southern Africa, possibly having spread from native *Protea* spp. to non-native *A. meurnsii* and *A. decurrens* trees. Although *C. albifundus* has been collected from *Protea* spp., these reports are based on limited records for which only aged herbarium specimens exist. During surveys of wound-infecting fungi on native tree species in South Africa, a fungus resembling *C. albifundus* was collected from *Protea gagedi*, *Acacia caffra*, *Burkea africana*, *Combretum molle*, *C. zeyheri*, *Faurea saligna*, *Ochna pulchra*, *Ozoroa paniculosa* and *Terminalia sericea*. The identity of the fungus was confirmed as *C. albifundus*, using comparisons of DNA sequence data for the ITS and 5.8S gene of the rRNA operon. In pathogenicity trials, lesions were produced on *C. molle* and *A. caffra*, with some trees beginning to die at the termination of the experiment. This study represents the first report of *C. albifundus* from native tree species in South Africa and provides unequivocal evidence that the fungus occurs naturally on native *Protea* spp. The wide host range of *C. albifundus*, as well as its abundance on these indigenous hosts lends further support to the view that it is a native African pathogen.

1 Introduction

Ceratocystis albifundus De Beer, Wingfield & Morris is considered to be the most important pathogen of non-native plantation-grown *Acacia meurnsii* de Wild trees in South Africa (ROUX and WINGFIELD 1997; ROUX et al. 1999a). Infection results in rapid wilting and death of susceptible trees. One-year-old trees can die within 6 weeks after artificial inoculation with *C. albifundus* (ROUX et al. 1999a). Initial symptoms of infection on more tolerant trees include stem cankers characterized by exudation of gum from swollen blisters under the bark. Internal symptoms include discolouration of the vascular tissues of the affected trees (MORRIS et al. 1993; ROUX et al. 1999a).

Ceratocystis albifundus was first reported as the cause of wilt and death of *A. meurnsii* trees in the late 1980s. At that stage the fungus was believed to represent the wilt pathogen *C. fimbriata* Ell. & Halst. (MORRIS et al. 1993). The pathogen was later shown, based on DNA sequence comparisons, to represent a new species and was assigned the name *C. albifundus* (WINGFIELD et al. 1996). *Ceratocystis albifundus* can be distinguished from *C. fimbriata* based on the light coloured, as opposed to dark ascomatal bases and divergent rather than convergent ostiolar hyphae. The fungi are also clearly different based on DNA sequence data (WINGFIELD et al. 1996; WITTHUHN et al. 1999; ROUX et al. 2004).

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Prior to the description of *C. albifundus*, there were three records of *C. fimbriata* from *Protea* spp. in South Africa. GORTER (1977), in his book listing plant pathogenic fungi of South Africa, recorded '*C. fimbriata*' from native *Protea gigantea* in the Mpumalanga Province (then Transvaal). Two other reports are based on dried herbarium material of '*C. fimbriata*' and a *Ceratocystis* sp. lodged in the National collection of fungi (PREM44932, PREM48263) collected from native *P. cynaroides* near Pretoria in 1974 and *P. grandiceps* near Nelspruit in 1985, respectively.

Although the morphology of the *Ceratocystis* sp. on herbarium specimens from *Protea* spp. strongly suggested that this fungus is *C. albifundus* and not *C. fimbriata*, it has not been possible to confirm this fact based on DNA sequence comparisons. No living cultures from native hosts exist and attempts to derive DNA from the dried cultures from *Protea* spp. housed at PREM have failed. The important assumption that *C. albifundus* is native to South Africa is thus equivocal and deserves further investigation.

Until 1998, *C. albifundus* was known only from South Africa. It has, however, now been reported from *A. mearnsii* trees in Uganda (ROUX et al. 2001a), Kenya (ROUX et al. 2004) and Tanzania (ROUX et al. 2005). The initial reports of the fungus only from South Africa, as well as its apparent occurrence on native *Protea* spp. led to the hypothesis that *C. albifundus* is native to South Africa (ROUX et al. 1999a). Substance was added to this view through the results of population genetic studies using molecular markers (ROUX et al. 2001b).

