Genetic recombination in *Teratosphaeria destructans* causing a new disease outbreak in Malaysia

Minette Havenga[1,2,*], Brenda D. Wingfield[1], Michael J. Wingfield[1], Seonju Marincowitz[1], Léanne L. Dreyer[3], Francois Roets[2], Yani Japarudin[4,5], Janneke Aylward[1,2]

[1]Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa

[2]Department of Conservation Ecology and Entomology, Stellenbosch University, Stellenbosch, South Africa

[3]Department of Botany and Zoology, Stellenbosch University, Stellenbosch, South Africa

[4]Sabah Softwoods Berhad, Tawau, Malaysia

[5]Forest Industries Research Centre, University of the Sunshine Coast, Sunshine Coast, QLD, Australia

*Correspondence to: Minette Havenga, Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Email: havenga.minette@fabi.up.ac.za

Funding information

The SARChI chair in Fungal Genomics, Grant/Award Number: 98353; South African Agency for Science and Technology Advancement, Grant/Award Number: - NRF Centre of Excellence in Tree Health Biotechn; Tree Protection Co- operative program (TPCP); University of Pretoria (UP) Doctoral Research Bursary; National Research Foundation

ABSTRACT

The *Eucalyptus* foliar pathogen *Teratosphaeria destructans* causes severe and widespread damage in South-East Asian and South African plantations. In 2016, leaf blight symptoms resembling those caused by *T*. *destructans* were observed in a plantation of a *Eucalyptus grandis* × *E*. *urophylla* hybrid in Sabah, Malaysia. The aims of this study were to confirm the identity of the causal agent as *T*. *destructans* and to investigate the genetic structure of isolates associated with this newly detected disease outbreak. Using sequence data of three gene regions, the identity of the pathogen was confirmed as *T*. *destructans*. The mating type and microsatellite genotypes of 41 isolates from this Malaysian population were identified and compared with those from previously characterized populations in South-East Asia and South Africa. The Malaysian population had the highest genotypic diversity of any *T*. *destructans* population thus far investigated. Both the mating types were found in the collection of isolates, and these were in approximately equal proportions. Although structures linked to a sexual state of the fungus have not been found, sexual reproduction is theoretically possible and could explain the high genetic diversity in the pathogen that must have been accidentally introduced into

Malaysia. This is the first record of *T*. *destructans* in Sabah and, to the best of our knowledge, in also other parts of Borneo.

Keywords: heterothallic, mating type, microsatellite markers, population genetics, sexual reproduction

1 INTRODUCTION

Until recently, the forestry industry in Malaysia has relied largely on natural forests for hardwood resources (Hii et al., 2017). In 2005, the Ministry of Plantation Industries and Commodity (KPPK) and the Malaysian Timber Industrial Board (MTIB) implemented an intensive forestry programme to relieve the pressure of logging on natural hardwood forests (Yahya, 2020; Zaiton et al., 2018). For this purpose, *Acacia* species, *Hevea brasiliensis*, *Paraserianthes falcataria* and *Neolamarckia cadamba* were approved for planting, with *Acacia mangium* being most widely planted (Yahya, 2020). However, a new disease, first discovered in Indonesia (Tarigan et al., 2011) and caused by the aggressive wilt pathogen *Ceratocystis manginecans* M. van Wyk, Al Adawi & M.J. Wingf., has spread widely in South-East Asian countries (Fourie et al., 2016), including Malaysia (Lee, 2018; Nambiar et al., 2018), creating a need for alternative plantation species.

Plantations of *Eucalyptus* species have rapidly replaced the *A*. *mangium* plantations devastated by *C*. *manginecans*. Specifically in Malaysia, various *Eucalyptus* species, including *E*. *grandis* × *E*. *urophylla* hybrids and *E*. *pellita*, have been planted in Sabah, Sarawak and Peninsular Malaysia (Yahya, 2020; Zaiton et al., 2018). These trees were first introduced in 2008 (Yahya, 2020), and the *Eucalyptus* industry in Malaysia is therefore relatively young. As such, little is known regarding the presence of *Eucalyptus* pathogens and the damage that they may cause in the region.

In South-East Asia, the leaf and shoot blight pathogen *Teratosphaeria destructans* (M.J. Wingf. & Crous) M.J. Wingf. & Crous is one of the most devastating non-native *Eucalyptus* pathogens (Andjic et al., 2019). Particularly susceptible hosts include *E*. *grandis*, *E*. *urophylla*, *E*. *pellita*, *E*. *camaldulensis* and their hybrids (Andjic et al., 2019). The fungus causes chlorotic lesions on the leaves, and these progress to large subcircular brown spots with diffused borders and red-brown margins (Burgess et al., 2006; Wingfield et al., 1996). Only the asexual state of *T*. *destructans* is known, and these are easily recognized as black pycnidia below the stomata on the abaxial leaf surfaces, exuding cirri of conidia (Wingfield et al., 1996). Young trees of susceptible genotypes are defoliated, resulting in shoot dieback, lower stem quality, stunted growth and, in severe cases, tree death (Burgess et al., 2006; Dell et al., 2008).

Teratosphaeria destructans was first reported and described from an *E*. *grandis* plantation in North Sumatra, Indonesia, in 1995 (Wingfield et al., 1996). Since then, it has been reported to cause severe disease in Thailand, East Timor, Vietnam (Old et al., 2003), China (Burgess et al., 2006), Lao (Barber et al., 2012) and, most recently, South Africa (Greyling et al., 2016). Similar to

other *Teratosphaeria* species, the pathogen most likely spreads with contaminated plant germplasm, including seeds (Andjic et al., 2019; Jimu et al., 2016). This is relevant considering that the first *E*. *grandis* × *E*. *urophylla* plantations in Sabah and Peninsular Malaysia were established from seeds acquired from southern China (Yahya, 2020), where *T*. *destructans* causes widespread damage in all *Eucalyptus*-growing regions (Dell et al., 2008).

