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Comparison of populations of the wilt pathogen *Ceratocystis albifundus* in South Africa and Uganda

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Ceratocystis albifundus is an important fungal pathogen of *Acacia mearnsii* trees in South Africa. In a previous study, a high level of gene diversity was demonstrated in a South African population of *C. albifundus*. This, together with the occurrence of the pathogen on native *Protea* species and its exclusive occurrence in South Africa, led to the hypothesis that *C. albifundus* is probably native to that country. More recently, *C. albifundus* has been reported from *A. mearnsii* in south-western Uganda. The aim of this study was to compare the populations of *C. albifundus* from Uganda and South Africa based on genetic diversity, population structure and possible gene flow. This was achieved using codominant microsatellite markers developed for the closely related species *Ceratocystis fimbriata*. Available isolates for comparison were from six different areas of South Africa and six jungle stands in Uganda. Eight of the 11 available markers amplified loci in *C. albifundus*. Gene diversity was higher in the Ugandan population, but genotypic diversity was greater for the South African isolates. There were no common genotypes between the two populations and they shared only 22% of the total alleles. The populations were genetically isolated from each other and highly substructured within. There was no association between isolates collected from the same geographic locations, and gene flow between the two populations was low. Results suggest that *C. albifundus* was probably not introduced into Uganda from South Africa but rather that an ancestral population, yet to be discovered, is the source of both populations.

Keywords: *Acacia mearnsii*, *Ceratocystis fimbriata*, fungi, genetic diversity, microsatellite markers, population structure

Introduction

The genus *Ceratocystis sensu stricto* includes many well known canker and wilt pathogens (Wingfield *et al*., 1993). During the past 15 years a number of species belonging to this genus have been identified as pathogens of commercial plantation forestry species. One such species is *Ceratocystis albifundus*, a serious wilt pathogen of Australian *Acacia* spp. This pathogen was first described in 1996 after it was found infecting *Acacia mearnsii* (black wattle) trees in South African plantations (Morris *et al*., 1993; Wingfield *et al*., 1996; Roux *et al*., 1999).

Ceratocystis albifundus causes a disease known as ceratocystis wilt, which is characterized by the formation of cankers and lesions on the bark, with gum exudation from these lesions. Internally the pathogen causes extensive discoloration of the sapwood and, in the final stages of disease development, wilting, dieback and death of trees ultimately occurs (Roux *et al*., 1999). The disease has resulted in economically significant losses in South African *A. mearnsii* plantations (Roux *et al*., 1999).

To test the hypothesis that *C. albifundus* is native to South Africa, Roux *et al*. (2001) determined the nuclear and mitochondrial gene diversities of this pathogen. They compared their results with published data for three other *Ceratocystis* spp. thought to be native in their respective areas of collection (Harrington *et al*., 1998). Gene diversity for *C. albifundus* was found to be high in comparison to those of the native *Ceratocystis* spp. Apart from the Australian *Acacia* species *A. mearnsii*, *A. dealbata* (silver wattle) and *A. decurrens* (green wattle) (Gorter, 1977; Morris *et al*., 1993), the only other hosts on which *C. albifundus* has been reported are two native South African *Protea* species (Gorter, 1977; Wingfield *et al*., 1996). The narrow host range, restricted geographic distribution, presence on native hosts and high genetic diversity of *C. albifundus* supported the hypothesis that it is native to South Africa.

More recently, isolates of *C. albifundus* were discovered on *A. mearnsii* in jungle stands and private woodlots in Uganda (Roux & Wingfield, 2001). This discovery raised questions regarding the relationship between the Ugandan and South African populations of the fungus.

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Table 1 Isolates of *Ceratocystis albifundus* from South Africa and Uganda used in this study

Origin	Sample size	Isolate number[a]	Collector
South African population			
Bloemendal	10	CMW 4059, 4061–4066, 4068, 4109–4110	J. Roux
Cintsa	1	CMW 4097	T.C. Harrington
Dalton	13	CMW 4079–4080, 4082–4090, 4103–4104	J. Roux
East London	4	CMW 4092–4095	T.C. Harrington
Umkomaas	1	CMW 4091	J. Roux
Vryheid	11	CMW 4069–4078, 4107	J. Roux
Ugandan population			
Kagalama	1	CMW 15760	J. Roux
Kakarome	4	CMW 4998, 7111–7112, 9181	G. Nakabonge
Mafuga	15	CMW 5329–5364, 7115, 7153–7162, 7268, 9178	G. Nakabonge
Kachwekano	5	CMW 7113–7114, 9174, 9177, 9377	G. Nakabonge
Karungu	6	CMW 7116, 9182–9184, 9375–9376	G. Nakabonge
Murutunga	5	CMW 9173, 9175–9176, 9179, 9180	G. Nakabonge

[a]CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

It also cast doubt on the original assumption that *C. albifundus* is native to South Africa.