Discovery of *C. albifundus* in Uganda presented an opportunity to compare populations of this pathogen from two countries and to consider further the question of its origin. NAKABONGE (2002) and BARNES et al. (2005) showed that the fungus is genetically equally diverse in both countries, when comparing populations of *C. albifundus* from Uganda and South Africa. No further conclusions could, however, be made at that time regarding the origin of *C. albifundus*.

Recent surveys following severe wind damage have enabled us to collect a *Ceratocystis* sp. from fresh wounds on a wide range of native woody plants in South Africa. The aim of this study was to identify a fungus resembling *C. albifundus* isolated from these trees. Use was made of both morphological characteristics and DNA sequence comparisons to confirm results, while inoculation tests were performed to consider the pathogenicity of the fungus.

2 Materials and methods

2.1 Isolates

Surveys were conducted on Leeuwfontein Collaborative Nature Reserve (S25°23.770 E028°37.506), approximately 60 km north-east of Pretoria, South Africa. Wounds created by strong winds were examined on approximately 40 damaged trees in the reserve. The bark directly adjacent to the wounds was removed and examined with a 10× magnification hand lens. Sections of wood and bark displaying fruiting bodies were placed in brown paper bags and transported to the laboratory for further examination and isolations.

Isolations were made directly from fruiting bodies on the wood and inside of the bark. Where the ascomata were not giving rise to spores, the material was incubated in moist chambers to induce sporulation. Single ascospore masses were transferred from the apices of ascomatal necks to malt extract agar (20 g malt extract, 15 g agar; Biolab, Midrand, South Africa) containing 0.001 g streptomycin sulphate (Sigma, Steinheim, Germany; MEAS) and incubated at 25°C until the onset of growth. Where fruiting bodies of a *Ceratocystis* sp. were observed, but overgrown by other fungi, a carrot baiting technique described by MOLLER and DEVAY (1968) was used as a selective isolation method.

Cultures were purified by transferring single colonies to separate Petri dishes containing MEAS. All cultures are maintained in the culture collection (CMW) of the Forestry and

Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa and a subset was deposited with the Centraalbureau voor Schimmelcultures (CBS), the Netherlands. Dried cultures were also deposited in the National Fungal Herbarium (PREM), Pretoria, South Africa (Table 1).

2.2 Morphology

Isolates were identified based on standard morphological characteristics for *C. albifundus* (WINGFIELD et al. 1996) using a Zeiss Axiovision microscope (Carl Zeiss, Jena, Germany). These included characters such as the colour of the ascomatal bases and the orientation of the ostiolar hyphae.

2.3 DNA extraction, amplification and sequencing

A set of six isolates was selected for further identification using DNA sequencing (Table 1). Cultures were grown on MEA plates for 2 weeks after which the mycelium was scraped from the plates and ground to a fine powder in liquid nitrogen using mortars and pestles. DNA was extracted using a slightly modified version of the method of MÖLLER et al. (1992). Using a pipette and sterile lab tips, 500 μ l of TES buffer (100 μ l Tris; 10 μ l EDTA; 2% SDS) and 200 μ l of Proteinase K were added to each sample and incubated for an hour at 60°C. The salt concentration was adjusted by adding 140 μ l of 5 M NaCl. An aliquot of 65 μ l of 10% CTAB was added to each centrifuge tube and incubated at 60°C for an additional 10 min. About 905 μ l of SEVAG (24 : 1 chloroform : isoamylalcohol) was added to the tubes and they were incubated for 10 min at 4°C. Tubes were centrifuged for 10 min at 12 000 rotations per minute (rpm), 15 300 g. Aqueous phases were transferred to fresh, sterile 1.5 ml microcentrifuge tubes. Isopropanol (440 μ l) was added to the tubes and incubated for 30 min on ice to precipitate the nucleic acids. After centrifugation (12 000 rpm, 15 300 g for 5 min) the aqueous phases were discarded and the DNA pellets dried by inverting the tubes onto a paper towel for 5 min. DNA pellets were washed in 1 ml ice-cold 70% ethanol and dried at 60°C in a Concentrator 5301 (Eppendorf, Hamburg, Germany). DNA pellets were resuspended in 50 μ l sterile water and 6 μ l RNase A added. Successful DNA extraction was confirmed by running 5 μ l of the DNA suspensions on a 1% agarose gel containing ethidium bromide and visualizing the results under ultraviolet (UV) light.

