Chapter 4

Polymorphic microsatellite markers for the *Eucalyptus* fungal pathogen *Colletogloeopsis zuluensis*

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ABSTRACT

Nine polymorphic microsatellite markers for the phytopathogenic fungus *Colletogloeopsis zuluensis*, the causal agent of an important stem canker disease of *Eucalyptus*, were isolated and characterised. Two methods, (RAMS) and fast isolation by AFLP of sequence containing repeats with modifications (M-FIASCO) were used to isolate the microsatellites. Primers for 28 prospective microsatellite regions were designed and nine of these were polymorphic for *C. zuluensis*. Allelic diversity ranged from 0.12 to 0.80 with a total of 37 alleles. These markers will be used in future to determine the population genetic structure of *C. zuluensis* isolates and to monitor their global movement.

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INTRODUCTION

Colletogloeopsis zuluensise causes a serious stem canker disease on *Eucalyptus* (Wingfield *et al.,* 1997; Cortinas *et al.*). The fungus was first discovered in South African plantations and has subsequently been found in many other tropical and sub-tropical countries. *Colletogloepsis zuluensis* is an ascomycete closely related to *Mycosphaerella* Johansson, a genus of more than 800 species, approximately 60 of which have been identified as the causal agents of *Eucalyptus* leaf diseases (Crous *et al.,* 2004). Interestingly, this pathogen occurs only on stems of trees and never infects leaves. Sexual structures have never been reported and in contrast to many other *Mycosphaerella* spp., it has never been observed in the native range of *Eucalyptus*. The aim of this study was to isolate polymorphic microsatellite markers for *C. zuluensis* to be used in future studies considering the genetic structure, mode of reproduction and relationships among individuals emerging from disease outbreaks in many parts of the world.

MATERIALS AND METHODS

Two methods were used to screen for microsatellite sequences in *C. zuluensis*. Random Amplified Microsatellite Sequences (RAMS) (Hantula *et al.,* 1996) with anchored 3' primers (Zietkiewicz *et al.,* 1994) were used. PCR reactions using 45 combinations of anchored di-, tri- and tetranucleotide primers were then undertaken. Six banding patterns generated by PCR were cloned using the cloning kit PGEM T Easy (Promega). Sequences containing microsatellites were recovered by Genome Walking (Siebert *et al.,* 1995). The other method used was FIASCO (Zane *et al.,* 2002) with modifications (M-FIASCO) using the biotinylated probes (TC)15, (CA)15, (TCC)7, and (ATA)7. A detailed protocol of M-FIASCO can be found as Appendix II in this thesis.

Genomic DNA was extracted according to Cortinas *et al.,* (2004). A total of 1µg genomic DNA was pooled from isolates CMW1048 and CMW1026 from South Africa and CMW5236 from Thailand to screen for microsatellites. All *C. zuluensis* isolates used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. Human DNA, was used in parallel with *C. zuluensis* DNA as a positive control.

Modifications of the FIASCO method included preparation of the digestion-ligation using *Mse*I restriction enzyme and a highly concentrated T4 DNA ligase (2000000 U/ ml) (Hamilton *et al.,* 1999). Both enzymes were acquired from New England BioLabs and the same buffer was used. Another modification included the addition of 10 µg of tRNA (Sigma), rather than unrelated PCR product, to the magnetic beads to minimize the non-specific binding of the genomic DNA before mixing with the hybridisation complexes (Zane, pers. com.). Furthermore, a number of A nucleotides, "A tailing", were extended at the 3'end of the PCR fragments immediately before cloning into the TOPO 4- TA Kit (Invitrogen), to increase the cloning efficiency. One µg of cleaned PCR product was mixed with 4 µL dATP (2 mM), 0.2 µL (1 U) *Taq* polymerase (Roche), 2.5 µL of 10x *Taq* polymerase buffer with $MgCl_2$ (500 mM Tris/HCl, 100 mM KCl, 50 mM $(NH4)_2SO_4$, 20 mM $MgCl_2$, pH 8.3) (Roche) and 10.3 µL distilled water. Fragments were incubated at 72 °C for 15 min in an iCycler PCR machine (Bio-Rad).

After cloning, colony-PCRs were carried out by diluting 5 µL of the cell culture suspension in 50 µL of distilled water. Dilutions were incubated for 7 min at 96 °C and 1 µL was used as template in the colony-PCR reactions together with M13 TOPO 4 primers (5'-GTAAAACGACGGCCAG-3'/ 5'-CAGGAAACAGCTATGAC-3') (Invitrogen) as described in Zane *et al.,* (2002). Three µL of PCR products, cleaned with Sephadex G-50 (Sigma), were used in 10 µL total sequencing reactions using Big Dye v3.1 (Applied Biosystems) and the previous TOPO 4 primers using the following thermal profile: 96 °C for 10 s, 56 °C for 30 s, and 60 °C for 4 min for a total of 25 cycles using an iCycler (Bio-Rad) PCR machine. The sequencing extension products were purified using the Ethanol/EDTA/Sodium Acetate precipitation protocol following the manufacturer's protocol. Electrophoresis was carried out on an ABI 3100 auto sequencer (Applied Biosystems).

Eight putative microsatellites were finally recovered after genome walking using RAMS and twenty putative microsatellites were obtained using M-FIASCO. Primers for these microsatellite regions were designed by eye and using Oligo Analyser 3 (Integrated DNA Technologies) available on the internet at http://www.idtdna.com/Home/Home.aspx, to adjust Tm, length and check for the

formation of hairpins, self-dimers and hetero-dimers. To test for polymorphisms, ten isolates were chosen to span a wide range of geographical origins of *C. zuluensis* from South Africa, Thailand and China. PCR using an iCycler (BioRad) were performed in 25 µL reactions containing 100 ng DNA template, 0.2 mM dNTPs (Promega), 0.15 µM of each primer, 0.2 µL Taq Polymerase (Roche), 1x buffer with $MgCl_2$ (50 mM Tris/HCl, 10 mM KCl, 5 mM $(NH4)_2SO_4$, 2 mM $MgCl_2$, pH 8.3) (Roche) and 18µL of distilled water under the following thermal conditions: 96°C 1min, 35 cycles of 94 °C for 30 s, annealing temperature according to Table 1 for 30 s, and extension at 72°C for 1 min. After PCR, products were run on 3 % agarose gels or sequenced to the detect polymorphisms. To sequence the amplicons, the specific designed primers were used using the same PCR sequencing conditions previously described. Two of the putative RAMS loci and seven of the M-FIASCO loci contained polymorphic microsatellites. The forward primers of the polymorphic loci were fluorescently labelled using NED, VIC, FAM or PET dyes for filter set G5 (Applied Biosystems) and tested on DNA from 30 additional isolates (CMW4518, CMW5236, CMW7411, CMW7420, CMW7425, CMW7426, CMW7435, CMW7438, CMW7440, CMW7442, CMW7443, CMW7447, CMW7459, CMW7460, CMW7463, CMW7470, CMW7491, CMW11239, CMW13324, CMW15833, CMW15963, CMW15970, CMW17315, CMW17317, CMW17320, CMW17322, CMW17404, CMW17406, CMW17476, CMW17477). The fragments were electrophoresed on an ABI 3100 auto sequencer (Applied Biosystems). Allele sizes for all the isolates were determined using ABI Genemapper, version 3.0 (Applied Biosystems) using LIZ 500 size standard. The allelic diversity of polymorphic alleles was evaluated according to Nei 1973. Linkage disequilibrium (LD) was calculated using MULTILOCUS 1.2 (Agapow & Burt 2001).

RESULTS AND DISCUSSION

The allelic diversity (Nei 1973) of nine polymorphic alleles ranged from 0.12 to 0.80 with a minimum of two, and a maximum of eight alleles per locus (Table 1). Thirty-seven alleles were observed across the nine loci. No Linkage disequilibrium (LD) was detected between any pair of loci. Cross-species amplification on *Mycosphaerella* spp. (*M. nubilosa*, *M. molleriana*, *M. vespa*, *M. ambiphylla*, *M. cryptica* and *M.*

suttonii) that are phylogenetically related to *C. zuluensis,* produced negative or non-specific amplifications for the nine polymorphic microsatellite loci. The results suggest the fact that these fungi have been reproductively isolated for a significant period of time.

The overall recovery efficiency of putative microsatellite loci, considering the total number of clones sequenced per method was 3.2 % using RAMS (250 clones) and 5.7 % with M-FIASCO (352 clones). In contrast, the human DNA control produced 68 % microsatellite sequences using M-FIASCO (100 clones). The nine polymorphic microsatellite markers developed in this study will now be used to consider the population genetic structure and the reproductive strategy of *C. zuluensis*. They will also be used to determine whether gene flow occurs among populations from different areas of occurrence of the pathogen.

ACKNOWLEDGEMENTS

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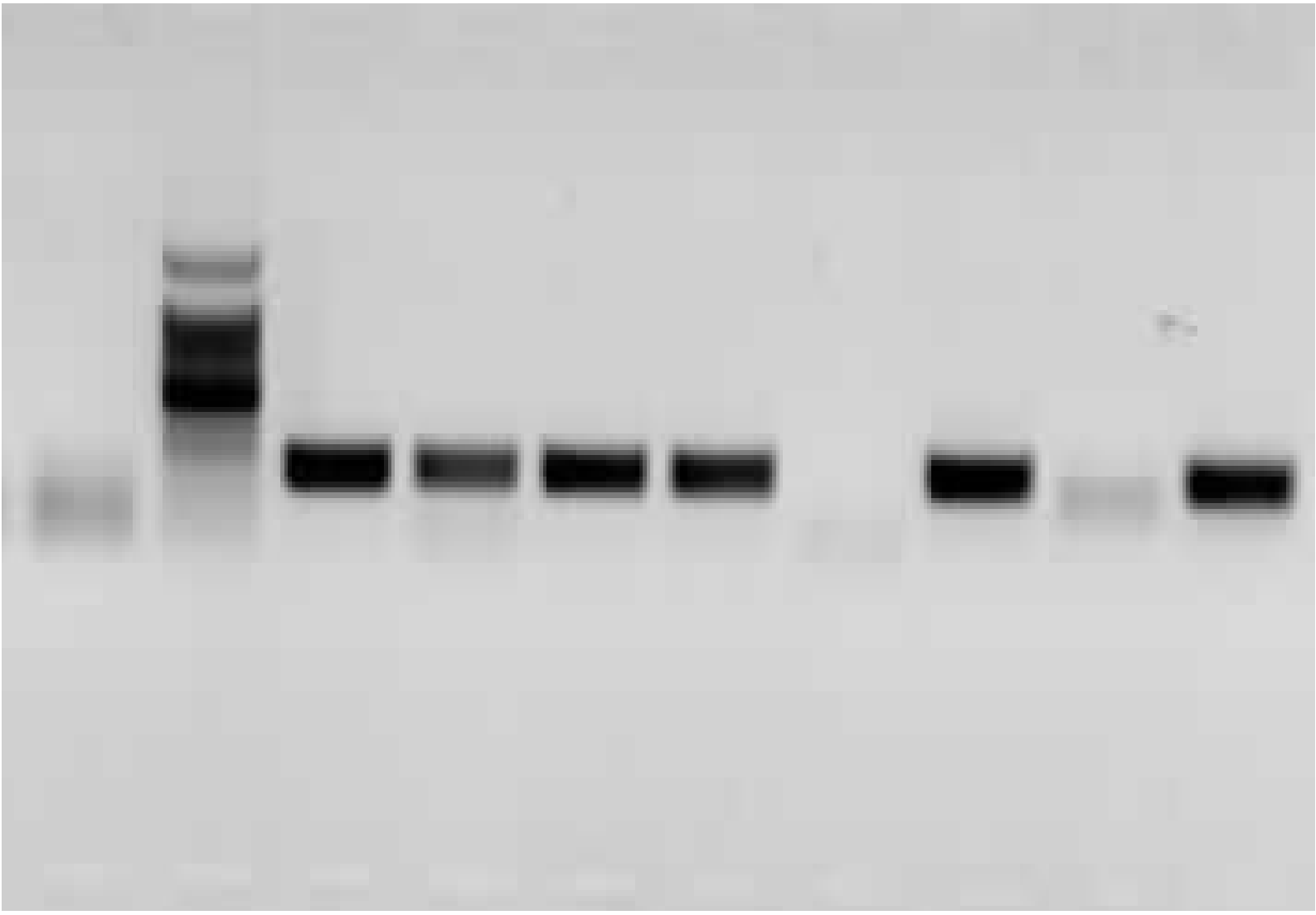
Table 1 Locus and primer names, GenBank Accession numbers, primer sequences, repeat motif, annealing temperature (*T*a), $MgCl_2$ concentration, number and size range of alleles and observed (*H*) allelic diversity (Nei 1973) of the nine polymorphic regions analysed in this study using 30 isolates of *Colletogloeopsis zuluensis*.

Locus name	Primer names	GenBank Accession no.	Primer sequences (5'-3')	Repeat motif	*T*a (^{0}C)	$MgCl_2$ (mM)	No. of alleles	Size range (bp)	Mean *H*	N°. of isolates tested
Czulu 1	Czulu 1F	DQ156110	PET – CTG ATG GCA ATG GGC GTG TGA C	(TG)8	58 °C	3.5	4	153-159	0.35	30
	Czulu 1R		GCC TCT TGC TCT GGC TGT AGG T							
Czulu 2	Czulu 2F	DQ156111	PET – AAG CAT GAA ACG GAC TCT GCG C	(TG)6	61 °C	3.5	4	185-188	0.69	30
	Czulu 2R		GAC GAG GGT GAT GGT CGT TGC							
Czulu 3	Czulu 3F	DQ156112	NED - GGA CAT TGA TTT CAC GCC GAC G	(TGG)9	58 °C	3.5	2	169-172	0.12	30
	Czulu 3F		CTG CAA CGA CAA ATC TCA ACC TG							
Czulu 4	Czulu 4F	DQ156113	FAM - GAC TTT GAC CAG CAT GTC GAC C	(TGG)5	62 °C	3.5	2	149-152	0.23	30
	Czulu 4R		GTG TGG AGG TGG GAA GTG GTG							
Czulu 5	Czulu 5F	DQ156114	FAM - GTT GTG TCC GAT CCT GCG AAG C	(CG)7(AG)21CA(AG)9	62 °C	2.0	7	174-196	0.80	30
	Czulu 5R		CAA GGG CGA AGT CGA GTA TGA GG							
Czulu 6	Czulu 6F	DQ156115	NED – CCA ACC CCA CCA TCA ACC TCA	(TCC)4… 125bp…(CAT)9	61 °C	3.5	5	322-339	0.48	30
	Czulu 6F		TAC CCC CTC CAA AGC TAA CCC							
Czulu7	Czulu 7F	DQ156116	NED – ACA ACC CAC TCC CTA CCC CGG	(ACCCC)6	65 °C	3.5	3	213-225	0.55	30
	Czulu 7R		AAT TGG GCT ATG CTG GTC ACT CG							
Czulu 8	Czulu 8F	DQ156117	VIC – AGC ACG CTG CAC GAG CAA CGG	(TCCC)6… 27bpTC-rich region…	65 °C	2.0	8	185-339	0.76	30
	Czulu 8R		TCG TTT GTG GGG GCC AGC GGC	(GTCTCCCTCTCT)8						
Czulu 9	Czulu 9F	DQ156118	PET - TTA GCC GTC TGG AGT GAA GAG G	(ACC)9 ATCACCACCGTT(ACT)14	58 °C	3.5	2	221-225	0.23	30
	Czulu 9R		GCT TTG TAA GCG CGG TAC GTG							

Chapter 5

Microsatellite markers for the E*ucalyptus* stem canker fungal pathogen *Kirramyces gauchensis*



Chapter 5

Microsatellite markers for the *Eucalyptus* stem canker fungal pathogen *Kirramyces gauchensis*

ABSTRACT

Ten microsatellite markers were developed for the fungus *Kirramyces gauchensis*, which causes an important stem canker disease of *Eucalyptus* trees in plantations. Primers for 21 microsatellite regions were designed from cloned fragments. Fourteen of the primer pairs provided single amplicons and 10 of these were polymorphic for *K gauchensis*. Allelic diversity ranged from 0.24 to 0.76 with a total of 30 alleles. None of the markers was able to amplify in the phylogenetically distinct but morphologically similar species *Kirramyces zuluensis.* The 10 characterized polymorphic microsatellite regions will be studied to determine the population structure of *K gauchensis* in plantations of different countries.