The aim of this study was to compare available isolates of *C. albifundus* from South Africa and Uganda to determine if either is a source or founder population of the other. This comparison was made using polymorphic microsatellite DNA markers previously developed for *C. fimbriata* (Barnes *et al*., 2001). These markers were chosen in view of the close phylogenetic relationship between *C. albifundus* and *C. fimbriata* (Witthuhn *et al*., 1999). The markers were tested on *C. albifundus* and subsequently used to determine the genetic diversity, population structure and gene flow between the two *C. albifundus* populations.

Materials and methods

Fungal isolates

Two populations of *C. albifundus* isolates were used in this study. The first population was collected from *A. mearnsii* trees showing typical symptoms of ceratocystis wilt in the Kabale district, south-western Uganda (Table 1). The 36 isolates obtained all originated from an area of ≈45 km^2. The second population, consisting of 40 isolates of *C. albifundus* from South Africa (RSA) (Table 1), was obtained from the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. These isolates were originally randomly isolated from diseased and dying *A. mearnsii* trees (each isolate originating from a different tree) in various plantations and naturally regenerated stands spanning a distance of approximately 200 and 30 km in the provinces of KwaZulu Natal and the Eastern Cape, respectively (Roux *et al*., 2001) (Table 1). All isolates used in this study are maintained in FABI's culture collection (CMW).

The Ugandan isolates were obtained from diseased pieces of wood collected randomly from the stems of *A. mearnsii* trees that had been wounded during harvesting for fuelwood in different woodlots. Isolation from the affected wood showing streaked discoloration was carried out using two methods. The first included wrapping pieces of wood in carrot slices to bait for *Ceratocystis* spp. (Moller & De Vay, 1968). These were incubated for 7–14 days in plastic bags at 25°C. The second method involved placing wood pieces in moist chambers consisting of Petri dishes lined with wet filter paper. The moist chambers were incubated at 25°C until sporulation occurred. After ascomata developed on the carrot or wood samples, masses of ascospores were transferred to malt yeast agar plates (20 g L^{-1} malt extract, 10 g L^{-1} yeast extract; Biolab, Midrand, South Africa) to obtain pure cultures.

DNA extraction

All isolates were grown on malt extract agar (20 g L^{-1} malt extract, 10 g L^{-1} agar; Biolab) at 25°C until sporulation was observed. Single masses of spores were lifted from the apices of ascomatal necks with sterilized toothpicks and transferred to Erlenmeyer flasks containing 50 mL malt extract broth (20 g L^{-1}; Biolab). Flasks were incubated at 25°C until a thick mat of mycelium had formed on the surface of the broth. The mycelium was collected and lyophilized, and DNA was isolated as previously described by Roux *et al*. (2001). An extra phenol : chloroform (1 : 1) step was included to purify the DNA further. RNaseA (10 mg mL^{-1}, Roche Molecular Biochemicals, Mannheim, Germany) was added to digest the RNA after DNA isolation.

Microsatellite PCR amplification

Eleven sets of PCR-based microsatellite primers, shown to be polymorphic for isolates of *C. fimbriata* (Barnes *et al*., 2001), were tested on five randomly selected South African *C. albifundus* isolates to determine whether they

would amplify the desired PCR products. The PCR reaction mix and conditions were the same as those described by Barnes *et al.* (2001). Primer sets that gave positive results for each of the five test isolates were used to amplify the remaining isolates of *C. albifundus* in both populations. For the primer sets that did not amplify the desired PCR product, a range of annealing temperatures and DNA concentrations were tested. Where primers failed to produce products after these manipulations, they were discarded. The DNA concentrations of the PCR products were measured visually against the intensity of a 100 bp marker (Roche Molecular Biochemicals) on a 2% agarose gel stained with ethidium bromide, exposed to UV illumination.