The *T*. *destructans* populations from Indonesia, China, Thailand, Vietnam and South Africa were recently shown to have been introduced independently from an unknown source (Havenga et al., 2020b). This conclusion was based on the low genotypic diversity, high levels of clonality and strong genetic structure in these populations. The fungus has an outcrossing mating system (Havenga, Wingfield, Wingfield, Roets, et al., 2020) and requires two individuals of opposite mating type for sexual reproduction to occur (Billiard et al., 2011). Even though both mating types are present in most South-East Asian populations, one mating type is dominant in all of these populations (Havenga, Wingfield, Wingfield, Roets, et al., 2020). This suggests a strong influence of asexual reproduction in areas where this non-native pathogen has become established (Havenga et al., 2020b).

In 2016, leaf blight with symptoms resembling those caused by *T*. *destructans* was observed in a newly established *E*. *grandis* × *E*. *urophylla* plantation in Sabah, Malaysia. The aims of this study were, firstly, to identify the pathogen, especially given that several closely related species such as *T*. *eucalypti* (Cooke & Massee) Crous and *T*. *pseudoeucalypti* Andjic & T. Burgess cause similar symptoms and have a morphology closely resembling *T*. *destructans*; and, secondly, to compare the genetic diversity and mating type ratio of this new population with those of previously characterized populations (Havenga et al., 2020b).

2 MATERIALS AND METHODS

2.1 Sampling and identification

Leaves with symptoms resembling those caused by *T*. *destructans* were collected from 41 *E*. *grandis* × *E*. *urophylla* hybrid trees in a single plantation located in the Sipitang District, Sabah, Malaysia, during March 2016. Cirri of conidia were lifted from the abaxial sides of the symptomatic leaves using a sterile dissection needle and spread across the surface of Malt Extract Agar (MEA, Merck, South Africa; 15 g/l agar, 30 g/l malt extract, 5 g/l mycological peptone) in Petri dishes. Colonies emerging from single conidia were transferred to MEA amended with 3 g/l yeast extract (MEA + Y; Oxoid, France) and grown at 25°C in the dark. DNA extractions and sequencing of the ribosomal RNA internal transcribed spacer gene region (ITS), partial β-tubulin gene (TUB) and elongation factor 1-alpha gene (EF-1α) regions, as well as maximum-likelihood phylogenetic analyses, followed Havenga, Wingfield, Wingfield, Roets, et al., (2020). Isolates considered in this study have been maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria.

2.2 Population genetic analyses

The *MAT* idiomorph in each of the 41 isolates was identified using primers T_Ma1-F; T_Ma1-R (Aylward et al., 2020) and TdMAT2_2; TdMAT2_1 (Havenga, Wingfield,

Wingfield, Roets, et al., 2020) following the methods described by Havenga, Wingfield, Wingfield, Roets, et al., (2020). The null hypothesis of a 1:1 ratio for occurrence of the *MAT1-1* vs. the *MAT1-2* idiomorph was calculated using Pearson's chi-square test in R 3.6.0 (R Core Team, 2013).

The Malaysian isolates were genotyped at ten *T. destructans* microsatellite loci (Td3, Td5, Td7, Td9, Td11, Td14, Td20, Td24, Td25 and Td27) according to Havenga et al., (2020b), using the South African isolate CMW44962 for which the genome has been sequenced, as a positive control. These isolates were evaluated as a single population and compared with previously genotyped populations (Havenga et al., 2020b). Two data sets were prepared in GenAlEx 6.5.1 (Peakall & Smouse, 2006). The full data set included all individuals from all populations (Table 1), whereas the clone-corrected data set consisted of one individual per multilocus genotype (MLG) that occurred in each population.

TABLE 1. *Teratosphaeria destructans* isolates used in this study

Population	Multilocus genotype (MLG)[a]	Number of individuals	Mating type		Total number of individuals per population	Number of MLGs per population
			MAT1-1	*MAT1-2*		
Malaysia	MLG19	1	0	1	41	20
	MLG20	2	1	1		
	MLG21	1	0	1		
	MLG22	11	4	7		
	MLG23	4	3	1		
	MLG24	2	1	1		
	MLG25	1	0	1		
	MLG26	1	0	1		
	MLG27	1	0	1		
	MLG28	1	1	0		
	MLG29	6	3	3		
	MLG30	1	1	0		
	MLG31	1	0	1		

Population	Multilocus genotype (MLG)[a]	Number of individuals	Mating type		Total number of individuals per population	Number of MLGs per population
			MAT1-1	*MAT1-2*		
	MLG32	1	0	1		
	MLG33	2	1	1		
	MLG34	1	0	1		
	MLG35	1	0	1		
	MLG36	1	0	1		
	MLG37	1	1	0		
	MLG38	1	1	0		
China	MLG14	1	0	1	43	3
	MLG15	2	0	2		
	MLG18	40	1	39		
North Sumatra, Indonesia	MLG5	3	0	5	33	9
	MLG6	1	0	1		
	MLG7	1	1	0		
	MLG9[b]	2	2	0		
	MLG10	19	19	0		
	MLG11	1	1	0		
	MLG12	1	1	0		
	MLG13	1	1	0		
	MLG17	4	3	1		

Population	Multilocus genotype (MLG)[a]	Number of individuals	Mating type		Total number of individuals per population	Number of MLGs per population
			MAT1-1	*MAT1-2*		
South Sumatra, Indonesia	MLG1	9	0	9	25	4
	MLG2	2	0	2		
	MLG3	2	2	0		
	MLG16	12	0	12		
South Africa	MLG9[b]	62	0	62	62	1
Thailand	MLG4	1	1	0	29	3
	MLG8	4	4	0		
	MLG9[b]	24	23	1		
Vietnam	MLG9[b]	3	3	0	3	1
Total		236				

[a] MLG1-MLG18 identified in Havenga et al., (2020a) and MLG19-MLG38 identified in this study.

