

## The *Eucalyptus* stem canker pathogen *Teratosphaeria gauchensis* represents distinct genetic groups in Africa and South America

By L. Jimu<sup>1</sup>, S. F. Chen<sup>2</sup>, M. J. Wingfield<sup>2</sup>, E. Mwenje<sup>3</sup> and J. Roux<sup>2,4</sup>

<sup>1</sup>Department of Plant Production and Soil Science, Forest Science Postgraduate Programme, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; <sup>2</sup>Department of Microbiology and Plant Pathology, FABI, University of Pretoria, Private Bag X20, Hatfield, Pretoria 0028, South Africa; <sup>3</sup>Bindura University of Science Education (BUSE), Bindura, Zimbabwe; <sup>4</sup>E-mail: Jolanda.roux@fabi.up.ac.za (for correspondence)

### Summary

*Teratosphaeria gauchensis* (Capnodiales) causes a serious stem canker disease on commercially propagated *Eucalyptus* species in South America. Recently, this pathogen was detected for the first time in Africa. Very little is known regarding the biology or origin of *T. gauchensis*, but it has been suggested that it is native to South America. The aim of this study was to compare isolates from Africa and South America using microsatellite markers. Bayesian analysis conducted in STRUCTURE, principal coordinates analysis and a UPGMA dendrogram revealed two distinct genetic groups for these isolates. The South American isolates were more genetically diverse than those from Africa. Patterns of genetic diversity in Africa suggest that *T. gauchensis* could have been introduced into Zimbabwe before spreading north-eastwards. The existence of the two genetic groups and high haplotype richness associated with the South American and Zimbabwean populations suggest that it will be more difficult to reduce the impact of disease caused by *T. gauchensis* in these regions than in those areas where there is limited genetic diversity.

### 1 Introduction

*Teratosphaeria gauchensis* (M.–N. Cortinas, Crous & M.J. Wingf.) M.J. Wingf. & Crous and *Teratosphaeria zuluensis* (M.J. Wingf., Crous & T.A. Cout.) M.J. Wingf. & Crous are two closely related fungal pathogens that cause a serious stem canker disease of *Eucalyptus* species outside the natural range of these trees. The first outbreak of this disease was in South Africa in 1989, and the causal pathogen was described as *Coniothyrium zuluensis* (Wingfield et al. 1997). Subsequently, the disease has been reported from several countries globally (Roux et al. 2002, 2005; van Zyl et al. 2002; Gezahgne et al. 2003, 2004, 2005; Old et al. 2003; Cortinas et al. 2004, 2006b; Chungu et al. 2010; Jimu et al. 2014, 2015a).

Multigene phylogenetic sequence analyses have shown that the fungus once considered to be a single species, *T. zuluensis*, represents two distinct species (Cortinas et al. 2006c), with *T. gauchensis* occurring in Africa (Ethiopia, Uganda) and South America (Argentina and Uruguay). *T. gauchensis* was recently reported in Zimbabwe, representing the first record of the pathogen in southern Africa, a region where only *T. zuluensis* was previously known (Jimu et al. 2015a). The pathogen was also recently reported for the first time in Europe (Silva et al. 2014).

*T. gauchensis* and *T. zuluensis* cause discrete sunken lesions that sometimes merge to form large necrotic patches on *Eucalyptus* stems (Wingfield et al. 1997; van Zyl et al. 2002; Gezahgne et al. 2003; Old et al. 2003). Dark brown to black pycnidia can usually be seen on the dead bark (Wingfield et al. 1997). Kino exudes from the lesions, staining stems and branches, and kino-pockets occur in the wood. Stem girdling results in the emergence of epicormic shoots below the cankers. In severe cases, the disease results in dead tops, brush-like crowns and tree death (Wingfield et al. 1997; van Zyl et al. 2002; Gezahgne et al. 2003; Old et al. 2003). The disease drastically reduces the quality of the wood, rendering it unsuitable for construction and sawn timber.

Little is known regarding the biology of *T. gauchensis* or *T. zuluensis*. The two pathogens are thought to reproduce asexually because a sexual morph has never been observed (Wingfield et al. 1997; van Zyl et al. 2002; Cortinas et al. 2006c, 2010, 2011). Although both species cause disease in a number of countries, their origins and pathways of dissemination are not known. Neither species has been observed in Australia, the centre of origin of eucalypts. It was originally believed that *T. zuluensis* was native in South Africa (Wingfield et al. 1997; van Zyl et al. 2002; Old et al. 2003), but recent population genetic studies have suggested a centre of diversity outside Africa (Cortinas et al. 2010; Chen et al. 2011; Jimu et al. 2015b).

Population genetic studies on *T. gauchensis* isolates from Argentina and Uruguay showed moderate genetic diversity and it was suggested that this species may be native to South America (Cortinas et al. 2011). In this study, we combined *T. gauchensis* microsatellite data for isolates collected in South America (Cortinas et al. 2011) and Africa to investigate the population structure and differentiation of this pathogen and thus to consider its possible origin and patterns of global movement. In addition, the genetic diversities for the previously uncharacterized isolates from Africa were computed to better understand its movement on this continent. The possible mode of reproduction of the pathogen was also considered.

## 2 Materials and methods

### 2.1 Isolate collection and identification

Isolates of *T. gauchensis* were collected from *Eucalyptus* trees showing symptoms of stem canker in Ethiopia, Uganda and Zimbabwe during 2011 and 2012 (Table 1). In Zimbabwe, hierarchical sampling was applied, where diseased bark samples were collected from a reference tree at the centre of a compartment or woodlot, followed by random selection of diseased trees along two perpendicular transects, extending outwards from the central tree. Diseased *Eucalyptus* trees were surveyed in five different sites representing three agro-ecological regions. In Ethiopia and Uganda, all observed diseased trees were sampled within a specific site because stem canker symptoms were rare in these sites. In Ethiopia, surveys were conducted in three regions and in Uganda in two areas, both in 2012. During sample collection, one to five small pieces of infected bark were collected from individual trees and these were labelled and placed in paper bags for subsequent laboratory processing.

Bark pieces were incubated in moist chambers for 1–2 days to induce sporulation after which spore masses were transferred to Petri dishes containing 2% MEA (20 g/l malt extract; 15 g/l agar, Biolab, Midrand, South Africa, and 1 l deionized water). After 5–7 days, cultures were purified by transferring single hyphal tips to fresh 2% MEA plates. The plates were incubated at 25°C for 4 weeks. Representative isolates for each country were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Additional isolates from Ethiopia and Uganda were sourced from this collection.

