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Distribution and population diversity of *Ceratocystis pirilliformis* in South Africa

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Abstract: *Ceratocystis pirilliformis* was first isolated from wounds on *Eucalyptus nitens* in Australia and subsequently found in a similar niche on *E. grandis* in South Africa. Artificial inoculation studies under field conditions in South Africa resulted in bark lesions and sap-stain, suggesting that the fungus is a pathogen of potential importance to forestry in South Africa. Because *Eucalyptus* spp. are native to Australia and *C. pirilliformis* was first found there in the absence of disease it has been assumed that the fungus is native to Australia. The aim of this study was to expand the base of knowledge regarding the distribution and population diversity of *C. pirilliformis* in South Africa. Wounds were examined on *Eucalyptus* spp. growing in seven of the most important forestry areas in South Africa. PCR-based microsatellite markers, developed for the closely related tree pathogen *C. fimbriata sensu lato* were used to assess the diversity of the isolates collected. *Ceratocystis pirilliformis* was found in four out of seven areas surveyed, substantially expanding its known distribution in South Africa. Of the 27 available microsatellite markers, 18 amplified the desired loci of *C. pirilliformis* and of these, seven were polymorphic. Measures of genetic diversity based on gene diversity, genotypic diversity as well as allelic richness indicated that the isolates from South Africa has a low level of genetic diversity and that the fungus is most likely not native to the country. Inclusion of available Australian isolates showed much higher diversity for *C. pirilliformis* in that country.

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INTRODUCTION

Genus *Ceratocystis* includes several economically important species that are pathogens of crop plants, including trees. Examples of tree pathogens include *C. albifundus* de Beer, Wingfield & Morris, the cause of wilt and canker on nonnative *Acacia mearnsii* in South Africa (Wingfield et al 1996), *C. fimbriata* Ellis & Halsted *sensu lato*, the cause of wilt and canker stain disease of *Eucalyptus* spp. in Africa and South America (Roux et al 1999, Roux et al 2004, Barnes et al 2003a), *C. platani* (Walter) Engelbrecht & Harrington, which causes canker stain disease of plane trees (*Platanus* spp.) in Europe (Walter et al 1952), *C. cacaofunesta* Engelbrecht & Harrington, which causes canker disease of cacao trees in Brazil (Engelbrecht and Harrington 2005), *C. fagacearum* (Bretz) Hunt, the causal agent of oak wilt in the USA (Bretz 1952) and *C. polychroma* M. van Wyk, M.J. Wingfield & E.C.Y. Liew, associated with dieback of clove trees (*Syzygium aromaticum*) in Indonesia (van Wyk et al 2004).

The so-called *Ceratocystis fimbriata sensu lato* represents a complex of species typified by *C. fimbriata sensu stricto*, the cause of sweet potato black rot (Halsted 1890). In recent years many strains, previously identified as host-specialized strains of *C. fimbriata*, called ''types'', ''races'' or ''forms'' detected within the complex (Baker et al 2003, Harrington and Baker 2002, Wellman 1972), have been shown to represent distinct species. Thorpe et al (2005) for example showed that *C. fimbriata* isolates from plants in the family Araceae, the only known monocotyledonous family host for *C. fimbriata*, represent three groups of cryptic species in the *C. fimbriata* complex, based on ITS sequence analysis. In recent years several new species, including some previously known as *C. fimbriata s.s.*, thus have been described. They are *C. albifundus* (Wingfield et al 1996), *C. pirilliformis* (Barnes et al 2003b), *C. polychroma* (van Wyk et al 2004), *C. cacaofunesta*, *C. platani* (Engelbrecht and Harrington 2005), *C. variospora* (Davids.) C. Moreau, *C. populicola* J.A. Johnson & Harrington, *C. caryae* J.A. Johnson & Harrington, *C. smalleyi* J.A. Johnson & Harrington (Johnson et al 2005) and *C. tsitsikammensis* Kamgan & Jol. Roux (Kamgan et al 2008). These species are phylogenetically and morphologically more similar to each other than to other *Ceratocystis* spp. and have been shown to represent a

distinct subclade within genus *Ceratocystis*, known as the *C. fimbriata* species complex (Witthuhn et al 1999, Wingfield et al 2006). Considerable work, however, still is required to elucidate this complex group of species.