GENESCAN analysis

PCR products were multiplexed for GENESCAN analysis based on the approximate size of the PCR product and type of fluorescent label attached to the primer (Table 2). Each sample mix contained 0·5 μL combined DNA (1·5 ng in total), 1·1 μL 1 × loading buffer and 0·4 μL internal standard GENESCAN-500 TAMRA (Applied Biosystems, Warrington, UK). Samples were separated on a 4·25% PAGE gel using an ABI Prism 377 DNA sequencer. The allele sizes for DNA fragments were determined using a combination of the GENESCAN 2·1 analysis software and GENOTYPER 3·0 (Applied Biosystems).

Statistical analyses

Genetic diversity

Every isolate was scored based on the presence or absence of an allele at a specific locus. The frequency of each allele was calculated by taking the number of times the allele was present in the population and dividing it by the population sample size. The allele frequencies were used to calculate the gene diversity (Nei, 1973): $H = 1 - \sum_k x_k^2$ where x_k is the frequency of the kth allele (Table 3) for each population.

Multilocus genotypes for all isolates were determined based on the combinations of alleles at each locus. The genotypic diversity was then calculated using the formula: $G = 1/\sum[fx(x/n)^2]$ where G is the effective number of equally frequent genotypes, n is the sample size, and f_x is the number of multilocus genotypes occurring x times in the sample (Stoddart & Taylor, 1988). Due to their similar sample size, the genotypic diversities between populations were compared using the percentage of maximum diversity, G_{max}, in each population by calculating $G_{max} = G/N \times 100$, where N is the sample size (McDonald *et al.*, 1994; Grünwald *et al.*, 2003). A t-test with a significance level of $P = 0{\cdot}05$ was used to determine if the difference between the percentages of maximum diversity obtained between populations was significant (Chen *et al.*, 1994). This test was confirmed by performing a bootstrap test of significance using the diversity index option in GENODIVE (Meirmans & Van Tienderen, 2004), with 1000 permutations. To determine whether sufficient loci had been used to score genotypic diversity, the genotypic diversity was plotted against the number of loci (1000 random samplings) using MULTILOCUS version 1·2 (Agapow & Burt, 2000).

Genetic distance

The genetic distance between all isolates of *C. albifundus* from RSA and Uganda was calculated based on Nei's (1972) unbiased genetic distance. The distance matrix was constructed using the program GENDIST in the Phylogeny Inference Package (PHYLIP) version 3·6b (Felsenstein, 1993), and a tree was produced using Neighbor-Joining in MEGA version 2·1 (Kumar *et al.*, 2001).

Genetic differentiation and gene flow

The level of genetic differentiation between populations was calculated in POPGENE version 1·31, using Nei's (1973) G_{ST} statistic which varies between zero and one. POPGENE was also used to estimate the level of gene flow, calculated as the number of migrants using private alleles (Slatkin & Barton, 1989) (N_m), between the populations from the estimate of G_{ST} where $N_m = 0{\cdot}5(1 - G_{ST})/G_{ST}$ (McDermott & McDonald, 1993). Populations that are completely genetically isolated would have N_m values of zero and G_{ST} values tending towards one (Hartl & Clark, 1989).

Results

Microsatellite PCR amplification

Eight of the 11 microsatellite primers designed for *C. fimbriata* (Barnes *et al.*, 2001) gave amplification products for *C. albifundus*. Primers CF11/12 and CF13/14 gave no amplification products, while primer AG1/2 produced multiple bands. These primers were thus discarded. Successful PCR amplifications were obtained for all isolates using the remaining eight pairs of microsatellite primers (Table 2).

Statistical analysis

Genetic diversity

A total of 41 different alleles were produced across the eight loci amplified for the two populations. The Ugandan population contained 26 alleles (from 36 isolates) while the RSA population contained 24 alleles (from 40 isolates). Only 22% of the alleles were shared between the two populations (Table 3). Sixty-five percent of the alleles in the Ugandan population and 63% of the alleles in the RSA population were unique. No alleles were shared between the two populations at loci A17/18 and C5/6 (Table 3). Locus A7/8 was the most polymorphic in both populations while loci A15/16 and CF15/16 were monomorphic in the Ugandan and RSA populations, respectively. Gene diversity (H), calculated using allele frequencies, was 0·41 for the Ugandan and 0·38 for the RSA population.