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INTRODUCTION

Species of *Kirramyces* include important pathogens of *Eucalyptus* leaves, shoots and stems (Andjic *et al.,* 2007). *Kirramyces* (= *Colletogloeopsis*) *gauchensis* is the casual agent of a serious stem canker disease on *Eucalyptus* trees (Cortinas *et al.,* 2006b, c). This fungus is very similar to but phylogenetically distinct from *Kirramyces* (= *Colletogloeopsis*) *zuluensis,* which is also an important *Eucalyptus* stem canker pathogen. *Kirramyces gauchensis* has a wide geographic distribution and has been recorded on *E. grandis, E. tereticornis, E. camaldulensis* and different hybrids in plantations of South American and African countries as well as in Hawaii. The fungus has never been found in the native range of *Eucalyptus* or infecting trees of other genera. At present, the origin of this fungus is unknown. The mycelia of *Kirramyces gauchensis* is haploid as well as the anamorph reproductive structures found in nature, the pycnidias. Like in *K. zuluensis*, sexual or teleomorph reproductive structures have never been reported and is thus *K. gauchensis* is considered an anamorph genus of the teleomorph genus *Mycosphaerella*. However, other closely related species of *Kirramyces* have *Mycosphaerella* sexual states and phylogenetic inference suggests that the same could be true for *K. gauchensis* and *K. zuluensis*.

Microsatellite markers have been useful in understanding the population biology of many fungal pathogens (e.g. McDonald 1997; Zhan & McDonald 2004; Feau *et al.,* 2005). Initial studies on *K. gauchensis* were frustrated by the fact that microsatellite primers developed for *K. zuluensis* did not amplify any amplicons (Cortinas *et al.,* 2006c). However, this fact and multilocus phylogenetic analyses led to the discovery that isolates initially treated as a single species actually represented distinct taxa (Cortinas *et al.,* 2006c). The objective of this study was, therefore, to isolate and characterize microsatellite loci that can be used to study the population structure of *K. gauchensis,* collected from diseased trees in different countries.

MATERIALS AND METHODS

The microsatellite-containing regions were isolated using a modified form (Cortinas *et al.,* 2006a) of the FIASCO technique of Zane *et al.,* (2002). All isolates used in this

study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. To screen for repetitive sequences, 1 µg of genomic DNA was pooled from the isolates CMW 7474, CMW 7300, CMW 7279 of *K. gauchensis*. Genomic DNA was extracted from cultures using phenol-chloroform following the method described by Cortinas *et al.,* (2006b, c). The biotinylated probes (TC)15, (CA)15 and (GATA)6 were used to enrich the genomic DNA. All PCR's were carried out using an iCycler (Bio-Rad) using the thermal profiles described in Cortinas *et al.,* (2006a).

Of 384 sequenced clones, 21 contained repetitive regions. Primers for these 21 loci were designed visually. OLIGO Analyser 3 (Integrated DNA Technologies) was used to check the melting temperature (Tm), formation of hairpins, self-dimers and hetero-dimers. When the designed primer pairs were tested, 14 primer pairs resulted in single amplicons of the expected size range. One primer from each of the 14 primer pairs was labeled with fluorescent dyes using NED, VIC, FAM or PET dyes for filter set G5 (Applied Biosystems), to allow detection on an ABI 3100 sequencer. PCR amplifications were performed in 25 µL reactions containing 100 ng DNA template, 0.2 mM dNTPs (Promega), 0.15 µM of each primer, 0.2 µL (1U) *Taq* Polymerase (Roche), 1x buffer with $MgCl_2$ (50 mM Tris/HCl, 10 mM KCl, 5 mM $(NH4)_2SO_4$, 2,0 mM $MgCl_2$ or 3,5 mM $MgCl_2$, pH (8.3) (Roche) (Table 1) and 18.0 µL of distilled water. The thermo-cycling conditions were as follows: initial denaturation at 96 °C for 4 min, followed by 10 cycles of denaturation at 94°C for 30 s, annealing temperature according to Table 1 for 30 s, and extension at 72°C for 1 min, followed by 30 cycle repetitions of denaturation at 94°C for 30 s, annealing temperature according to Table 1 for 30 s and extension at 72°C for 1 min (with 5 s increments every 2 repetitions). A final extension was carried out at 72 °C for 45 min. Fragment size analysis was carried out after electrophoresis using the software GENEMAPPER, version 3.0 (Applied Biosystems) and LIZ 500 (-250) size standard (Applied Biosystems).

To assess the level of polymorphism, 21 isolates from Argentina (CMW4915, CMW14336, CMW14337, CMW14338, CMW14339, CMW14343, CMW14345, CMW14347, CMW14348, CMW14349, CMW14351, CMW7345, CMW14510,

CMW14510, CMW14511, CMW14512,CMW14515, CMW14516, CMW7342, CMW1458, CMW15835), and an equal number of isolates from Uruguay (CMW17561, CMW1495, CMW1501, CMW1502, CMW7270, CMW7272, CMW7275, CMW7276, CMW7277, CMW7278, CMW7281, CMW7282, CMW7287, CMW7290, CMW7292, CMW7293, CMW7298, CMW7299, CMW7305, CMW7306, CMW7309) were genotyped. Of the 14 designed primers pairs, 10 loci were polymorphic, two were monomorphic and two yielded complex stutter patterns that were difficult to interpret.

RESULTS AND DISCUSSION

The allelic diversity (Nei 1973) of the 10 polymorphic loci ranged from 0.21 to 0.76 with a minimum of two, and a maximum of six alleles per locus (Table 1). Thirty alleles were found across the 10 loci. Linkage disequilibrium (LD) was calculated using MULTILOCUS 1.2 (Agapow & Burt 2001). Significant LD ($P< 0.05$) was detected for some loci pair comparisons (data not shown), suggesting little evidence for recombination. This indicates that clonal reproduction can be playing an important role in the reproductive structure of this species. Confirmation of this result will be needed with comprehensive population studies. Cross-species amplification between *K. gauchensis* and the closely related *K. zuluensis* (25 isolates) produced negative, incorrect size bands or smeared amplifications, suggesting that the two species no longer share these loci. These primers also failed to amplify amplicons when tested as diagnostic markers on two other related species, *M. nubilosa* and *M. molleriana.*The primers are thus not only useful as population markers but also have the potential to be used as species-specific markers to identify *K. gauchensis* in the development of a DNA-based identification technique.

In this study, 10 microsatellite loci have been characterized and shown to be specific for *K. gauchensis.* These markers can now be applied to populations of the pathogen from different parts of the world, as part of an effort to understand its global diversity and population biology. Such studies will hopefully also enhance efforts to reduce the impact of the pathogen on *Eucalyptus* forestry.

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Table 1 Locus and primer names, GenBank Accession numbers, primer sequences, repeat motif, annealing temperature (*Ta*), $MgCl_2$ concentration, number and size range of alleles and observed (*H*) allelic diversity (Nei 1973) of the ten polymorphic loci analysed in this study using 42 isolates of *Kirramyces gauchensis*.

Locus name	Primer names	GenBank Accession no.	Primer sequences (5'-3')	Repeat motif	*Ta*	$MgCl_2$ (mM)	No. of alleles	Size range (bp)	Mean *H*	No. of isolates tested
K. gauchensis 1	Kgauche 1F	DQ975190	NED - CTC CAT TGC ATC GGG TCT CAT G	(AG)24	59 °C	3.5	6	290-	0.76	42
	Kgauche 1R		GGT GGC AAG TTC GAG CTT CA					327		
K. gauchensis 2	Kgauche 2F	DQ975191	PET - CAA ATC CTC GGC TGC GTC ATG G	(GA)4 TA(GA)4	54 °C	3.5	3	148-	0.50	42
	Kgauche 2R		CAC TGC GCT TTC GTC TCT ACC GA					183		
K.gauchensis 3	Kgauche 3F	DQ975192	NED - AGA TGG CTG TAC GAA GAA TGT CC	(CT)7 AC(TC)17	60 °C	3.5	3	211-	0.42	42
	Kgauche 3F		AAG CCA ATC CAC GCG TCA AGG	TTTCT(GT)12				266		
K.gauchensis 4	Kgauche 4F	DQ975193	VIC - CCG CGA GAG AAA CAA CAT CC	(GA)10	59 °C	3.5	2	251-	0.24	42
	Kgauche 4R		GAT AGG AGG CAC ATA ACC CAA G					260		
K.gauchensis 5	Kgauche 5F	DQ975194	FAM - TTG GCC AGC AGG AAC ATG AGC	(GTGGT)GGT(GTGGT)	62 °C	2.0	2	288-	0.43	42
	Kgauche 5R		CAC TCA TTC ACT TGA CCG CCT C	3 (GGT)2(GTGGT)2				294		
K.gauchensis 6	Kgauche 6F	DQ975195	FAM - CGC CTT ATG CCT TTG ATG GTT GC	(GT)15	56 °C	3.5	4	165-	0.43	42
	Kgauche 6F		GAT TCC TAA ATC GAC CAT CCG C					203		
K.gauchensis7	Kgauche 7F	DQ975196	VIC - ACC AGG GAT GCC GTA TGT GCA G	(TG)9	60 °C	3.5	2	107-	0.46	42
	Kgauche 7R		CAT CAC ACA CCG TCC TCC CAC					109		
K.gauchensis 8	Kgauche 8F	DQ975197	PET - ATC ATC TGC CCT TGG ACG GAC G	(TG)9	59 °C	2.0	3	134-	0.21	42
	Kgauche 8R		CCA TCA CCA CAC GAA ACA TCA AG					150		
K.gauchensis 9	Kgauche 9F	DQ975198	FAM - GAT CAC GCA ATG AGA GTG TCT CC	(ACAG)5	54 °C	3.5	2	89-98	0.52	42

	Kgauche 9R		GGT TTC CGA CTG ATT GGT TCA TC								
K.gauchensis 10	Kgauche 10F	DQ975199	PET – ATA GTA AGA AGA TAA ATA AGG CG	(AAG)53	52°C	3.5	3	134-	0.40	42
	Kgauche 10R		GCG AAG TAG ACT ATA TAA GTA TC					143		

Chapter 6

Genetic diversity in the *Eucalyptus* stem pathogen *Teratosphaeria zuluensis*

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ABSTRACT

Coniothyrium canker caused by the fungal pathogen *Teratosphaeria (= Coniothyrium) zuluensis* is one of the most important diseases affecting plantation-grown *Eucalyptus* trees. Little is known regarding the pathogen and this study consequently considers the genetic diversity and population structure of *T. zuluensis*. Eleven microsatellites markers, of which six were developed in this study, were used to analyze two temporally-separated populations of *T. zuluensis* from South Africa, one population from Malawi and a population from China. Results showed that the populations of *T. zuluensis* have a moderate to high diversity and that clonal reproduction is predominant. There was also evidence that the genetic diversity of the pathogen in South Africa has increased over time. Comparison of *T. zuluensis* populations from South Africa, Malawi and China suggest that South Africa is most probably not the centre of origin of the pathogen as has previously been suggested.

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INTRODUCTION

Numerous new diseases have emerged in plantations of non-native *Eucalyptus* spp. during the course of the past three decades (Wingfield *et al.,* 2008). This largely coincides with global expansion of *Eucalyptus* plantations in the tropics and sub-tropics (Park *et al.,* 2000; Old *et al.,* 2003; Wingfield 2003). Amongst the most important of these new diseases is Coniothyrium canker (Wingfield *et al.,* 1997; Old *et al.,* 2003), which first appears as small necrotic spots on the young green bark of *Eucalyptus* trees. These can subsequently develop into large girdling stem cankers and in some cases cause tree death (Wingfield *et al.,* 1997; Van Zyl *et al.,* 2002a). The disease has spread rapidly in South Africa, and for a period of about ten years, seriously threatened the rapidly expanding clonal *Eucalyptus* plantations, particularly in the Zululand forestry area.

Coniothyrium canker was first discovered in plantations of *Eucalyptus grandis* in the Zululand forestry area of South Africa in 1991 and the causal agent was described as the new species, *Coniothyrium zuluense* (Wingfield *et al.,* 1997). Consistent with the complex taxonomy of *Coniothryium* that has limited and confusing morphological characteristics, this fungus has undergone various name changes. It was consequently transferred to *Colletogloeopsis* as *Colletogloeopsis zuluensis* (Cortinas *et al.,* 2006) and has more recently treated as *Kirramyces zuluensis* (Andjic *et al.,* 2007) and *Rederiella zuluensis* (Crous *et al.,* 2007). Based on phylogenetic inference, the pathogen was recognised as related to *Mycosphaerella* (Gezahgne *et al.,* 2005; Cortinas *et al.,* 2006) and it is now acknowledged as a member of the Teratosphaeriaceae (Crous *et al.,* 2007) and treated as *T. zuluensis* favouring the sexual (teleomorph) genus *Teratosphaeria* (Crous *et al.,* 2009). Assuming that a decision is made to recognise the value of anamorph characters in the Teratosphaeriaceae and where a single name is used for these, a revision of the taxonomy of this group will most likely favour the name *Colletogloeopsis zuluensis* for the Coniothyrium canker pathogen (unpublished data). However, for the present, the name *Teratosphaeria zuluensis* is most appropriate and it is consequently applied in this manuscript.

Based on DNA comparisons for multiple gene regions, two distinct species, *K. zuluensis* and *K. gauchensis* have been found to cause Coniothyrium canker in

different parts of the world (Cortinas *et al.,* 2006c). *Teratosphaeria zuluensis* occurs in South America, Africa and South-east Asia and has been reported from Thailand (Van Zyl *et al.,* 2002b), Mexico (Roux *et al.,* 2002), Vietnam (Gezahgne *et al.,* 2003; Old *et al.,* 2003), China (Cortinas *et al.,* 2006b) and Malawi (Roux *et al.,* 2005, Cortinas *et al*., 2006c). *Teratosphaeria gauchensis* occurs in South America and Africa and has been reported from Argentina (Gezahgne *et al.,* 2004) and Uruguay, (Cortinas *et al.,* 2006c), Ethiopia and Uganda (Gezahgne *et al.,* 2003, 2005). In contrast to *T. zuluensis*, *T. gauchensis* has never been reported from South-east Asian countries.

Coniothyrium canker caused by *T. zuluense* appeared unexpectedly and spread rapidly in South Africa, initially on a single highly productive *E. grandis* clone. The fact that the disease was first observed in South Africa and that it was unknown elsewhere in the world, led to the suggestion that that the pathogen might be native in the country, possibly having undergone a host shift (Slippers *et al.,* 2005) from native Myrtaceae. An origin on a native South African host and undergoing a subsequent host jump (Slippers *et al.,* 2005) would be similar to that reported for the *Eucalyptus* canker pathogen *Chrysoporthe austroafricana* in Southern Africa (Wingfield 2003; Gryzenhout *et al.,* 2004; Heath *et al.,* 2006; Nakabonge *et al.,* 2006).

The fact that *T. zuluensis* has not yet been observed in the native range of *Eucalyptus* lends support to the host jump hypothesis. However, the close phylogenetic relationship between these canker pathogens and other important leaf pathogens of *Eucalyptus* (Park & Keane, 1982; Carnegie *et al.,* 1998; Hunter *et al.,* 2004, Andjic *et al.,* 2007) which are known to occur in Australia, suggests that *T. zuluensis* is most likely a *Eucalyptus* pathogen that has yet to be discovered in its native range.

Almost nothing is known regarding the biology or genetics of *T. zuluensis*. In nature, asexual pycnidia (Wingfield *et al.,* 1997) are found on lesions on the young green bark and they produce large numbers of asexual mitospores. Sexual reproductive structures have never been observed (Wingfield *et al,* 1997; Cortinas *et al.,* 2006b). This suggests that the fungus is a haploid organism that reproduces

clonally, mainly as a result of mitotic events (Wingfield *et al.,* 1997; Crous 1998; Crous *et al.,* 2004; 2006).

The objective of this study was to consider the genetic structure of a population of *T. zuluensis* and thus to provide some support to tree breeders concerned about the durability of resistance in planting stock. Two temporally -separated populations from South Africa, and smaller available populations of isolates from Malawi and China, were analyzed using eleven microsatellite markers (Cortinas *et al.,* 2006a), of which six were developed in this study. More specifically, the aims were to i) determine whether there has been a change in the genetic variation between isolates sampled during 1997 and 2005 in South Africa, ii) determine whether the South African populations have a high diversity relative to populations from other countries supporting the hypothesis that South Africa might have been a source of *T. zuluensis* to those countries and iii) consider the genetic structure and distribution of variation within populations.