Ceratocystis pirilliformis is a recently described species residing in the larger *C. fimbriata s.l.* clade (Barnes et al 2003b). It first was described from Australia where it was found infecting artificially induced wounds on native *Eucalyptus nitens* trees (Barnes et al 2003b). The fungus subsequently was found in South Africa on stem wounds of *E. grandis* trees (Roux et al 2004). Studies by Roux et al (2004) showed that *C. pirilliformis* is capable of causing significant lesions on 1 y old *E. grandis* trees under field conditions. However, it has not yet been associated with naturally dying trees in either South Africa or Australia and its status as a pathogen remains unclear.

Introduced fungal pathogens, including species of *Ceratocystis*, have caused substantial losses to forest ecosystems around the world. The accidental introduction of *C. platani* into Europe is one such example and has lead to large-scale mortality of plane trees (*Platanus* spp.) in Italy (Anonymous 1986, Santini and Capretti 2000). However, for most of these fungi little is known regarding their areas of origin. In the case of *C. albifundus* and *C. platani* analyses of gene diversity with microsatellite markers has contributed to elucidating their possible origins in Africa (Barnes et al 2005) and the USA (Baker et al 2003, Engelbrecht et al 2004) respectively.

Little is known regarding the distribution, impact or origin of *C. pirilliformis* in South Africa. It has been reported from only a few locations, and it has been hypothesized that the fungus is native to Australia, where it would have co-evolved with its only known host, *Eucalyptus* (Barnes et al 2003b, Roux et al 2004). This view emerges from the fact that the fungus was isolated from native *E. nitens* in the absence of disease (Barnes et al 2003b). The aim of this study was to expand the base of knowledge regarding the host range and geographic distribution of *C. pirilliformis* in South Africa. An additional aim was to consider the population diversity of the fungus in this country in the hope that it might provide a more concrete indication as to whether it is native or introduced in South Africa.

MATERIALS AND METHODS

Distribution in South Africa.—Surveys for *C. pirilliformis* were conducted in seven areas of South Africa where *Eucalyptus* spp., native to Australia, are propagated commercially. These areas included those with subtropical climates (Bushbuckridge, Kwambonambi, Sabie, Tzaneen) and those with temperate climates (Lothair, Paulpietersburg, Pietermaritzburg) (FIG. 1). Tree species surveyed included *E. grandis*, a hybrid of *E. grandis* × *camaldulensis* (GC) and *E. nitens*. In all areas, other than in Pietermaritzburg and Lothair, wounds were made on the stems of *Eucalyptus* trees, as described by Barnes et al (2003b). Wounds were left for a minimum of 1 wk and in some cases up to 2 mo before pieces of bark and wood were collected from them. The length of time between wounding and sampling depended on the presence of fungal structures (ascomata, spore drops, mycelia) on the surfaces of the wounds, which was influenced by climatic conditions. Samples from Pietermaritzburg and Lothair were collected from the stumps (1 mo old) of felled trees. For the Bushbuckridge, Kwambonambi, Paulpietersburg, Sabie and Tzaneen areas both stumps and artificially induced wounds were sampled.

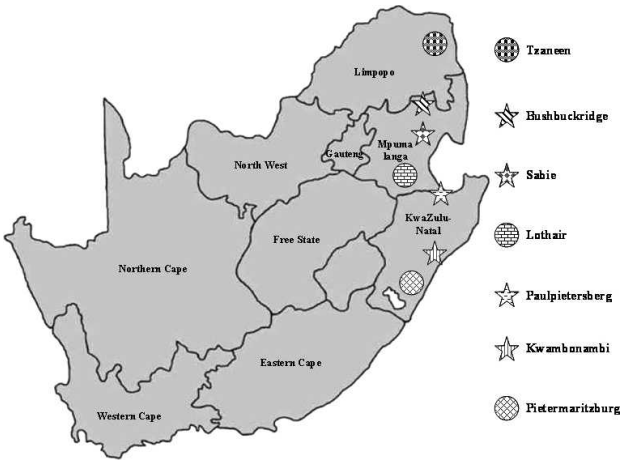

FIG. 1. South Africa showing the seven areas surveyed for *C. pirilliformis*. Stars indicate areas where *C. pirilliformis* was present.

