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 analyse two temporally separated populations of *T. zuluensis* from South Africa, one population from Malawi and one 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.

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 subtropics (Park *et al*. 2000; Old *et al*. 2003; Wingfield 2003). Among 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 10 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 *Colletogloeopis* as *Colletogloeopsis zuluensis* (Cortinas *et al*. 2006b) and has more recently been treated as *Kirramyces zuluensis* (Andjic *et al*. 2007) and *Readeriella zuluensis* (Crous *et al*. 2007). Based on phylogenetic inference, the pathogen was recognised as related to *Mycosphaerella* (Gezahgne *et al*. 2005; Cortinas *et al*. 2006b) 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 (M. J. Wingfield, unpubl. 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, *T. zuluensis* and *T. 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. zuluensis*, appeared unexpectedly and spread rapidly in South Africa initially on a single highly productive *E. grandis* clone. The fact 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 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 and Keane 1982; Carnegie *et al*. 1998; Hunter *et al*. 2004; Andjic *et al*. 2007) that 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 analysed using 11 microsatellite markers (Cortinas *et al*. 2006a), six of which were developed in this study. More specifically, the aims were: (i) to 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 ~9 years later (end of 2005) in remnant plantations of a highly susceptible *E. grandis* clone.

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

Country	Host	Collection date	Collector	No. of isolates
South Africa 1997 (SA1997)	*Eucalyptus grandis*	1997	L. van Zyl	75
South Africa 2005 (SA2005)	*E. grandis*	2005	M. J. Wingfield, M. N. Cortinas	110
Malawi	*E. grandis*	2004	J. Roux	41
China	*E. urophylla*	2004	T. Burgess	22
Total	–	–	–	248

For the South African collections, a hierarchical sampling strategy was used. Infected bark pieces were taken 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 ~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 of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa.

DNA extraction

Isolates were grown on 2% malt extract agar plates, for 30 days at 25°C (5–6 cm diameter). Mycelium from these actively growing single-conidial cultures was scraped from the 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 characterised 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, CA, 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 11 loci. Isolates with the same haplotype were

considered to be clones.

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

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 and 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 and Taylor (1988) and different sample sizes were compensated for by calculating the maximum percentage of genotypic diversity as G/N × 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 and 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 β calculated by regression (r^2) (the –1 × 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 11 loci was tested using Chi-square tests (Workman and Niswander 1970).

The differentiation among populations was measured as theta (θ) (Weir 1996), which is a modification of FST (Wright 1978). Theta (θ) values were calculated using MULTILOCUS, version 1.3 (Agapow and Burt 2001) using the equation $\theta = Q - q/1 - q$, 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 randomisations in which individuals were randomised 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 (burnin set at 10 000 runs) with 10 iterations. The analysis was repeated for the most likely K obtained using 1 000 000 runs (burnin 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 and 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 randomisations 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 and Belknir 2007; Rozenfeld *et al*. 2007) between the two temporally separated South African populations (SA1997 and SA2005) 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 SA1997 and SA2005 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 GENCLONE 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 3, 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 3). The Ritland (1996) co-ancestry coefficients (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.

Table 3. 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[A]	x coordinates	y coordinates	No. 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

[A]Refer to Fig. 2.

Results

Isolates

A total 248 isolates of *T. zuluensis* were obtained from the isolations from trees in South Africa. Of these, 75 were from SA1997 and 110 were from SA2005. 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 4). In the two South African populations, 41 and 50 different alleles were observed for SA1997 and SA2005, 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 3 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 4) was monomorphic in the Chinese and Malawian populations.

Table 4. Allelic frequencies and other diversity indices of clone-corrected populations of *Teratosphaeria zuluensis* from Malawi, China and South Africa (SA1997 and SA2005) at 11 microsatellite loci.