To confirm the identities of the *Teratosphaeria* isolates, actively growing mycelium was scraped from four-week-old cultures on MEA plates using sterile surgical blades and transferred into 2-ml Eppendorf tubes, freeze-dried and ground to a fine powder. The method described by Möller et al. (1992) was used to extract DNA from the mycelium. Each sample was treated with 2  $\mu$ l of RNase (1 mg/ml) and left overnight to digest contaminating RNA. DNA concentrations were measured using a NanoDrop<sup>®</sup>ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA). The internal transcribed spacer (ITS) and translation elongation factor 1- $\alpha$  (TEF1 $\alpha$ ) gene regions of representative samples were sequenced as described by Cortinas et al. (2006c). DNA sequences of the isolates were compared with previously published *T. gauchensis* and *T. zuluensis* sequences obtained from GenBank [National Centre for Biotechnology Information (NCBI), USA National Institute of Health Bethesda]. Alignments of sequence data were made online using MAFFT v. 7 (<http://mafft.cbrc.jp/alignment/software/>) after which the program MEGA 6 (Tamura et al. 2013) was used to check the alignments. Parsimony analyses were conducted and most parsimonious trees were generated using PAUP v. 4.0b10 (Swofford 2002). *Teratosphaeria nubilosa* was used as an outgroup. Isolates confirmed to be those of *T. gauchensis* were used for population genetic studies.

### 2.2 Loci amplification and allele size determination

A total of 13 microsatellite loci were amplified for *T. gauchensis* isolates using 13 pairs of markers, ten (Kgau1 to Kgau10) of which had been developed for *T. gauchensis* (Cortinas et al. 2008) and the other three (Czulu3, Kzulu13 and Kzulu14) for *T. zuluensis* (Cortinas et al. 2006a, 2010). The PCR mixtures consisted of 5  $\mu$ l of 5 $\times$  MyTaq reaction buffer, 0.5  $\mu$ l MyTaq DNA polymerase, 0.5  $\mu$ l forward primer, 0.5  $\mu$ l reverse primer, 16.5  $\mu$ l sabax water and 2  $\mu$ l DNA template giving a total volume of

Table 1. Geographical location and isolate details of *T. gauchensis* isolates from Africa. Details for isolates collected from Argentina and Uruguay were summarized by Cortinas et al. (2011).

Country	Province	Site	Host	Collection year	Collectors	Number of trees	Number of isolates	Multilocus genotypes per site
Ethiopia	Guragae	Wolkite	<i>E. camaldulensis</i>	2012	Gezaghne A, Jimu L	16	16	1
	Jima	Omanado	<i>E. camaldulensis</i>	2012	Gezaghne A, Jimu L	1	1	1
	West Shewa	Illo	<i>E. camaldulensis</i>	2012	Gezaghne A, Jimu L	4	4	1
Uganda	Eastern	Jinja	<i>E. grandis</i>	2012,	Jimu L,	23	35	3
	Western	Fort Portal	<i>E. grandis</i>	2011	Roux J	6	6	2
Zimbabwe	Bulawayo	Bulawayo	<i>E. grandis</i> $\times$ <i>camaldulensis</i> hybrid, <i>E. propinqua</i> , <i>E. paniculata</i>	2011	Jimu L	19	30	20
	Midlands	Gweru	<i>E. camaldulensis</i>	2011	Jimu L	18	27	14
	Mashonaland Central	Madziwa	<i>E. camaldulensis</i> , <i>E. grandis</i> $\times$ <i>camaldulensis</i> hybrid	2011	Jimu L	17	24	9
		Rushinga	<i>E. camaldulensis</i> , <i>E. grandis</i> $\times$ <i>camaldulensis</i> hybrid	2011	Jimu L	10	14	8
		Masvingo	Masvingo	<i>E. grandis</i>	2012	Jimu L, Roux J	7	10

25  $\mu$ l. PCRs and annealing temperatures for the microsatellite markers were as described by Cortinas et al. (2006a, 2008, 2010). PCR products were checked for amplification on 2% agarose gels stained with Gel Red (Biotium, Hayward, CA, USA) in  $1 \times$  TAE buffer (Tris base 0.4 M, acetic acid 1%, EDTA 0.5 M, pH 8.0) and visualized under UV illumination.

The PCR amplicons were size-separated on an ABI PRISM™ 3500 Automated DNA Sequencer (Applied Biosystems, Foster City, CA, USA) together with the internal size standard LIZ 500 (–250) (Applied Biosystems). Amplicon size analyses were made using the software GENEMAPPER version 4.3 (Applied Biosystems). Each product length (rounded to the nearest base pair) was considered to represent a different allele and every allele was assigned an alphabetical letter. Microsatellite data for South American populations were sourced from the study of Cortinas et al. (2011) and combined with the data from African countries generated in this study. Both data sets were generated using an ABI PRISM™ Automated DNA Sequencer (Applied Biosystems) and GENEMAPPER (Applied Biosystems). Fresh DNA was extracted from twenty isolates used by Cortinas et al. (2011) and re-analysed in this study. This allowed confirmation of the combination of data from that study, with data generated in the current study. The data were subjected to downstream analyses after several rounds of quality control.

## 2.3 Data analyses

Data generated for African isolates and those obtained from population studies conducted by Cortinas et al. (2011) for the South American isolates were used for genetic analyses. Prior to data analyses, the mean genotypic diversity was plotted against the number of loci (not shown) using MULTILOCUS 1.3 (Agapow and Burt 2001) to ascertain that the numbers of isolates and loci were sufficiently large to ensure statistical significance. The combined data set was clone-corrected using GENCLONE version 2.0 (Arnaud-Haond and Belkhir 2007), retaining only one isolate per genotype for further analyses. This was performed to avoid bias due to the occurrence of repeated genotypes (Grünwald et al. 2003).

### 2.3.1 Population structure and differentiation

Assignment analyses of data for the microsatellite loci common to both African and South American populations were conducted using the Bayesian clustering algorithm in STRUCTURE version 2.2 (Pritchard et al. 2000; Falush et al. 2003), principal coordinates analysis (PCoA) and analysis of molecular variance (AMOVA) using GenAlEx 6.4 (Peakall and Smouse 2006). STRUCTURE was used to assign individuals into '*K*' populations based on allelic frequencies. The algorithm excludes prior information regarding the geographical origin of the isolates. The program was run twice; with the first run to determine the optimal *K* using an admixture ancestry model and an independent allele frequency model. The admixture model considers that individual *T. gauchensis* isolates could have mixed ancestry, that is isolate *i* inherited a certain percentage of its genome from ancestors in population *k*. The independent allele frequency model allows for the fact that allele frequencies in different populations should be reasonably different from each other (Pritchard et al. 2000). The runs were set at 20 for *K* = 1–10. The algorithm was executed at 300 000 Markov chain Monte Carlo (MCMC) repetitions following a burn-in of 30 000 iterations. The most likely value for *K* was estimated using Evanno's  $\Delta K$  method (Evanno et al. 2005) using STRUCTURE HARVESTER (Earl and VonHoldt 2012). The second analysis involved assigning individual *T. gauchensis* isolates into the optimal *K* obtained in the first stage. This was run at 1 000 000 MCMC repetitions following a burn-in of 100 000 iterations. Results from 20 runs for each *K* were collated in CLUMPP v1.1.2 (Jakobsson and Rosenberg 2007). The output was then visualized in DISTRUCT v1.1 (Rosenberg 2004). Additionally, a genetic distance matrix generated using GenAlEx 6.2 was imported into MEGA 6 to construct a dendrogram using the unweighted pair-group method with arithmetic mean (UPGMA).