Pieces of bark and wood, bearing fungal structures typical of *Ceratocystis* spp. (mycelium and/or ascomata, often bearing spore drops), were collected from wounds and stored in separate brown paper bags for each tree/stump sampled. Samples from a specific compartment were placed together in larger plastic bags that also served as moist chambers and transported to the laboratory. Isolations were made directly from samples on which freshly developed *Ceratocystis* fruiting bodies were found. Samples that were too dry were sprayed with sterile distilled water, sealed in plastic bags, one per compartment, and incubated at room temperature (~25 C) to induce sporulation of *Ceratocystis* spp. Samples were inspected daily for the presence of fruiting bodies (mycelium and/or ascomata bearing spore drops).

Single drops of spores produced at the apices of ascomata were transferred to Petri dishes containing 2% malt extract agar (MEA: 20 g malt extract, 15 g agar, Biolab, Midrand, South Africa, and 1 L deionized water) with 0.05 g/L streptomycin (SIGMA-ALDRICH, Steinheim, Germany). Plates were incubated at 24 C for 7 d to obtain cultures. Isolates of *C. pirilliformis* were identified based on the description provided by Barnes et al (2003b) as well as by DNA sequencing. Duplicates of all isolates have been deposited in the culture collection (CMW) of the Forestry

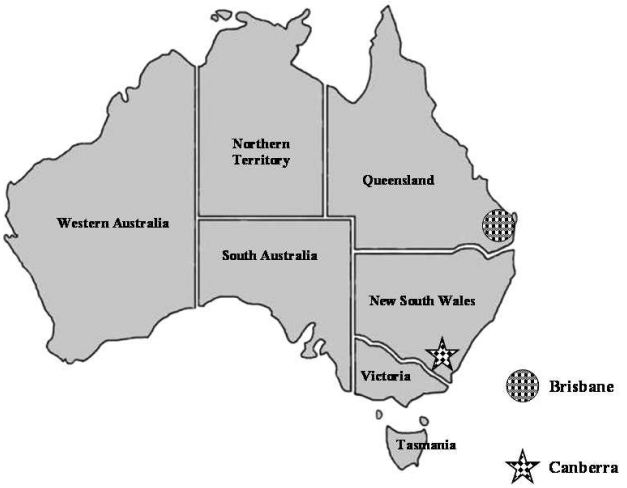

FIG. 2. Australia showing the two areas where *C. pirilliformis* was collected.

and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. A number of *C. pirilliformis* isolates collected from Canberra and Brisbane (FIG. 2) during studies in Australia (Barnes et al 2003b, Fouche et al 2007) (TABLE I) also were included in the study.

DNA extraction.—Cultures were grown on 2% MEA for 7–10 d. Mycelium collected by scraping the surface of the agar plates with a sterile scalpel was placed in 1.5 mL Eppendorf tubes. DNA was extracted with the protocol described by Möller et al (1992), with the exception that 10 μL of RnaseA was added at the final step, and the samples were incubated overnight at room temperature to digest RNA. The presence of DNA was verified by running aliquots (5 μL) of the extraction mixture on 1% agarose gels stained with ethidium bromide and viewing it under ultraviolet (UV) light.

Microsatellite amplification and allele scoring.—Twenty-seven sets of microsatellite primers, shown to be polymorphic for *C. fimbriata* (Barnes et al 2001, Steimel et al 2004) and *C. albifundus* (Barnes et al 2005), were tested on three randomly selected isolates (CMW12680, CMW16521, CMW16471) of *C. pirilliformis* from South Africa. The PCR reaction mixes and thermal cycling conditions were the same as those described by Barnes et al (2001) and Steimel et al (2004) for all primers tested. Primers that successfully amplified the desired size fragments were used to amplify DNA for the remaining isolates of *C. pirilliformis* collected from South Africa and Australia. Primers that did not amplify, produced double bands or were inconsistent with amplification were discarded. To verify the approximate sizes of the amplicons, 5 μL aliquots of the PCR products were separated on 2% agarose gels stained with ethidium bromide and viewed under UV light.