Loci	Alleles	Malawi	China	SA1997	SA2005	Total	Loci	Alleles	Malawi	China	SA1997	SA2005	Total
Czulu1	A	0.737	0.181	0.2434	0.091		Kzulu12	A	0.398	0.227	0.854	0.773	
	B	0.184	0.455	0.659	0.523			B	0.526	0.727		0.068	
	C			0.024	0.261			C	0.026	0.045	0.049	0.1591	
	D		0.364		0.114			D	0.053		0.049		
	E	0.079		0.073				E			0.049		
	F				0.011		Kzulu13	A	0.158	0.524	0.200	0.049	
Czulu2	A		0.183	0.122	0.012			B	0.053	0.191	0.318	0.602	
	B	0.263	0.366	0.390	0.391			C	0.553	0.191	0.366	0.349	
	C	0.737	0.455	0.488	0.598			D	0.237	0.095	0.098		
Czulu3	A				0.250		Czulu6	A	0.394	0.455	0.634	0.840	
	B			0.097	0.031			B	0.026	0.364		0.159	
	C	1.000	1.000	0.902	0.716			C	0.316	0.182			
Kzulu5	A	0.027		0.024	0.114			D	0.263				
	B	0.108	0.050	0.024				E			0.293		
	C	0.216	0.100					F			0.024		
	D			0.024	0.273			G			0.049		
	E				0.011		Czulu7	A	0.316	0.190	0.146	0.023	
	F	0.027	0.050					B	0.553	0.810	0.781	0.716	
	G	0.270	0.200	0.195	0.273			C	0.053		0.073	0.216	
	H	0.027						D	0.079			0.046	
	I	0.027					Kzulu14	A	0.053	0.227	0.195	0.0342	
	J	0.027		0.024	0.068			B	0.026	0.227			
	K	0.243	0.550	0.707	0.114			C	0.026	0.091	0.342	0.568	
	L	0.027	0.050		0.023			D	0.026	0.091		0.011	
	M				0.091			E		0.046			
	N				0.034			F		0.046			
Kzulu10	A		0.095	0.049	0.205			G	0.579	0.189	0.366	0.364	
	B	0.447	0.191	0.220	0.398			H	0.263	0.091	0.097	0.011	
	C	0.237	0.286	0.7073	0.2273			I	0.026			0.011	
	D		0.238	0.024	0.0909		N^A		41	22	75	110	248
	E	0.316	0.095				$N_c{}^B$		37 (7.5%)	22 (0%)	43 (22.9%)	86 (11.1%)	188
	F		0.095		0.011		$N_a{}^C$		45	42	41	50	
	G				0.068		No. of private alleles		3	2	4	9	18
Kzulu11	A				0.034		H^D		0.51	0.53	0.44	0.51	
	B	0.158	0.091	0.024	0.056		No. of polymorphic loci		10	10	11	11	
	C	0.737	0.818	0.927	0.738		G^E		34.48	22	18.18	47.61	
	D				0.045		$\hat{G}^F$		84%	100%	24%	43%	
	E	0.105	0.091	0.049	0.091		S^G		3.68	3.09	3.4	4.35	
	F				0.034		V'^H		0.98	0.99	0.90	0.97	
							β parameter^I				1.658	2.779	

[A] N = Number of isolates (non-clone-corrected).
[B] N_c = Number of haplotypes in the clone-corrected populations.
[C] N_a = Observed number of alleles.
[D] H = Nei's gene diversity (Nei 1973).
[E] G = Genotypic diversity (Stoddart and Taylor 1988).
[F] $\hat{G}$ = G/N% = percentage maximum diversity.
[G] S = Shannon–Weiner index.
[H] V' = evenness index derived from Shannon–Weiner.
[I] β parameter = Pareto distribution.

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 SA2005 (Table 4). 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 4). 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 4). 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 (1658) than that determined for the SA2005 (2779) 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 African populations (Table 5). These results suggest that the South African (RSA) populations belong to different gene pools.

For the θ calculations, only the Malawian population showed significant Chi-square values ($P < 0.05$) when compared with other populations (Table 6). The differentiation between the South African populations ($\theta = 0.10$) was the smallest. The largest differentiation was observed between Malawi and the SA2005 ($\theta = 0.18$) populations.

Table 5. 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.

Pairwise populations		Czulu1	Czulu2	Czulu3	Kzulu5	Kzulu10	Kzulu11	Kzulu12	Kzulu13	Czulu6	Czulu7	Kzulu14	No. of significantly different loci
Malawi and China	χ^2	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	χ^2	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	χ^2	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	χ^2	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	χ^2	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	χ^2	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 6. 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 ($P < 0.05$).

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 group 1 (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 LD in the populations of *T. zuluensis* (Table 7). 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 I_A results were comparable to the LD results obtained by the pairwise analyses (Table 7). Significant departures from gametic equilibrium were detected for all populations (0.41–0.75) 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.