[b] Genotypes identified in more than one population.

The number of unique MLGs, as well as the expected frequency of each MLG, was determined and compared with that of the previously characterized populations (Havenga et al., 2020b). The hypothesis that identical genotypes in a population arose independently through recombination was tested using the MLGsim 2.0 (Stenberg et al., 2003). The significance of P_{sex} statistics was calculated based on a simulation of 1000 random permutations at $p < 0.05$.

Genotypic diversity was calculated utilizing the Shannon–Wiener index (H; Shannon, 2001), Stoddart and Taylor's G index (Stoddard & Taylor, 1988), Simpson's corrected lambda estimation (lambda x N/(N-1); Simpson, 1949) and the percentage genotypic diversity ($\hat{G} = G/N*100$; McDonald et al., 1994). The evenness of each MLG per population was calculated with Pielou's E.5 index (Pielou, 1975). Private alleles (Np) in each population were detected in GenAlEx. To confirm the number of repeats of private alleles, DNA from one representative isolate and a positive control (CMW44962) were amplified and sequenced as described by Havenga et al., (2020b). The null hypothesis of random recombination was tested using rbarD (Agapow & Burt, 2001) with 999 permutations.

Analysis of molecular variance (AMOVA) was calculated to test for significant population structure. Three methods, each applying a different mathematical model, were used as predictors of the genetic relationship among the isolates in the full data set. A minimum spanning network (MSN) was constructed based on Bruvo's genetic distance (Bruvo et al., 2004). The optimal number of clusters was calculated using the Bayesian information criteria (BIC) with the adegent (Jombart, 2008) package in R and by calculating L(*K*) and Δ*K* with CLUMPAK (Clustering Markov Packager Across K; Kopelman et al., 2015) using the best *K* function. Individuals were assigned to clusters (*K*) using the multivariate discriminant analysis of principal component (DAPC) method (Jombart, 2008), as well as a Bayesian, model-based method implemented in STRUCTURE 2.3.4 (Falush et al., 2003).

2.3 In vitro pairings of isolates on Eucalyptus leaves

The sexual state of *T*. *destructans* is unknown even though both *MAT* idiomorphs co-occur in several populations (Havenga, Wingfield, Wingfield, Roets, et al., 2020). Because previous attempts failed to induce the sexual state *in vitro* (Havenga, Wingfield, Wingfield, Roets, et al., 2020), we modified previously described experiments. Malaysian isolates of *T*. *destructans* with opposite mating types (*MAT1-1*: CMW48638 and CMW48640; *MAT1-2*: CMW48624 and CMW48646) were paired. As previous experiments have indicated rapid growth of *T*. *destructans* on nitrogen-deficient media (Havenga, Wingfield, Wingfield, Roets, et al., 2020), isolates were grown on nitrogen-deficient medium for two weeks at 25°C, after which ~15 mg of mycelia was scraped from the surface and mixed with 1.5 ml ddH_2O in a 2.0-ml Eppendorf tube. The tube was briefly agitated with a vortex mixer, poured into 150 ml nitrogen-deficient liquid medium (nitrogen-deficient medium excluding bacteriological agar) and incubated for one week at 25°C in the dark.

Fungal growth (50 ml) of the two test isolates in each pairing were mixed in Eppendorf bottles. Pairings included four *MAT1-1* × *MAT1-2* and two control pairings (*MAT1-1* × *MAT1-1* and *MAT1-2* × *MAT1-2*). The fungal mixture was used to inoculate young, asymptomatic *E*. *grandis* leaves collected from a nursery tree. Leaves were surface-disinfested by soaking in 70% ethanol for 30 s and left to air-dry. They were either left unwounded or were wounded artificially on the adaxial surface of the leaf with a sterile scalpel blade and inoculated by fully soaking the leaves in 30 ml of fungal mixture for 30 s. The leaves were incubated in separate Petri dishes (one leaf per dish) with either the adaxial or abaxial leaf side on damp tissue paper. The dishes were incubated for 24 days at 25°C in the dark. Pairings were repeated three times for each combination of wounded/unwounded and adaxial/abaxial incubation. To determine whether sexual structures (ascomata and ascospores) had formed, microscopic slide mounts were made from (i) the fungal growth on the leaves, (ii) cross-sections through diseased leaves and (iii) fungal fruiting structures.

3 RESULTS

3.1 Sampling and identification

A total of 41 isolates, one isolate per tree, were obtained from the *E*. *grandis* × *E*. *urophylla* plantation in Sabah, Malaysia (Table S1). In the Maximum-likelihood (ML) phylogeny, the representative isolates from Malaysia formed a well-supported (93%) monophyletic clade that included the ex-type isolate

of *T*. *destructans* (Figure S1). The polymorphic microsatellite markers amplified all *T*. *destructans* loci in the 41 isolates, further confirming their identity as *T*. *destructans* (Havenga et al., 2020a).