Differentiation among populations was tested using theta ( $\theta$ ) (Weir 1996) and Nei's (1978) unbiased genetic identity (*I*) and genetic distance (*D*). POPGENE version 1.31 (Yeh et al. 1999) was used to compute genetic identities and distances, while MULTILOCUS Version 1.3 was used to calculate theta. Theta was computed 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 different populations are the same. Theta was tested for significance through a comparison of the observed value with that of 1000 randomizations.

### 2.3.2 Genetic diversity within populations

Genetic characterization of populations was achieved using gene diversity (Nei 1973) and allelic richness (Stoddart and Taylor 1988). The software program POPGENE was used to compute gene diversity for each clone-corrected population. The diversity was based on the equation  $H = 1 - \sum x_k^2$ , where  $x_k$  is the *k*th allele frequency (Nei 1973). Allelic richness ( $A_{\text{rare}}$ ) was inferred after correcting for unequal sample sizes using a rarefaction statistical technique implemented in HP-RARE 1.1 (Kalinowski 2005). As Ethiopia had the lowest number of isolates, the number of sampled alleles per locus was adjusted to 22 for rarefaction.

### 2.3.3 Mode of reproduction

Index of association ( $I_A$ ) (Maynard-Smith et al. 1993; Agapow and Burt 2001), a statistical tool in MULTILOCUS version 1.3, was used to assess the reproductive modes of the clone-corrected populations from Africa. The  $I_A$  values were computed using a comparison of observed values and expected values of 1000 randomly mating datasets (Taylor et al. 1999). The

likelihood that each population is undergoing recombination was ascertained where the observed values were within the distribution range of the recombined values. Conversely, the population was considered clonal where the observed values were outside the distribution range of the recombined values.

### 3 Results

#### 3.1 Isolates and microsatellite loci

A total of 167 isolates from Africa were obtained for this study (Table 1). These included isolates from various *Eucalyptus* species, including *Eucalyptus camaldulensis*, *Eucalyptus grandis*, *Eucalyptus paniculata* and *Eucalyptus propinqua*. Phylogenetic analyses conducted using ITS and TEF1 $\alpha$  DNA sequences of representative isolates confirmed the identity of the isolates as *T. gauchensis* (Fig. 1).

Microsatellite analyses were conducted on data acquired from 298 isolates, 167 from Africa and 131 from South America. A total of 13 microsatellite loci were selectively amplified in the entire (African and South America) *T. gauchensis* dataset. Six loci were common among all populations (Kgau2, Kgau5–Kgau9), while four (Kgau1, Kgau3, Kgau4 and Kgau10) were unique to South American populations, having failed to amplify in African populations. Three additional loci (Czulu1, Kzulu13 and Kzulu14) were amplified for *T. gauchensis* populations from Africa using markers designed for *T. zuluensis*.

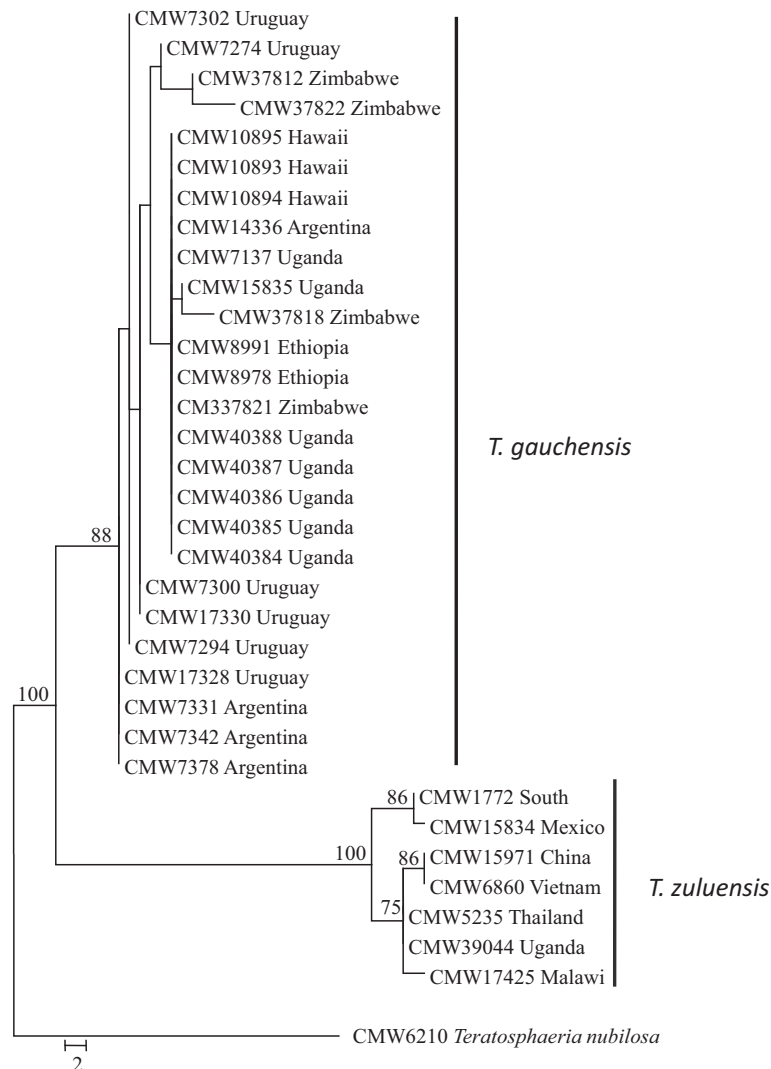


Fig. 1. First of 1000 equally most parsimonious trees obtained from a heuristic search with 35 random taxon additions of combined ITS and TEF1  $\alpha$  sequences aligned using PAUP v4.0b10. Bootstrap support values (after 1000 replicates) above 75% are shown at the nodes. Representative isolates of *T. gauchensis* populations from Argentina, Ethiopia, Uganda, Uruguay and Zimbabwe as well as selected *T. zuluensis* isolates. *Teratosphaeria nubilosa* was used as an outgroup.

