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Genetic variation in the wattle wilt pathogen *Ceratocystis albofundus*

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***Ceratocystis albofundus* is an important wilt pathogen on exotic *Acacia mearnsii* trees in South Africa. It is known only from this country and has also been reported from native *Protea* spp., but it is not clear if the pathogen is native or introduced to South Africa. This study was conducted to determine the nuclear and mitochondrial gene diversity in a population of *C. albofundus* and to compare this diversity with that of other *Ceratocystis* species. Isolates were collected from a number of geographic regions in South Africa. Total genomic DNA was extracted from each isolate, digested with *Pst*I and probed with the radioactively labelled oligonucleotide marker $(CAT)_5$ to determine nuclear DNA diversity. For the determination of mitochondrial DNA diversity, the RFLPs of *Hae*III digests were scored directly without probing. Nei's gene diversity (*H*) was determined and a distance matrix was developed for each set of markers and analyzed using UPGMA. The *C. albofundus* population was found to have a high level of both nuclear and mitochondrial gene diversity when compared with published data of populations of other *Ceratocystis* spp. This further supports the hypothesis that *C. albofundus* is native to South Africa.**

Key Words——*Acacia mearnsii*; $(CAT)_5$; population.

Ceratocystis albofundus De Beer, Wingfield & Morris causes Ceratocystis wilt (wattle wilt) of exotic *Acacia mearnsii* de Wild. trees in South Africa (Morris et al., 1993; Wingfield et al., 1996b). This disease yearly results in large-scale losses and is of great concern to the South African wattle growers industry. Infection by this pathogen leads to rapid wilt and die-back of trees, often resulting in death within a few weeks after symptom development (Morris et al., 1993; Roux et al., 1999). Ceratocystis wilt was first recorded in 1989 from the KwaZulu-Natal Midlands where *A. mearnsii* trees were found dying of an unknown cause. Since then, regular outbreaks of the disease have been reported in the last decade (Roux and Wingfield, 1997).

Ceratocystis albofundus has been recorded only from South Africa. No reports are known from native *A. mearnsii* in Australia. The only recorded hosts of this fungus, apart from the exotics *A. mearnsii, A. decurrens* and *A. dealbata*, are *Protea gigantea* L. in the Mpumalanga Province (Gorter, 1977) and *P. cynaroides* L. (herbarium specimen PREM44932). These two collections were as *C. fimbriata* Ell. & Halst., but a re-examination has shown that both have perithecia with light-coloured bases and dark necks that distinguish *C. albofundus* from *C. fimbriata* (Wingfield et al., 1996b). The fungus from *Protea* also has hat-shaped ascospores and divergent ostiolar hyphae, similar to sequenced isolates of *C. albofundus* from *A. mearnsii* (Wingfield et al., 1996b). Morphological differences between *C. albofundus* and the more geographically widespread, but closely related, *C. fimbriata* have been supported by rDNA sequence data and RFLPs (Wingfield et al., 1996b; Witthuhn et al., 1999).

Species of *Ceratocystis*, especially strains of *C. fimbriata*, have been introduced to new ecosystems with dramatic effect. For example, the introduction of the *Platanus* strain into Europe (Panconesi, 1981) and the rubber tree strains into Malaysia (Sharples, 1936) have lead to large-scale tree mortality. We wanted to test the hypothesis that *C. albofundus* might have been introduced into South Africa. Such knowledge would be useful for quarantine purposes and help with the management of the disease.

We tested this hypothesis by determining the nuclear and mitochondrial gene diversity of the South African population of *C. albofundus* to see if there is evidence for a genetic bottleneck. Such a bottleneck would suggest that the pathogen had been recently introduced. Populations that have been subjected to genetic bottlenecks are characterized by low levels of genetic diversity. We used published genetic markers that had been used successfully with other *Ceratocystis* species (Harrington et al., 1998), which enabled us to compare the gene diversity of *C. albofundus* with other endemic species of *Ceratocystis*. These markers included a multi-locus, microsatellite probe, $(CAT)_5$, for nuclear gene diversity (DeScenzo and Harrington, 1994) and digestion of mitochondrial DNA with the restriction enzyme *Hae*III for

mitochondrial gene diversity (Wingfield et al., 1996a).