MATERIALS AND METHODS

Sampling and isolation

Isolates of *T. zuluensis* were obtained from cankers on the stems of severely infected *E. grandis* trees, from different localities (Table 1) including those in South Africa, Malawi and China. One population of isolates from South Africa was collected during the initial outbreak of the disease in 1997. Almost all susceptible trees were replaced in South African plantations subsequent to the outbreak of this disease. A second population of isolates was collected approximately nine years later (end of 2005) in remnant plantations of a highly susceptible *E. grandis* clone.

For the South African collections, a hierarchical sampling strategy was used. Infected bark pieces were collected from a single diseased tree at the centre of a plantation selected as the central point for the collection. Samples were taken only from diseased branches showing cankers at approximately 2 m above the ground. This was done as a precaution to avoid possible height differences in the distribution of haplotypes. Additional samples were taken from randomly chosen trees following transects, extending outwards from the central tree. Samples

collected from Malawi and China were from single *E. grandis* trees randomly collected during routine disease sampling.

Single conidial isolates were generated from the bark samples as described previously (Van Zyl *et al.,* 1997; Cortinas *et al.,* 2006b; 2006c). Cultures obtained from the samples were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA extraction

Isolates were grown on 2% malt extract agar (MEA) plates, for 30 days at 25°C (5 to 6 cm diameter). Mycelium from these actively growing single-conidial cultures was scraped from surface of the agar in the Petri dishes. The fungal material was freeze-dried, immersed in liquid nitrogen until frozen and ground to a fine powder. DNA was extracted using a phenol-chloroform method described by Cortinas *et al.,* (2006b).

Polymorphic microsatellite loci

Eleven polymorphic loci for all samples were amplified using five pairs of fluorescently labelled primers designed previously (Cortinas *et al.,* 2006a) and an additional six primer pairs developed as part of this study (Table 2). The additional primers were developed and characterized using the same methods as described by Cortinas *et al.,* (2006a). Amplicons obtained by PCR were size separated on an ABI 3100 Automated DNA Sequencer (Applied Biosystems, Foster City, USA) together with the internal size standard GENSCAN LIZ 500 (-250) (Applied Biosystems). Fragment size analysis was carried out using the software GENEMAPPER, version 3.0 (Applied Biosystems). Different alleles at each locus were identified based on the size of each amplicon and each allele was given an alphabetical designation. Multilocus haplotypes were generated by using the letters assigned to each isolate across the eleven loci. Isolates with the same haplotype were considered to be clones.

In order to check whether increasing the number of loci would modify the values of genotypic diversity, a plot of Mean Genotypic Diversity against the number of loci was performed using MULTILOCUS 1.3 (Agapow & Burt 2001). The

program samples randomly from 1 to m-1 loci (m= number of loci) from the dataset and calculates the number of different genotypes and the genotypic diversity.

Population genetic analysis

Genetic diversity, richness and evenness

Gene diversity was calculated in POPGENE using the algorithm (*H*) of Nei (1973) (Yeh *et al.,* 1999). Genotypic diversity (*G*) was calculated using Stoddart & Taylor (1988) and different sample sizes were compensated for by calculating the maximum percentage of genotypic diversity as G/N x 100. A *t*- test was used to determine whether the genotypic diversities of the populations were significantly different from each other (Chen *et al.,* 1994).

GENCLONE 2.0 (Arnaud-Haond & Belknir 2007; Rozenfeld *et al.,* 2007) was used to describe the clonal diversity of the populations in terms of richness, evenness and heterogeneity. This program was specially developed to deal with clonal organisms and uses a 'round-robin' method to calculate the allelic frequencies in order to avoid the overestimation of the low frequencies alleles. The Shannon -Weiner index for calculating richness and the corresponding evenness index (V') were used and Pareto distributions (richness and evenness integrated) were constructed to calculate heterogeneity.

The Shannon index is 0 for populations with only a single haplotype and increases in populations with many different haplotypes. For evenness (V), values between 0 and 1 are expected. The clonal evenness is used to describe the equal distribution of sampling units (haplotypes). The log-log transformation of the Pareto distribution gives an integrated representation of both richness and evenness (heterogeneity). The parameter □ calculated by regression (r^2) (the -1 x regression slope) from the Pareto distribution, increases exponentially with increasing evenness.

Population differentiation and assignment tests

Differences in allele frequencies between populations of *T. zuluensis* were calculated from clone corrected datasets using POPGENE. The significance of

differences in allelic frequencies between populations across the eleven loci was tested using Chi square tests (Workman & Niswander 1970).

The differentiation among populations was measured as theta (θ) (Weir 1996), which is a modification of F_{ST} (Wright 1978). Theta (θ) values were calculated using MULTILOCUS, version 1.3 (Agapow & Burt 2001) using the equation $\theta = Q\text{-}q / 1\text{-}q$, where Q is the probability that two alleles from the same population are the same and q is the probability that two alleles from differing populations are the same. For multiple loci, Q and q are summed across the evaluated loci. The significance of θ was evaluated by comparing the observed value to that of 1000 randomizations in which individuals were randomized across populations.

STRUCTURE version 2.2 (Pritchard *et al.,* 2000; Falush *et al.,* 2003) was used to carry out the assignment of individuals into 'K' number of clusters/populations based on their allelic frequencies for the South African, China and Malawi populations. The analyses were carried out in two steps. An initial analysis was carried out to determine the optimal 'K' using an admixture ancestry model and an independent allele frequency model. A hundred thousand runs were carried out (burn-in set at 10 000 runs) with 10 iterations. The analysis was repeated for the most likely K obtained using 1 000 000 runs (burn-in was set at 100 000). In both cases, the likelihood values were plotted against the delta likelihood values to determine the K with lower standard deviation and higher likelihood (Evanno *et al.,* 2005).

Recombination analyses

The random association of alleles was tested by calculating linkage disequilibrium (LD) for all pairs of loci and as a multilocus measure using the Index of Association (I_A), both implemented in MULTILOCUS version 1.3 (Maynard Smith *et al.,* 1993; Agapow & Burt 2001). The LD for all pairs of loci and I_A values were determined for all populations using clone corrected data matrices. The significance of the LD for all pairs of loci and the I_A observed values were determined by comparing the observed values with that of a distribution of a randomly mating population using 1000 randomizations of the allelic frequencies.

Analyses of clonal structure in the temporally-separated South African populations

Pairwise genetic differences among individuals were studied using GENCLONE 2.0 (Arnaud-Haond & Belknir 2007; Rozenfeld *et al.,* 2007) between the two temporally separated South African populations (SA 1997 and SA 2005) to determine clonal lineages that might constitute clusters of slightly different multilocus haplotypes, possibly derived from an original individual by mutation. The program makes use of microsatellite motif length differences to calculate a genetic distance index using a stepwise model of mutation and builds a histogram showing the distribution of pairwise genetic distances. The genetic distance index matrix generated for the two South African populations (SA 1997 and SA 2005) was imported into MEGA 4 (Tamura *et al.,* 2007) to perform cluster analyses using the unweighted pair group method with arithmetic mean (UPGMA). Furthermore, P_{gen}, the likelihood that two individuals with the same multilocus genotypes are the same clone and P_{sex}, the likelihood that individuals sharing the same multilocus genotype were derived from a distinct sexual reproductive event, were calculated using GENECLONE 2.0.

An examination was made as to whether pairs of individuals in the SA1997 and SA2005 populations that were separated by a defined spatial-interval, were more similar, or dissimilar to that expected from pairs of individuals that were randomly associated using spatial autocorrelation analysis as implemented in GENCLONE 2.0. A grid was superimposed on the localities sampled within South Africa (Table 7, Fig 2). Geographical x and y coordinates were assigned to the South African localities for both the SA1997 and SA2005 populations according to the position on the grid (Table 7). The Ritland (1996) co-ancestry coefficients (Fij) (the average genetic distance between pairs of individuals) were calculated using GENCLONE 2.0. Six distances classes were arbitrarily chosen and the grouping of the isolates determined.

RESULTS

Isolates

A total 248 isolates of *T. zuluensis* were obtained from the isolations from trees in South Africa. Of these, 75 were from the 1997 and 110 were from the 2005 South

African collections. From the single plantation in Malawi, 41 isolates were collected and 22 isolates were obtained from a single plantation in China (Table 1).

Polymorphic microsatellite loci

From the collection of 248 isolates of *T. zuluensis,* the 11 species-specific polymorphic microsatellite markers amplified a total of 68 different alleles (Table 3). In the two South African populations, 41 and 50 different alleles were observed for the 1997 and 2005 populations respectively. Forty five alleles were found for the Malawian isolates and 42 alleles were detected in the Chinese collection of isolates. The number of alleles per individual locus ranged from three to 14. Private alleles were observed in all populations. In total, 18 private alleles were identified, of which four were detected in the SA1997 population and nine in the SA population collected in 2005. Three private alleles were found in the Malawian population and two were observed in the Chinese collection of isolates. The majority of private alleles showed frequencies ranging from 3.5% to 10%. No monomorphic loci were detected in the South African populations although the locus Czulu3 (Table 3) was monomorphic in the Chinese and Malawian populations.

Genetic analysis of populations from South Africa, Malawi and China

Genetic diversity, richness and evenness

The plot of mean genotypic diversity against the number of loci constructed using MULTILOCUS 1.3 showed that a plateau of genotypic diversity was reached using the set of 11 microsatellite markers developed for *T. zuluensis* (data not shown). This provided statistical support for the assumption that the total diversity of the populations had been adequately sampled.

The levels of gene diversity in the *T. zuluensis* populations were moderate. Values were H= 0.51 for Malawi, H= 0.53 for China, H= 0.44 for SA1997 and H= 0.51 for SA 2005 (Table 3). One hundred and eighty-eight genotypes were identified across all the *T. zuluensis* isolates. The levels of clonality within populations ranged from 0% in China to 43% in SA1997.

The maximum genotypic diversity ranged from a minimum of $\hat{G}$ = 24% for the SA1997 population to a maximum value of $\hat{G}$ =100% for the Chinese population.

The populations from Malawi ($\hat{G}$ = 84%), and SA2005 ($\hat{G}$ = 43%) showed intermediate values (Table 3). No significant difference (P < 0.05) in genotypic diversities was found between the populations from South Africa and Malawi. Only one genotype was shared between the populations studied and this was for the South African population sampled in 1997 and 2005.

The relative richness, evenness and heterogeneity (richness and evenness integrated) gave Shannon–Weiner index values ranging from 3.09 for China and 4.35 for SA2005. The corresponding evenness index, V', ranged from 0.90 for SA1997 to 0.99 for China (Table 3). These values indicated moderate to high heterogeneity for all the populations in the study with the SA2005 population having the highest level of heterogeneity, and groups of clones within populations, had a similar size. The evenness index was also high for all the populations. The highest level of evenness was observed in the Chinese and Malawian populations.

The Pareto distributions determined for SA1997 and SA2005 showed good regression fits. The slopes of the regression lines were different for both populations (r^2=9.99, p<0.0001 in SA1997; r^2=0.97, p<0.0001 in SA2005). They suggested high diversity and low heterogeneity (low dominance of haplotypes relative to other haplotypes within populations). Nevertheless, the slope obtained for the SA1997 population was shallower (1,658) than that determined for the SA2005 (2,779) population, indicating lower heterogeneity among the haplotypes obtained in 1997 than those in 2005. It was not possible to calculate the Pareto distribution and the associated parameters for the China and Malawi populations. This was due to the haplotypes in both populations having approximately the same number of replicates (maximum evenness) which would not produce sufficient pairwise point comparisons between haplotypes to calculate the parameter β by regression (Arnaud-Haond *et al.,* 2007; Rozenfeld *et al.,* 2007).

Population differentiation and assignment tests

Significant differences were found between loci for the clone-corrected populations in the majority of the pairwise comparisons, including the two temporally-separated South Africans populations (Table 4). These results suggest that the RSA populations belong to different gene pools.

For the theta (θ) calculations, only the Malawian population showed significant Chi-square values ($P < 0.05$) when compared with other populations (Table 5). The differentiation between the SA populations ($\theta = 0.10$) was the smallest. The largest differentiation was observed between Malawi and the SA2005 ($\theta = 0.18$) populations.

The assignment tests indicated that the number of groups obtained with the highest likelihood and lowest standard deviation was K= 5.The majority of isolates from SA2005 were assigned to G1 (Fig 1). Groups G2 and G3 also consisted of mainly South African isolates while G4 and G5 were assigned the majority of isolates from China and Malawi respectively.

Recombination analyses

Pairwise comparisons between loci detected linkage disequilibrium (LD) in the populations of *T. zuluensis* (Table 6). The values were moderate with a maximum in the SA2005 population where almost half of the loci were in LD (values ranging from 8/49 to 21/49). The multilocus Index of Association (I_A) results were comparable to the LD results obtained by the pairwise analyses (Table 6). Significant departures from gametic equilibrium were detected for all populations (0.41 to 0.75) except China (0.17). The observed values of I_A for all the populations except the China population was significantly different to the value expected from a randomised distribution of allelic frequencies, suggesting that recombination has occurred only in the China population. This is also the only population that showed 100% genotypic diversity.

Analyses of clonal structure in the temporally-separated South African populations

Differences were found in the distribution of haplotypes between the temporally-separated South African populations SA1997 and SA2005. Using GENCLONE, 43 different haplotypes were identified in the SA1997 population, 12 of which were repeated in the population (replicates of the same haplotype). These identical haplotypes formed clusters containing two to 12 replicates each (Fig 3). In contrast, 86 different haplotypes were identified in SA2005 and 13 haplotypes formed clusters with between two to four replicates. The P_{gen} calculated for both

populations suggested that the majority of haplotypes were most likely a result of clonal reproduction (all $P_{gen} < 0.002$). In addition, within the different haplotype clusters, the probability that the haplotype replicates originated from different sexual events (P_{sex}) was very low ($P_{sex} < 0.03$) in the majority of cases.

The distribution of clones and haplotypes in the populations was further evaluated by plotting histograms to show the frequency distribution of genetic distances among haplotypes. A bimodal distribution pattern of frequencies was obtained for SA1997 and SA2005 indicating there are two main groups of clones within these populations (Fig 3). The global shape of the histograms was also informative as it was possible to visualize a decreased homogeneity of SA2005 population relative to SA1997 population. The bimodal pattern observed was further analysed using UPGMA analysis (Figs 4, 5) to examine whether there was an association between the groups and localities. The trees generated resulted in two main clusters for both the SA1997 and SA2005 populations. The trees showed no association between localities and clusters.

The overall results of tests for correlation between genetic and geographic distance of the SA1997 and SA2005 populations were significant ($p < 0.05$). Using the complete data set, the values were 1 for SA1997 and SA2005 and using the clone corrected data, 0.99 for SA1997 and 1 for SA2005. In both cases, the results suggested genetic structuring by means of gene flow restrictions at the scale at which the isolates were sampled.

DISCUSSION

In this study, eleven microsatellite markers were used to consider the population biology and structure of the *Eucalyptus* stem canker pathogen *T. zuluensis* in South Africa. Despite an observable reduction of pathogen population size on *Eucalyptus* across plantations in South Africa, there was an increase in genetic diversity during the period between 1997 and 2005. Two small populations collected from Malawi and China for comparative purposes were more diverse compared with two temporally-separated populations from South Africa. This result does not support the hypothesis (Wingfield *et al.,* 1997) that South Africa represents the original source of *T. zuluensis.*

Because the majority of susceptible trees in South African plantations were replaced with trees resistant to *T. zuluensis,* it was expected that the genetic diversity of the pathogen would be substantially lowered in the population of isolates collected nine years after the onset of the disease. Further, that the population diversity of *T. zuluensis* collected in 2005 would either reflect the one collected in 1997 or show a reduction of genetic diversity due to increased random genetic drift (Wright 1931; Young *et al.,* 1996). Results of this study showed no evidence of such a decrease in genetic diversity. Populations of *T. zuluensis* collected in 1997 and 2005 showed significant levels of differences in genetic diversity including allelic richness and evenness (homogeneity) and a shift of allelic frequencies. Recent studies have shown that the capacity of populations to recover genetic diversity after a reduction in population size is not easily predicted (Young *et al.,* 1996; Lowe *et al.,* 2005). The outcome depends on a combination of factors that are frequently unknown such as the original population size and other parameters related to the life history and reproductive structure of the populations (Young *et al.,* 1996; Edwards *et al.,* 2005; Lowe *et al.,* 2005; Reusch 2006). For instance, the reduction of population size from an original, highly diverse population can produce enhanced opportunities for a different group of haplotypes (including better adapted haplotypes) to replace those that were there in the first place (McNelly & Roose 1984; Watkinson & Powell 1993; Hughes & Stachowicz 2004; Kohn 2005).