For Genescan analyses, PCR products for each isolate were multiplexed based on the expected size of amplicons and the type of fluorescent label attached to the primer (TABLE II). Each sample mix included 2 μL of the combined DNA, 0.14 μL internal standard Genescan-500 Liz (Applied Biosystems, Foster City, California) and 10 μL formamide. Sample mixes were separated on a 36 cm capillary with POP^{TM4} polymer on an ABI Prism 3100 sequencer. Allele sizes for PCR products were determined with GENOMAPPER version 3.0 (Applied Biosystems, Foster City, California).

Alleles that scored a difference of one base pair as determined by GENOMAPPER were sequenced and compared with each other to determine the validity of the extra base pair in the length of the allele. This was due especially to the fact that most of the microsatellite repeat units within these loci are di-, tri- or tetranucleotide repeats. PCR products were purified with Sephadex G-50 Gel (SIGMA-ALDRICH, Steinheim, Germany), as recommended by the manufacturer. An accurate concentration of the purified PCR product was determined with a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Rockland, Delaware). Sequencing reactions were performed with the Big Dye cycle sequencing kit with Amplitaq DNA Polymerase, FS (Perkin-Elmer, Warrington, UK), according to the

TABLE I. Isolates of *C. pirilliformis* from South Africa and Australia used in this study

Origin	Sample size	Isolate number (CMW)	Hosts	Collectors
South Africa				
Sabie	19	16511, 16512, 16514–16526, 16746, 16747, 16749, 16750	*E. grandis* × *camaldulensis*	G. Kamgan & J. Roux
Paulpietersburg	10	16463, 16466, 16467, 16469–16471, 16527–16529, 12281	*E. grandis*	G. Kamgan & R. Heath
	1	12671	*E. grandis*	J. Roux & H. Hatting
Bushbuckridge	8	12673, 12675–12677, 12679, 12680, 11699, 17914	*E. grandis* × *camaldulensis*	J. Roux
Kwambonambi	1	11722	*E. grandis* × *camaldulensis*	J. Roux
Australia				
Canberra	13	6566, 6574, 6575, 6579, 6583, 6670, 6569, 6556, 6586, 6576, 6577, 6571, 6563	*E. nitens*	M.J. Wingfield
Brisbane	3	19341, 19344, 19334	*E. tereticornis*	J. Roux & G. Pegg

TABLE II. Organization of PCR-based microsatellite markers that amplify in *C. pirilliformis* into two lanes for GENESCAN analyses based on the expected size of the amplicons and the type of fluorescent label attached to the primer

Primers	Fluorescent label and expected sizes (base pairs)	Authors
Lane 1		
CAG900	PET (196)	Steimel et al 2004
GACA6K	VIC (212)	Steimel et al 2004
CF11/12	FAM (216–230)	Barnes et al 2001
CF15/16	VIC (218–267)	Barnes et al 2001
CAA38	NED (240)	Steimel et al 2004
CF17/18	PET (266–292)	Barnes et al 2001
CCAG15	FAM (269)	Steimel et al 2004
AG7/8	VIC (284–304)	Barnes et al 2001
CAGDL2-5	PET (342)	Steimel et al 2004
CCAA15	NED (342)	Steimel et al 2004
CF13/14	PET (402–415)	Barnes et al 2001
Lane 2		
CF23/24	PET (154–168)	Barnes et al 2001
AAG8	NED (187)	Steimel et al 2004
CF21/22	NED (250–259)	Barnes et al 2001
AG1/2	PET (255–266)	Barnes et al 2001
CCAA9	FAM (253)	Steimel et al 2004
DBVCAT	VIC (272)	Steimel et al 2004
AAG9	VIC (413)	Steimel et al 2004

manufacturer's protocol on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California). PCR products 60–100 ng was used to prepare a 10 μL sequencing PCR that contained 2 μL of ready reaction mixture (Big dye), 2 μL of 5× reaction buffer, 1 μL of either the reverse or forward, nonfluorescent primer (10 mM) being tested and Sabax water. The same primers were used as those described for the PCR amplifications. Both DNA strands were sequenced. Sequence data were aligned and compared manually with Sequence Navigator version 1.01 (ABI PRISM, Perkin Elmer).