The internal transcribed spacer regions (ITS 1 and 2) and the 5.8S gene of the ribosomal DNA operon was amplified using the polymerase chain reaction (PCR). Primers ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATGATATGC-3') were used for the amplification (WHITE et al. 1990). PCR mixtures and amplification conditions were the same as those previously described by ROUX et al. (2004). PCR products were visualized on 1% agarose gels under UV light.

DNA sequencing was carried out using the same primers as those for the PCR with the Big Dye Cycle Sequencing kit and Amplitaq[®] DNA polymerase, FS (Perkin-Elmer, Warrington, UK). The protocol provided by the manufacturer was used for the sequencing and was conducted on an ABI PRISM 3100 Autosequencer (Applied BioSystems, Foster City, CA, USA). Sequences were visualized and edited with SEQUENCE NAVIGATOR version 1.0.1.[™] (Applied Biosystems, Inc., Foster City, CA, USA). Sequences were compared with those published by WINGFIELD et al. (1996), as well as those available in GenBank (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov>).

Sequences were aligned manually against an existing database and adjusted by the insertion of gaps, where required. Analyses were made using the heuristic search option in PAUP* 4.0b10 (Phylogenetic Analysis Using Parsimony; SWOFFORD 2002). Gaps were treated as a 'fifth character' (newstate), with the simple addition of taxa, Tree Bisection

Table 1. List of isolates used in DNA sequence analyses

| Taxon | Culture number ¹ | CBS/PREM number | Host | Origin | GenBank accession number | Reference | Collector |
|-----------------------------------|-----------------------------|-----------------|----------------------------|------------------|--------------------------|-------------------------|--------------|
| <i>Ceratocystis albifundus</i> | CMW2148 | PREM51639 | <i>Acacia mearnsii</i> | South Africa | AF264910 | WINGFIELD et al. (1996) | MJ Morris |
| | CMW2475 | | <i>A. mearnsii</i> | South Africa | AF043605 | WITTHUHN et al. (1999) | S McLennan |
| | CMW17271 ² | PREM58859 | <i>Combretum zeyheri</i> | South Africa | DQ250383 | WITTHUHN et al. (1999) | J Roux |
| | CMW17275 ² | PREM58860 | <i>Faurca saligna</i> | South Africa | DQ250384 | WITTHUHN et al. (1999) | J Roux |
| | CMW17773 ² | PREM58856 | <i>Combretum molle</i> | South Africa | DQ250382 | WITTHUHN et al. (1999) | RN Heath |
| | CMW17774 ² | PREM58857 | <i>Acacia caffra</i> | South Africa | | WITTHUHN et al. (1999) | RN Heath |
| | CMW17775 ² | PREM58858 | <i>Ozoroa paniculosa</i> | South Africa | | WITTHUHN et al. (1999) | RN Heath |
| | CMW9044 ² | | <i>Protea gaudeti</i> | South Africa | DQ250381 | | J Roux |
| | CMW15049 | | <i>Ipomoea batatas</i> | USA | DQ520629 | JOHNSON et al. (2005) | CF Andrus |
| | CMW1547 | | <i>I. batatas</i> | Papua New Guinea | AF264904 | JOHNSON et al. (2005) | ECH McKenzie |
| <i>Ceratocystis pirilliformis</i> | CMW6569 | | <i>Eucalyptus nitens</i> | Australia | AF427104 | BARNES et al. (2003) | MJ Wingfield |
| | CMW6574 | | <i>E. nitens</i> | Australia | AF427106 | BARNES et al. (2003) | MJ Wingfield |
| <i>Ceratocystis polychroma</i> | CMW11449 | CBS115775 | <i>Syzygium aromaticum</i> | Indonesia | AY528972 | VAN WYK et al. (2004) | MJ Wingfield |
| | | PREM57821 | | | | | |
| | CMW11455 | CBS115774 | <i>S. aromaticum</i> | Indonesia | AY528973 | VAN WYK et al. (2004) | MJ Wingfield |
| <i>C. virescens</i> | | PREM57822 | | | | | |
| | CMW11164 | | <i>Fagus americanum</i> | USA | DQ520639 | VAN WYK et al. (2007) | D.Houston |