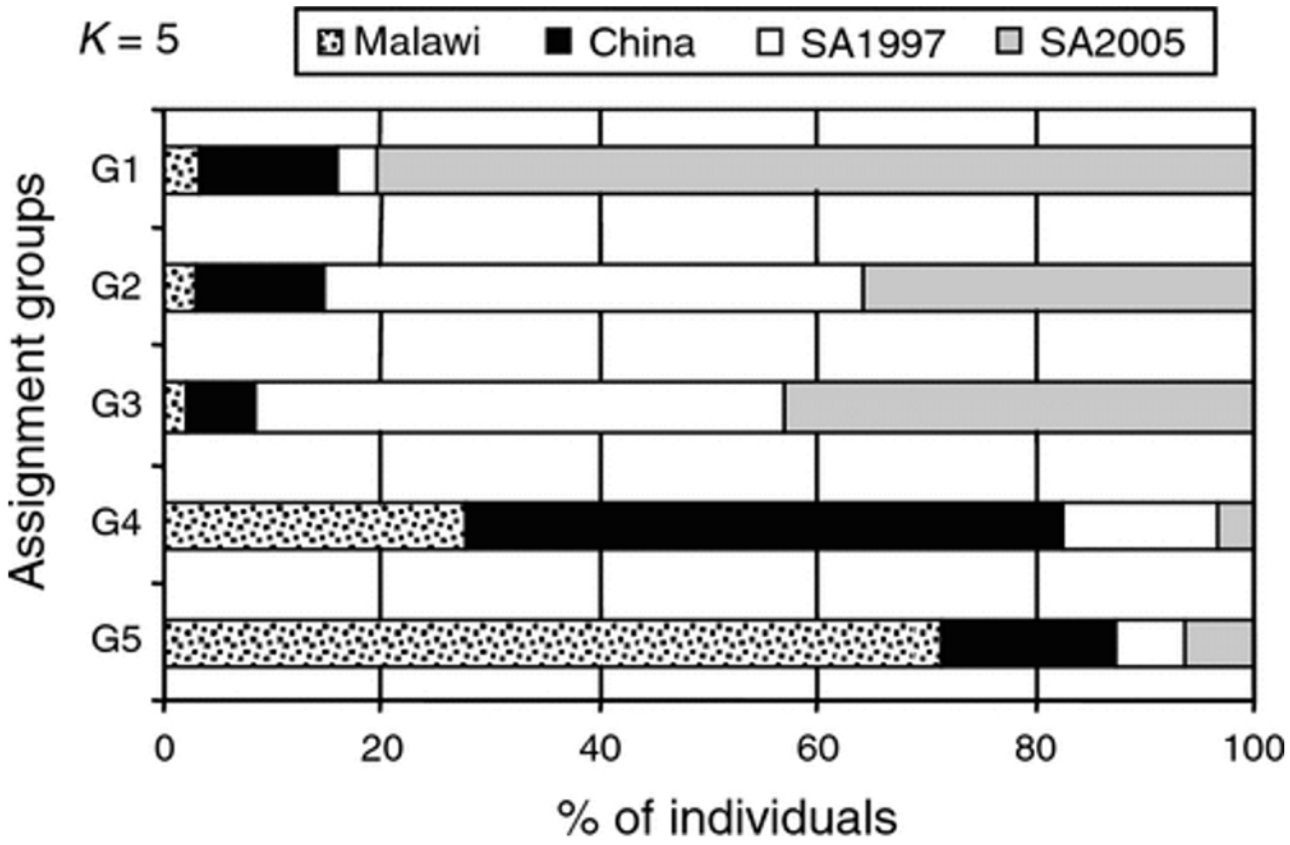


Fig. 1. Proportion of individuals from each geographical population assigned to the K = 5 groups (G1 to G5). In 3 out of the 5 groups (G1 to G3), the majority of SA1997 individuals group with SA2005 individuals. The majority of the Chinese and Malawi individuals group in distinctive groups (G4, G5).

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 2–12 replicates each (Fig. 3). In contrast, 86 different haplotypes were identified in SA2005 and 13 haplotypes formed clusters with between two and 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 visualise 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 generated trees revealed two main clusters for both the SA1997 and SA2005 populations. The trees showed no association between localities and clusters.