### 3.2 Population structure and differentiation

Assignment tests conducted on the six microsatellite loci amplified in all populations showed significant global spatial structure among the *T. gauchensis* isolates. MCMC performed in STRUCTURE showed that the highest Delta  $K$  value coincided with a  $K$  value of two (Fig. 2). There were thus two genetic groups with the highest likelihood and lowest standard deviation. Assignment of all isolates into these groups resulted in the majority of isolates from Africa (99%) being assigned to Group 1 and those from South America (98.6%) to Group 2. Principal coordinates analysis (Fig. 3) and UPGMA analyses

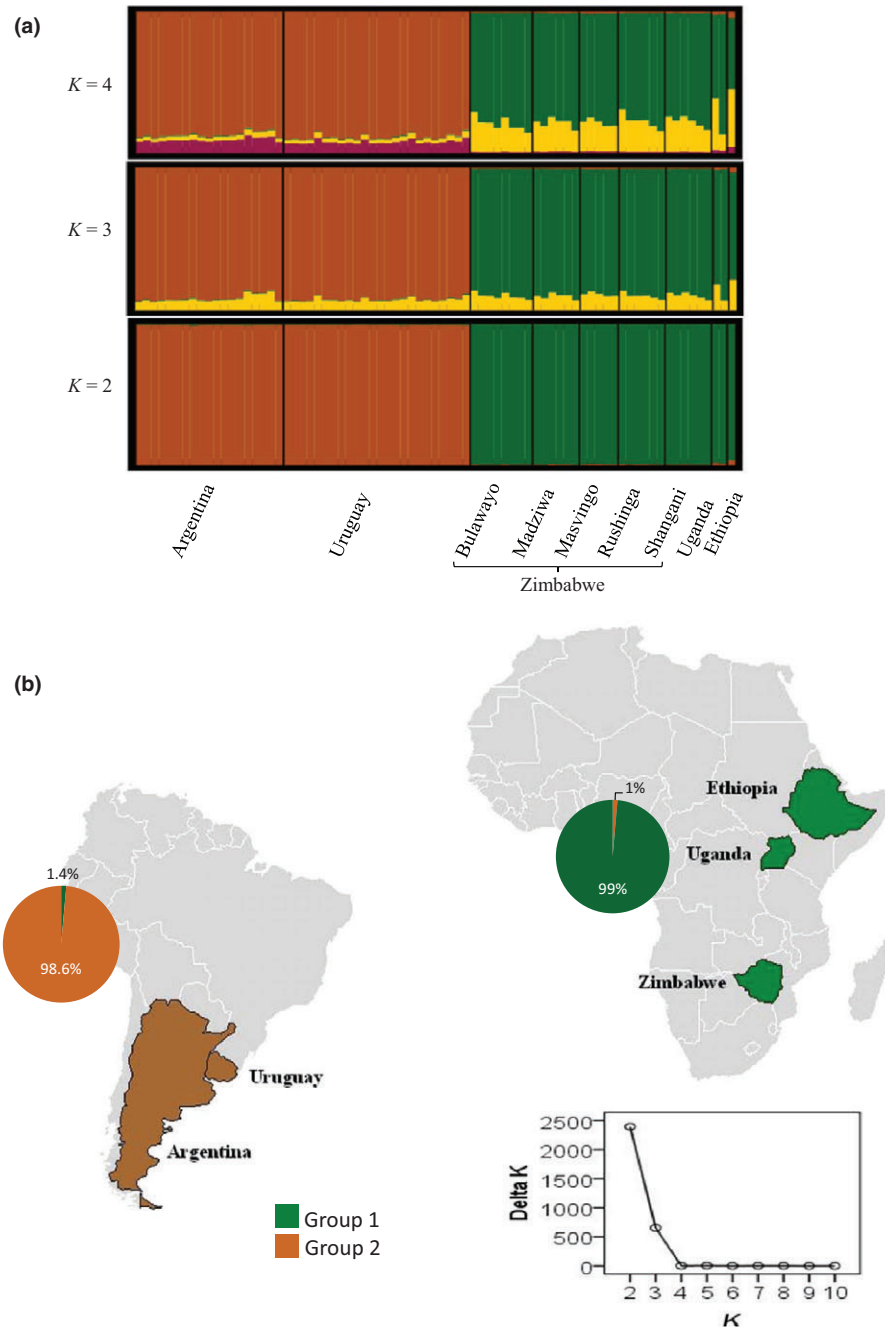


Fig. 2. Genetic clustering of *T. gauchensis* genotypes from Africa and South America. (a) Individual admixture proportion for genotypes at  $K=2$  to  $K=4$ . Each individual is represented by a thin vertical line, which is partitioned into  $K$  colour segments that represent the individual's estimated  $Q$  fractions in  $K$  clusters. Black lines separate different sites. (b) Map showing countries where *T. gauchensis* were obtained from. Pie charts show admixture proportions for the African and the South American populations at  $K=2$ , the best  $K$  inferred using the delta  $K$  method (insert).



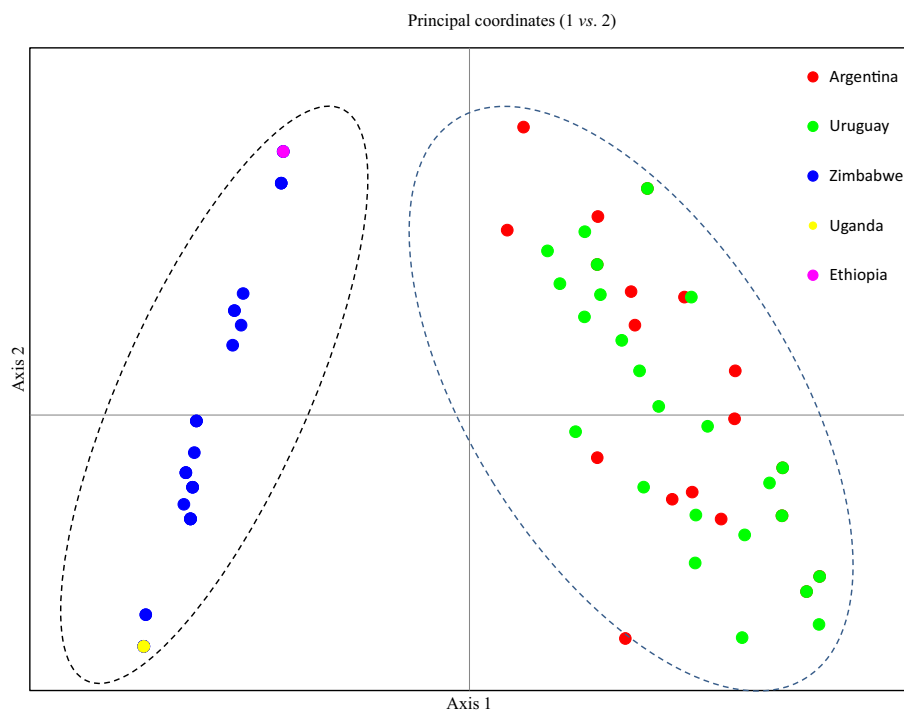


Fig. 3. Principal coordinates analysis (PCoA) of the haplotypes recovered from genotypes from Argentina, Ethiopia, Uganda, Uruguay and Zimbabwe.

(Fig. 4) also revealed two geographically separated clusters for *T. gauchensis* isolates, confirming the results obtained with STRUCTURE. Analysis of molecular variance ( $_{AMOVA}$ ) between the African and the South American groups of isolates showed that most of the variance was attributable to among-group variation (77% total variation (Table 2).