¹CMW represents cultures from the culture collection of the Forestry and Agricultural Biotechnology Institute, PREM those from the National Fungal Herbarium, Pretoria, South Africa and ATCC those from the American Type Culture Collection.

²Isolates sequenced in this study.

Reconnection (TBR) branch swapping and MULPAR options in effect. Branch support was estimated using the heuristic search option and 1000 replicates (FELSENSTEIN 1988). *C. virescens* (DQ520639) was used as an outgroup and was treated as a monophyletic sister group to the ingroup.

2.4 Pathogenicity trials

Twenty, three-year-old *Acacia caffra* (Thunb.) Willd. and 20 *Combretum molle* G. Don sapling plants were obtained from a commercial wholesale nursery for artificial inoculation trials. For each tree species, 15 trees were inoculated with a 10-day-old *C. albifundus* culture (CMW14168) grown on MEA and five trees were inoculated with sterile agar discs to serve as controls. For inoculations, a small square of bark (5 × 5 mm) was removed from the stem of each tree to expose the cambium. An agar plug of equal size was placed into each wound with the mycelium of *C. albifundus* facing the xylem. All inoculation sites were closed with Parafilm (Pechiny Plastic Packaging, Chicago, IL, USA) to protect the wounds and inoculum from desiccation.

Inoculated plants were examined after 7 weeks and lesion lengths measured on each tree. Re-isolations were made from the lesions to fulfil Koch's postulates. Data were analysed using programs in SAS (SAS Institute Inc. 1999). All data were tested for normal distribution and analysed according to the General Linear Model (GLM).

3 Results

3.1 Isolates

Isolates of a *Ceratocystis* sp. resembling *C. albifundus* were obtained from wounds on 22 different trees. These trees were of nine different species including *A. caffra* (Mimosaceae), *Burkea africana* Hook. (Caesalpiniaceae), *C. molle*, *C. zeyheri* Sond. (Combretaceae), *Faurea saligna* Harv., *Protea gaguei* J.F.Gmelin (Proteaceae), *Ochna pulchra* Hook (Ochnaceae), *Ozoroa paniculosa* (Sond.) R.&A.Fern (Anacardiaceae) and *Terminalia sericea* DC (Combretaceae). Fresh mycelium and ascomata of a *Ceratocystis* sp. (Fig. 1a) were commonly found between the bark and the wood of broken branches and stems (Fig. 1b). This was especially true where the bark had slightly peeled away from the xylem, to provide a protective flap for the development of mycelial mats (Fig. 1c). Dried fruiting bodies were also evident on exposed cambium.

3.2 Morphology

The fungus collected from the indigenous tree species showed morphological characteristics typical of *C. albifundus*. Sexual fruiting structures had light-coloured ascomatal bases with dark necks (Fig. 1a). Cultures produced perithecia containing hat-shaped ascospores and *Thielaviopsis* anamorphs with two types of conidia (cylindrical- and barrel-shaped). Ascomata further produced necks with divergent ostiolar hyphae. No chlamydospores were observed.

3.3 DNA extraction and sequencing

DNA was extracted from six cultures and the ITS and 5.8S gene regions amplified to produce fragments of approximately 700 nucleotides in size. After manual adjustment of sequence alignments the data set consisted of 544 characters were used in the final analysis. Analysis resulted in three most parsimonious trees of which one, with a consistency index (CI) of 0.8925 and a retention index (RI) of 0.9381, was chosen for presentation (Fig. 2).