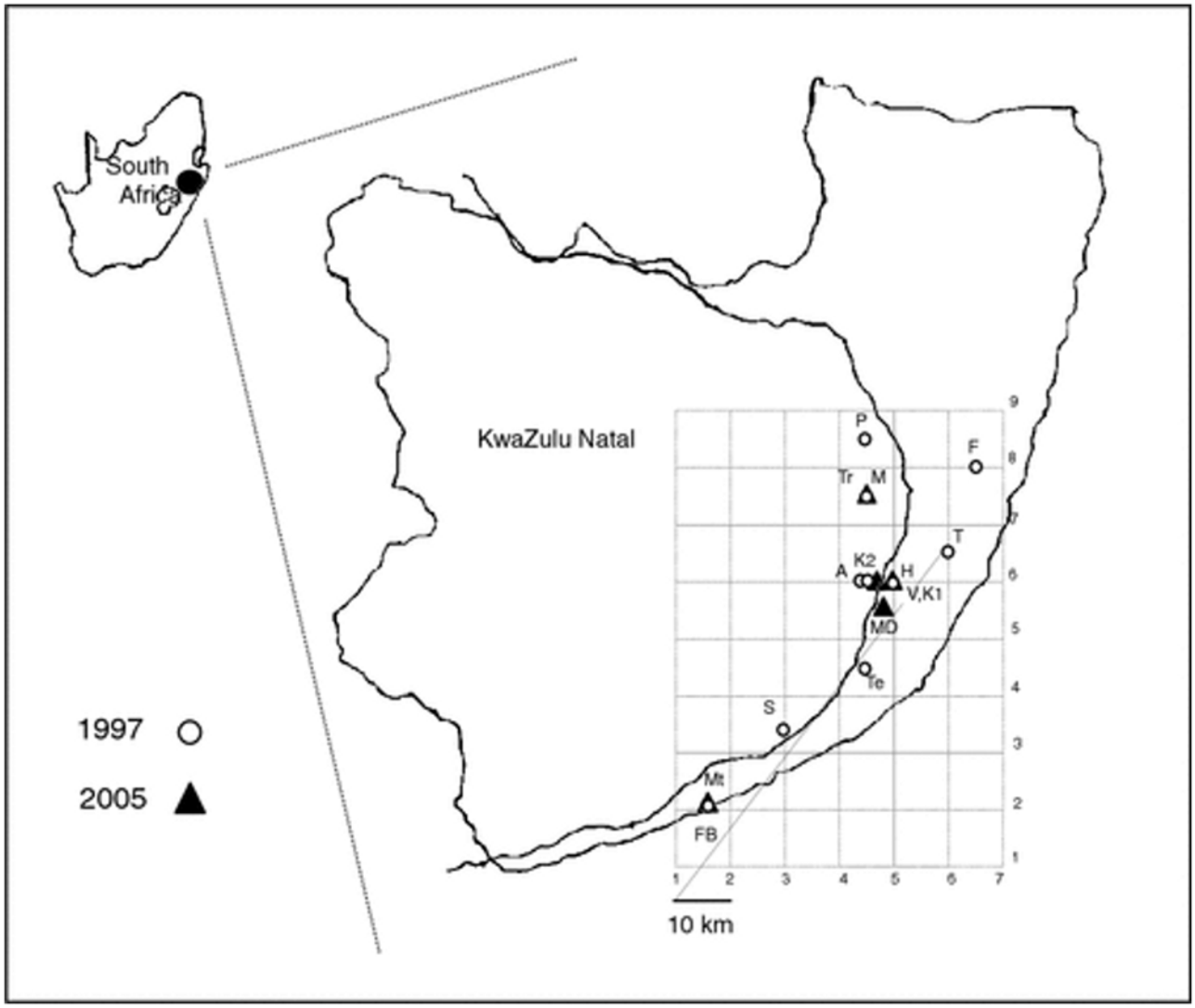


Fig. 2. Location of sites sampled in 1997 and 2005 in KwaZulu Natal, South Africa.

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 dataset, 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, 11 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*.

Table 7. 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 randomisations.

	LD between pairs of loci	Obs. I_A	Range of obtained I_A values after 1000 randomisations	Obs. I_A within randomised 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*	–0.0008–0.17	No
All	14/49	0.37*	–0.0033–0.15	No

In the last column recombination is indicated as a 'yes' based on the observation that the observed I_A value falls within the randomised dataset values. *Significant at $P < 0.05$.