The two genetic groups identified using STRUCTURE, PCoA and UPGMA analyses were significantly differentiated by  $\theta$ , as well as *I* and *D*. Pairwise comparison of the African and South American groups showed a high ( $p < 0.05$ ) value of  $\theta$  (0.48). Theta values were similarly high and significant when Ugandan and Zimbabwean populations were compared with Argentinean and Uruguayan populations (Table 3). When populations within each continent were compared, low  $\theta$  ( $p > 0.05$ ) values were obtained. The low genetic identity (0.29) and high genetic distance (1.24) further support differentiation between the African and South American groups of isolates. Similarly, low genetic identities and large distances were found when Ugandan and Zimbabwean populations were compared with Argentinean and Uruguayan populations (Table 3). In contrast, higher genetic identities and lower distances were obtained when populations within each continent were compared.

### 3.3 Genetic diversity within populations

Nei's gene diversity was higher for the South American population, compared to that of the African population (Table 4). The numbers of alleles and genotypes were highest in the South American populations (Table 4; Fig. 5). Only five of a total of 25 alleles (20%) recovered from six microsatellite loci were common between the African and South American *T. gauchensis* groups (Fig. 5). There were five (20%) private alleles for the African collection and 15 (60%) for the South American population. There were no common genotypes between the two groups.

For the African populations, Nei's genetic diversity was 0, 0.30 and 0.32 for the Ethiopian, Ugandan and Zimbabwean populations, respectively. Similarly, allelic richness ( $A_{rare}$ ) as estimated in HP-RARE 1.1 was 1, 1.99 and 2 for Ethiopian, Ugandan and Zimbabwean populations, respectively (Table 4). Within Africa, the number of sample size corrected private alleles was highest for Zimbabwe. The number of multilocus genotypes was highest for the Zimbabwean population (42 genotypes from 81 isolates) followed by Uganda (3 from 35 isolates) and Ethiopia (1 from 22 isolates). Of the three genotypes recovered from Uganda, two were shared with Zimbabwe and one with Ethiopia. The genotype common to Ethiopia and Uganda accounted for 83% of isolates from Uganda. The two genotypes common in Uganda and Zimbabwe accounted for 17 and 6% of the total isolates from each country, respectively. The Ethiopian genotype was not shared with isolates representing the Zimbabwean population.

Multiple genotypes were recorded on single *Eucalyptus* trees in Zimbabwe compared to Ethiopia and Uganda, where all isolates from a single tree represented clones of the same genotype. For Zimbabwe, some isolates collected from the same tree had different genotypes. The greatest number of genotypes recovered from a single tree was obtained for isolates collected from Gweru with all four isolates representing different genotypes, followed by Bulawayo and Masvingo, each with

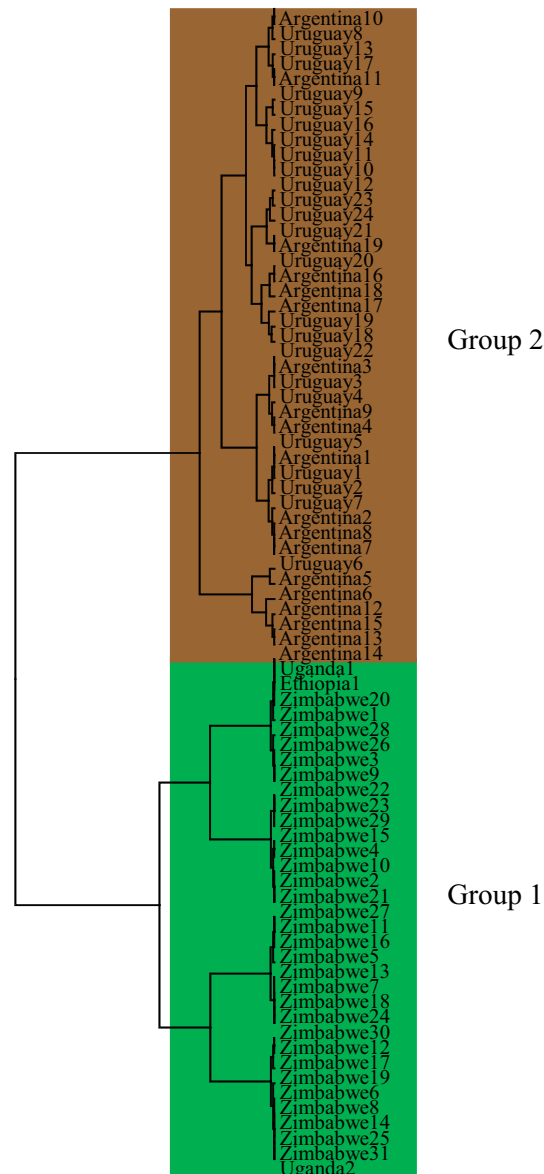


Fig. 4. UPGMA tree for *T. gauchensis* genotypes from Africa and South America constructed in MEGA 6 using the distance matrix calculated in GenAlEx 6.2.

Table 2. Analysis of genetic variation among *T. gauchensis* groups and populations.

Source	df	SS	MS	Est. Var.	%Var
Among groups <sup>1</sup>	1	70695.62	70695.62	1847.38	77
Within groups <sup>1</sup>	75	40746.56	543.29	543.29	23
Total	76	111442.18		2390.66	100
Among American populations <sup>2</sup>	1	779.79	779.79	17.68	4
Within American populations <sup>2</sup>	41	16595.65	404.77	404.77	96
Total	42	17375.44		422.45	100
Among African populations <sup>3</sup>	1	2.51	2.51	0	0
Within African populations <sup>3</sup>	31	22654.40	730.79	730.79	100
Total	32	22656.91		730.79	100

<sup>1</sup>African and South American groups as defined by STRUCTURE.  
<sup>2</sup>The South American group of isolates.  
<sup>3</sup>The African group of isolates.

Table 3. Differentiation through Nei's genetic identity, Nei's genetic distance and theta among pairs of *T. gauchensis* populations from Africa and South America.

	Argentina	Uruguay	Uganda	Zimbabwe
Argentina	***	0.97 0.01 <sup>ns,1</sup>	0.33 0.33 <sup>a</sup>	0.27 0.49 <sup>a</sup>
Uruguay	0.03	***	0.33 0.36 <sup>a</sup>	0.27 0.50 <sup>a</sup>
Uganda	1.11	1.12	***	1.09 0.01 <sup>ns</sup>
Zimbabwe	1.31	1.31	0.01	***

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).  
 $\theta$  values end with superscripts where <sup>a</sup>represents significance and <sup>ns</sup>non-significance ( $p = 0.05$ ).  
<sup>1</sup>Cortinas et al. (2011).

Table 4. Genetic diversity of *T. gauchensis* isolates collected from Africa and South America characterized through microsatellite markers.