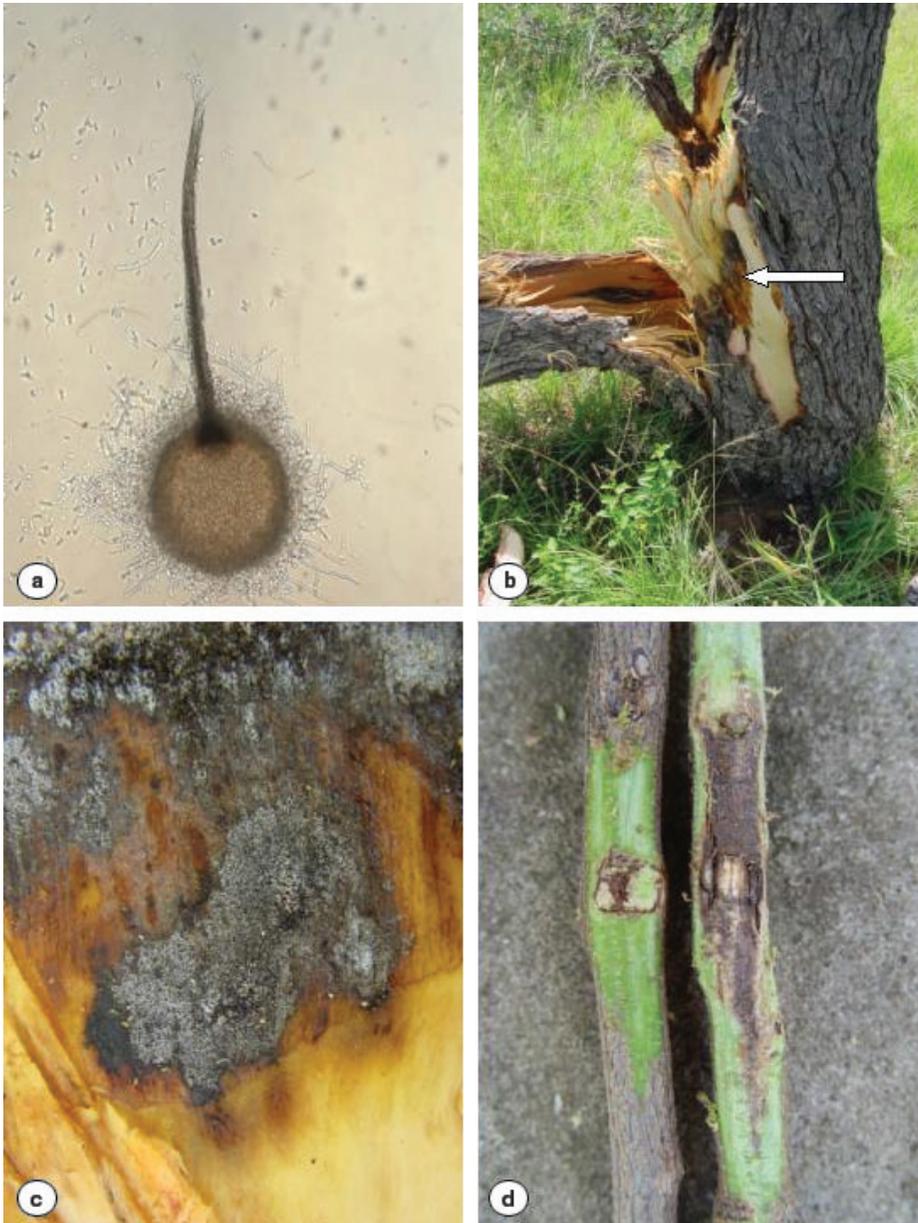


Fig. 1. Symptoms of *Ceratocystis albifundus* infection on native South African trees. (a) *Ceratocystis albifundus* sexual fruiting body showing light-coloured ascum base, (b) typical wound from which *C. albifundus* was isolated, note grey mycelial growth on wound (arrow), (c) *C. albifundus* mycelium growing in the cambium of a *Combretum* tree, (d) control (left) and bark lesion (right) produced by *C. albifundus* after artificial inoculation

Isolates from Leeuwfontein Collaborative Nature Reserve grouped with *C. albifundus* isolates previously collected from non-native *A. mearnsii* trees in a strongly supported clade with a Bootstrap of 100%.