A total of 53 different genotypes were identified within the two populations of *C. albifundus*. Twenty-eight of

Table 2 PCR-based microsatellite markers (Barnes *et al.*, 2001) that successfully amplified isolates of *Ceratocystis albifundus*, used to study populations from South Africa and Uganda

Primers	Forward primer sequence	Reverse primer sequence	Fluorescent label (expected size range, bp)[a]
AG7/8	CGA GAC AGC AAC ACA AGC CC	GGG GCG GTG GTG CAA TTG TC	TET (284–323)
AG15/16	CTT GAC CGA CCT GCC GAT TG	GGA TAG CAG CGA CAA GGA CC	FAM (274–288)
AG17/18	GTC GGT GGT GGA GAC GGT C	CGG CCC TGC CAA CGG ATG	FAM (304–313)
CF5/6	GAC CAA AGA TGG TGG CGA GC	CAT GGG CAT GCC TAG CCT TG	TET (359–385)
CF15/16	CGT TTG CAA GGC AAG GCA GC	CAG GGA CTA GGG TCT GCC AG	FAM (218–267)
CF17/18	GAA ACC GAG AGT CAT CGT CC	CGA GCC AAG ACG TTC ATT GAA G	TET (267–292)
CF21/22	GCG TTG AAA GAT GTG GCG TG	GCA CTA GCA GAA TAG AAT GCA G	TET (250–285)
CF 23/24	CAT GAT CGA CAA GGG CGC TG	CAG GGA ATT CCC GAT GGC AG	TET (154–168)

[a]Fragment sizes based on those calculated by Barnes *et al.* (2001).

these were from the RSA population and 25 originated from the Ugandan population. The frequency of these genotypes varied. Twenty-one isolates in the RSA population and 20 in the Ugandan population contained unique genotypes (Fig. 1). Genotypic diversity was calculated at $G = 25$, which is 62% of the maximum possible value of 40, for the RSA population; and $G = 15{\cdot}8$, 44% of the maximum possible value of 36, for the Ugandan population. The probability values for the bootstrap tests were: P (RSA ≥ Uganda) = 0·27 and P (RSA ≤ Uganda) = 0·73. Both values are >0·025 (Meirmans & Van Tienderen, 2004) and thus not significant. The *t*-test based on maximum genotypic diversity and the bootstrap test of significance both showed that the null hypothesis – that there is no significant difference in genotypic diversity between the two populations – cannot be rejected. Although no identical genotypes were shared between the RSA and Ugandan populations, isolates within the two populations having the same genotypes came from different plantations and plots and were therefore not geographically exclusive.

A P value <0·001 was obtained for the graphs plotted for genotypic diversity *vs* the number of loci, and a plateau had been reached. This indicated that additional sampling would have had a negligible effect on the genotypic diversity obtained for either the RSA or Ugandan population (data not shown).

Genetic distance

A neighbour-joining tree using Nei's (1972) genetic distance showed distinct separation of all the Ugandan isolates from the RSA isolates (Fig. 1). Although the Ugandan and RSA isolates were structured according to geographic location with respect to country, there was no clustering of isolates originating from the same plantation, plot or farm. Particularly in Uganda, different genotypes were evenly distributed throughout the populations sampled.

Genetic differentiation and gene flow

The measure of genetic differentiation reflects the amount of substructuring occurring within populations. The G_{ST} value of 0·31 was high, given the level of genotypic diversity within populations, and indicated that 31% of the variation observed was found among populations, while 69% was found within populations. Effective gene flow between the populations was estimated at an N_m value of 1·11, but as there were no shared genotypes, this indicates a relatively high degree of isolation.

Discussion

This study has shown that the PCR-based microsatellite markers developed to study the population and evolutionary biology of *C. fimbriata*, can be equally effectively used for the closely related species *C. albifundus*. The results also show that populations of *C. albifundus* from Uganda and South Africa exhibit high levels of genetic diversity. It is unlikely that either of these populations was a source or founder population of the other. The hypothesis that *C. albifundus* was recently introduced into Uganda from South Africa can be rejected.