The populations of *T. zuluensis* showed a broad global range of genotypic diversity (between 24% and 100%) but the South African populations had the lowest levels of genotypic diversity (SA1997, 24%; SA2005, 43%). In comparison, high genotypic diversities (84% to 100%) were detected in the Malawian and Chinese populations, despite the fact that the sample size for these populations was relatively small. Native populations typically have higher diversity than introduced populations (McDonald 1997; Stukenbrock *et al.,* 2007; Hunter *et al.,* 2008). Thus, our results fail to support the view that *T. zuluense* originated in South Africa (Wingfield *et al.,* 1997). This speculative view emerged due to the fact that the pathogen first appeared in South Africa and that it had never been found in the native range of *Eucalyptus* spp. (Wingfield 2003).

On a global scale, the allele frequency theta (θ) and assignment tests indicated significant differentiation across the *T. zuluensis* populations. Multiple clusters were formed according to the assignment tests showing that the majority of individuals from the populations in China and Malawi are different to the individuals reflecting the two South African populations. The large numbers of private alleles in the populations, together with the genetic diversity results, negate the possibility that South Africa represents a centre of origin for *T. zuluensis.* What is, however, clear is that there is no significant gene flow between the populations that were examined in this study. This suggests that *T. zuluensis* in South Africa, Malawi and China have originated independently of each other but from an unknown source.

The observed differentiation between the two temporally separated populations from South Africa (SA1997 and 2005) was unexpected. The genetic distances and cluster analyses within these populations revealed a level of population structure. Two major groups of intermingled haplotypes from different localities were recovered as bimodal distributions in both populations. The spatial correlation analysis provided additional evidence of structure at the "with-in" population level indicating there were restrictions to gene exchange at the sampled scale. The best explanation for these observations is that the two populations arose as the result of loss of haplotypes and subsequent introduction of new haplotypes. The restricted gene exchange also provides evidence that dispersal occurs mainly by conidia as is the case with other closely related fungi (Feau *et al.,* 2005; Milgate *et al.,* 2005; Hunter *et al.,* 2008) that show predominantly clonal population structure.

Linkage disequilibrium analyses showed significant departure from random mating for all populations studied with the exception of the population from China. The fact that sexual structures have never been observed for *T. zuluensis* in South Africa or elsewhere does not preclude the existence of cryptic sexual recombination. Results of this study, however, suggest that sexual recombination is not the predominant form of reproduction in the *T. zuluensis* populations in South Africa and Malawi. The fact that evidence for recombination was observed in the Chinese population, which is also the most genetically diverse, is enigmatic as this fungus has only recently been observed in that country and on *Eucalyptus* which is

not native to this region. While, *T. zuluensis* might therefore have its origin in South-east Asia, the fact that is not known in Australia does not imply that it is not present also there. This would be consistent with the fact that there are growing numbers of examples of *Eucalyptus* pathogens being reported for the first time in plantations outside Australasia and thus before they are detected in that country (Wingfield *et al.,* 1996; Burgess *et al.,* 2007) and this could also be the case for *T. zuluensis*.

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Table 1 *Teratosphaeria zuluensis* isolates used in this population study.

Country	Host	Collection date	Collector	No. Isolates
South Africa 1997 (SA1997)	*E. grandis*	1997	L Van Zyl	75
South Africa 2005 (SA2005)	*E. grandis*	2005	MJ Wingfield/ MN Cortinas	110
Malawi	*E. grandis*	2004	J Roux	41
China	*E. urophylla*	2004	T Burgess	22
Total				248

Table 2 Locus and primer names, primer sequences with florescent labels, repeat motif, annealing temperature (T_a), $MgCl_2$ concentration and size ranges of the alleles for six additional species-specific *Teratosphaeria zuluensis* microsatellites loci developed in this study and used in the population analyses of *T. zuluensis* isolates.

Locus name	Primer names	Primer sequences (5'- 3')	Repeat motif	T_a (°C)	$MgCl_2$ (mM)	Size range (bp)
Kzulu5	F18F/ F18RC1	FAM- GTT GTG TCC GAT CCT GCG AAG C GGATCTCCTCAATCACTTACTGC	$(CG)_7(AG)_{21}CA(AG)_9$	56	3.5	192-265
Kzulu10	F19W2/ F19FL2	PET- CCG CTG TGG CAT CCA AAT TCC GGC GCT CTG TCA CTG CTA AGG C	$(TATCAACACC)_8$	59	3.5	321-426
Kzulu11	F25F1/ F25RC2	PET- CGC TAT TTG CTG CTT TTG GAA CC AGG GGC TGT ATG TAG ATG CCG	$(AG)_7$	59	3.5	101-124
Kzulu12	F27F/ F27RC1	PET- GGA TCA GAA ATG CGA GGA CGA GG CTACCACGACTTTCCTCACTACG	(TG)rich	63	3.5	275-304
Kzulu13	F33F1/ F33RC1	VIC- AGT GAG ACA TAG GCA CGG GTA GG GGT ACG CTT GAA CAC ACA CA	$(TG)_{12}$	58	3.5	123-154
Kzulu14	Ms42RC1/ Ms42F1	VIC- GCT CGA CCA CGC CTG ACT TAA GG ACG ATG GCG GCA GTG AAG GAG	$(TG)_{12}$	59	3.5	254-282

Table 3 Allelic frequencies and other diversity indices of clone-corrected populations of *Teratosphaeria zuluensis* from Malawi, China and South Africa at 11 microsatellite loci.

Loci	Alleles	Malawi	China	SA-1997	SA-2005	Total
Czulu1	A	0.737	0.181	0.2434	0.091	
	B	0.184	0.455	0.659	0.523	
	C			0.024	0.261	
	D		0.364		0.114	
	E	0.079		0.073		
	F				0.011	
Czulu2	A		0.183	0.122	0.012	
	B	0.263	0.366	0.390	0.391	
	C	0.737	0.455	0.488	0.598	
Czulu3	A				0.250	
	B			0.097	0.031	
	C	1.000	1.000	0.902	0.716	
Kzulu5	A	0.027		0.024	0.114	
	B	0.108	0.050	0.024		
	C	0.216	0.100			
	D			0.024	0.273	
	E				0.011	
	F	0.027	0.050			
	G	0.270	0.200	0.195	0.273	
	H	0.027				
	I	0.027				
	J	0.027		0.024	0.068	
	K	0.243	0.550	0.707	0.114	
	L	0.027	0.050		0.023	
	M				0.091	
	N				0.034	
Kzulu10	A		0.095	0.04 9	0.205	
	B	0.447	0.191	0.220	0.398	
	C	0.237	0.286	0.7073	0.2273	
	D		0.238	0.024	0.0909	
	E	0.316	0.095			
	F		0.095		0.011	
	G				0.068	
Kzulu11	A				0.034	
	B	0.158	0.091	0.024	0.056	
	C	0.737	0.818	0.927	0.738	
	D				0.045	
	E	0.105	0.091	0.049	0.091	
	F				0.034	
Kzulu12	A	0.398	0.227	0.854	0.773	
	B	0.526	0.727		0.068	
	C	0.026	0.045	0.049	0.1591\	
	D	0.053		0.049		
	E			0.049		
Kzulu13	A	0.158	0.524	0.200	0.049	
	B	0.053	0.191	0.318	0.602	
	C	0.553	0.191	0.366	0.349	
	D	0.237	0.095	0.098		
Czulu6	A	0.394	0.455	0.634	0.840	

	B	0.026	0.364		0.159	
	C	0.316	0.182			
	D	0.263				
	E			0.293		
	F			0.024		
	G			0.049		
Czulu7	A	0.316	0.190	0.146	0.023	
	B	0.553	0.810	0.781	0.716	
	C	0.053		0.073	0.216	
	D	0.079			0.046	
Kzulu14	A	0.053	0.227	0.195	0.0342	
	B	0.026	0.227			
	C	0.026	0.091	0.342	0.568	
	D	0.026	0.091		0.011	
	E		0.046			
	F		0.046			
	G	0.579	0.189	0.366	0.364	
	H	0.263	0.091	0.097	0.011	
	I	0.026			0.011	
N		41	22	75	110	248
Nc		37 (7.5%)	22 (0%)	43 (22,9%)	86 (11.1%)	188
Na		45	42	41	50	
Number of private alleles		3	2	4	9	18
H		0.51	0.53	0.44	0.51	
Number of polymorphic loci		10	10	11	11	
G		34.48	22	18,18	47,61	
Ĝ		84%	100%	24%	43%	
S		3.68	3.09	3.4	4.35	
V’		0.98	0.99	0.90	0.97	
β				1.658	2.779	

N = Number of isolates (non clone-corrected)

Nc=Number of haplotypes in the clone-corrected populations

Na = Observed number of alleles

H = Nei’s Gene Diversity according (1973)

G = Genotypic Diversity (Stoddart & Taylor, 1988)

Ĝ = G/N% = percentage maximum diversity

S = Shannon–Weiner index

V’ = Evenness index derived from Shannon-Weiner

β= β parameter of Pareto distribution

Table 4 Pairwise Chi-square comparisons of allelic frequencies between *Teratosphaeria zuluensis* populations from Malawi, China, South Africa 1997 and South Africa 2005. The total number of loci whose frequency differ significantly from each other (as indicated by *), in the pairwise comparison, is shown in the last column.

Pairwaise populations		Czulu 1	Czulu2	Czulu3	Kzulu5	Kzulu10	Kzulu11	Kzulu12	Kzulu13	Czulu6	Czulu7	Kzulu14	Number of significantly different loci
Malawi and China	chi2	27.19*	9.13*	0.000	7.79	21.69*	0.61	3.42	14.45*	17.41*	4.93	22.76*	6 out of 11
	df	3	2	0	9	5	2	3	3	3	3	8	
Malawi and SA1997	chi2	21.20*	7.61*	3.90*	25.41*	27.91*	5.64	30.26*	11.49*	40.89*	7.37	21.67*	9 out of 11
	df	3	2	1	10	4	2	4	3	6	3	6	
Malawi and SA2005	chi2	67.61*	2.46	13.46*	5.67*	42.20*	7.481	41.31*	44.39*	62.35*	26.35*	44.69*	9 out of 11
	df	5	2	2	13	6	5	3	3	3	3	6	
China and SA1997	chi2	18.31*	0.41	2.29	10.44	19.25*	1.92	40.81*	6.19	31.22*	1.71	21.98*	5 out of 11
	df	4	2	1	8	5	2	4	3	5	2	7	
China and SA2005	chi2	14.21*	11.77*	8.08*	46.14*	20.60*	2.96	47.78*	41.00*	23.12*	14.35*	55.70*	10 out of 11
	df	4	2	2	11	6	5	2	3	2	3	8	
SA1997 and SA2005	chi2	25.6*	7.85*	13.58*	54.45*	28.98*	7.17	14.35*	20.19*	40.26*	12.25*	17.69*	10 out of 11
	df	5	2	2	9	5	5	4	3	4	3	5	

Table 5 Population differentiation values, represented as Theta (*θ),* for the *Teratosphaeria zuluensis* populations.

T. zuluensis	China	SA1997	SA2005
Malawi	0.11*	0.17*	0.18*
Thailand	0.10	0.20	0.18
China		0.13	0.16
SA1997			0.10

*significant Chi-square values (P < 0.05)

Table 6 Two-locus linkage disequilibrium analysis (LD) expressed as the number of loci with significant differences over the total pairwise loci comparisons, observed value of Index of Association (I_A) and range of I_A values obtained after 1000 randomizations. In the last column recombination is indicated as a 'yes' based on the observation that the observed I_A value falls within the randomized dataset values.

	LD between pairs of loci	Obs. I_A	Range of obtained I_A values after 1000 randomizations	Obs. I_A within randomized the data range. (i.e. evidence for recombination)
T. zuluensis				
China	8/49	0.17	-0.003- 0.24	yes
Malawi	15/49	0.75*	-0.002- 0.13	no
SA1997	8/49	0.70*	-0.02- 0.28	no
SA2005	21/49	0.41*	-00008- 0.17	no
All	14/49	0.37*	-0.0033- 0.15	no

*significant $p<0.05$

Table 7 Localities sampled from in South Africa in 1997 and 2005 including x and y coordinates and number of isolates obtained from each location.

Locality	Locality abbreviation	X Coord.	Y Coord.	Number of isolates
1997				
Aboyoni	A	4,5	6,0	4
Honey Farm	H	5,0	6,0	9
Palm Ridge	P	4,5	8,5	11
Shire	S	3,0	3,5	4
Teranera	Te	4,5	4,5	5
Teza	T	6,0	6,5	17
Trust	Tr	4,5	7,5	6
Fair Breeze	FB	1,5	2,0	1
Kwambonambi	K1	5,0	6,0	7
2005				
Kwambonambi	K2	4,5	6,0	8
Venters	V	4,5	6,0	42
Mtubatuba	M	4,75	7,5	44
Mtunzini	Mt	1,5	2,0	14
Moba Dam	MD	4.5	5,5	3

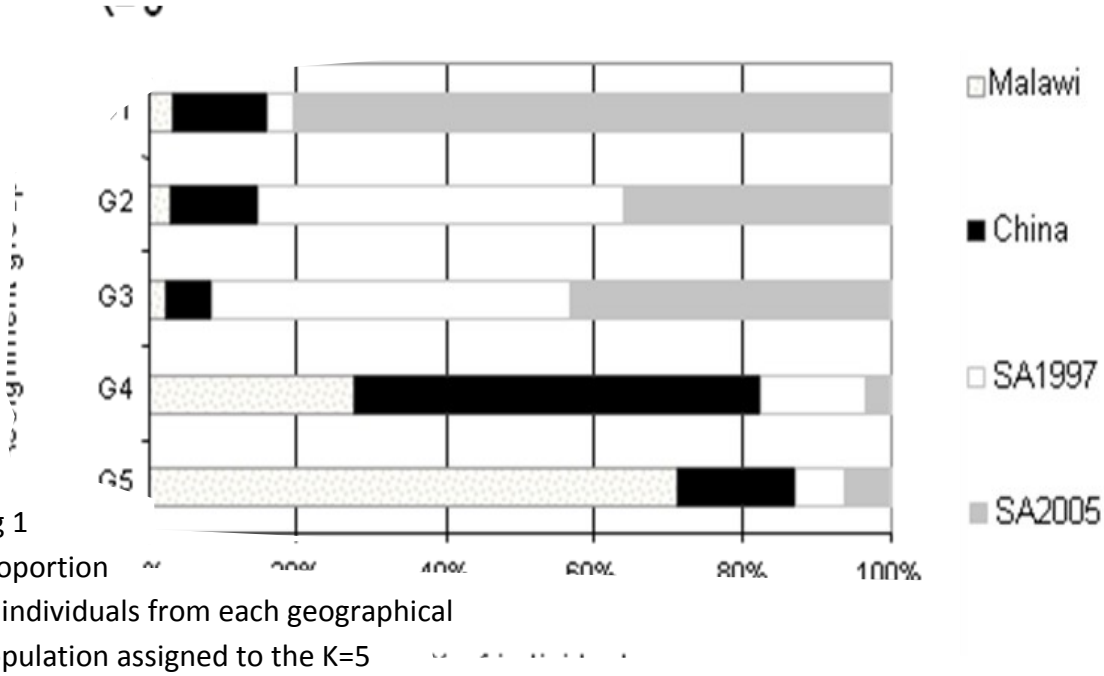


Fig 1 Proportion of individuals from each geographical population assigned to the K=5 groups (G1 to G5). In three out of the 5 groups (G1to G3), the majority of SA1997 individuals group with SA2005 individuals. The majority of the Chinese and Malawi individuals group in distinctive groups (G4, G5).