Parameter	Africa			South America	
	Ethiopia	Uganda	Zimbabwe	Argentina <sup>1</sup>	Uruguay <sup>1</sup>
Ni <sup>2</sup>	22	35	81	38	93
Ng <sup>3</sup>	1	3	42	28	63
Na <sup>4</sup>	6	12	12	31	35
H <sup>5</sup>	0	0.30	0.32	0.43	0.42
Npl <sup>6</sup>	0	6	6	10	10
A <sub>Rare</sub> <sup>7</sup>	1	1.99	2	–	–

<sup>1</sup>Cortinas et al. (2011).  
<sup>2</sup>Ni Number of isolates (non-clone corrected).  
<sup>3</sup>Ng Number of genotypes.  
<sup>4</sup>Na Number of alleles.  
<sup>5</sup>H Gene Diversity (Nei 1973).  
<sup>6</sup>Npl Number of polymorphic loci.  
<sup>7</sup>Mean number of alleles with rarefaction.

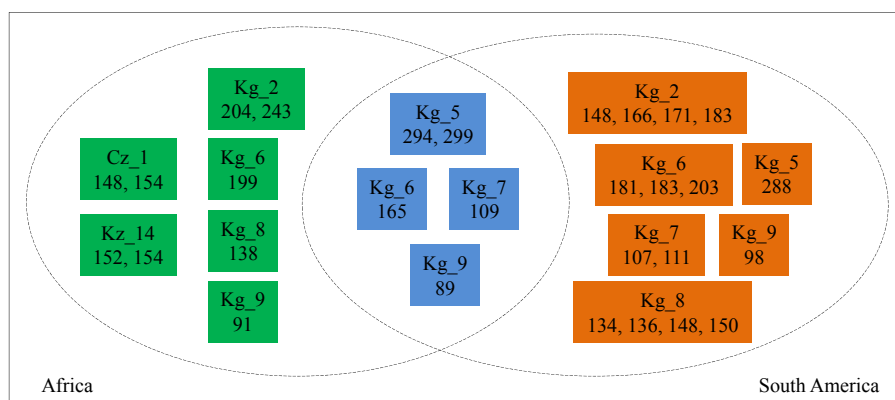


Fig. 5. Allele distribution and differentiation between Africa and South America. Private alleles for each locus for each continent are shown in green, whilst common alleles are shown in brown. Allele designation represents allele sizes as determined by GENEMAPPER version 4.3 (Applied Biosystems) software package. Africa was represented by MLHs from Ethiopia, Uganda and Zimbabwe, whilst South America was represented by MLHs from Argentina and Uruguay.

three genotypes from among the same number of isolates. Single genotypes were obtained from five isolates obtained from single trees in Ethiopia and Uganda.

Genetic analyses of the five populations of *T. gauchensis* in Zimbabwe showed variable genetic diversities. Isolates from Bulawayo had the highest gene diversity (0.32) and number of genotypes (20), followed by Gweru with a gene diversity of 0.31 and 14 genotypes. Lower numbers of genotypes were recorded for Madziwa (9), Masvingo (5) and Rushinga (8) with gene diversities of 0.28, 0.28 and 0.29, respectively. No genotypes were common across all five sites. However, single genotypes were shared by isolates from Bulawayo, Gweru and Masvingo as well as Bulawayo, Gweru and Rushinga. Five geno-



types were shared in isolates from Bulawayo and Gweru, four from Bulawayo and Rushinga, three from Gweru and Masvingo, two from Gweru and Rushinga, one from Bulawayo and Madziwa; Bulawayo and Masvingo, Gweru and Madziwa and Madziwa and Masvingo.

### 3.4 Mode of reproduction

The  $I_A$  values for all *T. gauchensis* populations fell within the distribution ranges of recombining populations (Table 5), suggesting that sexual reproduction occurs in the fungus. However, for some populations, the observed recombination lacked statistical support. Index of association was not computed for isolates from Ethiopia because only one genotype was recovered.

## 4 Discussion

*Teratosphaeria* stem canker is one of the most important diseases of *Eucalyptus* trees grown as non-natives in plantations globally. Despite its importance, little is known regarding the origin and mechanisms of dispersal of the two causal agents of the disease. This is especially true for *T. gauchensis*, for which only populations from South America were previously available for study. In the present study, a sufficient number of isolates from three African countries were obtained to undertake a population genetic investigation, and thus to compare and contrast populations of the pathogen from two distinct regions of the world.

Two genetically differentiated groups were found for isolates of *T. gauchensis*. One of these represented populations from Africa and the other South America. The geographic distance between the two regions should provide sufficient isolation to allow population structure to develop, while limiting genetic admixture between the two groups. Our population structure results showed that the African and the South American populations are distinctively different from each other, suggesting that both are alien and invasive. In this case, both groups would have originated from an unknown source, possibly Australia, and dispersed independently to South America and Africa. The fact that neither *T. gauchensis* nor *T. zuluensis* has been reported from Australia does not preclude their existence there. This has been demonstrated in various studies where invasive alien *Eucalyptus* pathogens were known only in their invasive range but were later found in their Australian native range (Wingfield et al. 1997; Burgess et al. 2007).

The higher genetic diversity recorded for the Zimbabwean compared to the Ethiopian and Ugandan populations provides strong evidence to suggest that the pathogen could have been introduced into Zimbabwe before spreading to Uganda and Ethiopia, most likely through trade in *Eucalyptus* seed (Jimu et al. 2015c). This possibility is supported by the moderate genetic diversities, coupled with haplotype richness characteristics of the Zimbabwean population and the clonal nature of the Ugandan and Ethiopian populations. Furthermore, the occurrence of multiple genotypes on some *Eucalyptus* trees recorded for the Zimbabwean population shows that the fungus has most likely been in that country for a longer period of time. This would have allowed for genetic recombination to occur, most likely through sexual recombination. This is in comparison with the populations in Ethiopia and Uganda that were represented by only single genotypes on individual trees. The clonal nature of the populations in East Africa could be explained by a ‘founder effect’ where a few isolates representing a small fraction of the diversity in Zimbabwe could have been moved northwards to Ethiopia and Uganda. The moderate genetic diversities of the Zimbabwean population could be a result of earlier or multiple introductions and admixture (Smart and Fry 2001; Dutech et al. 2010; Pérez et al. 2012).

*T. zuluensis* is thought to have moved globally through trade in *Eucalyptus* seed (Cortinas et al. 2006c, Jimu et al. 2015c). The genetic characteristics of the African populations of *T. gauchensis* are consistent with the history of *Eucalyptus* seed production and trade. Zimbabwe, through its forest research centres has produced and exported seed to countries in southern and eastern Africa for a very long time. For example, a Regional Seed Centre (RSC) was established in 1984 to provide member countries, including Ethiopia, Kenya, Uganda and Zambia with seed. This would have provided a clear pathway for *T. gauchensis* to spread via *Eucalyptus* seed from Zimbabwe to countries in east Africa during the last two to three decades.

Table 5. Indices of association used to measure recombination in *T. gauchensis* populations.