3.4 Pathogenicity trials

After 7 weeks, the majority of the *A. caffra* trees showed signs of wilting, necessitating the termination of the experiment so that lesion lengths could be measured accurately. Both

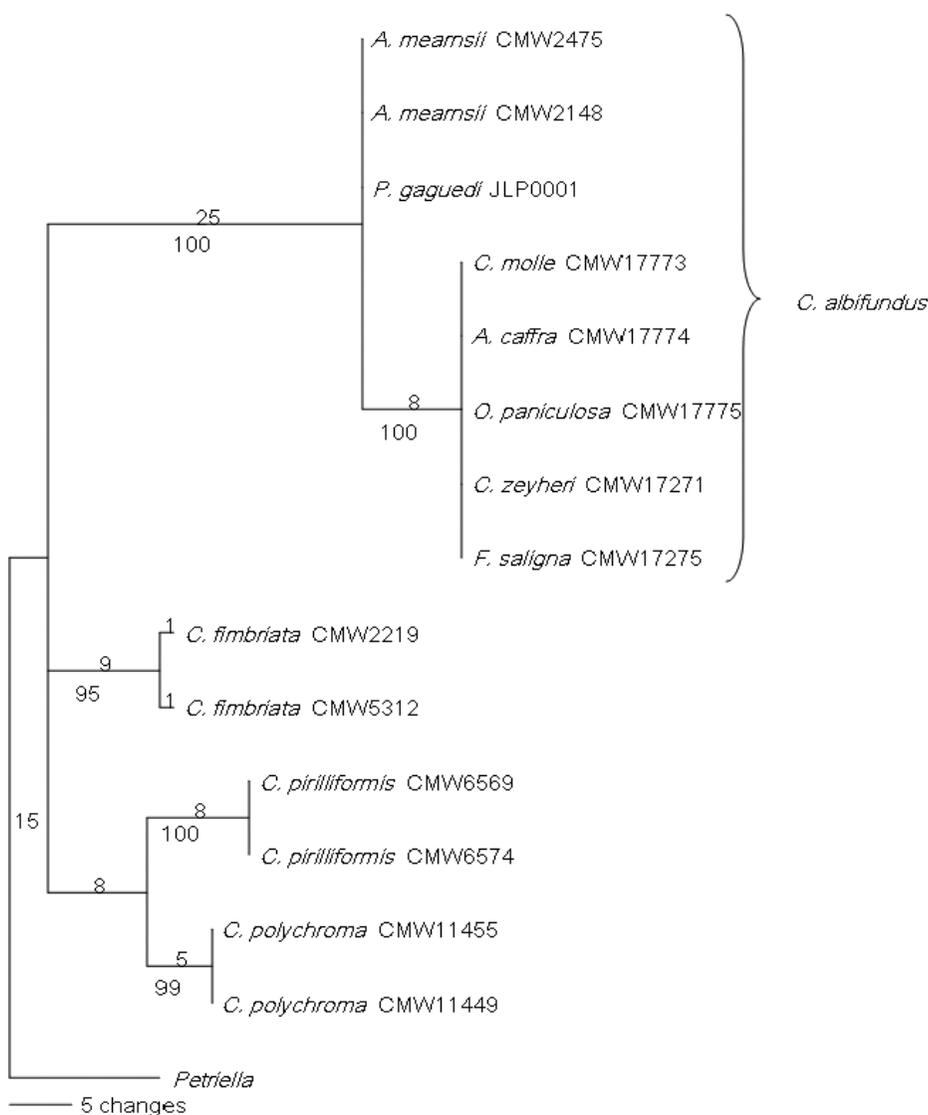


Fig. 2. Most parsimonious tree produced using the Heuristic search option in PAUP. Bootstrap values are indicated below the branches and branch lengths above. CI: 0.8925, RI: 0.9381