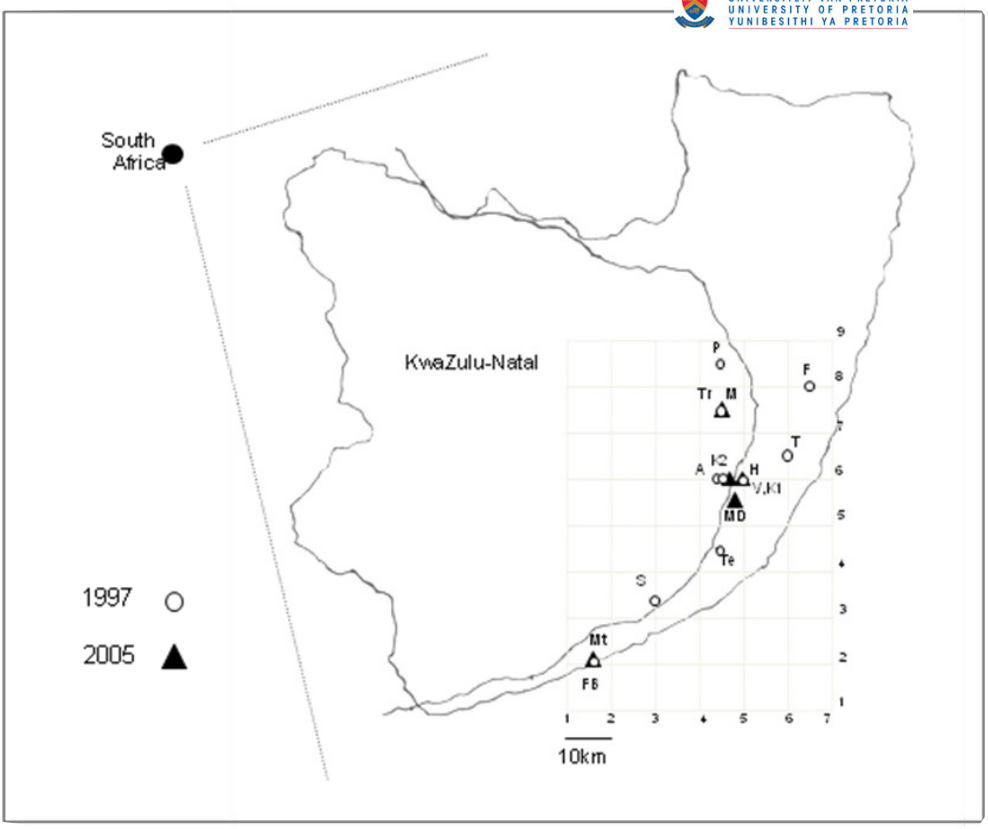


Fig 2 Location of sites sampled in 1997 and 2005 in KwaZulu Natal, South Africa (see Table 7).

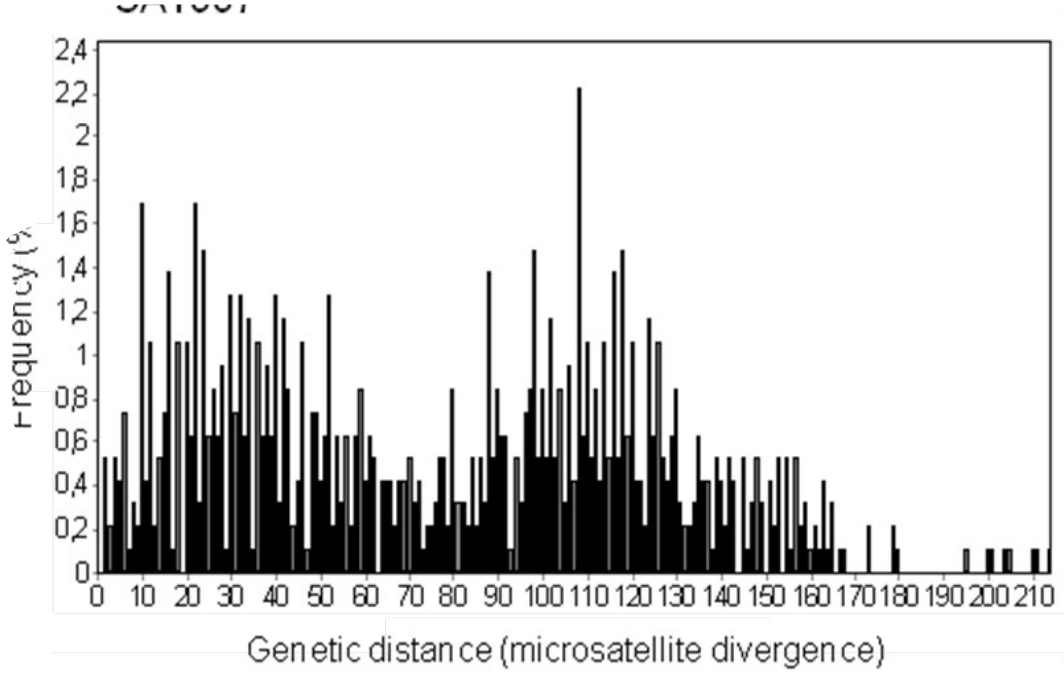
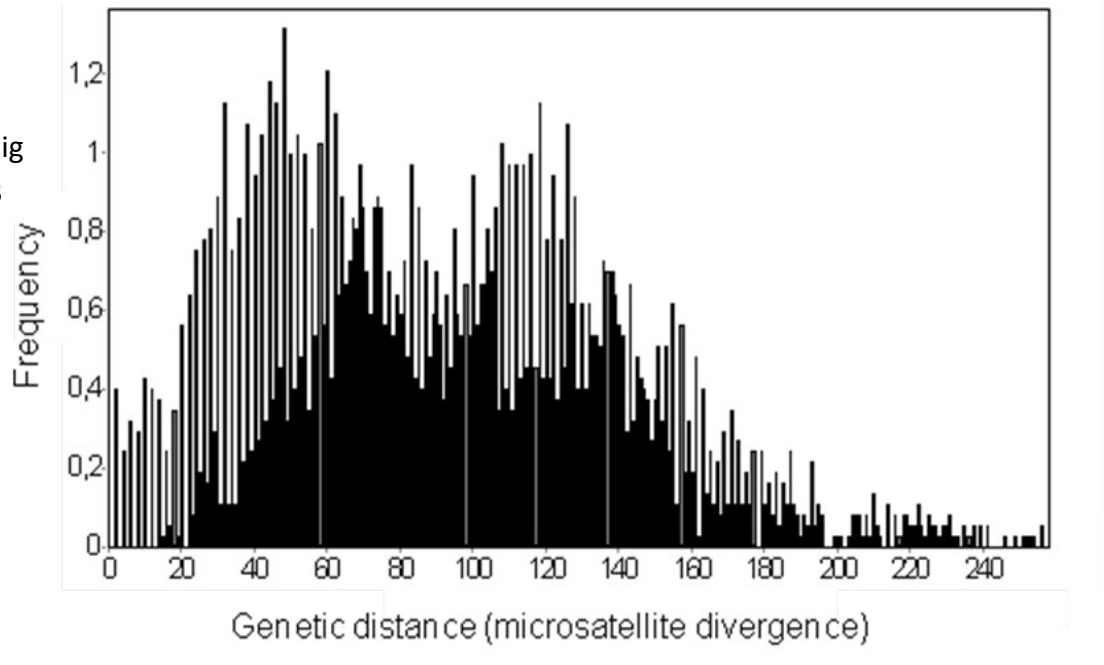


Fig 3



Frequency distribution of microsatellite divergence amongst pairs of isolates A. for the SA1997 population and B. for the SA2005 population.

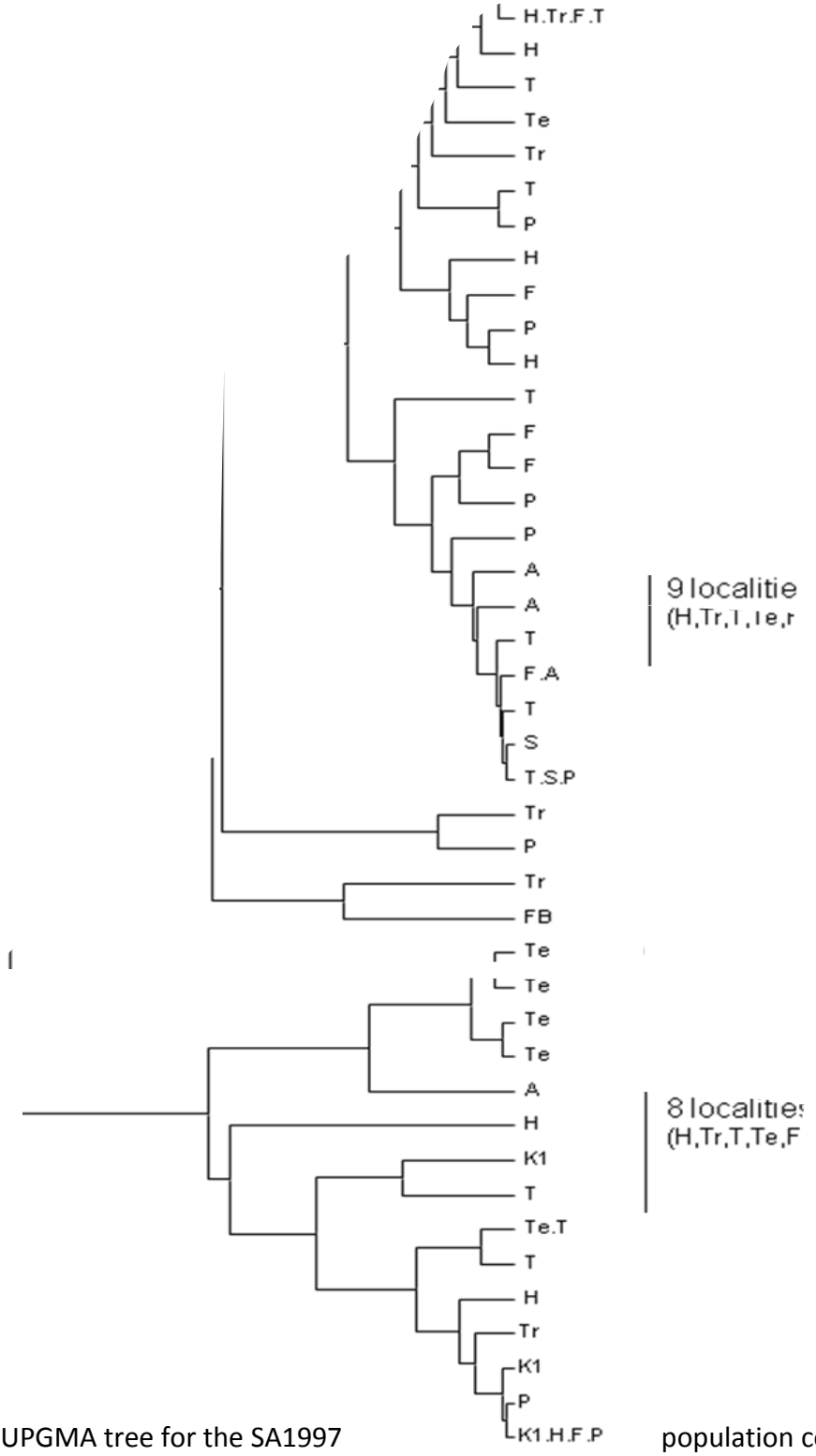


Fig 4 UPGMA tree for the SA1997 population constructed in MEGA 4 using the distance matrix calculated in GENCLONE 2.0. The branches include samples recognized as part of the clone in GENCLONE 2.0. The letters indicate the original sampling location of the clones included in the branch as indicated

in Table 3. Two main clusters of clones emerge in the tree. Each cluster includes clones from the majority of the sampled locations.

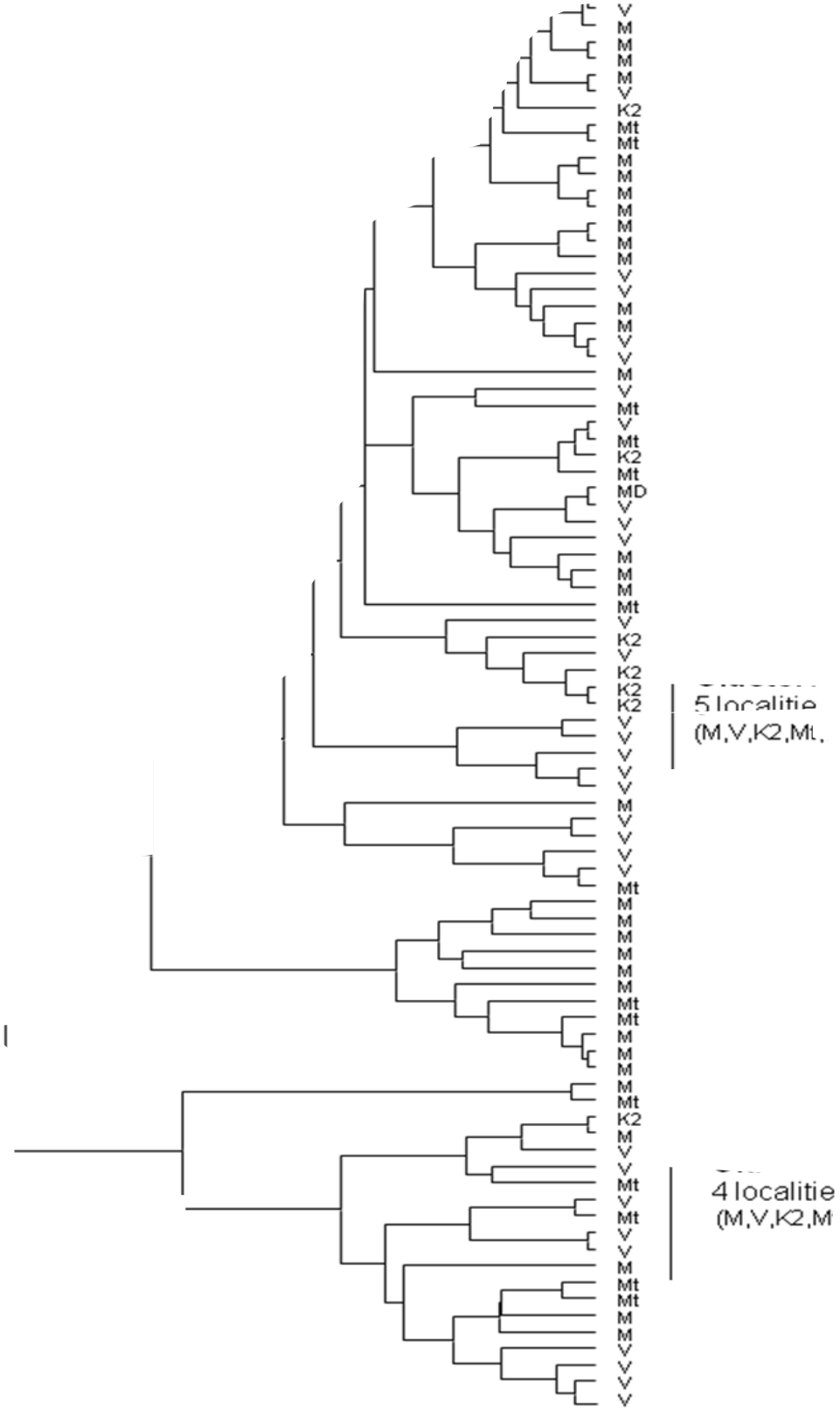


Fig 5 UPGMA tree for the SA2005 population constructed in MEGA 4 using the distance matrix calculated in GENECLONE 2.0. The branches include samples recognized as part of the clone in GENCLONE 2.0. The letters indicate the original sampling location of the clones included in the branch as indicated in Table 3. Two main clusters of clones emerge in the tree. Each cluster includes clones from the majority of the sampled locations.

Chapter 7

Unexpected genetic diversity revealed in the *Eucalyptus* canker pathogen *Teratosphaeria gauchensis*

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ABSTRACT

Teratosphaeria gauchensis causes a serious canker disease on *Eucalyptus* spp. in plantations in South America and Africa. The pathogen is closely related to, but distinct from *T. zuluensis* that causes a similar stem canker disease on *Eucalyptus.* The objective of this study was to use 10 previously developed polymorphic microsatellite markers to study the population diversity of *T. gauchensis*, based on collections of the fungus made in Argentina and Uruguay. The alleles were size -analyzed to determine population genetic parameters of the *T. gauchensis* populations. The results showed that isolates from the two collection sites represent the same population. Overall, the genetic diversity amongst isolates was higher than expected and inconsistent with the notion that the pathogen represents a recent introduction into South America.

INTRODUCTION

Teratosphaeria gauchensis (M.N. Cortinas, Crous & M.J. Wingf.) M.J Wingf. & Crous Andjic & M.J. Wingf. and the related *Teratosphaeria zuluensis* (M.J. Wingf., Crous & T.A. Cout.) M.J. Wingf. & Crous cause a disease known as Coniothyrium canker on *Eucalyptus* spp. *Teratosphaeria zuluensis* was the first of these fungi to be described after it was discovered causing serious damage to the stems of clonally propagated *Eucalyptus grandis* in the Kwa-Zulu Natal province of South Africa (Wingfield *et al.,* 1997). The disease spread rapidly in the 1990's and became one of the most serious impediments to in *Eucalyptus* plantation forestry in that country (Old *et al*., 2003).

