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.

Teratosphaeria gauchensis (M.N. Cortinas, Crous & M.J. Wingf.) M.J Wingf. & Crous 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

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these fungi to be described after it was discovered causing serious damage to the stems of clonally propagated *Eucalyptus grandis* in the KwaZulu-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 *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 described as *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 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 trunk. 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; Crous et al. 2006). In nature, only asexual pycnidia are found on the bark lesions. These structures 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 species 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* has been found and that *T. gauchensis* has a close phylogenetic relationship to other *Teratosphaeria Eucalyptus*-specific pathogens, it is likely, that this fungus is yet be discovered in the native range of *Eucalyptus*. 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 *T. nubilosa* (= *M. nubilosa*) (Pérez et al. 2009a; Pérez et al. 2009b; Pérez et al. 2009c).

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.

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 lesions 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 of 23 trees in Argentina and 43 trees in Urugauy (Table 1) 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.

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

Country	Province/Department	Host	Collection Period	Collectors	Number of trees	Number of isolates
Argentina	Total 3 provinces	*E. grandis*	2001/2003/2004	MJ Wingfield/	23	38
	Entre Ríos		2003, 2004	MN Cortinas	12	26
	Corrientes		2001		1	1
	Misiones		2001		7	8
	Undefined within the 3 provinces		1999		3	3
Uruguay	Total 2 departments	*E. grandis*	1999/2001/2005	MJ Wingfield/	43	93
	Paysandú		1999, 2001, 2005	MN Cortinas	23	33
	Rivera		1999, 2001, 2005		20	60

DNA extraction and amplification of microsatellite loci

Single conidial isolates were obtained and DNA from these isolates was extracted as described by Cortinas et al. (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 originally deposited in Gen-Bank as "*K. gauchensis*" followed by a number. In this publication they are abbreviated in the tables as "Kg" loci, followed by a number. The microsatellite loci were amplified by Polymerase Chain Reaction (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 as described in Cortinas et al. (Cortinas et al. 2008).

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 an undefined source within these provinces. Ninety-three isolates were obtained and analysed from Uruguay including 33 from the Paysandú department and 60 from the Rivera department (Table 1). GENEMAP-PER, 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.

The gene diversity (H), genotypic diversity (G), richness and evenness, population differentiation and trecombination analyses using linkage disequilibrium (LD) and Index of Association (I_A) were performed as in (Cortinas et al. 2008).

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 private 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 *T. 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.

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 the Argentinean and Uruguayan populations.

No admixture patterns were detected using STRUC-TURE 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.

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

Loci	Alleles	Argentina	Uruguay
Kg-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	
Kg-2	A	0.643	0.429
	B	0.215	0.427
	C	0.143	0.127
	D		0.016
Kg-3	A	0.500	0.508
	B	0.036	
	C	0.429	0.429
	D	0.036	0.032
	E		0.032
Kg-4	A	0.964	0.968
	B	0.036	
	C		0.016
	D		0.016
Kg-5	A	0.679	0.740
	B	0.286	0.222
	C	0.036	0.032
Kg-6	A	0.607	0.571
	B	0.143	0.397
	C		0.032
	D	0.250	
Kg-7	A	0.679	0.6825
	B	0.286	0.2857
	C	0.036	0.0317
Kg-8	A	0.071	
	B	0.036	
	C	0.893	0.984
	D		0.016
Kg-9	A	0.464	0.333
	B	0.536	0.667
Kg-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

Table 2 (continued)

Loci	Alleles	Argentina	Uruguay
Number of different genotypes (haplotypes)		28	63
G		20.41	46.29
Ĝ		54%	50%
S		3.29	3.96
V'		0.963	0.967
β paramter		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 (Nei 1973), *G* Genotypic Diversity (Stoddart & Taylor 1988) $\hat{G} = G/N\%$ percent maximum diversity, *S* Shannon–Weiner index, *V'* Evenness index derived from Shannon-Weiner (V'), β *parameter* Pareto distribution parameter

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 to high level 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. Because of its occurrence on *Eucalyptus* trees, it was thought to have been introduced into these countries relatively recently, probably from the native range of *Eucalyptus*. Thus, a low genetic diversity and a small number of predominant haplotypes (clones) were expected in the populations of *T. gauchensi*s. This would be similar to a number of other closely related *Eucalyptus* pathogens recently reported in Uruguay (Balmelli et al. 2004; Pérez et al. 2009a; Pérez et al. 2009b; Pérez et al. 2009c). 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. 2009a; Pérez et al. 2009b; Pérez et al. 2009c).

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; Hayden et al.

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

Locus/clone correctedpopulations		Kg-1	Kg-2	Kg-3	Kg-4	Kg-5	Kg-6	Kg-7	Kg-8	Kg-9	Kg-10
Argentina and Uruguay	Chi^2	6.63	4.73	3.15	3.13	0.45	20.60[a]	0.009	7.36	1.42	1.89
	df	7	3	4	3	2	3	2	3	1	3

[a] significant Chi-square values ($P < 0.05$)

2005; Zandjanakou-Tachin et al. 2009), *M. fijiensis* (Carlier et al. 1996; Hayden et al. 2003a) and *T. nubilosa* (Hunter et al. 2008; Hunter et al. 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; (Cortinas et al. 2004; Cortinas et al. 2006a; Cortinas et al. 2006b; Hunter et al. 2008; Pérez et al. 2010a; Pérez et al. 2010b; Pérez et al. 2010c)).

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; Hunter et al. 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 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) and members of Botryosphaeriaceae (Pérez et al. 2010b). It would not be unusual for *T. gauchensis* to have behaved in a similar fashion.

Table 4 Two-locus linkage disequilibrium analysis (LD) expressed as the number of loci with significant differences over the total pairwise loci comparisons, range of I_A values after 1000 randomisations and observed Index of Association (I_A). 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	Range of obtained I_A values after 1000 randomizations	Obs. I_A	Obs. I_A within the randomized data range. (i.e. evidence for recombination)
Argentina	0/45	−0.0005–0.33	0.22	Yes
Uruguay	4/45	−0.0066–0.13	0.08[a]	Yes
All	4/45	−0.00015–0.15	0.13	Yes

[a] significant $p < 0.05$

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