3.2 Population genetic analyses

3.2.1 Genetic diversity

A total of 29 alleles were amplified across 10 loci in the Malaysian and previously investigated *T*. *destructans* isolates (Table S2). Four new alleles were observed in this study, specifically in loci A, C, J and L. Loci J and K had the greatest number of alleles (n = 4), and locus J was also the most diverse (H_{exp} = 0.67). Consistent with the results of Havenga et al., (2020b), locus D had the most evenly distributed alleles (E.5 = 1.00), both of which were present in the Malaysian population.

Twenty Malaysian genotypes were newly identified in this study and were not shared with any of the previously characterized populations (Table 1). Three genotypes dominated the Malaysian population (occurring in 11, 6 and 4 individuals, respectively), whereas the remaining genotypes occurred only once (14 MLGs) or twice (3 MLGs) in the data set (Table 1). Both *MAT* idiomorphs were present, and the observed distribution of 17 *MAT1-1* isolates and 24 *MAT1-2* isolates did not deviate significantly (p > 0.05) from the expected 1:1 ratio.

Six Malaysian genotypes (MLG20, MLG22, MLG23, MLG24, MLG29 and MLG33) occurred more than once and were associated with both *MAT* idiomorphs (Table 1). Isolates that had genotypes MLG20, MLG24 and MLG33 each included one *MAT1-1* and one *MAT1-2* isolate. Four *MAT1-1* and seven *MAT1-2* isolates had an MLG22 genotype. While isolates that had MLG23 and MLG29 genotypes, each included three *MAT1-1* isolates and one *MAT1-2* isolate. The P_{sex} values of these six genotypes supported the hypothesis (p > 0.05) that some of these isolates were the product of sexual reproduction (Table S2).

Of all *T*. *destructans* populations investigated to date, the one from Malaysia had the highest genotypic richness (eMLG = 14.08) and genotypic diversity according to the H (2.57), G (8.45) and corrected lambda (0.90) indices (Table 2). The observed percentage genotypic diversity in Malaysia was 20.61%, nearly double what was previously described in South Sumatra ($\hat{G}$ = 10.72%; Table 2). The evenness of genotypes in the Malaysian population (E.5 = 0.62) was higher than that of those in most other populations (Table 2), but lower compared with that of those in the South Sumatran population (E.5 = 0.81), due to the dominance of three genotypes (Table 1).

TABLE 2. Genotypic diversity, evenness and private alleles calculated for seven[a] *Teratosphaeria destructans* populations

Population	N[b]	MLG[c]	eMLG ± SE[d]	H[e]	G[f]	Corrected Ĝ (%)[h] lambda[g]		E.5[i]	Np[j]	Before clone correction[k]		After clone correction[k]	
										rbarD[l]	*P*	rbarD[l]	*p*
China	43	3	2.41 ± 0.61	0.30	1.15	0.14	2.67	0.44	0	0.661	0.001	0.280	0.076
Indonesia, Northern Sumatra	33	9	7.72 ± 0.91	1.49	2.76	0.66	8.36	0.51	1	0.239	0.001	−0.031	0.796
Indonesia, Southern Sumatra	25	4	4.00 ± 0.00	1.12	2.68	0.65	10.72	0.81	4	0.449	0.001	−0.014	0.646
Malaysia	41	20	14.08 ± 1.48	2.57	8.45	0.90	20.61	0.62	4	0.034	0.022	−0.014	0.734
South Africa	62	1	1.00 ± 0.00	0.00	1.00	0.00	1.61	N/A	0	-	-	-	-
Thailand	29	3	2.86 ± 0.35	0.55	1.42	0.31	4.90	0.58	0	−0.065	1.000	−0.5	1.000
Vietnam[a]	3	1	-	-	-	-	-	-	0	-	-	-	-
Total	236	41	10.37 ± 1.85	2.40	5.33	0.82	2.29	0.44	9	0.205	0.002	0.082	0.001

[a] Populations with less than 10 individuals are excluded from diversity calculations.

[b] Number of individuals observed.

[c] Number of multilocus genotypes (MLGs) observed.

[d] Number of expected multilocus genotypes (eMLGs) and standard error at the smallest sample size of 25 individuals based on rarefaction.

[e] Shannon–Wiener index of MLG diversity (Shannon, 2001); H’ = - Σ(pi) (log2 pi).

[f] Stoddard and Taylor's index of MLG diversity (Stoddard & Taylor, 1988); G = 1/Σ[f_x(x/n)2].

[g] Corrected Simpson's index (Simpson, 1949); corrected lambda = (lambda × N/(N-1)).

[h] Percentage genotypic diversity (McDonald et al., 1994); $\hat{G}$ = G/N*100.

[i] Evenness, E.5 index (Pielou, 1975); E.5 = (H’/logS).

[j] Number of private alleles.

[k] Only populations with more than one MLG.

[l] Modified version of index of association Ia (Agapow & Burt, 2001).

The Malaysian population of isolates included four private alleles occurring in 2, 23, 31 and 36 of the 41 Malaysian isolates, respectively (Table 2; Table S4). All three alleles previously identified only from China (Havenga et al., 2020b) were present in nine Malaysian MLGs. The Malaysian clone-corrected data set represented 48.87% of the full data set. For both data sets, the hypothesis of random mating in the Malaysian population could not be rejected at $p = 0.01$, providing evidence of sexual recombination (Table 2). However, the value of rbarD was at the edge of the normal distribution for the full data set (Figure S2). Together with clones observed in Malaysia, this suggests that asexual reproduction also plays a significant role in reproduction.