Analyses of diversity.—For analyses of diversity (gene diversity, genotypic diversity and allelic richness) we computed a clone-corrected dataset. Isolates that contained the same allele for each locus were considered clones and duplicate clones were removed. The frequency of each allele within South Africa and Australia was calculated by taking the number of times the allele was present in the collection and dividing it by the collection sample size. The allele frequencies were used to calculate gene diversity using the formula described by Nei (1973): $H = 1-\Sigma_k x_k^2$, where x_k is the frequency of the Kth allele. Due to the difference in sample size between the South African and Australian collections of *C. pirilliformis*, we computed allelic richness and private allelic richness based on the rarefaction method as described by Hurlbert (1971) to compare richness estimates of alleles between the two collections. Under this method allelic richness is obtained with the computer program HP-RARE 1.0 (Kalinowski 2005).

Multilocus genotypes for all isolates were determined based on the combination of alleles at each polymorphic locus (TABLE III). The genotypic diversity was thus calculated with the formula: $\hat{G} = 1/\Sigma\ [f_{(x)}(x/n)^2]$, where $\hat{G}$ is the effective number of frequent genotypes, n is the sample size and $f_{(x)}$ is the number of multilocus genotypes occurring × times in the population (Stoddart and Taylor 1988). The percentage of maximum genotypic diversity in each collection was determined with the formula $G_{max} = \hat{G}/N \times 100$ (McDonald et al 1994), and the significance of the differences between them was determined with a *t*-test (Chen et al 1994). To confirm that sufficient numbers of isolates and markers were used in the population analyses to make sound conclusions the software program Multilocus v1.3b (Agapow and Burt 2001) was used to plot genotypic diversity against the number of loci, with 1000 resampling repetitions.

RESULTS

Distribution in South Africa.—A total of 39 isolates of *C. pirilliformis* were collected in four areas of South Africa (TABLE I). Of these, 19 were from Sabie (Bergvliet Plantation) (S25°03.242′ E030°51.680′), 11 were from Paulpietersburg (Eersteling Plantation) (S27°31.843′ E030°48.123′), eight were from the Bushbuckridge (Waterhoutboom) (S24°56.829′ E030°55.213′) area and one was from Kwambonambi (FIG. 1, TABLE I). Isolates from the Sabie area were collected from *E. grandis* × *camaldulensis* hybrid clones. Isolates from Paulpietersburg, Kwambonambi and Bushbuckridge were from artificially induced wounds on *E. grandis* and *E. grandis* × *camaldulensis* respectively. No *C. pirilliformis* isolates were obtained from Lothair, Pietermaritzburg and Tzaneen. Isolates obtained from wounds were associated in some cases with blue to brown streaks in the cambium, spreading upward from the wounds. No other disease symptoms were observed.

All isolates were identified as *C. pirilliformis* based on the morphology of pure cultures as described by Barnes et al (2003b). An additional 16 isolates from Australia were obtained from the culture collection of the Forestry and Agricultural Biotechnology Institute (FIG. 2, TABLE I). These included 13 isolates from *E. nitens* growing near Canberra and three isolates from *E. nitens* near Brisbane.

Microsatellite amplification and allele scoring.—Eighteen of the 27 sets of microsatellite primers (TABLE II) amplified products within the expected size ranges. However, only seven of these were polymorphic in all isolates tested (TABLE IV). At four of the loci some alleles were scored (based on length) that were only one base pair different in length. In locus AG1/2 alleles 274 and 275 were scored, in locus AG7/8

TABLE III. Multilocus genotypes across the seven polymorphic loci of *C. pirilliformis* from South Africa and Australia