Continent	Population	$I_A^1$	$RI_A^2$	$OI_A^3$	p-Value
Africa	Uganda	2.50	−1.00 to 5.00	Yes	p > 0.05
	Zimbabwe	0.03	−0.05 to 0.08	Yes	p > 0.05
	All	0.04	−0.05 to 0.09	Yes	p < 0.05
South America	Argentina <sup>4</sup>	0.22	−0.01 to 0.33	Yes	p > 0.05
	Uruguay <sup>4</sup>	0.08	−0.01 to 0.13	Yes	p < 0.05
	All	0.13	−0.01 to 0.15	Yes	p > 0.05

<sup>1</sup> $I_A$  Observed  $I_A$   
<sup>2</sup> $RI_A$  Range of obtained  $I_A$  values after 1000 randomizations.  
<sup>3</sup> $OI_A$  Observed  $I_A$  within randomized data range (i.e. evidence for recombination).  
<sup>4</sup>Cortinas et al. (2011).

Genetic diversity of *T. gauchensis* isolates collected from five sites in Zimbabwe suggests that Chesa Forest Research Station (CFRS), from which isolates for the Bulawayo population were collected, could have been the point of entry of *T. gauchensis* into Zimbabwe. This is possible because CFRS imports *Eucalyptus* seed, mainly from Australia, for species trials and orchards. Seeds from these orchards are then collected and distributed to farmers in hotter and drier regions of Zimbabwe. CFRS produces *E. camaldulensis*, *E. camaldulensis/grandis* hybrid and *E. propinqua* seed, all species from which *T. gauchensis* was isolated in this study. Furthermore, these findings are consistent with the history of rural afforestation in Zimbabwe. *Eucalyptus* woodlots in communal areas, such as Rushinga and Madziwa, are a relatively recent development. They were introduced in the 1980s to 1990s under the rural afforestation programme, hence the lower gene diversities and haplotype richness found in isolates of *T. gauchensis*.

The observed indices of association for the African populations support the view that genetic recombination has occurred in *T. gauchensis*. Evidence for recombination observed in this study suggests that *T. gauchensis* could be reproducing by both asexual and sexual means. A similar situation was found for the Argentinean and Uruguayan populations (Cortinas et al. 2011). Chen et al. (2011) also provided evidence of sexual reproduction in a Chinese population of the closely related *T. zuluensis*. Although the sexual morphs of the two pathogens have never been observed, this does not preclude their existence and the fact that cryptic sex is occurring such as has recently been shown for the well-known pine pathogen *Diplodia sapinea* (Bihon et al. 2012). The presence of sexual reproduction would have implications for the management of the disease where durability of resistance in host trees would more easily be overcome.

### Acknowledgements

This work is based on research supported by the National Research Foundation of South Africa (Grant specific unique reference number UID83924). The grant holders acknowledge that opinions, findings and conclusions or recommendations expressed in any publication generated by the NRF supported research are that of the authors and that the NRF accepts no liability what so ever in this regard. We thank members of the Tree Protection Co-operative Program (TPCP), the THRIP initiative of the Department of Trade and Industry and the DST/NRF Centre of Excellence in Tree Health Biotechnology (CTHB) for financial support. We also thank Dr. Alemu Gezahgne of the Ethiopian Institute of Agricultural Research; Paul Jacovelli and Andrew Akasibayo of Sawlog Production Grant Scheme (SPGS), forestry companies in Fort Portal and Jinja in Uganda and forest companies in Zimbabwe for assistance with sample collection.

### References

- Agapow, P. M.; Burt, A., 2001: Indices of multilocus linkage disequilibrium. *Mol. Ecol. Notes* **1**, 101–102.
- Arnaud-Haond, S.; Belkhir, K., 2007: GENCLONE: a computer program to analyse genotypic data, test for clonality and describe spatial clonal organization. *Mol. Ecol. Notes* **7**, 15–17.
- Bihon, W.; Slippers, B.; Burgess, T.; Wingfield, M. J.; Wingfield, B. D., 2012: Diverse sources of infection and cryptic recombination revealed in South African *Diplodia pinea* populations. *Fungal Biol.* **116**, 112–120.
- Burgess, T. I.; Andjic, V.; Wingfield, M. J.; Hardy, G. E. St J., 2007: The eucalypt leaf blight pathogen *Kirramyces destructans* discovered in Australia. *Australas. Plant Dis. Notes* **2**, 141–144.
- Chen, S. F.; Barnes, I.; Chungu, D.; Roux, J.; Wingfield, M. J.; Xie, Y. J.; Zhou, X. D., 2011: High population diversity and increasing importance of the *Eucalyptus* stem canker pathogen, *Teratosphaeria zuluensis*, in South China. *Australas. Plant Pathol.* **40**, 407–415.
- Chungu, D.; Muimba-Kankolongo, A.; Wingfield, M. J.; Roux, J., 2010: Plantation forestry diseases in Zambia: contributing factors and management options. *Ann. For. Sci.* **67**, 1–9.
- Cortinas, M. N.; Koch, N.; Thain, J.; Wingfield, B. D.; Wingfield, M. J., 2004: First record of the *Eucalyptus* stem canker pathogen, *Coniothyrium zuluense* from Hawaii. *Australas. Plant Pathol.* **33**, 309–312.
- Cortinas, M. N.; Barnes, I.; Wingfield, B. D.; Wingfield, M. J., 2006a: Polymorphic microsatellite markers for the *Eucalyptus* fungal pathogen *Colletogloeopsis zuluensis*. *Mol. Ecol. Notes* **6**, 780–783.
- Cortinas, M. N.; Burgess, T.; Dell, B.; Xu, D.; Crous, P. W.; Wingfield, B. D.; Wingfield, M. J., 2006b: First record of *Colletogloeopsis zuluense* comb.nov., causing a stem canker of *Eucalyptus* in China. *Mycol. Res.* **110**, 229–236.
- Cortinas, M. N.; Crous, P. W.; Wingfield, B. D.; Wingfield, M. J., 2006c: Multi-gene phylogenies and phenotypic characters distinguish two species within the *Colletogloeopsis zuluensis* complex associated with *Eucalyptus* stem cankers. *Stud. Mycol.* **55**, 133–146.
- Cortinas, M. N.; Wingfield, B. D.; Wingfield, M. J., 2008: Microsatellite markers for the *Eucalyptus* stem canker fungal pathogen *Kirramyces gauchensis*. *Mol. Ecol. Resour.* **8**, 590–592.
- Cortinas, M. N.; Barnes, I.; Wingfield, M. J.; Wingfield, B. D., 2010: Genetic diversity in the *Eucalyptus* stem pathogen *Teratosphaeria zuluensis*. *Australas. Plant Pathol.* **39**, 383–393.
- Cortinas, M. N.; Barnes, I.; Wingfield, B. D.; Wingfield, M. J., 2011: Unexpected genetic diversity revealed in the *Eucalyptus* canker pathogen *Teratosphaeria gauchensis*. *Australas. Plant Pathol.* **40**, 497–503.
- Dutech, C.; Fabreguettes, O.; Capdevielle, X.; Robin, C., 2010: Multiple introductions of divergent genetic lineages in an invasive fungal pathogen, *Cryphonectria parasitica*, in France. *Heredity* **105**, 220–228.
- Earl, D.; VonHoldt, B., 2012: STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv. Genet. Resour.* **4**, 359–361.
- Evanno, G.; Regnaut, S.; Goudet, J., 2005: Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol. Ecol.* **14**, 2611–2620.
- Falush, D.; Stephens, M.; Pritchard, J. K., 2003: Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics* **164**, 1567–1587.
- Gezahgne, A.; Roux, J.; Wingfield, M. J., 2003: Diseases of exotic *Eucalyptus* and *Pinus* species in Ethiopian plantations. *S. Afr. J. Sci.* **99**, 29–33.
- Gezahgne, A.; Roux, J.; Thu, P. Q.; Wingfield, M. J., 2004: *Coniothyrium* stem canker of *Eucalyptus*, new to Argentina and Vietnam. *S. Afr. J. Sci.* **99**, 587–588.
- Gezahgne, A.; Cortinas, M. N.; Wingfield, M. J.; Roux, J., 2005: Characterisation of the *Coniothyrium* stem canker pathogen on *Eucalyptus camaldulensis* in Ethiopia. *Australas. Plant Pathol.* **34**, 85–90.