3.2.2 Population structure

The distance-based MSN revealed that the 20 Malaysian genotypes were closely related (Figure 1a). These genotypes did not intersperse with those from any other locality and were separated from the other populations by a larger genetic distance than typically found within populations. This was consistent with the results of the AMOVA, which also revealed high molecular variation among populations (77%), but low variation within populations (26%; Table 3). The isolate allocation of DAPC and STRUCTURE largely corresponded to the MSN arrangement of genotypes and their geographical location (Figure 1). Inclusion of 20 genotypes with those previously identified by Havenga et al., (2020b) significantly increased the genetic diversity of the global population and therefore changed the population groupings slightly.

TABLE 3. Analysis of molecular variance (AMOVA) for seven *Teratosphaeria destructans* populations

	d.f.	Sum of squares	Mean squares	Estimate of variance	Total variation (%)	*p*
Among populations	6	321.54	53.59	1.64	74	0.001
Within populations	229	135.08	0.59	0.59	26	0.001
Total	235	456.627		2.23	100	

The DAPC and STRUCTURE analyses allocated *T. destructans* individuals into the same four clusters (Δ*K*) (Figure 1; S3; S4). Only isolates obtained from Indonesia and Malaysia belonged to cluster 1 and cluster 3, respectively (Figure 1b, c). Cluster 2 comprised the same South African, Thai and Vietnamese isolates reported by Havenga et al., (2020b), and five additional isolates from North Sumatra (previously in the Indonesian cluster), one isolate from China (previously in the Chinese cluster) and one isolate from Malaysia. Most of the Chinese isolates and one isolate from Malaysia comprised cluster 4. The Malaysian cluster had high levels of admixture, particularly with the Chinese and South Africa–Thailand–Vietnamese clusters, while the Indonesian cluster had low signs of admixture from the Malaysian cluster (Figure 1c). The *MAT* idiomorph, MLG identity and DAPC cluster group for each individual investigated in this study are presented in Table S1. Amplicon size, repeat motif and number of repeats for each *T. destructans* isolate are presented in Table S5.