Materials and Methods

Strains studied The 49 isolates used in this study were obtained from dying *A. mearnsii* trees throughout South-Eastern South Africa (Table 1). Isolations were made from diseased trees using the carrot slice technique described by Moller and DeVay (1968). Each isolate originated from a different tree and was transferred from a single drop of ascospores on one perithecium. It has been shown for the closely related *C. fimbriata* that the progeny of a single perithecium differs only in mating type (Harrington and McNew, 1997; Witthuhn et al., 2000). All isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria and in the culture collection of T. C. Harrington, Iowa State University. Herbarium material has been deposited in the culture collection of FABI and the National Collection of fungi at the Plant Protection Research Institute (PPRI), Pretoria, South Africa.

DNA Isolation and Restrictions Total genomic, high molecular weight DNA was extracted from all isolates by using a slightly modified version of the technique described by DeScenzo and Harrington (1994). This was repeated to ensure the accuracy of the results obtained. Restriction digests of dilute preparations of the total genomic DNA (25 μg) using the enzymes *Hae*III or *Pst*I (5 U/μg genomic DNA) (GIBCO BRL) followed the procedures described by DeScenzo and Harrington (1994). This was followed by separation of the bands on 1% agarose (Biorad analytical grade) gels (20 cm × 25 cm × 5 mm) in 1X TBE. DNA samples loaded on the gels were standardized for each isolate (2–10 μg) to ensure uniform loading of the gels. Gels were run with constant stirring of the buffer at 88 V for 17.5 h for *Pst*I gels and at 80 V for 17 h for *Hae*III gels. Markers (1 μg λ *Hind*III DNA, GIBCO BRL) were included in the outside lanes. Gels were stained for ~15 min in ethidium bromide on a rotary shaker, destained in water for ~30 min, and visualized under UV light. The *Pst*I gels were dried using a gel drier for 60 min at 50°C, sealed and stored at 4°C until further use. Digital images of the *Hae*III gels were used to analyze the mtDNA polymorphisms.

Table 1. List of *Ceratocystis albofundus* isolates from wilted *Acacia mearnsii* used to determine gene diversity in South Africa.

Culture number[1]	Origin[2]
CMW4059–CMW4068	Bloemendal, KZN
CMW4069–CMW4078	Vryheid, KZN
CMW4079–CMW4085	Dalton, KZN
CMW4087–CMW4090	Dalton, KZN
CMW4092–CMW4096	East London, EC
CMW4097	Cintsa, EC
CMW4102	Bloemendal, KZN
CMW4103–CMW4104	Dalton, KZN
CMW4105–CMW4106	Piet Retief, MP
CMW4107	Vryheid, KZN
CMW4109–CMW4110	Bloemendal, KZN
CMW4757–CMW4758	Umtata, EC
CMW4905–CMW4906	Kataza, KZN

[1] CMW numbers represent cultures maintained in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

[2] All isolates were collected from diseased *Acacia mearnsii* in South Africa. KZN refers to KwaZulu-Natal Province, EC to the Eastern Cape Province and MP to Mpumalanga Province.

DNA probing The 15 bp oligonucleotide $(CAT)_5$ was end-labelled with ^{32}P and used as probe for in-gel hybridization of the *Pst*I gels (DeScenzo and Harrington, 1994). The λ *Hind*III marker was ^{32}P-labelled using the Prime-a-Gene Labelling System (PROMEGA). The labelled marker was denatured and added to the labelled $(CAT)_5$ probe. Hybridization and washing conditions were as described by DeScenzo and Harrington (1994). Gels were wrapped between two layers of plastic and

Table 2. Number of phenotypes, polymorphic loci and average gene diversity in *Ceratocystis albofundus* and other *Ceratocystis* species based on nuclear DNA fingerprinting with *Pst*I restrictions and the oligonucleotide probe $(CAT)_5$.

Species/populations	No. Isolates	No. Phenotypes	No. Markers	No. polymorphic markers	Gene diversity (H)
C. albofundus	38	38	50	47	0.2137
C. eucalypti[1]	10	9	19	17	0.3747
C. virescens[1]	16	2	4	1	0.0935
Chalara australis[1]	30	3	22	2	0.0111
C. albofundus					
Bloemendal	12	12	50	27	0.202
Dalton	12	12	50	35	0.258
Vryheid	8	8	50	30	0.282
Piet Retief	2	2	50	7	0.159
East London	4	4	50	20	0.278

[1] Data from Harrington et al. (1998).