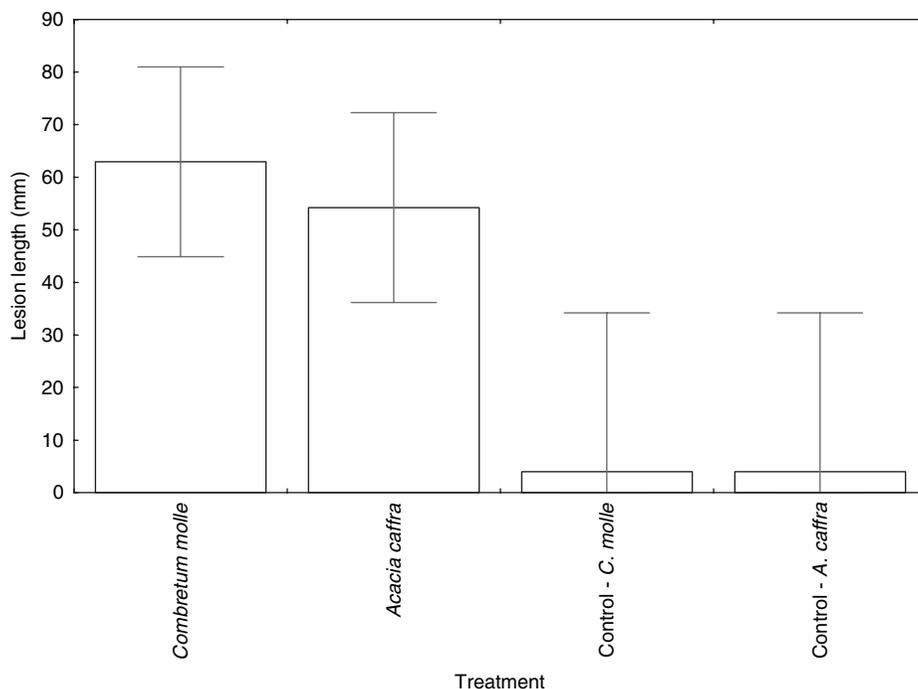


Fig. 3. Histogram showing results of artificial inoculation trials with *Ceratocystis albifundus* on *Combretum molle* and *Acacia caffra* in the greenhouse. $Pr < 0.0010$; $CV = 75.17$; $R^2 = 0.37$, confidence limit = 95%

A. caffra and *C. molle* trees inoculated with *C. albifundus* had obvious dark-coloured lesions in the bark (Fig. 1d) and xylem lesions. None of the inoculated control trees showed signs of wilting or death, nor did they develop lesions in the bark or xylem. Wounding sites were covered with callus tissue in the control inoculations. *Ceratocystis albifundus* was recovered from the lesions on the inoculated trees but not from control plants. Statistical analysis showed clear differences between the control trees and those inoculated with *C. albifundus* (Fig. 3; $Pr > 0.0010$; $CV = 75.17$; $R^2 = 0.37$; 95% confidence level).

4 Discussion

Results of this study provide the first unequivocal evidence that *C. albifundus* occurs naturally on native trees in South Africa. While morphological evidence from two herbarium specimens on *Protea* spp. had previously supported this view, we have now been able to confirm, based on DNA sequence data, that the fungus occurs naturally on these plants. This is important as numerous cryptic species of *Ceratocystis* are emerging (BARNES et al. 2003; VAN WYK et al. 2004,2006; BAKER ENGELBRECHT and HARRINGTON 2005; THORPE et al. 2005) and morphological characteristics distinguishing them are becoming increasingly less clear.

Other than confirming that *C. albifundus* occurs on native *Protea* spp. in South Africa, the fungus was also found on a relatively large number of other native tree species. These included *F. saligna*, which is also a member of the Proteaceae, as well as tree species

representing five other families. This wide host range on native South African tree species, and no reports from other continents, in combination with the lack of tree mortality in the country, provides substantial additional evidence that the fungus is native to southern Africa.