Isolate (CMW)	Origins	Multilocus genotypes							Genotype number
South African isolates									
11722	KW	B	B	B	D	B	B	B	**1**
12677, 16522	BU, SA	A	C	F	C	D	A	D	**2**
16521	SA	A	E	B	C	B	A	D	**3**
12676, 12680, 16469, 16514	SA, BU, PA	A	E	B	C	D	A	D	**4**
16515	SA	A	E	F	C	D	A	D	**5**
11699, 12281	BU, PA	A	B	B	C	B	A	D	**6**
16523	SA	A	B	D	C	B	A	D	**7**
16527	PA	A	B	D	C	D	A	D	**8**
16463, 16517, 16520, 16525, 16526, 16529	PA, SA	A	B	F	C	D	A	D	**9**
16466	PA	A	C	B	C	D	A	D	**10**
16516	SA	A	D	H	C	D	A	D	**11**
12673, 12675	BU	A	D	F	C	D	A	D	**12**
12671	PA	A	D	E	C	B	A	D	**13**
16470, 16528, 16749	SA, PA	A	D	B	C	B	A	D	**14**
16747	SA	A	D	F	C	B	A	D	**15**
12679, 16467, 16471, 16511, 16512, 16518, 16519, 16524, 16746, 16750, 17914	BU, PA, SA	A	D	B	C	D	A	D	**16**
Australian isolates									
6670	CA	A	A	B	E	B	A	C	**17**
6566, 6571	CA	B	C	B	E	B	A	C	**18**
6574, 6575, 6576, 6577, 6579	CA	B	B	C	A	B	B	A	**19**
6556, 6583, 6586	CA	B	B	D	C	B	B	D	**20**
6569	CA	B	B	D	E	B	A	D	**21**
6563	CA	A	C	C	B	A	A	D	**22**
19334, 19344	BR	A	B	G	C	C	A	C	**23**
19341	BR	A	B	A	C	C	A	D	**24**

SA, refers to Sabie; PA, refers to Paulpietersburg; BU, refers to Bushbuckridge; KW, refers to Kwambonambi; CA, refers to Canberra and BR, refers to Brisbane.

alleles 276 and 277 were scored, in locus CF17/18, alleles 273 and 274 were scored and in locus CF21/22, alleles 248 and 249 were scored. These alleles were sequenced but no differences were observed in sequence within the microsatellite or flanking regions. This is due most probably to the A-tailing effect or plus 1 effect during PCR amplification (Clark 1988, Magnuson et al 1996). They were thus treated as single alleles with sizes 249, 274, 275 and 277 at loci CF21/22, CF17/18, AG1/2 and AG7/8 respectively.

After correction and confirmation of alleles a total of 30 alleles (2–8 alleles per locus) were obtained from the seven polymorphic loci amplified for the South African and Australian isolates of *C. pirilliformis* (TABLE IV). Eleven alleles were shared by isolates from the two countries, eight were unique to the South African isolates and 11 were unique to the Australian collection. Locus CF15/16 was the most polymorphic containing a total of eight alleles. Allele frequencies obtained for all loci were recorded (TABLE IV) and ranged from the least frequent allele at 0.06 to the most frequent allele at 0.94.

Analyses of diversity.—The gene diversity per locus ranged from H = 0.12 to H = 0.78 with an average gene diversity value of H = 0.34 and H = 0.57 for the South African and Australian isolates respectively (TABLE V). In all loci except one (CF11/12), the allelic richness and unique allele richness was greater in the Australian collection than in the South African collection with an average allele richness of 3.14 and 2.49 respectively (TABLE V).

From a collection of 55 isolates, 24 different genotypes were identified including 16 from South Africa and eight from Australia (TABLE III). No shared genotypes occurred for the South African and Australian isolates. Thirteen (81.3%) of the South African genotypes differed at only one allele at each of the seven polymorphic loci. The genotypic diversity was $\hat{G} = 7.49$ and $\hat{G} = 5.56$ for the South African and Australian isolates of *C. pirilliformis* respectively with a maximum genotypic diversity of 19.2% and 34.78% respectively. The *t*-test indicated that there was no significant difference between the genotypic diversities obtained for the South African and Australian

TABLE IV. Allele frequency per locus and number of unique alleles for South African (RSA) and Australian (AUS) collections of *C. pirilliformis* using seven polymorphic microsatellite markers