- Grünwald, N. J.; Goodwin, S. B.; Milgroom, M. G.; Fry, W. E., 2003: Analysis of genotypic diversity data for populations of microorganisms. *Phytopathology* **93**, 738–746.
- Jakobsson, M.; Rosenberg, N. A., 2007: *CLUMPP*: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* **23**, 1801–1806.
- Jimu, L.; Wingfield, M. J.; Mwenje, E.; Roux, J., 2014: First report of *Teratosphaeria zuluensis* causing stem canker of *Eucalyptus grandis* in Uganda. *Forest Pathol.* **44**, 242–245.
- Jimu, L.; Wingfield, M.J.; Mwenje, E.; Roux, J., 2015a: Diseases on *Eucalyptus* species in Zimbabwean plantations and woodlots. *South. For.* **77**, 221–230.
- Jimu, L.; Chen, S.; Wingfield, M.J.; Mwenje, E.; Roux, J., 2015b: Three genetic groups of the *Eucalyptus* stem canker pathogen *Teratosphaeria zuluensis* introduced into Africa from an unknown source. *Antonie Van Leeuwenhoek*. doi: 10.1007/s10482-015-0606-0.
- Jimu, L.; Kemler, M.; Wingfield, M.J.; Mwenje, E.; Roux, J., 2015c: The *Eucalyptus* stem canker pathogen *Teratosphaeria zuluensis* detected in seed samples. *Forestry*. doi:10.1093/forestry/cpv037.
- Kalinowski, S. T., 2005: HP-RARE: a computer program for performing rarefaction on measures of allelic diversity. *Mol. Ecol. Notes* **5**, 187–189.
- Maynard-Smith, J.; Smith, N. H.; O'Rourke, M.; Spratt, B. G., 1993: How clonal are bacteria? *Proc. Natl Acad. Sci. USA* **90**, 4384–4388.
- Möller, E. M.; Bahnweg, G.; Sandermann, H.; Geiger, H. H., 1992: A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. *Nucleic Acids Res.* **20**, 6115–6116.
- Nei, M., 1973: Analysis of gene diversity in subdivided populations. *Proc. Natl Acad. Sci. USA* **70**, 3321–3323.
- Nei, M., 1978: Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89**, 583–590.
- Old, K.M.; Wingfield, M.J.; Yuan, Z.Q., 2003: *A Manual of Diseases of Eucalypts in South-East Asia*. Jakarta, Indonesia: CIFOR.
- Peakall, R.; Smouse, P. E., 2006: GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Notes* **6**, 288–295.
- Pérez, G.; Slippers, B.; Wingfield, M. J.; Wingfield, B. D.; Carnege, A. J.; Burgess, T. I., 2012: Cryptic species, native populations and biological invasions by a eucalypt forest pathogen. *Mol. Ecol.* **21**, 4452–4471.
- Pritchard, J. K.; Stephens, M.; Donnelly, P., 2000: Inference of population structure using multilocus genotype data. *Genetics* **155**, 945–959.
- Rosenberg, N. A., 2004: Distruct: a program for the graphical display of population structure. *Mol. Ecol. Notes* **4**, 137–138.
- Roux, J.; Wingfield, M. J.; Cibrian, D., 2002: First report of *Coniothyrium* canker of *Eucalyptus* in Mexico. *Plant. Pathol.* **51**, 382.
- Roux, J.; Meke, G.; Kanyi, B.; Mwangi, L.; Mbaga, A.; Hunter, G. C.; Nakabonge, G.; Wingfield, M. J., 2005: Diseases of plantation forestry tree species in Eastern and Southern Africa. *S. Afr. J. Sci.* **101**, 409–413.
- Silva, M.R.C.; Diogo, E.; Bragança, H.; Machado, H.; Phillips, A.J.L., 2014: *Teratosphaeria gauchensis* associated with trunk, stem and foliar lesions of *Eucalyptus globulus* in Portugal. *Forest Pathol.* **45**, 224–234.
- Smart, C. D.; Fry, W. E., 2001: Invasions by the late blight pathogen: renewed sex and enhanced fitness. *Biol. Invasions* **3**, 235–243.
- Stoddart, J. A.; Taylor, J. F., 1988: Genotype diversity: estimation and prediction in samples. *Genetics* **118**, 705–711.
- Swofford, D.L., 2002: PAUP\*. Phylogenetic Analysis Using Parsimony (\*and other Methods). Version 4. Sunderland, MA: Sinauer Associates.
- Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S., 2013: MEGA 6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.* **30**, 2725–2729.
- Taylor, J. W.; Jacobson, D. J.; Fisher, M. C., 1999: The evolution of asexual fungi: reproduction, speciation and classification. *Annu. Rev. Phytopathol.* **37**, 197–246.
- Weir, B. S., 1996: *Genetic Data Analysis II*. Sunderland, MA: Sinauer Associates.
- Wingfield, M. J.; Crous, P. W.; Coutinho, T. A., 1997: A serious new canker disease of *Eucalyptus* in South Africa caused by a new species of *Coniothyrium*. *Mycopathologia* **136**, 139–145.
- Yeh, F.C.; Yang, R.C.; Boyle, T., 1999: 'POPGENE. Version 1.31. Microsoft Windows Based Freeware for Population Genetic Analysis.' Alberta: University of Alberta.
- van Zyl, L. M.; Coutinho, T. A.; Wingfield, M. J.; Pongpanich, K.; Wingfield, B. D., 2002: Morphological and molecular relatedness of geographically diverse isolates of *Coniothyrium zuluense* from South Africa and Thailand. *Mycol. Res.* **106**, 51–59.