Due to the serious economic impact of Coniothyrium canker on plantations in South Africa, there were various studies undertaken to better understand the relevance and biology of *T. zuluensis* (Van Zyl, 1999, Van Zyl *et al.,* 2002). Some years later, a very similar disease was discovered on *E grandis* clones in Argentina and Uruguay and surprisingly, the causal agent was found to be different to *T. zuluense* (Cortinas *et al*., 2006b). The causal agent of the disease was a fungus that was provided with the name *Teratosphaeria gauchensis*. *Teratosphaeria gauchensis* and *T. zuluensis* are morphologically almost indistinguishable and they give rise to the same symptoms after infection. Thus, the only reliable means to distinguish between the two fungi is via DNA sequence comparisons. Both fungi were initially described as mitotic species and residing in the teleomorph genus *Mycosphaerella* based on phylogenetic inference (Cortinas *et al.,* 2006b; Andjic *et al.,* 2007) but recent taxonomic re-evaluation has relegated them to anamorphs of *Teratosphaeria* in the Teratosphaeriaceae (Crous *et al.,* 2007; Crous *et al.,* 2009).

Teratosphaeria gauchensis causes cankers on young branches and on tree trunks although it has also been isolated from leaf spots on *E. maidenii* and *E. tereticornis* in Uruguay (Pérez *et al.,* 2009a). The typical stem and trunk lesions caused by this fungus are necrotic and have a characteristic dark oval shape (Cortinas *et al.,* 2006b). The extent of the lesions varies depending on the susceptibility of the infected trees. Severe infections arise from small cankers that merge to cover large areas of the trunks. Both the soft tissue and wood become malformed resulting in retarded growth and girdling can be observed at the tree

tops. Kino pockets are formed as part of the defence response of the trees. Kino that exudes from the cankers can cause the stems to become a black colour. In some cases, diseased trees also produce epicormic shoots alongside the cankers that can cause the terminal parts of the branches and stems to die (Wingfield *et al.,* 1997; Cortinas *et al*., 2006b).

Very little is known regarding the biology of *T. gauchensis*. It is presumed that the fungus exists in a haploid state (Wingfield *et al.,* 1997; Crous, 1998; Crous *et al.,* 2004; 2006). In nature, only asexual pycnidia are found on the bark lesions. These structues give rise to mitospores (conidia) that are presumably responsible for short distance dispersal, as is the case for closely related fungi (Feau *et al.,* 2005; Milgate *et al.,* 2005; Hunter *et al.,* 2008). Sexual structures have never been observed in nature nor have they been produced in culture.

The origin of *T. gauchensis* is not known. Its distribution is limited to Uganda and Ethiopia (Gezahgne 2003; Gezahgne *et al.,* 2005), Argentina and Uruguay (Gezahgne *et al.,* 2004; Cortinas *et al.,* 2006b) and Hawaii (Cortinas *et al.,* 2004). It is has also never been found on any host other than *Eucalyptus* species, which is an exotic in all these countries. The current distribution of *T. gauchensis* does not overlap with the distribution of the sibling species *T. zuluensis* (Cortinas *et al.,* 2006b). The fact that *Eucalyptus* species are not native to any of the countries where *T. gauchensis* is found, and its close phylogenetic relationship to other *Terathosphaeria* species on *Eucalyptus,* suggests that it is a *Eucalyptus*-specific pathogen, which has yet to be discovered in its native range. If that is the case, then one would expect to find fungal populations with low genetic diversity in areas where it has been introduced, which is true for the related *M. nubilosa* (Pérez *et al.,* 2009).

The aim of this study was to investigate the population diversity and structure of *T. gauchensis* found on non-native *Eucalyptus* in plantations of Argentina and Uruguay where the associated disease has been particularly serious. To achieve this goal, ten polymorphic microsatellite markers, recently developed for this species (Cortinas *et al.,* 2008), were used to calculate estimates of haplotype richness and evenness, haplotypic diversity and genetic differentiation for isolates collected in Argentina and Uruguay.

MATERIALS AND METHODS

Sampling and isolations

Necrotic lesions on the bark of infected *Eucalyptus* clones were sampled from plantations in the neighbouring provinces of Entre Ríos, Corrientes and Misiones in Argentina and from two areas (Rivera and Paysandú), in the Northern part of Uruguay. The sampling area covered a range of approximately 450 km in a North-South direction and 300 Km in an East- West direction (Table 1). Samples were collected as part of a disease evaluation project in Uruguay and Argentina between 1999 and 2005. Samples were taken from one lesion per tree on the stems of randomly chosen trees approximately 2 m above the ground.

One hundred and thirty one single conidial isolations were made from lesions as described previously (Cortinas *et al*., 2006a). These single conidial cultures were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, where they are maintained in long-term storage facilities.

DNA extraction and microsatellite loci

Single conidial isolates were grown on 2% malt extract agar (MEA) in Petri dishes for 30 days at 25°C. The fungal mycelium was scraped from the colonies, freeze dried, immersed in liquid nitrogen until frozen and ground to a fine powder. DNA extraction followed, from a total of one hundred and thirty-one isolates of *T. gauchensis,* using the phenol-chloroform method as described by Cortinas *et al.,* (2006a).

Ten pairs of fluorescently labelled primer sets for 10 polymorphic microsatellite loci of *T. gauchensis* (Cortinas *et al.,* 2008) were used in this study. The microsatellite loci were amplified by PCR and the amplified products were size-separated on an ABI 3100 Automated DNA Sequencer (Applied Biosystems, Foster City, USA) using GENSCAN LIZ 500 (-250) (Applied Biosystems) as internal size standard. Thirty-eight isolates were analysed from Argentina. These included 10 isolates from the Entre Rios province, 17 from the Corrientes province, eight from the Misiones of Argentina and three from undefined source within these provinces.

Ninety-three isolates were obtained/ analysed from Uruguay including 33 from the Paysandú department and 60 from the Rivera department (Table 1). GENEMAPPER, version 3.0 (Applied Biosystems) software package was used to carry out the fragment size analysis. Based on size differences of the amplicons produced for each locus, different alleles were identified. For further analyses, each allele was designated by their size in nucleotides or by a letter of the alphabet.

Genetic diversity

Gene diversity (*H*) was estimated using the algorithm of Nei (1973) as implemented in POPGENE (Yeh *et al*., 1999). Genotypic diversity (*G*) was calculated (Stoddart and Taylor 1988). To compensate for differences in sample size, the maximum percentage of genotypic diversity (G/N x 100) was used. The significance of differences in haplotypic diversity between populations was determined using a *t*-test (Chen *et al.,* 1994).

Richness and evenness

The clonal diversity of the populations in terms of richness and evenness was studied using GENCLONE 2.0 (Arnaud-Haond & Belknir, 2007; Arnaud-Haond *et al.,* 2007). This program was specially developed to deal with clonal organisms. The program has a 'round-robin' algorithm implemented to avoid the overestimation of the rare allele frequencies. As implemented in GENECLONE 2.0, richness and evenness of the populations were studied using the Shannon -Weiner index (richness), the complementary index (V') (evenness) and Pareto distributions to examine richness and evenness as a whole.

Population differentiation and assignment tests

The program POPGENE was used to determine differences in allele frequencies between populations of *T. gauchensis.* Clone-corrected datasets were analysed to avoid over representation of genotypes produced by asexual reproduction or by sampling at different spatial scales. Differences in allelic frequencies between

populations across the ten loci were tested using Chi square tests (Workman & Niswander, 1970).

The program MULTILOCUS version 1.3 (Agapow & Burt, 2001) was used to estimate the amount of differentiation among populations. The program estimates theta (θ) (Weir, 1996); a modification of the original F_{ST} of Wright (1978). An evaluation of the level of significance of θ was carried out by comparing the observed value to the values obtained by a thousand randomizations of the individuals across populations.

Assignment of individuals into a number of clusters/populations (K) was carried out for the Uruguayan and Argentinean populations using STRUCTURE version 2.2 (Falush *et al.,* 2003). Individuals are assigned to one (K=1), two or more populations where their allelic frequencies were indicative of admixture. To determine the "optimal K", one hundred thousand runs were performed with 10 iterations using an admixture ancestry model and an independent allele frequency model. The burn-in was set at 10 000 runs. Assignments of individuals to the optimal "K" populations was carried out using 1 000 000 runs with a burn-in of 100 000.

Recombination analyses

MULTILOCUS version 1.3 (Agapow & Burt, 2001) was used to test for random association of alleles by calculating linkage disequilibrium (LD) for all pairs of loci and the Index of Association (I_A), using clone corrected data matrices. To determine the significance of the LD and I_A observed values, a distribution of values from a randomly mating population was simulated by performing 1000 randomizations of the allelic frequencies. The LD and I_A observed values were then compared with those obtained for the simulated distribution.

RESULTS

Allele and genetic diversity

Forty- three different alleles were recovered for the 131 isolates of the *T. gauchensis* collected and analysed. Individually, 31 different alleles were recovered from the Argentinean samples and 35 from the Uruguayan population (Table 2). The

number of alleles at individual loci, for both populations, ranged from two to eight. Private alleles were observed in both populations; five from Argentina and nine from Uruguay. The majority of these alleles were present with frequencies higher than 3%. No monomorphic loci were observed.

The gene diversity (H) calculated for *T. gauchensis* was 0.43 in Argentina and 0.42 in Uruguay (Table 2). Ninety-one different genotypes were identified across the two *K. gauchensis* populations (Table 2). One genotype was found to be shared between the Argentinean and Uruguayan populations. The number of repeated genotypes was 26.3% for the Argentinean population and 33.3% for the Uruguayan population. The maximum genotypic (haplotype) diversity was similar for Uruguay ($\hat{G}$ = 50%) and Argentina ($\hat{G}$ = 54%) (Table 2). The *t* test (P < 0.05) showed no significant differences between the genotypic diversities of the Argentinean and Uruguayan populations.

Richness and evenness

The heterogeneity within the populations (relative richness and evenness) values obtained were S= 3.29 and V'= 0.965 for Argentina and S= 3.96 and V'= 0.967 for Uruguay, very similar for both populations. Both had regression values = 1 and similar slopes (β= 2.64 for Argentina and β = 2.43 for Uruguay). Together, these results showed moderate to high haplotype heterogeneity and a high level of evenness (groups of clones of similar membership size). The majority of repeated haplotypes in Argentina and Uruguay formed groups of two individuals.

Population differentiation and assignment tests

The allelic frequencies across populations were compared by calculating the differences in allelic frequencies per locus and between pairwise populations (Table 3). The analysis of the loci showed that the frequencies of the alleles between the populations of Argentina and Uruguay were only significantly different at one (Locus 6) of 10 loci. The theta value of 0.011 (P< 0.05) indicated no differentiation among populations.

No admixture patterns were detected using STRUCTURE as clusters were not detected. The assignment diagrams showed that the majority of individuals

assigned to all different K groups in similar proportions in the tested range between K=1 to K=10.

Recombination analyses

In *T. gauchensis,* low LD was found using two-locus pairwise analyses: zero out of 45 comparisons in the Argentinean population and four of 45 comparisons in the Uruguayan population showed linkage disequilibrium (Table 4). The results obtained from the multilocus Index of Association (I_A) analyses were comparable to the LD results calculated using the pairwise method (Table 4). The observed values of I_A in *T. gauchensis* fell within the randomized distribution of allelic frequencies suggesting that recombination could be occurring in both *T. gauchensis* populations.

DISCUSSION

Teratosphaeria gauchensis is a pathogen of growing importance to a rapidly expanding *Eucalyptus* plantation industry in South America. This study provides the first consideration of its genetic diversity and thus, long term durability of resistance in intensively propagated planting stock. As such, populations of *T. gauchensis* from Argentina and Uruguay showed a genetic structure that is very different to one expected for a recently introduced pathogen. These populations contained moderate levels of genetic variation, homogeneous distribution of haplotypes, no differentiation between populations and indications that recombination is occurring.

The moderate to high levels of genetic diversity found in the *T. gauchensis* populations from South America were unexpected as the disease was only discovered in Argentina and Uruguay in the last two decades. Thus, a low genetic diversity and a small number of predominant haplotypes (clones) were expected in the populations of *T. gauchensis*. This would be similar to a number of other closely related *Eucalyptus* pathogens recently reported in Uruguay (Balmelli *et al.,* 2004; Pérez *et al.,* 2009). For example, the *Eucalyptus* leaf blotch pathogen *T. nubilosa* was found to be clonal, which suggests a recent, localized introduction in the area (Pérez *et al.,* 2009).

The levels of genetic diversity of *T. gauchensis* found in this study were comparable with the genetic diversities of other phylogenetically related *Mycosphaerella* and *Teratosphaeria* species from their native ranges. These species include *M. musicola* (Hayden *et al.,* 2003b; 2005; Zandjanakou-Tachin *et al.,* 2009), *M. fijiensis* (Carlier 2004; Hayden *et al*., 2003a) and *T. nubilosa* (Hunter *et al.,* 2008; 2009). Interestingly, with the exception of *T. gauchensis*, all these species have well characterized sexual states that would promote their genetic diversity.

Results of this study showed evidence of recombination in the studied *T. gauchensis* population from Argentina. This result was unexpected as sexual structures have never been found in the field for this fungus. Nonetheless, there is precedence for finding evidence of recombination in apparently asexual fungi (Taylor *et al.,* 1999; Zhou *et al.,* 2007). From this study we can conclude that *T. gauchensis* in all likelihood has a mixed mode of reproduction and has asexual and sexual reproductive structures similar to the most closely related *Mycosphaerella* spp. (Cortinas *et al.,* 2010; Crous *et al.,* 2004; 2006; Hunter *et al.,* 2008; Pérez *et al.,* 2010). A more exhaustive survey should be conducted in the future to find the teleomorph in the field.

Population genetic analyses showed that the two collections of isolates from Argentina and Uruguay can be considered as part of the same genetic pool, rather than two separate and unrelated populations. Thus, the differentiation tests showed weak to no differentiation between the two *T. gauchensis* populations. These results were further supported by the assignment tests whereby the individuals from Argentina and Uruguay, regardless of the number of clusters tested, were separated in equal proportions among clusters, indicating a lack of population structure for the isolates (Pritchard *et al.,* 2000).

Analyses of *T. gauchensis* isolates from Argentina and Uruguay are not compatible with the hypothesis that this is a recently introduced pathogen. One possible explanation for this result is that the fungus originated in Australasia where *Eucalyptus* is native, as in the case of *T. nubilosa* (Hunter *et al.,* 2008; 2009). This would be consistent with recent well documented examples of new *Eucalyptus* pathogens first being described from plantations outside the native range of *Eucalyptus* and later being discovered in Australia (Wingfield *et al.,* 1996; Burgess *et*

al., 2007). An alternative interpretation is that the pathogen has undergone a host shift from native ad Myrtaceae in Argentina and Uruguay. There are a growing number of *Eucalyptus* pathogens that have undergone host jumps (Slippers *et al.*, 2005) from native Myrtaceae and Melastomataceae (Myrtales) in countries where *Eucalyptus* spp. have been planted as exotics (Wingfield 2003; Wingfield *et al.,* 2008; Glen *et al*., 2007) Many of these examples are from South America including Uruguay (Pérez 2008). The most recent examples are *Quambalaria eucalypti* (Pérez *et al.,* 2008), *Neofusicoccum eucalyptorum* (Pérez *et al.,* 2009b), *Puccinia psidii* (Pérez *et al.,*2010a, in press) and members of Botryosphaeriaceae (Pérez *et al.,*2010b). It would not be unusual for *T. gauchensis* to have behaved in a similar fashion.

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Table 1 List of T. *gauchensis* isolates included in this population study.

Country	Province /Department	Host	Collection Period	Collector	Number of isolates
Argentina	Total 3provinces	*E. grandis*	2001/2003/2004	MJ Wingfield/ MN Cortinas	38
	Entre Ríos				10
	Corrientes				17
	Misiones				8
	Undefined within the 3 provinces				3
Uruguay	Total 2 departments	*E. grandis*	1999/2001/2005	MJ Wingfield/ MN Cortinas	93
	Paysandú				33
	Rivera				60

Table 2 Allelic frequencies and other diversity indices of the clone-corrected populations from Argentina and Uruguay at 10 microstellite loci.