	AG1/2		CF11/12		CF15/16		AAG8		CCAA15		CAGDL2-5		CCAG15	
Allele/Locus	RSA	AUS	RSA	AUS	RSA	AUS	RSA	AUS	RSA	AUS	RSA	AUS	RSA	AUS
A	0.94	0.50		0.13		0.13		0.13		0.13	0.94	0.75		0.13
B	0.06	0.50	0.31	0.63	0.44	0.25		0.13	0.44	0.63	0.06	0.25	0.06	
C			0.13	0.25		0.25	0.94	0.38		0.25				0.38
D			0.38		0.13	0.25	0.06		0.56				0.94	0.50
E			0.19		0.06			0.38						
F					0.31									
G						0.13								
H					0.06									
No unique alleles	0	0	2	1	3	3	1	3	1	2	0	0	1	2

collections. The genotypic diversity versus the number of loci gave rise to a curve that is approaching a plateau (data not shown), suggesting that the sample size for the South African collection of *C. pirilliformis* was large enough for a population study.

DISCUSSION

Results of this study demonstrate that *C. pirilliformis* occurs in several *Eucalyptus*-growing areas of South Africa. In addition the genetic diversity was lower in South Africa on introduced hosts than on native hosts in Australia. These results are consistent with the view that *C. pirilliformis* probably was introduced into South Africa.

Ceratocystis pirilliformis was collected widely in this study, but it was never associated with cankers or dying trees, although it was isolated from discolored areas within the wood. The areas sampled in South Africa included those with temperate and subtropical climates, suggesting that *C. pirilliformis* can exist effectively under a wide range of environmental conditions. Likewise Australian isolates of the fungus originate from Canberra, which has a cold winter (temperatures less than 0 C and regular frosts), and Brisbane, which has a subtropical climate. It thus appears that *C. pirilliformis* could become established in most *Eucalyptus* growing areas of South Africa. The absence of the fungus from Pietermaritzburg and Tzaneen is best explained by the fact that it might not yet have spread to those areas.

Surveys for *C. pirilliformis* in this study gave rise to large numbers of isolates of *C. fimbriata s.l.* These isolates were used in a population diversity study (van Wyk et al 2006). *Ceratocystis fimbriata s.l.* isolates from these collections had a higher gene diversity (H = 0.36) than that for *C. pirilliformis* (H = 0.34) in the present study. Furthermore *C. fimbriata s.l.* was found in all areas surveyed and it also was much more common than *C. pirilliformis*. For example it was found commonly in the Tzaneen area in the absence of *C. pirilliformis*. In the Kwambonambi area, where only one isolate of *C. pirilliformis* was collected, 31 isolates of *C. fimbriata s.l.* were obtained. These

TABLE V. Number of isolates and alleles and measures of genetic diversity per locus for the Australian and South African collections of *C. pirilliformis*

	No. of isolates[1]		No. of alleles		H[2]		Allelic richness		Private allelic richness	
	RSA	AUS	RSA	AUS	RSA	AUS	RSA	AUS	RSA	AUS
AG1/2	16	8	2	2	0.12	0.50	1.76	2.00	0.00	0.24
CF11/12	16	8	4	3	0.71	0.53	3.94	3.00	1.99	1.05
CF15/16	16	8	5	5	0.69	0.78	4.47	5.00	2.52	3.05
AAG8	16	8	2	4	0.12	0.69	1.76	4.00	0.76	3.00
CCAA15	16	8	2	3	0.49	0.53	2.00	3.00	1.00	2.00
CAGDL2-5	16	8	2	2	0.12	0.38	1.76	2.00	0.00	0.24
CCAG15	16	8	2	3	0.12	0.59	1.76	3.00	0.76	2.00
Total/Mean	16	8	19	22	0.34	0.57	2.49	3.14	1	1.65

[1] Clone corrected.
[2] H = Nei's (1973) gene diversity.

results suggest that *C. fimbriata s.l.* has been present on *Eucalyptus* spp. in South Africa longer than *C. pirilliformis*.