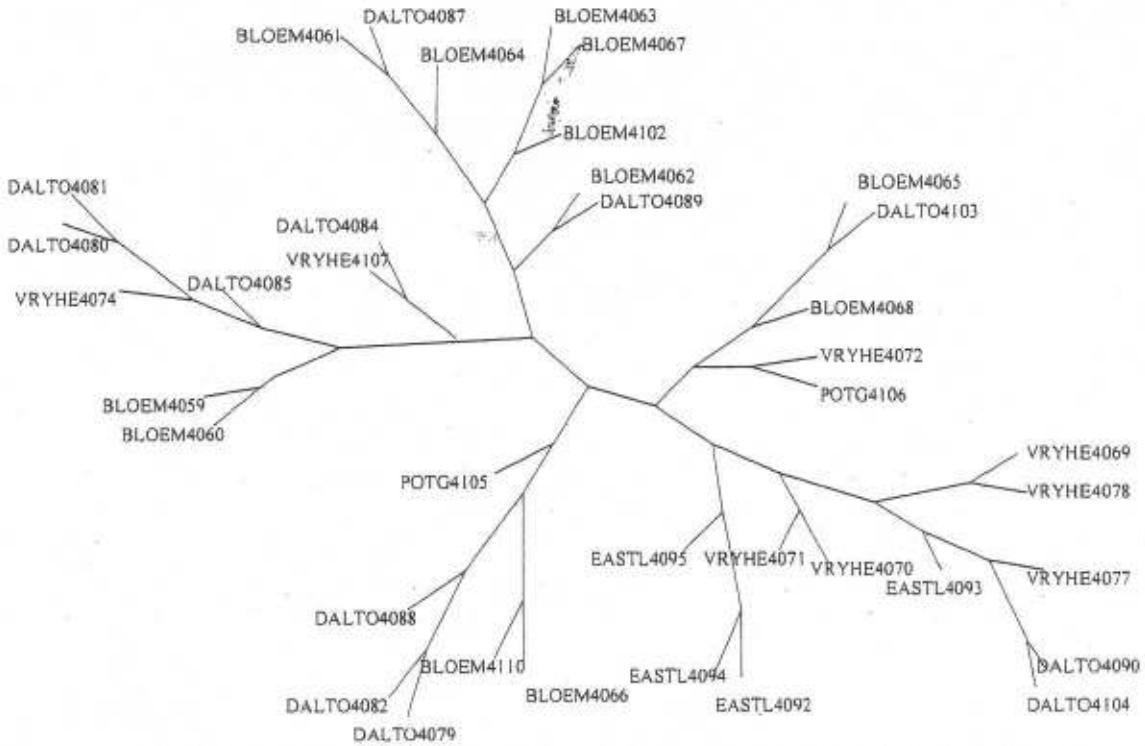

Fig. 1. Dendrogram of UPGMA cluster analysis of nuclear DNA genetic distance matrixes of *C. albofundus* isolates after probing *Pst*I restricted DNA with $(CAT)_5$. Letters refer to the area from which the isolate was obtained, while the numbers refer to the CMW number of the isolate. These areas are abbreviated as follows: BLOEM, Bloemendal; DALTO, Dalton; VRYHE, Vryheid; EASTL, East London; and POTG, Piet Retief.

visualised in either of two ways. Some gels were exposed to Kodak X-ray film for one to two wk, while other gels were exposed to a Phoshor Imager (Molecular Dynamics, Sunnyvale, California, USA) screen for 1–2 d. All experiments were repeated and only bands present on all runs were scored.

Analysis of data Each band greater than 2 kb was scored as either present (1) or absent (0) for each isolate tested. Band sizes were determined using the program GelReader 2.0.5 (NCSA, University of Illinois, Champagne, Urbana, IL). Only bands that were clearly visible in all runs were used in analyses. Nei's (1973) gene diversity (H) was calculated and a distance matrix and dendrogram generated using Neighbour-joining and the Unweighted Pair-Group Mean Arithmetic Analysis (UPGMA) (Felsenstein, 1993). Although only a small number of isolates were available per geographic area, gene diversity values were also calculated for each area separately.

Results

Nuclear DNA diversity The nuclear bands resolved with $(CAT)_5$ hybridization were highly variable for *C. albofundus* when compared to other *Ceratocystis* spp. (Table 2). For *C. albofundus*, 47 of the 50 markers scored were found to be polymorphic. Each of the 37 isolates had a unique nuclear fingerprint. The average gene diversity (H) using the $(CAT)_5$ markers for *C. albofundus* was 0.2137. Gene diversity values for individual plantations were similar to those for the country as a whole (Table 2), with the highest value found for the Vryheid area (0.282). Dendrograms obtained from the UPGMA and Neighbour-joining analysis of the distance matrix were similar to each other and showed a tendency for isolates from different plantations to group together in clusters (Fig. 1), that is, most clusters consisted of isolates from more than one geographic area.