Loci	Alleles	Argentina	Uruguay
K. gauchensis 1	A	0.036	0.064
	B	0.179	0.302
	C	0.536	0.508
	D	0.179	0.079
	E	0.036	0.016
	F		0.016
	G		0.016
	H	0.036	
K. gauchensis 2	A	0.643	0.429
	B	0.215	0.427
	C	0.143	0.127
	D		0.016
K. gauchensis 3	A	0.500	0.508
	B	0.036	
	C	0.429	0.429
	D	0.036	0.032
	E		0.032
K. gauchensis 4	A	0.964	0.968
	B	0.036	
	C		0.016
	D		0.016
K. gauchensis 5	A	0.679	0.740
	B	0.286	0.222
	C	0.036	0.032
K. gauchensis 6	A	0.607	0.571
	B	0.143	0.397
	C		0.032
	D	0.250	
K. gauchensis 7	A	0.679	0.6825
	B	0.286	0.2857
	C	0.036	0.0317
K. gauchensis 8	A	0.071	
	B	0.036	
	C	0.893	0.984
	D		0.016
K. gauchensis 9	A	0.464	0.333
	B	0.536	0.667
K. gauchensis 10	A	0.679	0.571
	B	0.321	0.381
	C		0.032
	D		0.016
N		38	93

Nc	28	63
Na	31	35
Number of private alleles	5	9
H	0.43	0.42
Number of different genotypes (haplotypes)	28	63
G	20.41	46.29
$\hat{G}$	54%	50%
S	3.29	3.96
V'	0.963	0.967
β	2.64	2.43

N= Number of isolates (non clone-corrected)
Nc= Number of haplotypes in the clone-corrected populations
Na= Observed number of alleles
H = Gene Diversity according to Nei (1973)
G = Genotypic Diversity (Stoddart and Taylor, 1988)
$\hat{G}$ = G/N% = percent maximum diversity
S= Shannon–Weiner index
V'= Evenness index derived from Shannon-Weiner (V')
β= β parameter of pareto distribution

Table 3 Pairwise Chi-square comparisons of allelic frequencies between *T. gauchensis* populations of Argentina and Uruguay.

Locus/clone corrected populations		*T. gauch.* 1	*T. gauch.* 2	*T. gauch. 3*	*T. gauch.* 4	*T. gauch.* 5	*T. gauch.* 6	*T. gauch.* 7	*T. gauch.* 8	*T. gauch.* 9	*T. gauch.* 10
Argentina and Uruguay	Chi^2	6.63	4.73	3.15	3.13	0.45	20.60*	0.009	7.36	1.42	1.89
	df	7	3	4	3	2	3	2	3	1	3

*significant Chi-square values (P < 0.05)

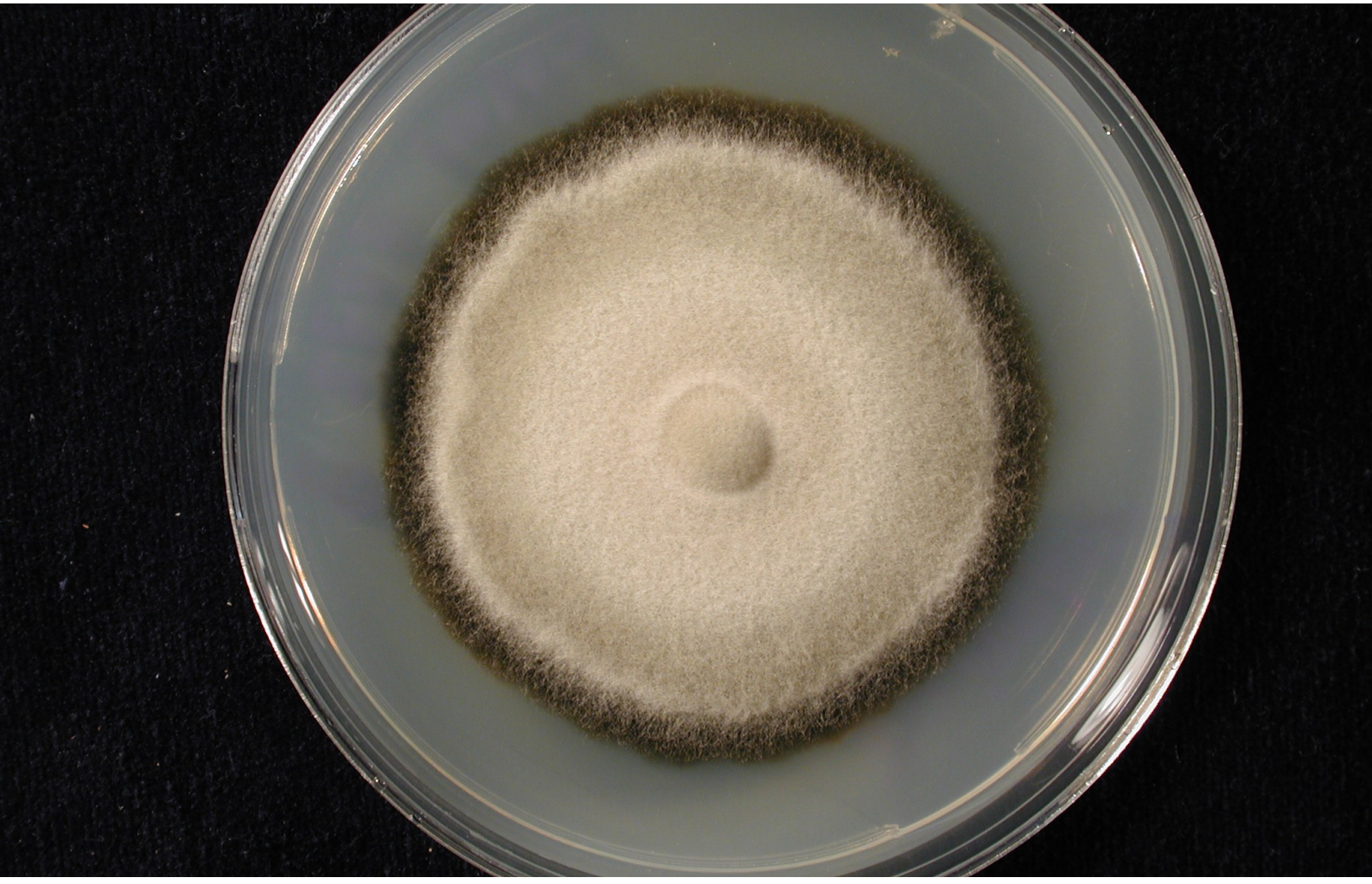
Table 4 Two-locus linkage disequilibrium analysis (LD) expressed as the number of loci with significant differences over the total pairwise loci comparisons, observed Index of Association (I_A) value and range of I_A values after 1000 randomisations. In the last column recombination is indicated as a 'yes' based on the observation that the observed I_A value falls within the randomized dataset values.

	LD between pairs of loci	Obs. I_A	Range of obtained I_A values after 1000 randomizations	Obs. I_A within the randomized data range. (i.e. evidence for recombination)
Argentina	0/45	0.22	-0.0005- 0.33	Yes
Uruguay	4/45	0.08*	-0.0066- 0.13	Yes
All	4/45	0.13	-0.00015- 0.15	Yes

*significant p<0.05

Appendix I

First record of the *Eucalyptus* stem canker pathogen, *Coniothyrium zuluense* from Hawaii



APPENDIX I

First record of the *Eucalyptus* stem canker pathogen, *Coniothyrium zuluense* from Hawaii

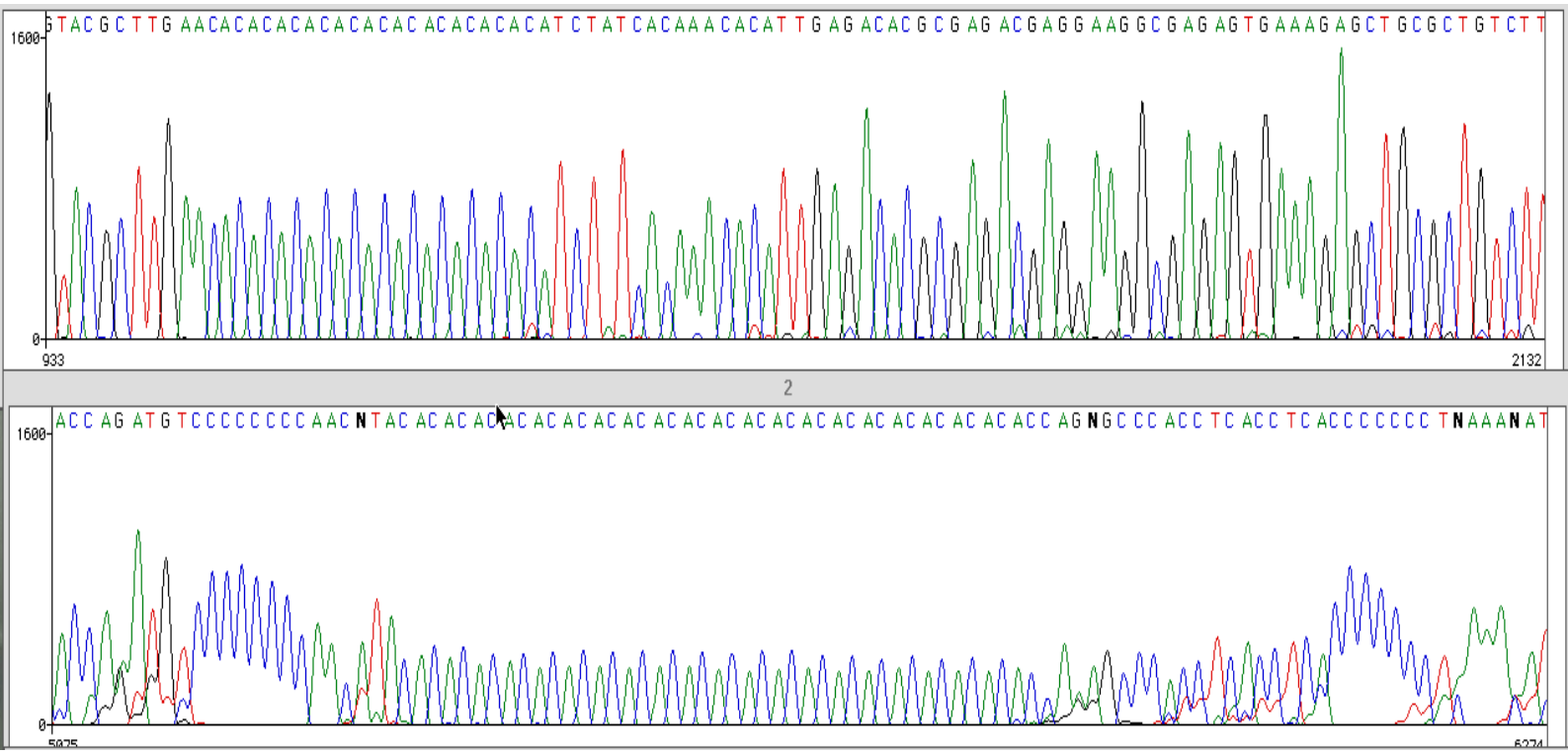
ABSTRACT

A new stem canker disease on *Eucalyptus grandis* in Hawaii is recorded. Symptoms are similar to those of *Coniothyrium* canker on *Eucalyptus* in South Africa. A fungus resembling *Coniothyrium zuluense* was found on lesions and analysis of ITS sequences confirmed this identification. *Coniothyrium* canker is a serious disease of *Eucalyptus* in South Africa and strategies to reduce its impact in Hawaii may be required.

Published as: Cortinas MN, Koch N, Thane J, Wingfield BD, Wingfield MJ (2004d). First record of the *Eucalyptus* stem canker pathogen, *Coniothyrium zuluense* from Hawaii *Australasian Plant Pathology* **33**, 309–312.

Appendix II

M - FIASCO protocol @ FABI



APPENDIX II

M - FIASCO at FABI

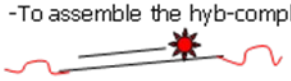
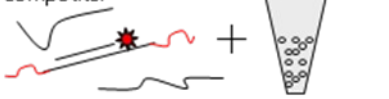
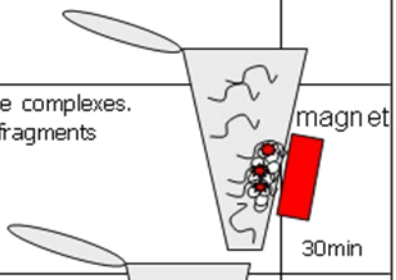
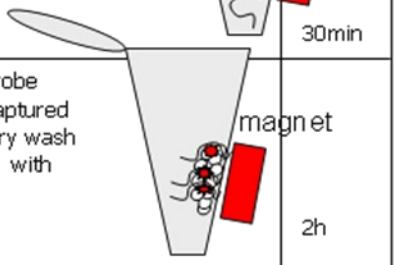
Capture of microsatellite sequences by enrichment procedures.

Version 1.4, May 2007.

This protocol was compiled as part of the PhD project of María Noel Cortinas. It was developed from a combination of pre-existent protocols included in the references section of the protocol.

The protocol was described in: Cortinas MN, Barnes I, Wingfield BD, Wingfield MJ (2006a). Polymorphic microsatellite markers for the *Eucalyptus* fungal pathogen *Colletogloeopsis zuluensis. Molecular Ecology Notes* **6**, 780–783.

Flow chart of microsatellites capture procedure in 5 steps

	Steps	Why do you do this? What is the result?	Timetable min, hs, days	
1.	**DNA Preparations**			1 or 2 days
	1.1 Pre-selection of RE	-To ensure your digestion was succesfull (smear)	3h	
	1.2 Digestion-ligation of adaptors	-To be able to amplify the fragments of DNA	3h	
	1.3 PCRs of **D**igested **D**NA with **A**daptors	-To increase amount of fragments that can be probed -To check ligation of adaptors -To repair nicks -To verify if size selection is needed	3h 1h (gel) 3h 1h (gel)	
2.	**Hybridizing DDA with µsat probes**			1 or 2 days
	2.1 Incubation of DNA together with biotinylated probes	-To assemble the hyb-complexes (DAA/DNA-probe) 	1h	
3.	**Capture of microsatellites (enrichment)**			
	3.1 Incubation with streptavidin-coated magnetic beads and competitor 	-Formation of µsats capture complexes. Non complementary DNA fragments remain in solution 	30min	
	3.2 Washes - 3 non stringent - 3 stringent	-Only complexes ssDNA-probe containing µsats remain captured with the beads during every wash (separation is acomplished with the aid of the magnet) 	2h	
	3.3 Elution	-To dismantle the complexes formed by ssDNA, probes, and beads complexes	15min	
	3.4 PCR post-capture. -Maybe you have to try more than once	-Back to double stranded DNA -To Increase amount of DNA -To verify quality of enrichment. You are looking for an homogeneous representation of fragments = smears	3h 1h (gel)	
	3.5 Freeze enrichments: optional	-If not prepared for cloning or you want to make more enrichments before cloning.		
4.	**Cloning: e.g. TOPO4 Kit**			2 days
5.	**Screening**			2 days
	5.1 Colony PCR		3h, 1h (gel)	
	5.2 Sequencing PCR		3h, 1h (purif)	
			O.N.	6 to 8

M-FIASCO @ FABI

Fundamentally Based on Hamilton *et al.* 1999 and Zane *et al.* 2002

http://fabinet.up.ac.za/personnel/showperson.php?id=marianoel

			Volume (µl)		Temp. (oC)	For how long? (Cycles or h)	
	1.	**DNA preparations**					
		Adaptor Preparation					
		Fiasco1 A (10 µM)	100,0 µl		96	2min	
		Fiasco2 B (10 µM)	100,0 µl		94	1min	
		Total	200,0 µl		Bench	until RT	
	1.1 y 1.2	**Genomic DNA Digestion**					
			Digestion Test				
		Enzyme:	**MSE I**				
		DNA (aprox. 1 µg)	10,0 µl				
		Enzyme buffer (NEB 2)	2,0 µl		37	O.Night	
		BSA 100X	0,2 µl				
		H20	7,0 µl				
		Enzyme	1,0 µl				
		Total	20,0 µl				
		Gel 0.8% agarose	Run gel				
		if test is OK, procede with the definitive digestion-ligation reaction					
		DNA (aprox. 1 µg)	80,0 µl				
		10x Enzyme buffer (NEB 2)	10,0 µl	Enzymes from New England,			
		BSA 100X	1,0 µl	buffer NEB2 compatible with both			
		ddH20	6,0 µl				
		Enzyme	2,0 µl				
		Ligase (High conc. 2000 U/ µl)	1,0 µl				
		ATP (1mM final)	10,0 µl				
		Adaptor (10 µM)	10,0 µl				
		Total	100,0 µl				
		Incubation			37	O.Night	
		Inactivation			65	20min	
		Gel 0.8% agarose	Run gel				
		Cleaning of Lig-reactions with Sigma Purification columns for high MW DNA "GenElute" (NA 1020)					
		Make PCR dilutions in ddH2O	1:5	or 1:10			
	1.3	**PCR post- ligation**					
		DNA	5,0 µl		94	2min	
		Buffer 10X with 15mM MgCl2	2,5 µl		94	30s	17, 20
		MgCl2	2,0 µl		53	1min	25, 30
		Primer Fiasco MseI- N (4 bases)	3,0 µl		72	1min	cycles
		dNTPs (10 µM)	4,0 µl		72	7min	
		H2O	8,1 µl		4	infinite	
		Taq (Normal or Expand Roche)	0,4 µl				
		Total	25,0 µl				