The fact that microsatellite markers produced for *C. fimbriata* (Barnes et al 2001, Steimel et al 2004) were effective on *C. pirilliformis* is not surprising. This fungus resides in the *C. fimbriata s.l.* clade and the two fungi are clearly closely related. Various of these markers also have been applied effectively to studies on *C. albifundus* (Barnes et al 2005), showing that they have a broad potential application within the *C. fimbriata s.l.* clade of *Ceratocystis*.

Ceratocystis pirilliformis isolates showed relatively low levels of gene diversity and allelic richness among the South African collections. In comparison the small collection of Australian isolates originating from fewer than 10 trees in two different areas, displayed much more gene diversity and allelic richness (TABLE V). This supports the hypothesis that *C. pirilliformis* is not native to South Africa.

The genotypic diversity of the South African collection was much higher ($\hat{G} = 7.49$) compared to that generated from the Australian isolates ($\hat{G} = 5.56$). This probably is misleading and could be due to differences in sample size between the two collections. However in the analyses the difference in sample size was compensated for by calculating the measure of allelic richness and private allelic richness based on rarefaction as well as calculating the maximum genotypic diversity of the two collections of isolates. These calculations showed that the allelic richness for the Australian isolates was much higher compared to those of the South African collection. In addition there were more alleles in Australian isolates (22) than in South African collection (19). The maximum genotypic diversity of the Australian isolates was also higher ($G_{max} = 34.78\%$) compared to those of the South African collection ($G_{max} = 19.2\%$). This difference however was not significant based on the *t*-test (Chen et al 1994) performed. These confirmed the fact that the Australian isolates of *C. pirilliformis* had more diversity than the South African collection and further support the view that the fungus is not native to South Africa. More isolates from Australia in particular is required to investigate this further.

Recently introduced populations are generally recognized by their low gene diversity in contrast to native populations that typically have very high gene diversity in their natural environment (Gordon et al 1996, McDonald 1997). However, multiple introductions of an organism such as a fungus into a new ecosystem also would result in high gene diversity (Burdon and Roelfs 1985, Burgess et al 2001). The genetic diversity exhibited by the South African population of *C. pirilliformis* in this study was low when compared with that of, for example *C. albifundus* in Uganda (H = 0.41) and South Africa (H = 0.38) (Barnes et al 2005) and the South African population (H = 0.36) of *C. fimbriata s.l.* (van Wyk et al 2006). For these reasons we assume that *C. pirilliformis* has been introduced into South Africa only recently and also that there have been few introductions. This is also supported by the relatively limited geographic distribution of the fungus in South Africa.

Ceratocystis pirilliformis is a relatively newly discovered fungus in South Africa and little is known regarding its biology or origin. Species of *Ceratocystis*, particularly those in the *C. fimbriata s.l.* species complex, are known to have close associations, mainly with casual insects (Jewell 1956, Juzwik and French 1983, Juzwik et al 1998). *Ceratocystis fimbriata s.l.* for example has been shown to be vectored by drosophylid flies (*Chymomyza procnemoides*) and nitidulid beetles (*Carpophilus freemani*) (Moller and Devay 1968). The spread of *C. pirilliformis* within South Africa thus could be aided by insects and the movement of infected timber. Fresh mycelium and fruiting bodies of *C. pirilliformis* were found abundantly under bark flaps during the survey. Under favorable conditions such as high humidity the fungus thus could be spread easily from one geographic area to another, under bark that has not been completely removed from cut timber.

Ceratocystis pirilliformis was not found to be associated with tree mortality or canker formation. The fungus however has been shown to be capable of causing significant bark and cambium lesions on inoculated 1 y old *E. grandis* trees under field conditions (Roux et al 2004). Whether *C. pirilliformis* is able to kill trees naturally remains unclear. The same is also true for *C. fimbriata s.l.* on *Eucalyptus* in South Africa, but this fungus has resulted in serious disease of *Eucalyptus* trees in Uruguay and Congo (Barnes et al 2003a, Roux et al 1999). Further studies clearly are required to clarify the potential impact that *C. pirilliformis* and *C. fimbriata* may have in South Africa.

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