Native populations are generally expected to have higher diversities, and hence numbers of private alleles, than newly found or introduced populations. Both the South African and the Ugandan population, however, had similar gene diversities and numbers of private alleles. Only 22% of the total number of alleles was shared, and thus over 60% of the alleles present in each population were unique to that population. The South African population did not have sufficient diversity or shared alleles for it to be the source of the Ugandan population. This lack of shared alleles suggests that both populations are evolving independently of each other, and that they have been separated for an extended period. This would allow gene frequencies and the genotypic constitution of the populations to change through drift and selection. It is also possible that an ancestral population, which has not yet been discovered, is the source of both these populations.

The multilocus genotypic diversity for the South African population of *C. albifundus* was higher than that for the Ugandan population although this difference was not significant. The rate at which a pathogen can evolve is directly related to the amount of genetic variation within the population (McDonald & McDermott, 1993). Many of the genotypes within the South African and Ugandan populations differed at only one allele at a specific locus,

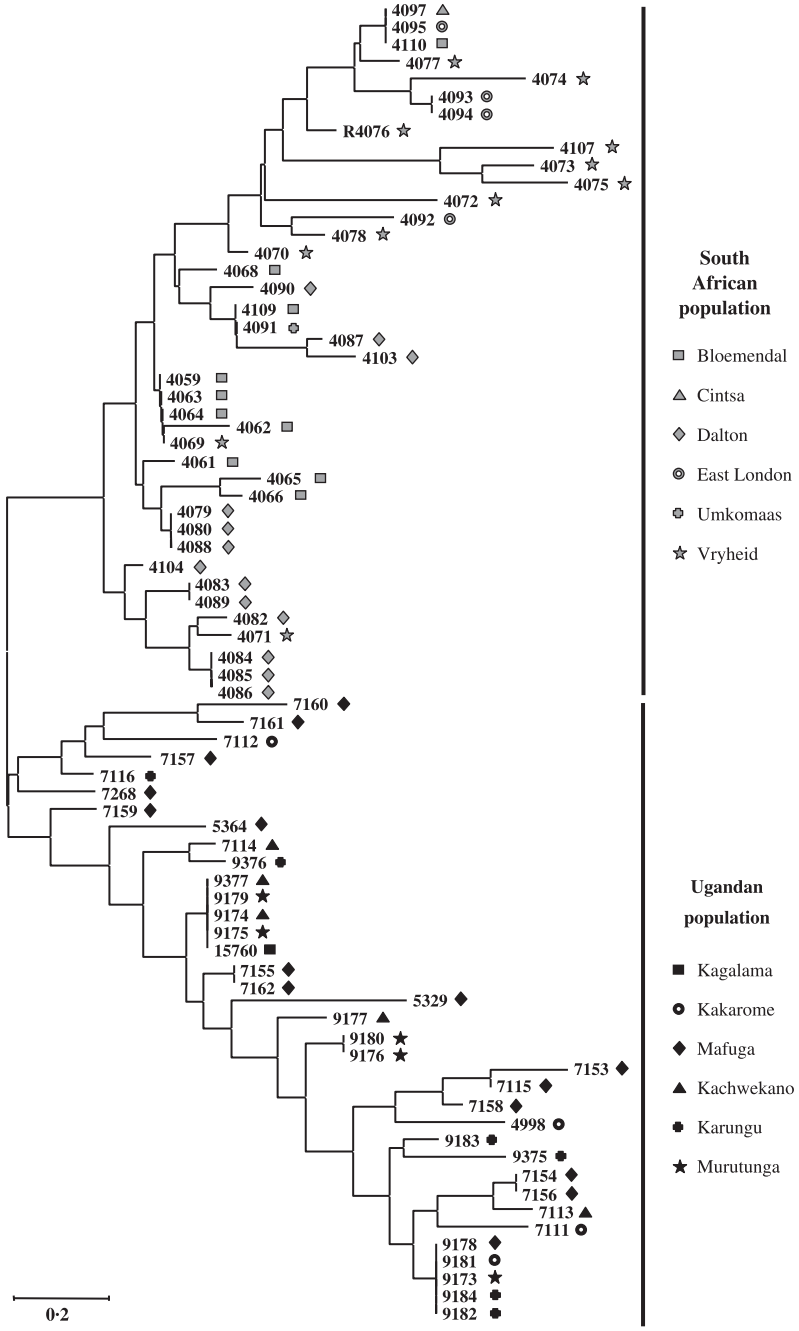

Figure 1 Neighbour-joining tree showing genetic identities among isolates from South African and Ugandan populations of *Ceratocystis albifundus*. The two populations are genetically distinct from one another and the isolates within populations are highly substructured. The distribution of genotypes is not restricted to any particular geographic location. Grey and black symbols represent the different geographic locations from which isolates were obtained in each population.