Leeuwfontein Collaborative Nature Reserve was selected for this survey, because it is close to one of the sites from which the fungus suspected to be *C. albifundus* was collected in the 1970s. During January 2004, strong winds occurred at this nature reserve causing considerable damage to several tree species. These wounds provided ideal infection sites for wound pathogens and were commonly infected by *C. albifundus*. Infection with *C. albifundus* first became visible as blue-grey mycelial mats approximately 2 weeks after the wind storm. In some cases, ascomata of *C. albifundus* were already visible, producing abundant hat-shaped ascospores at this time.

The identity of the fungus found on tree wounds at Leeuwfontein was confirmed based on both morphological characteristics and comparisons of DNA sequence data. ITS sequences for the selected isolates from Leeuwfontein grouped together with those of *C. albifundus* from *A. mearnsii* in South Africa. The fact that the morphology of isolates from native hosts is consistent with that described for *C. albifundus* confirms that the light-coloured bases and convergent ostiolar hyphae represent reliable diagnostic characteristics for this fungus.

The phylogenetic tree emerging from DNA sequence comparisons clearly grouped isolates from native trees with those of *C. albifundus* from non-native *A. mearnsii*. However, subclades were produced within the larger *C. albifundus* clade, with the recently collected isolates from Leeuwfontein grouping together but apart from other isolates. This raises a question as to whether *C. albifundus* might represent a species complex including a number of cryptic species. Studies are currently underway to collect a population of *C. albifundus* isolates from Leeuwfontein Nature Reserve and to survey other areas of the country for additional populations of isolates from native tree species. These populations will then be compared with those from non-native *A. mearnsii* to better understand the variation and population genetics of *C. albifundus* in South Africa.

Pathogenicity trials in this study showed that *C. albifundus* has the potential to cause significant lesions on two of the native hosts tested. It is unfortunate that additional hosts could not be tested for susceptibility but plants of the native trees encountered in this study have seeds that germinate with difficulty and are not commonly available. One option might have been to inoculate trees in the field but permission to do so could not be obtained from the relevant authorities. For the present, it is necessary to rely on the limited inoculations that yielded clear and statistically significant results. These showed that *C. albifundus* infection can result in disease and death of at least the two native tree species evaluated. Very high levels of variance were found between individual trees of the two genera tested ($CV = 75.17$; $R^2 = 0.37$). This is not surprising as a relatively high degree of variance in resistance would be expected from a native host and pathogen. The fact that some trees were dying and others were less affected suggests that *C. albifundus* might play a role in killing susceptible individuals and ensuring selection and future propagation of those that are more fit.

Various surveys have been conducted on native *A. mearnsii* in several areas of Australia in an attempt to find *C. albifundus* (M. J. Wingfield, unpublished data; J. Roux, unpublished data). These studies, including artificial wounds inflicted on trees (BARNES et al. 2003) have yielded no evidence that *C. albifundus* occurs in that country. Currently, wounds on a large number of different tree species have been established in South Africa and other African countries to further clarify the geographic distribution and host range of *C. albifundus*. Studies are also under way to determine the genetic diversity of isolates of *C. albifundus* from native hosts in South Africa. These studies will all contribute to a better understanding of this important pathogen.

It is clear from this study that *C. albifundus* has a wide host range, spanning several different families. The fact that this fungus has jumped to non-native *A. mearnsii* on which it causes a very serious disease is curious. Clearly, anthropogenic hosts jumps commonly occur in tree pathogens although these are poorly recognized and little understood (SLIPPERS et al. 2005). A more complete understanding of these processes would contribute considerably to global biosecurity.

The potential threat of *C. albifundus* to other non-native woody plants in South Africa and many trees related to its hosts in countries such as Australia should be closely examined. There is good evidence to show that *Ceratocystis* spp. move easily between countries and continents (ROUX et al. 1999b; THORPE et al. 2005; VAN WYK et al. 2005) and the chance of *C. albifundus* reaching nearby continents such as Australia seems high. It is, thus, important to maintain strict quarantine measures, not only regarding the movement of exotic plantation forestry species, but also Proteaceae and all other tree species, to prevent the movement of *C. albifundus* from areas where it occurs naturally.

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