Mitochondrial DNA diversity Variation in the mitochondrial DNA of *C. albofundus* as indicated by *Hae*III digestion of total genomic DNA was much higher than that of any of the three *Ceratocystis* spp. with which it was compared (Table 3). Forty-one of the 46 scored bands were polymorphic for *C. albofundus*. Thirty different phenotypes were found for the 31 isolates tested. The average gene diversity value for *C. albofundus* using the *Hae*III marker was 0.249.

Gene diversity for individual plantations was similar to that for the country as a whole (Table 3). Phylograms of the mtDNA distance matrix showed a grouping of isolates from different geographic areas (Fig. 2). Isolate CMW4084 from Dalton, for example, grouped with isolate CMW4758 from Umtata. These two areas are approximately 400 km apart, the one a commercial *A. mearnsii* growing area and the other an area with only "jungle" stands (natural regeneration) of *A. mearnsii*. Isolates CMW4093 and CMW4094 from East London in the Eastern Cape Province ("jungle" stands) grouped

Table 3. Number of phenotypes, polymorphic loci and average gene diversity in *Ceratocystis albofundus* and other *Ceratocystis* species based on mtDNA fingerprints generated with *Hae*III restriction fragments.

Species/populations	No. Isolates	No. Phenotypes	No. markers	No. polymorphic markers	Gene diversity (H)
C. albofundus	31	30	46	41	0.2490
C. eucalypti[1]	10	6	33	9	0.1115
C. virescens[1]	16	10	40	13	0.0928
Chalara australis[1]	30	2	28	1	0.0023
C. albofundus					
Bloemendal	9	9	46	26	0.251
Dalton	9	9	46	22	0.207
Vryheid	7	7	46	33	0.296
Piet Retief	2	2	46	13	0.125
East London	2	1	46	0	0

[1] Data from Harrington et al. (1998).

with isolate CMW4102 from Piet Retief (700 km distant in a commercial plantation) in south-eastern Mpumalanga Province.

Discussion

Our results show a level of nuclear and mtDNA diversity in *C. albofundus* higher than those of any of the other three *Ceratocystis* species to which it was compared. The high level of gene diversity suggests that the South African population is genetically diverse and has not gone through a recent genetic bottleneck. This, together with reports of *C. albofundus* from indigenous *Protea* spp. (Gorter, 1977; Wingfield et al., 1996b), leads us to reject the hypothesis that *C. albofundus* has been introduced to South Africa.

The three *Ceratocystis* spp. with which data for *C. albofundus* were compared have three reproductive strategies (Harrington et al., 1998). *Ceratocystis eucalypti* Yuan & Kile is an obligate outcrossing fungus, producing perithecia only when two strains of opposite mating type are crossed. It is a weak, wound-colonising pathogen of *Eucalyptus* spp. in Australia, where it is reported to be native (Kile et al., 1996). The second