resulting in a low level of genetic distance between isolates. High mutation rates might account for this result. Any mutations that occurred would automatically change the genotype and hence increase the diversity of the population. Recombination could also account for the high genotypic diversities observed, although this was not tested in the present study. In this respect, however, recombination resulting in new genotypes is not expected in *C. albifundus* due to its homothallic nature (De Beer, 1994). Although *C. albifundus* does have the ability to outcross (Harrington & McNew, 1997; Witthuhn *et al*., 2000), the extent to which this occurs in nature is not known.

A correlation between population differentiation and geographical distance between populations was evident. Thus all the South African and Ugandan isolates clustered together according to geographic location with respect to their country. The substantial physical distance between the two countries accounts for the low level of gene flow, and the high levels of differentiation might suggest that sufficient time has elapsed for the onset of genetic drift. This could change frequencies and eliminate alleles from a population resulting in the variation observed between the two *C. albifundus* populations. The isolates that were sampled from these two countries had identical genotypes

Table 3 Allele frequency and gene diversity (*H*) values calculated for South African and Ugandan populations of *Ceratocystis albifundus*

Locus	Allele length	Allele configuration	Allele frequencies	
			South Africa	Uganda
AG7/8	296	A	–	0·056
	299	B	–	0·056
	308	C	–	0·056
	309	D	0·025	–
	319	E	–	0·139
	320	F	–	0·639
	322	G	0·350	–
	323	H	0·050	–
	325	I	0·020	–
	326	J	0·100	–
	327	K	0·325	–
	331	L	0·075	0·028
	332	M	0·025	0·028
	334	N	0·025	–
			H = 0·751	*H* = 0·562
AG15/16	288	A	0·650	1·000
	293	B	0·350	–
			H = 0·455	*H* = 0·000
AG17/18	304	A	–	0·361
	305	B	–	0·639
	310	C	0·725	–
	311	D	0·275	–
			H = 0·399	*H* = 0·461
CF5/6	380	A	0·950	–
	382	B	–	0·861
	384	C	–	0·139
	387	D	0·050	–
			H = 0·095	*H* = 0·239
CF15/16	260	A	1·000	0·250
	261	B	–	0·750
			H = 0·000	*H* = 0·375
CF17/18	283	A	–	0·056
	284	B	–	0·389
	288	C	0·075	–
	290	D	–	0·056
	291	E	0·625	0·472
	292	F	0·300	0·278
			H = 0·514	*H* = 0·619
CF21/22	250	A	–	0·056
	251	B	–	0·028
	254	C	–	0·028
	283	D	0·400	0·667
	284	E	0·425	0·222
	285	F	0·175	–
			H = 0·629	*H* = 0·502
CF23/24	160	A	–	0·500
	166	B	0·100	0·500
	168	C	0·900	–
			H = 0·180	*H* = 0·502
Mean			*H* = 0·378	*H* = 0·407

present in the different plantations or plots, and most of the isolates with similar genetic distances originated from more than one geographic area. This indicates that the fungus is being spread randomly within each country.

Results of this study preclude any clear indication of the origin of *C. albifundus* on *A. mearnsii*. To fully explain the origin of this pathogen, attempts will need to be made to obtain populations from other African countries and hosts. Populations from countries between Uganda and South Africa might provide a link between populations or make it possible to trace the movement of the pathogen. *Acacia mearnsii* originates in Australia, and tracing the origin of seed and other planting stock of this tree could also lead to the discovery of additional populations of *C. albifundus*. The fact that *C. albifundus* occurs on native *Protea* spp. and causes a disease on an exotic tree species in South Africa is unusual, but supports the view that the fungus is well established in this country. Isolates of *C. albifundus* on native tree species, and especially *Protea* spp., are not available but would be most useful in gaining a better understanding of the origin of the fungus.

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