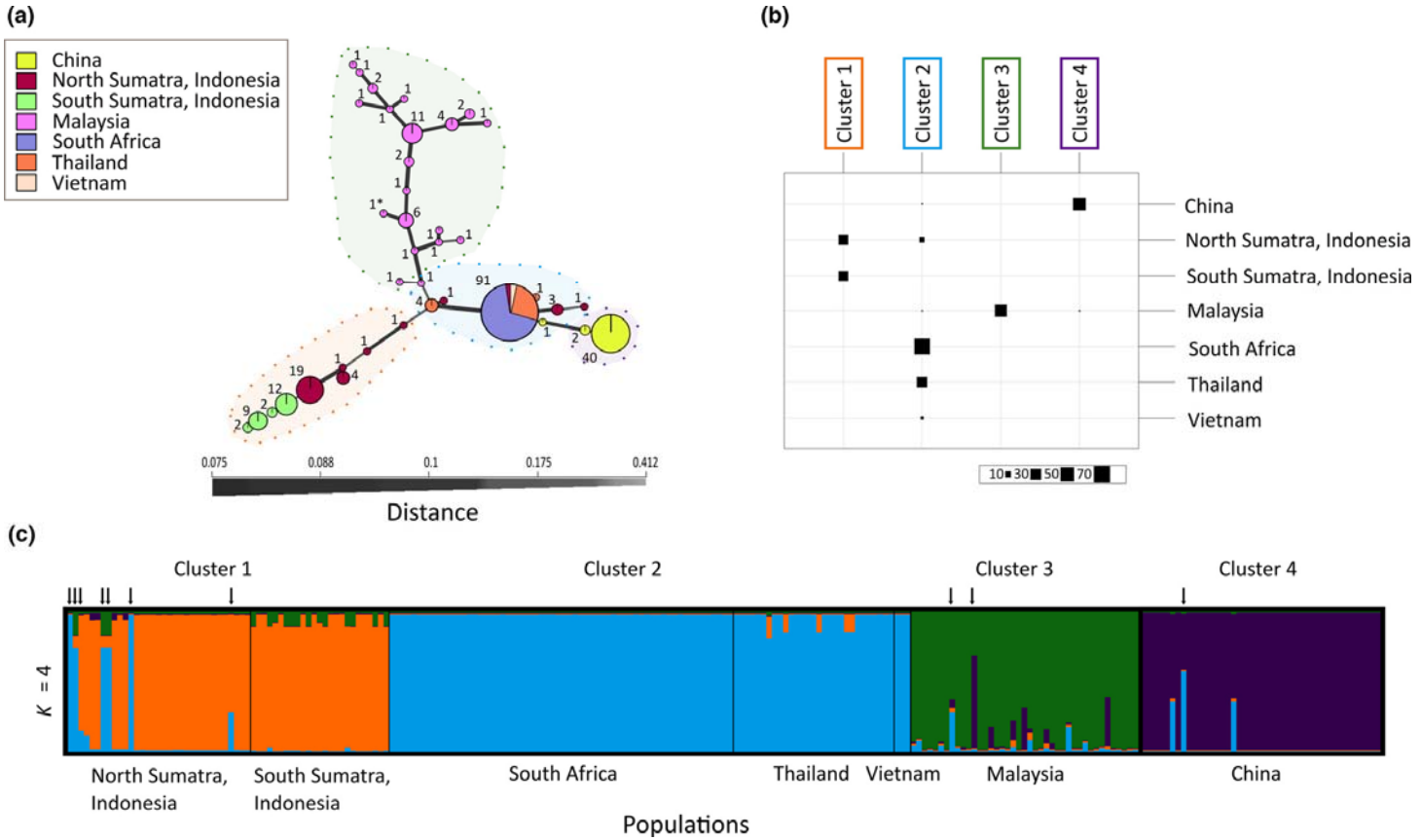

FIGURE 1. Population structure of seven *Teratosphaeria destructans* populations. Isolates were allocated to clusters based on (a) a minimum spanning network (MSN) calculated with Bruvo's genetic distance (Bruvo et al., 2004), (b) a discriminant analysis of principal components (DAPC) and (c) Bayesian inference applied in STRUCTURE. Each node in the MSN (a) represents a single multilocus genotype (MLG). Node size is proportional to the sample size (indicated next to the node). Branch thickness and shading between genotypes represent genetic distance, with branch thickness decreasing with increasing genetic distance. Dashed circles and shading on the MSN indicate the clusters calculated in b and c. The Malaysian MLG assigned to cluster 4 in the DAPC (b) is indicated with an asterisk (*) on the MSN. In the DAPC (b), the sample size of inferred clusters is proportional to the box size. Columns correspond to inferred clusters and rows to geographical locations. The optimal number of genetic clusters (Δ*K*) calculated by STRUCTURE (C) was four. Each vertical bar represents one individual. Isolates assigned to a different cluster than their population of origin in B are indicated with an arrow

3.3 In vitro pairings of isolates on Eucalyptus leaves

Mycelial growth and black necrotic lesions were observed on all leaf treatments. Additionally, asexual pycnidia were observed on the abaxial side of the wounded leaf treatments (Figure S5). Pycnidia were removed from the stomata, crushed and observed under the microscope, revealing asexual conidia (Figure S5). Crushed pycnidia were grown on MEA, and the mating type and genotype were identified as described in Section 2.1 and Section 2.2. The population genetic analyses confirmed that these structures belonged to isolate CMW48624 (*MAT1*-2). Asexual pycnidia were not found for the *MAT1-1* isolate. No sexual structures were observed.

4 DISCUSSION

This study presents the first confirmation of leaf blight caused by *T*. *destructans* in Malaysia. The Malaysian population had an even distribution of mating types and the highest observed genetic diversity for any *T*. *destructans* population studied to date. All 20 genotypes were unique to Malaysia, and population genetic analysis suggested the presence of a cryptic sexual cycle. These results were surprising, since they contradict what is known for other non-native populations of *T*. *destructans* (Havenga et al., 2020b; Havenga, Wingfield, Wingfield, Roets, et al., 2020), as well as for the related pathogens (Aylward et al., 2019; Taole et al., 2015), all of which appear to reproduce only asexually. The sexual state of *T*. *destructans* has never been found. Evidence of recombination in this study therefore suggests the existence of a cryptic sexual state in the Malaysian plantation sampled.

The Malaysian population of *T*. *destructans* had the highest genetic diversity (20.6%) of any known population of this pathogen. *Eucalyptus* plantations have been established in Malaysia relatively recently (Yahya, 2020). In contrast, the industry in Indonesia has been established for a much longer time period (Nambiar et al., 2018) and has the longest history of a *T*. *destructans* presence (Wingfield et al., 1996). Despite this fact, the genetic diversity of the pathogen in Malaysia was nearly double that found in Indonesia. It is also relevant that the diverse Malaysian population of isolates was collected from a single plantation, whereas the studied North Sumatra population in Indonesia was comprised of samples collected at different times and from different geographical locations (Havenga et al., 2020b).

Native populations of pathogens typically have higher genetic diversities and a greater number of private alleles compared with their introduced counterparts (McDonald, 1997). This is because introduced populations usually emerge from a small number of individuals, whereas native populations have had long periods of time to evolve (McDonald, 1997). Despite the high level of genetic diversity of the Malaysian population and the presence of both mating types, Malaysia is unlikely to represent the native range of this pathogen. According to current knowledge, this pathogen is specific to *Eucalyptus* (Andjic et al., 2019) and there are no *Eucalyptus* species native to Malaysia (Rejmánek & Richardson, 2011).

The first *Eucalyptus* hybrid plantation in Sabah was established with seeds obtained from southern China (Yahya, 2020), but the population from southern China (Havenga et al., 2020b) did not share any genotypes with the Malaysian isolates in

the present study. Malaysian and Chinese isolates, however, shared alleles that were previously thought to be limited to China (Havenga et al., 2020b). The hypothesis that *T. destructans* was introduced into Malaysia from China was therefore not conclusively supported. *Teratosphaeria destructans* causes widespread destruction in China (Burgess & Wingfield, 2017; Dell et al., 2008), but Havenga et al., (2020b) considered only a single site in southern China. An in-depth study of *T. destructans* in China would be required to further test this hypothesis.

High genetic diversities in the populations of two other exotic *Teratosphaeria* pathogens are believed to be the result of multiple introductions. *Teratosphaeria zuluensis* (M.J. Wingf., Crous & T.A. Cout.) M.J. Wingf. & Crous populations have an uneven distribution of mating types (Aylward et al., 2020), whereas *T. epicoccoides* (Cooke & Massee) Rossman & W.C. Allen genotypes are present in multiple populations with only a few unique alleles in the global collection (Taole et al., 2015). Although the global collection of *T. destructans* is primarily characterized by highly skewed mating type ratios and low genetic diversities, the Malaysian population had an even distribution of mating types, unique genotypes that grouped in the MSN and evidence that identical genotypes emerged through separate sexual events. This suggests that sexual recombination, rather than multiple introductions, is responsible for the genetic diversity in Malaysia.

The Malaysian population also consisted of a high number of clones. Only the asexual state of this pathogen is known (Burgess et al., 2006; Wingfield et al., 1996), and it is also the dominant form for many leaf-infecting *Teratosphaeria* species, including *T. eucalypti*, *T. pseudoeucalypti*, *T. epicoccoides* and *T. cryptica* (Cooke) Crous & U. Braun (Andjic et al., 2019; Burgess & Wingfield, 2017; Taole et al., 2015). Regardless of sexual reproduction, our results indicated that the asexual state remains important in the reproduction and dispersal of this pathogen.