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 9 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 difference 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 present in the first place (McNeilly and Roose 1984; Watkinson and Powell 1993; Hughes and 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 (84–100%, respectively), 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. zuluensis* 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).

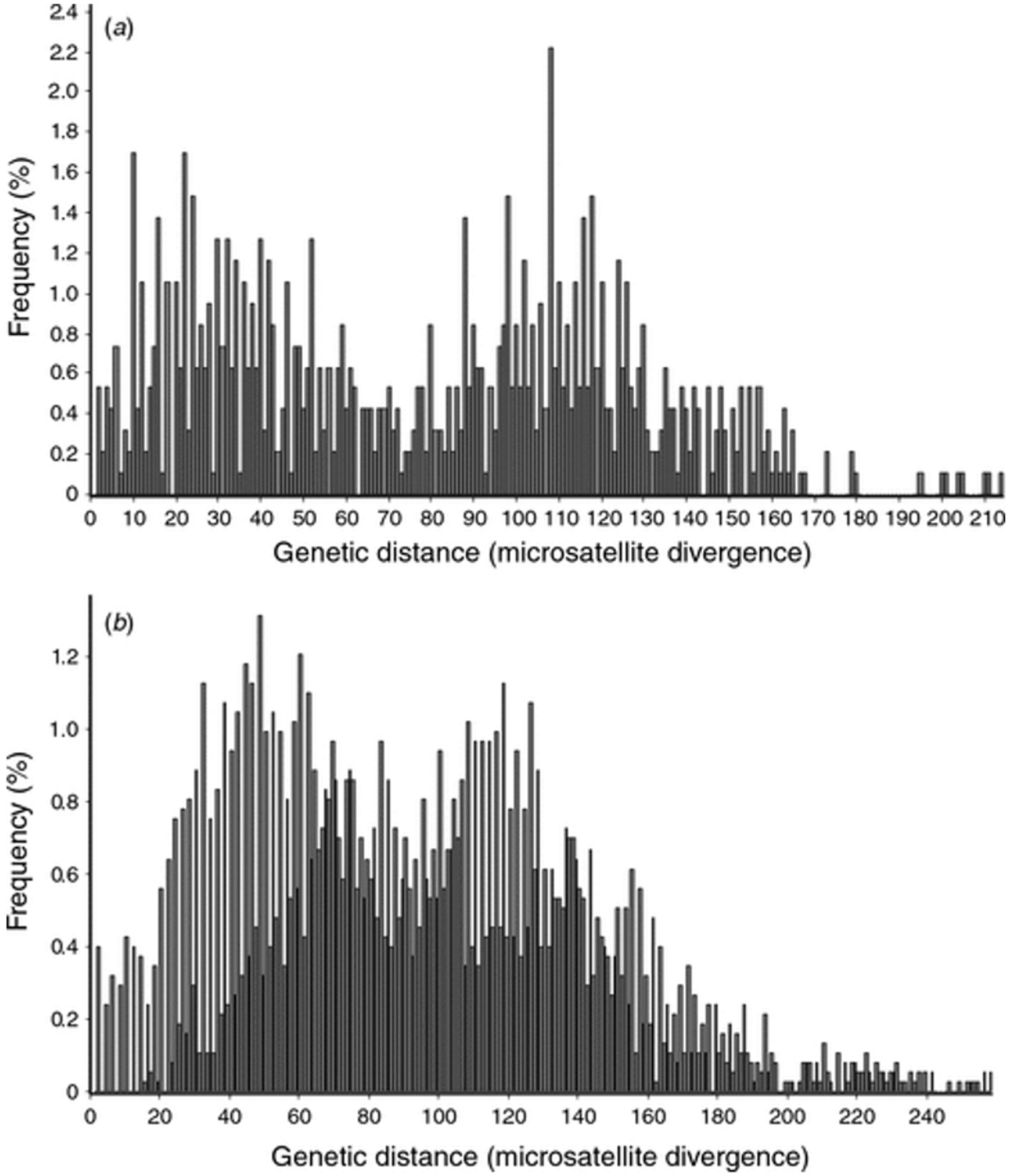
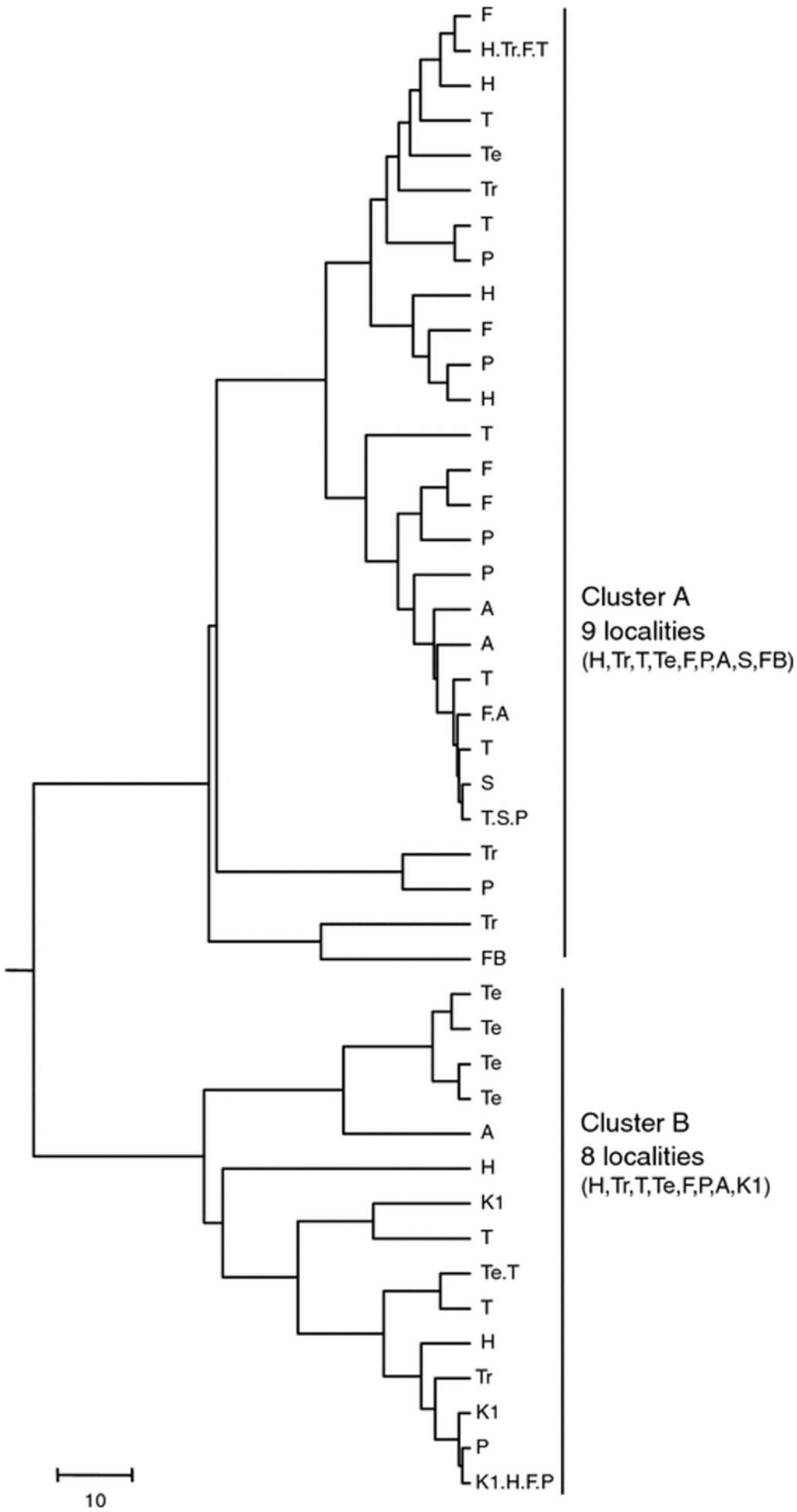