	2.	**Hybridizing your genomic DNA**					
	2.1	**Probing reactions (adjusted to 100 µl)**			in a termocycler		
					you can try different hyb. profiles		
		in eppe 0,5 ml add:			96	10min	
					62	1h	
		DNA	10,0 µl				
		Biotinylated Probes (10 µM)	6,0 µl		96	10min	
		Hybridization solution	82,0 µl		40	1h	
		H2O	0,0 µl				
		Total	100,0 µl		96	10min	
					RT	1h	
		before capturing, cleaning through Sephadex is also possible at this step					
	3.	**Capture of microsatellites (enrichment)**					
	3.1	**Incubation with the beads**					
		Use 1mg of beads per each hybridization mix you want to enrich					
		1 mg of beads (DYNAL, 1mg =100 µl)					
		Wash together all the beads you will use 3 to 5 times					
		Wash adding buffer TEN100	100,0 µl	for each 1mg of beads			
		Magnetize, remove supernatant		3 to 5 times			
		After the last wash resuspend in					
		same buffer	40,0 or 50,0 µl	for each 1mg of beads			
		Add to the resuspended beads:					
		tRNA (Sigma, R- 5636)	5-10 µl	(10 µg)			
		Mix well!!					
		and add:					
		hyb mixes	100,0 µl				
		TEN 100	300,0 µl				
		Incubate @			RT with agit.	30-60 min	
					(150-200rpm)		
				or	33	3h	
	3.2	**Enriching Washes (with gentle agitation)**					4-6h
		1 non Stringent TEN1000	400 µl			5min	
		2 non Stringent TEN1000	400 µl			5min	
		3 non Stringent TEN1000	400 µl			5min	
		4 Stringent Solution	400 µl			5min	
		5 Stringent Solution	400 µl			5min	
		6 Stringent Solution	400 µl		42	5min	
	3.3	**Elution**					
		Add 150 µl TLE or ddH2O			95	10min	
		After magnetizing collect in a clean tube					
		Precipitation					
		Add 1 vol isopropanol	150,0 µl				
		NaOAc 3M	7,5 µl				
		O.N -20oC				O.N	
		Centrifuge				15-30 min	
		Wash with EtOH 70%					
		Centrifuge				5-10 min	

		Resuspend in H2O	30,0 µl				
		Store at -20oC					
	3.4	**PCR post-capture**					
					94	2min	
		DNA	2,0 µl		94	30s	
		Buffer 10X	2,5 µl		53	1min	30 cycles
		MgCl2	2,0 µl		72	1min	
		Primer Fiasco N (4 bases)	3,0 µl		72	7min	
		dNTPs (10 µM)	4,0 µl		4	infinite	
		ddH2O	11,1 µl				
		Taq (normal or Expand, Roche)	0,4 µl				
		Total	25,0 µl				
		Agarose Gel		0,8 to 1%			
		Cleaning of PCR products					
		Sephadex G-50					
		Taq 3' tailing					
		DNA (Clean PCR product)	8,0 µl				
		2mM dATP	4,0 µl				
		Buffer 10X with 15mM MgCl2	2,5 µl		72	30min	
		Normal Taq polymerase	0,2 µl (1,0U)				
		ddH2O	10,3 µl				
		Total	25 µl				
	4.	**Cloning**					
		Ligations	**PGEM**		**TOPO**		
		DNA	2,5 µl	DNA	2,5 µl		
		Ligase Buffer	5,0 µl	Salt	1,0 µl		
		Vector	0,5 µl	Vector	1,0 µl		
		ddH20	0,5 µl	ddH2O	1,5 µl		
		Ligase	1,5 µl				
		Total	10,0 µl		6,0 µl		
					incubate for 30min		
		Follow the instrcutions of manufacturers for transforming and growing cells					
	5.	**Screening**					
	5.1	**Colony preparation**					
		Pick 20 colonies and grow in tubes in 2ml media			37	grow O.N	
		with antibiotics (LB or terrific Broth)					
		alternative:					
		Grow in 96 microtitre plates with 150- 200 µl LB + antibiotic			37	grow O.N	
		in each well					
		(add Glycerol for long term storage after colony PCR)					
		Dilute O.N cultures with ddH2O					
		Cell suspention	5,0 µl				
		H2O	45,0 µl	It depends on concentrations of cells			
		Total	50,0 µl	obtained in the O.N growth			
		Alternative: you can try growing the bacteria for only 3 h and make the Colony PCR					
		using the cell suspentions directly without dilutions					

		Denaturation in termocycler			96	7- 10min	
		(to open cells and liberate the DNA)			On ice until PCR		
	5.2	**Do colony PCR**					
		DNA	1,0 µl		96	5min	
		Buffer 10X	2,5 µl		94	30s	
		dNTPs (10 µM)	2,5 µl		53	1min	30 cycles
		MgCl2 (25mM)	2,0 µl		72	1min	
		Primer M13 TopoF (10 µM)	1,0 µl		72	7min	
		Primer M13 TopoR (10 µM)	1,0 µl		4	infinite	
		Taq (normal or Expand, Roche)	0,12 µl				
		H2O	16,38 µl				
		Total	25,0 µl				
		Cleaning of the PCR products before sequencing					
		Sephadex G-50 or Exo-Sap treatment					
	5.3	**Sequencing**					
		DNA	3,0 µl				
		Big Dye v3.1	2,0 µl		96	10s	
		Buffer 5X	2,0 µl		50	5s	25 cycles
		Primer (10 µM)	1,0 µl		60	4min	
		ddH2O	2,0 µl		4	infinite	
		Total	10,0 µl				
		References					
		Hamilton *et al.* protocol 1999					
		Hamilton MB, Pincus EL, Di Fiore A, Fleischer C (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *BioTechniques* **27**, 500-507.					
		Zane *et al.* 2002 protocol					
		Zane L, Baegelloni L, Patarnello T (2002) Strategies for microsatellite isolation: a review. *Molecular Ecology* **11,** 1-16.					
		Apendix					
		Topo (M13) primers					
		5' GTA AAA CGA CGG CCA G	16bp				
		5' CAG GAA ACA GCT ATG AC	17bp				
		Sephadex G-50 Recipe to clean PCR and Sequencing products					
		Disolve 2g in 30ml ddH2O					
		boil in microwave for 30 seconds					
		Use @ RT. Mix well before use					
		Store @ 4oC					
		Procedure:					
		Fill CentriSep plastic columns with					
		Sephadex G-50	650,0 µl				
		Centrifuge* with a collector tube				2 min*	
		and discard ddH2O					
		Add PCR or Seq products to the	10,0 µl - 60,0 µl				
		centre of the packed column					
		Centrifuge* and collect purif. DNA				2 min*	
		in a new clean tube		* 0.7, 0.8 g = 2800 rpm in eppendorf 5415D			
		Additional for Sequencing…					
		Dry in a vacuum centrifuge				aprox. 15 min	

		Exo-SAP					
		Prepare a solution of 1:1 Exonuclease I					
		and Shrimp Alkaline Phosphatase					
		mixing the enzymes in ddH2O. Store @ -20oC					
		Use 0.5-1 U of each enzyme for every 20 ul of PCR reaction product					
		incubate			37	15min	
					80	15min	
		PCR product ready to use					
		Cleaning of sequencing reactions					
		Sephadex G-50 or					
		96 well Ethanol precipitation (Ethanol/EDTA/Sodium Acetate precipitation protocol from ABI					
		(Applied Biosystems, Protocol booklet 4337035 Rev. A, CA, USA)					
		Solutions as in Zane *et al.* 2002					
		TEN 100	(10mM Tris-HCl, 1mM EDTA, 100mM NaCl, pH 7.5)				
		TEN 1000	(10mM Tris-HCl, 1mM EDTA, 1M NaCl, pH 7.5)				
		Stringent solution	(SSC 0.2X, 0.1% SDS				
		Hybridization solution	(SSC 4.2X, SDS 0.7%)				

SUMMARY

Coniothyrium canker is a fungal disease of *Eucalyptus* spp. grown in plantations. It was first discovered in South Africa in 1989 on *Eucalyptus grandis* trees in plantations of Kwa-Zulu Natal. The pathogen was only described in 1997 when it became economically important to the forestry industry. Since this first report in South Africa, the disease has been reported from other African, South-east Asian and South American countries and the island of Hawaii. The fungus has the capacity to infect a wide range of new clones, hybrids and *Eucalyptus* species. Isolates obtained from single conidia are pleomorphic and lack definitive morphological characteristics. DNA sequence comparisons are therefore, essential for identification. In this study taxonomic questions regarding the causal agents of Coniothyrium canker are addressed using morphological and multilocus phylogenetic sequence analyses. Furthermore, this work includes the first studies on the population genetics on the causal agents of Coniothyrium canker. Polymorphic miscrostellites DNA regions were isolated and pairs of fluorescent primers were designed to amplify the microsatellites alleles using PCR technology. The analyses of the alleles showed that isolates from Coniothyrium canker represent two major independent lineages. During the course of this study, the taxonomic status of the Coniothyrium canker pathogens changed in several occasions including placement in genera such as *Coniothyrium*, *Colletogloeopsis*, *Kirramyces* and *Teratosphaeria*. Morphological and DNA phylogenetic studies identified differences to justify the separation of two major lineages that are now treated as *Teratosphaeria zuluensis* and *Teratosphaeria gauchensis*. The allelic analyses of the microsatellites regions confirmed the separation of lineages as there was no cross amplification between the species. Moderate levels of variation were found for both species but important differences were found regarding the composition and distribution of the genetic variation. Sexual recombination appeared not to be important in *T. zuluensis* but important in the population biology of *T. gauchensis.* Both species most probably did not originate in the areas where they were found and studied. Overall, this study has provided the methodological and theoretical foundation that will promote future work aimed at

understanding Coniothyrium canker and reducing damage due to this important disease.

CONCLUSIONS

Current scientific contributions of this study and future research directions

The taxonomic contribution of this work was to provide evidence that Coniothyrium canker on *Eucalyptus* is caused by two cryptic species, *Teratospaheria zuluensis* and *T. gauchensis* and not by one as previously thought. A re-evaluation of morphological characteristics revealed only minuscule differences in the conidiogeneous cells and conidial size that can be used to discriminate between these species. Temperature growth studies and DNA sequence analyses, however, allowed a clear separation between these two taxa.

Phylogenetic results showed that both *T. zuluensis* and *T. gauchensis* can be accommodated within the genus *Teratospaheria.* Beyond the pure taxonomic interest, the clarification of the taxonomic status of the cause of Coniothyrium canker was important to help interpret further results within an accurate historical and biological context. Interestingly, the closest known phylogenetic relatives of these two species are also pathogens of *Eucalyptus.* Evidence emerged to support the fact that some of these relatives are native Australian species associated with *Eucalyptus* trees in their centre of origin.

The development of microsatellites markers provided tools to gain additional evidence to support the separation of both these *Teratosphaeria* species. The flanking primers developed to amplify the microsatellite regions of *T. zuluensis* could not be used to amplify microsatellites regions on *T. gauchensis.* Likewise primers developed for *T. gauchensis,* when applied to *T. zuluensis,* often did not result in any amplification. This reinforced the conclusion that there is enough genetic divergence between these species to consider them as two different taxa.

The phylogenetic analysis using the ATP6 DNA region detected a common mitochondrial ancestor between both species. This probably reflects the speciation events leading to the final separation of these species. It would be interesting to further explore the sequence information contained in the flanking sequences of

the microsatellite regions and also to find appropriate nuclear information to investigate the historical connections between these two taxa.

Population studies with both *T. zuluensis* and *T. gauchensis* using the DNA microsatellites regions identified in this study produced results that were different than expected. Globally, the *T. zuluensis* populations were shown to contain moderate levels of genetic variation. Sexual recombination seems not to be frequent and there is thus no genetic support for the notion that *T. zuluensis* from South Africa is the source of the *T. zuluensis* populations in the other countries where it was reported. The *Teratospaheria gauchensis* populations in South America were initially thought to be recently introduced into these regions. Results from the population analysis, however, revealed these populations to be well established with high genetic variation. In addition, there was evidence of recombination. Consequently, it is most likely that the pathogen is native to South America.

The population genetic data for *T. zuluensis* from different countries showed that the different populations were in different epidemiological phases. Whereas there are indications that the populations are shrinking in South Africa, it is possible that populations are expanding in China. *Teratospaheria zuluensis* in Asia showed high variation and recombination which is compatible with a scenario of a species experiencing a population expansion phase. Additional collections and appropriate genetic analyses will be necessary to refine and test these new scenarios in the future.

Future research on *T. zuluensis* and *T. gauchensis* will require special attention to the sampling strategies. It will be crucial to chose the right scale and conduct adequate samplings in accordance with the questions that need to be answered. In this way it will be possible to increase the level of confidence of the analyses that are done.

The DNA regions studied and microsatellite markers developed in this study proved to be sensitive enough to detect good levels of singularities within populations and to be useful in the investigation of some of the population dynamics of these populations. In this regard there are two aspects that I feel would be worthy of further study. The first is the bimodal distribution in the South African

T. zuluensis populations. This is also reflected in the phylogenetic data. It would be very interesting to uncover the reasons for the persistence of this internal structure. For example, the bimodal distribution could indicate some sort of ecological adaptation, distribution of mating types or even pathogenicity differences.

The second aspect that is particularly worthy of further study is the fact that both species have been reported in the African continent: *T. zuluensis* in the south and *T. gauchensis* in the north. It would be interesting to investigate whether these two species have an overlapping geographical range and if so, to study the populations in those areas. Finding these species coinciding in one region would not be entirely unexpected as other related *Teratosphaeria* species have been found co-infecting *Eucalyptus* plantations. If these two species co-occur within the same niche, it would be important to investigate to what extend these two species are sharing the same resource. This is important information that would have vital consequences in making global quarantine decisions and also would contribute in the field of ecology to address questions in the context of the niche theory.

The question of the origin, sources and dispersal for the populations of the *T. zuluensis* and *T. gauchensis* changed substantially as a result of this study. Based on the phylogenetic results, the most logical explanation would be that these species originated in the native range of *Eucalyptus* as has been shown for other pathogens of the same phylogenetic group. It is, however, not always trivial to find pathogens in their native range. In addition, *Eucalyptus* trees have been present in South America and Southern Africa for more than one hundred years, originally introduced as ornamentals or used as wind breaks. This situation could have favoured the development of large saprophytic populations of fungi from which some could jump hosts and infect the more recently established *Eucalyptus* plantations. With time, an additional problem is that the historical signal is most probably tainted in all these countries by the sporadic introduction of genetic material by humans in the form of informal exchanges of seeds, infected plant material or transmission of material via clothes and shoes. The importance of transportation of new inoculum in this ways is difficult to measure and is generally agreed that it is underestimated. Therefore, it is possible that the starting

populations in the countries receive multiple introductions from time to time from a different population sources.

Future studies should aim at narrowing down the number of alternative hypothesis relating to the origin, establishment and dispersal *of T. zuluensis* and *T. gauchensis* populations. This could be achieved, in part, by finding answers for some of the basic biological questions regarding these fungi and by achieving a closer cooperation with the forestry sector to collect relevant information on frequency and routes of exchanges of plant material. From the biological point of view, it would be very important to locate the teleomorphs of these species. This would provide the opportunity to re-evaluate how important clonality is for these species and to get better insights as to how variation is created and maintained in these populations. Another important question will be to determine to what extent these organisms can survive as saprobes or, as demonstrated recently in Uruguay, whether in some circumstances they only cause mild symptoms making these pathogens more difficult to detect. Understanding these basic biological questions is particularly important in terms of quarantine.

By the time this study was started, only five articles were published on Coniothyrium canker disease. During the period of this study this number was doubled. These studies showed that the disease is caused by two symptomatically indistinguishable species, *T. zuluensis* and *T. gauchensis*. Although morphological and phylogenetically closely related, they showed that the establishment of populations worldwide was very different and that both species have very different population structures. The polymorphic microsatellite markers that are now available should make it easier to perform additional studies aimed at acquiring a profound knowledge on the population genetic of these species. As these two species are closely related, I envision new contributions going beyond the individual species level to perform comparative studies. Thus, studies of *T. zuluensis* and *T. gauchensis* offer excellent opportunity to those who want to contribute to the field of emergent pathogen diseases, query about the movement of mitotic fungi around the world and answer questions on the speciation process of fungal species.