The Malaysian population had the highest recorded genotypic diversity of *T. destructans* populations investigated to date. It also displayed evidence of recombination, strong genetic structure and a high number of clones. We conclude that both the sexual and asexual states serve as modes of reproduction for this pathogen in Malaysia. Exclusively asexual species are rare (Sun & Heitman, 2011) because sexual reproduction produces genetically unique offspring that may be better adapted to certain conditions (Ni et al., 2011). Sexually driven evolution of *T. destructans* in Malaysia is concerning, because it may lead to a more adaptable pathogen (McDonald, 1997) and one that is less responsive to disease management practices. The extent to which *T. destructans* has spread within Malaysia is unknown. An in-depth study, investigating multiple sampling sites, would be required to evaluate the current distribution of *T. destructans* in Sabah, Malaysia and Borneo as a whole. Care must be taken to prevent further spread, which could easily occur if new plantations are established with plant material from Sabah.

ACKNOWLEDGEMENTS

We are grateful to various colleagues and members of the Borneo Forestry Co-operative who made it possible for us to collect material used in this study. In this regard, we especially thank Mr. David Boden and Mr. Edmund Gan for their support.

This work was supported by the National Research Foundation of South Africa (NRF) (Grant Number: 11824); the Department of Science and Technology (DST)—NRF Centre of Excellence in Tree Health Biotechnology (CTHB); the Tree Protection Co-operative Program (TPCP); the SARChI Chair in Fungal Genomics (Grant number: 98353); and a University of Pretoria (UP) Doctoral Research Bursary.

REFERENCES

- Agapow, P. M., & Burt, A. (2001). Indices of multilocus linkage disequilibrium. *Molecular Ecology Notes*, 1, 101– 102. https://doi.org/10.1046/j.1471-8278.2000.00014.x.

- Andjic, V., Carnegie, A. J., Pegg, G. S., Hardy, G. E. S. J., Maxwell, A., Crous, P. W., Pérez, C., Wingfield, M. J., & Burgess, T. I. (2019). 23 years of research on *Teratosphaeria* leaf blight of *Eucalyptus*. *Forest Ecology and Management*, 443, 19– 27. https://doi.org/10.1016/j.foreco.2019.04.013.

- Aylward, J., Dreyer, L. L., Havenga, M., Roets, F., Wingfield, B. D., & Wingfield, M. J. (2020). Genomic characterization of mating type loci and mating type distribution in two apparently asexual plantation tree pathogens. *Plant Pathology*, 69, 28– 37. https://doi.org/10.1111/ppa.13094.

- Aylward, J., Roets, F., Dreyer, L. L., & Wingfield, M. J. (2019). Teratosphaeria stem canker of *Eucalyptus*: two pathogens, one devastating disease. *Molecular Plant Pathology*, 20(1), 8– 19. https://doi.org/10.1111/mpp.12758.

- Barber, P. A., Thu, P., Hardy, G. E., & Dell, B. (2012). *Emerging disease problems in Eucalypt plantations in LAO PDR*. Paper presented at the The Impacts of Climate Change to Forest Pests and Diseases in The Tropics, Yogyakarta, Indonesia.

- Billiard, S., Lopez-Villavicencio, M., Devier, B., Hood, M. E., Fairhead, C., & Giraud, T. (2011). Having sex, yes, but with whom? Inferences from fungi on the evolution of anisogamy and mating types. *Biological Reviews*, 86, 421– 442. https://doi.org/10.1111/j.1469-185X.2010.00153.x.

- Bruvo, R., Michiels, N. K., D'Souza, T. G., & Schulenburg, H. (2004). A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. *Molecular Ecology*, 13, 2101– 2106. https://doi.org/10.1111/j.1365-294X.2004.02209.x.

- Burgess, T. I., Andjic, V., Hardy, G. E. S. J., Dell, B., & Xu, D. (2006). First report of *Phaeophleospora destructans* in China. *Journal of Tropical Forest Science*, 18(2), 144– 146. https://doi.org/10.1071/DN07056.

- Burgess, T. I., & Wingfield, M. J. (2017). Pathogens on the move: A 100-year global experiment with planted Eucalypts. *BioScience*, 67(1), 14– 25. https://doi.org/10.1093/biosci/biw146.

- Dell, B., Hardy, G., & Burgess, T. (2008). Health and nutrition of plantation eucalypts in Asia. *Southern Forests: a Journal of Forest Science*, 70(2), 131– 138. https://doi.org/10.2989/south.for.2008.70.2.8.536.

- 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.PMCID: PMC1462648. PMID: 12930761.

- Fourie, A., Wingfield, M. J., Wingfield, B. D., Thu, P. Q., & Barnes, I. (2016). A possible centre of diversity in South East Asia for the tree pathogen, *Ceratocystis manginecans*. *Infection, Genetics and Evolution*, 41, 73– 83. https://doi.org/10.1016/j.meegid.2016.03.011.

- Greyling, I., Wingfield, M. J., Coetzee, M. P. A., Marincowitz, S., & Roux, J. (2016). The *Eucalyptus* shoot and leaf pathogen *Teratosphaeria destructans* recorded in South Africa. *Southern Forests*, 78(2), 123– 129. https://doi.org/10.2989/20702620.2015.1136504.