Fig. 3. Frequency distribution of microsatellite divergence among pairs of isolates (a) for the SA1997 population and (b) for the SA2005 population.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Cluster A
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Cluster B
8 localities
(H,Tr,T,Te,F,P,A,K1)
10

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 recognised 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 4. Two main clusters of clones emerge in the tree. Each cluster includes clones from the majority of the sampled locations.

On a global scale, the allele frequency 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 and from an unknown source.

The observed differentiation between the two temporally separated populations from South Africa 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 a 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.

The LD 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 have its origin in South-East Asia, the fact that is not known in Australia does not imply that it is not present 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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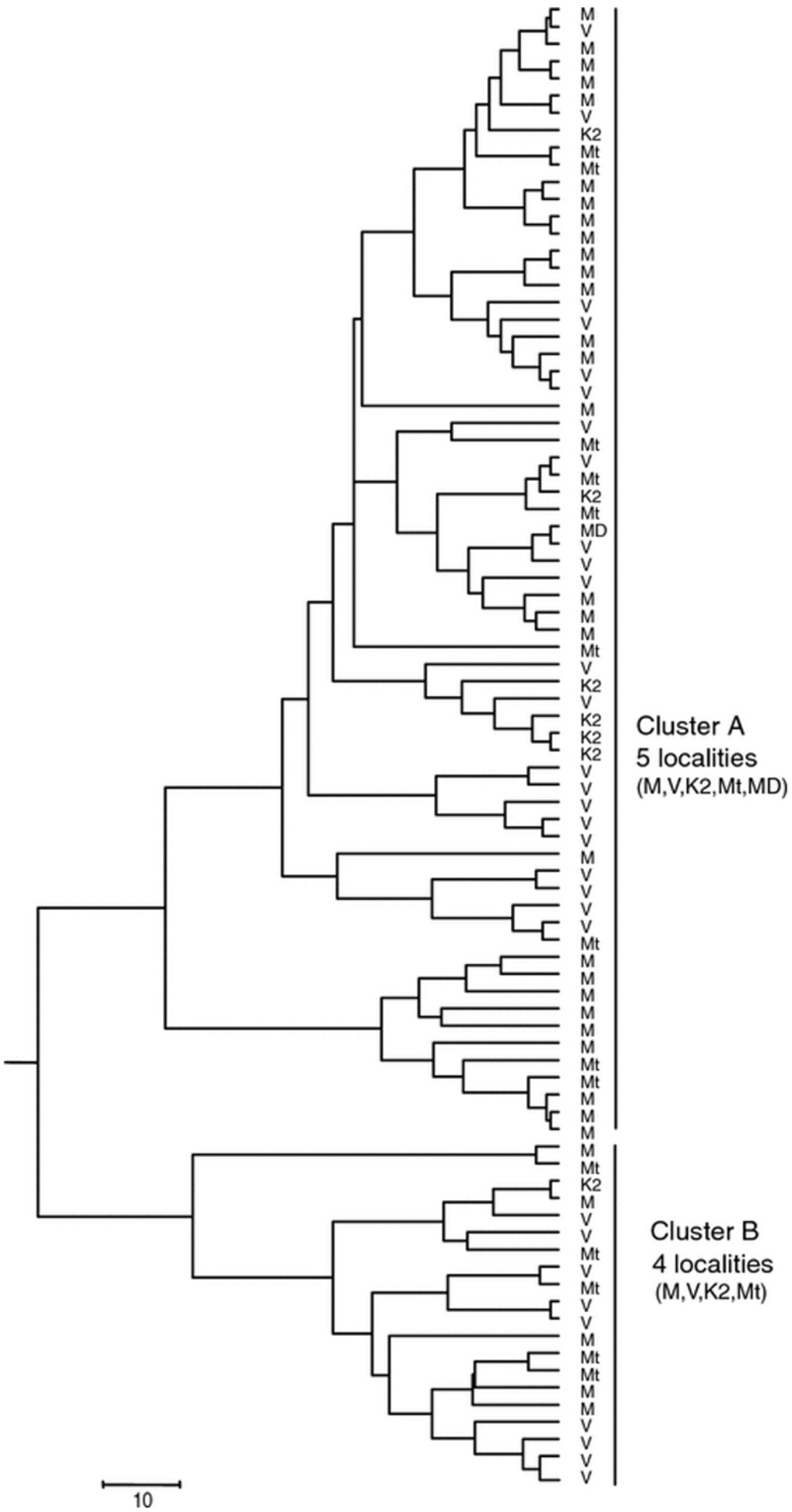
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V
V
V
Cluster A
5 localities
(M,V,K2,Mt,MD)
Cluster B
4 localities
(M,V,K2,Mt)
10

Fig. 5. UPGMA tree for the SA2005 population constructed in MEGA 4 using the distance matrix calculated in GENCLONE 2.0. The branches include samples recognised 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 4. Two main clusters of clones emerge in the tree. Each cluster includes clones from the majority of the sampled locations.

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