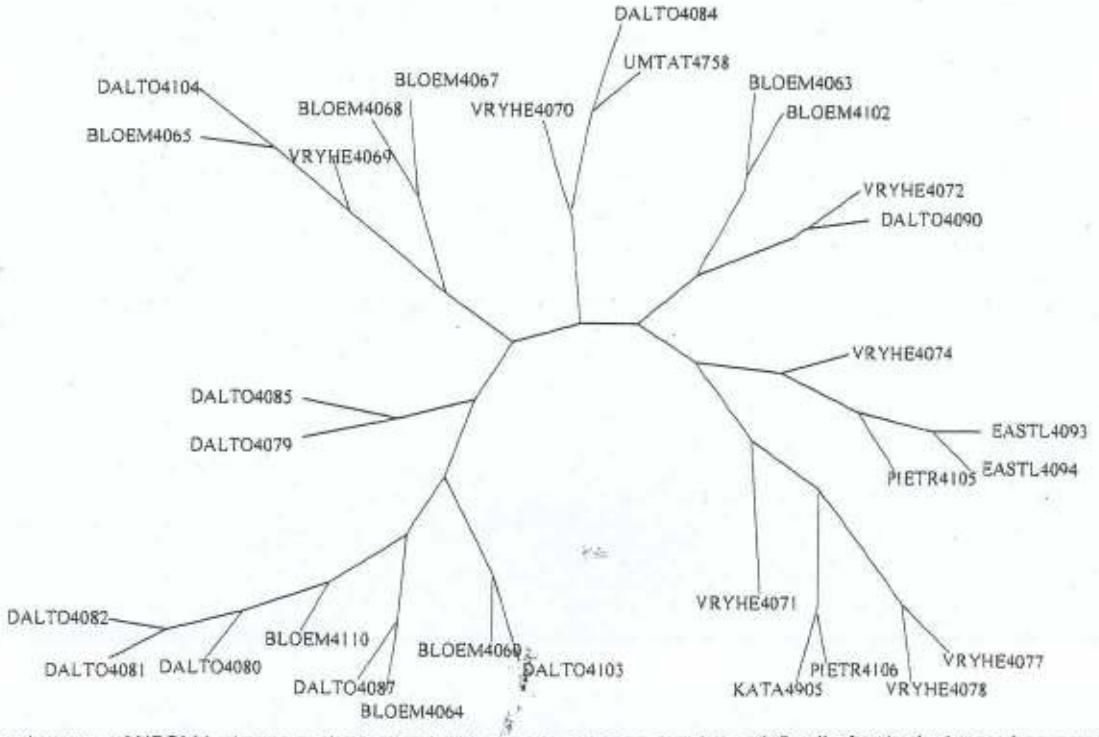

Fig. 2. Dendrogram of UPGMA cluster analysis of mtDNA genetic distance matrixes of *C. albofundus* isolates after restriction with *Hae*III. Letters refer to the area from which the isolate was obtained, while the numbers refer to the CMW number of the isolate. These areas are abbreviated as follows: BLOEM, Bloemendal; DALTO, Dalton; VRYHE, Vryheid; EASTL, East London; POTG, Piet Retief; KATA, Kataza and UMTAT, Umtata.

species, *Chalara australis* Walker & Kile, is an asexually reproducing fungus, also believed to be native to Australia, where it causes a serious wilt disease of *Nothofagus cunninghamii* (Hook.) Oerst. It has only one mating type (MAT-2) (Kile and Walker, 1987; Harrington et al., 1998). *Ceratocystis virescens* (Davids) C. Moreau causes sap-streak disease of maple (*Acer* spp.) and tulip poplar (*Liriodendron tulipifera*) in the eastern USA, where it is believed to be native. It has two mating types, with one of the mating types capable of unidirectional mating type switching and, thus, selfing (Harrington and McNew, 1997).

Ceratocystis albofundus is a homothallic fungus with two mating types: MAT-1 (self-sterile), requiring outcrossing, and MAT-2 (self-fertile), which is capable of selfing through uni-directional mating type switching (De Beer, 1994; Harrington and McNew, 1997). Thus, even though *C. albofundus* has a sexual state, it may be essentially clonal with much of its reproduction occuring through selfing. It has been shown for its closest relative, *C. fimbriata*, and other *Ceratocystis* spp. that the progeny of these fungi differ only in their mating types (Harrington and McNew, 1997, Witthuhn et al., 2000).

It was expected that the gene diversity values for *C. albofundus* would most closely resemble those for *C. virescens*, which has a similar reproductive strategy, if *C. albofundus* is native to South Africa. The fact that *C. albofundus* has nuclear DNA diversity values higher than those for *C. virescens*, and closer to those of the obligately outcrossing *C. eucalypti*, strongly suggests that it is either native to South Africa or has been in the country for an extended period of time.

The maternal inheritance of mtDNA makes this more sensitive than nuclear DNA to severe reductions in gene diversity such as those caused by introductions to new areas (Cann et al., 1987). High levels of mitochondrial gene diversity could also be attributable to a high mutation rate and large effective population sizes (Taylor, 1986). Most mutations in animal mtDNA take place through point mutations or nucleotide substitutions or deletions, but in fungi, a high number of length mutations (due to insertions and deletions) have been found in the mitochondrial genome (Taylor, 1986). *Cryphonectria parasitica* (Murrill) Barr is an introduced fungus to the USA, where an extremely high mtDNA diversity is hypothesised to have occurred due to high mutation rates (Milgroom and Lipari, 1993). Thus, the relatively high level of mtDNA gene diversity found in *C. albofundus* in South Africa may be due to a high mutation rate, in addition to its not having gone through a recent genetic bottle-neck, such as an introduction into the country.

Surveys to find other hosts, both native and exotic, for *C. albofundus* will continue. As mentioned earlier, *C. albofundus* has, thus far, been found only in South Africa, with no living cultures available from *Protea* spp. In this study, we have only been able to study the variation in a population obtained from the exotic plantation tree *A. mearnsii*. However, this sampled population was highly diverse compared to other endemic *Ceratocystis* species. It would appear that *C. albofundus* is well established on some native hosts in South Africa, while commonly infecting and killing the exotic *A. mearnsii* in plantations and naturalized stands.

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