- Havenga, M., Wingfield, B. D., Wingfield, M. J., Dreyer, L. L., Roets, F., & Aylward, J. (2020a). Diagnostic markers for *Teratosphaeria destructans* and closely related species. *Forest Pathology*, 50(6), e12645. https://doi.org/10.1111/efp.12645.

- Havenga, M., Wingfield, B. D., Wingfield, M. J., Dreyer, L. L., Roets, F., & Aylward, J. (2020b). Low genetic diversity and strong geographic structure in six introduced populations of the *Eucalyptus* foliar pathogen *Teratosphaeria destructans*. *Plant Pathology*, 69, 1540– 1550. https://doi.org/10.1111/ppa.13235.

- Havenga, M., Wingfield, B. D., Wingfield, M. J., Roets, F., Dreyer, L. L., Tatham, C. T., Duong, T. A., Wilken, P. M., Chen, S. F., & Aylward, J. (2020). Mating strategy and mating type distribution in six global populations of the *Eucalyptus* foliar pathogen *Teratosphaeria destructans*. *Fungal Genetics and Biology*, 137, 103350. https://doi.org/10.1016/j.fgb.2020.103350.

- Hii, S. Y., Ha, K. S., Ngui, M. L., Penguang Jnr, S. A., Duju, A., Teng, X. Y., & Meder, R. (2017). Assessment of plantation-grown *Eucalyptus pellita* in Borneo, Malaysia for solid wood utilisation. *Australian Forestry*, 80(1), 26– 33. https://doi.org/10.1080/00049158.2016.1272526.

- Jimu, L., Kemler, M., Wingfield, M. J., Mwenje, E., & Roux, J. (2016). The *Eucalyptus* stem canker pathogen *Teratosphaeria zuluensis* detected in seed samples. *Forestry*, 89(3), 316– 324. https://doi.org/10.1093/forestry/cpv037.

- Jombart, T. (2008). adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics*, 24, 1403– 1405. https://doi.org/10.1093/bioinformatics/btn129.

- Kopelman, N. M., Mayzel, J., Jakobsson, M., Rosenberg, N. A., & Mayrose, I. (2015). CLUMPAK: a program for identifying clustering modes and packaging population structure inferences across K. *Molecular Ecology Resources*, 5, 1179– 1191.

- Lee, S. S. (2018). Observations of the successes and failures of *Acacia* plantations in Sabah and Sarawak and the way forward. *Journal of Tropical Forest Science*, 30, 468– 475. https://doi.org/10.26525/jtfs2018.30.5.468475.

- McDonald, B. A. (1997). The population genetics of fungi: tools and techniques. *Phytopathology*, 87, 448– 453. https://doi.org/10.1094/PHYTO.1997.87.4.448.

- McDonald, B. A., Miles, J., Nelson, L. R., & Pettway, R. E. (1994). Genetic variability in nuclear DNA in field populations of *Stagonospora nodorum*. *Phytopathology*, 84, 250– 255. https://doi.org/10.1094/Phyto-84-250.
- Nambiar, E. K. S., Harwood, C. E., & Mendham, D. S. (2018). Paths to sustainable wood supply to the pulp and paper industry in Indonesia after diseases have forced a change of species from acacia to eucalypts. *Australian Forestry*, 81(3), 146– 161. https://doi.org/10.1080/00049158.2018.1482798.
- Ni, M., Feretzaki, M., Sun, S., Wang, X., & Heitman, J. (2011). Sex in fungi. *Annual Review of Genetics*, 45, 405. https://doi.org/10.1146/annurev-genet-110410-132536.
- Old, K. M., Wingfield, M. J., & Yuan, Z. Q. (2003). *Phaeophleospora* leaf diseases. In M. Dudzinski (ed.), *A Manual of Diseases of Eucalypts in South-East Asia* (pp. 25- 31). Center for International Forestry Research.
- Peakall, R., & Smouse, P. E. (2006). GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, 6, 288– 295. https://doi.org/10.1111/j.1471-8286.2005.01155.x.
- Pielou, E. C. (1975). *Ecological diversity*. Wiley, New York, 165, https://doi.org/10.4319/lo.1977.22.1.0174b.
- R Core Team (2013). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. http://www.R-project.org/.
- Rejmánek, M., & Richardson, D. M. (2011). Eucalypts. In D. Simberloff, & M. Rejmánek (Eds.), *Encyclopaedia of Biological Invasions* (pp. 203– 209). University of California Press.
- Shannon, C. E. (2001). A mathematical theory of communication. *ACM SIGMOBILE Mobile Computing and Communications Review*, 5, 3– 55. https://doi.org/10.1145/584091.584093.
- Simpson, E. H. (1949). Measurement of diversity. *Nature*, 163, 688. https://doi.org/10.1038/163688a0.
- Stenberg, P., Lundmark, M., & Saura, A. (2003). MLGsim: a program for detecting clones using a simulation approach. *Molecular Ecology Resources*, 3, 329– 331. https://doi.org/10.1046/j.1471-8286.2003.00408.x.
- Stoddard, J. A., & Taylor, J. F. (1988). Genotypic diversity: estimation and prediction in samples. *Genetics*, 118, 705– 711.
- Sun, S., & Heitman, J. (2011). Is sex necessary? *BMC Biology*, 9(56), 1– 4. http://www.biomedcentral.com/1741-7007/9/56.
- Taole, M., Bihon, W., Wingfield, B. D., Wingfield, M. J., & Burgess, T. I. (2015). Multiple introductions from multiple sources: invasion patterns for an important Eucalyptus leaf pathogen. *Ecology and Evolution*, 5(18), 4210– 4220. https://doi.org/10.1002/ece3.1693.
- Tarigan, M., Roux, J., van Wyk, M., Tjahjono, B., & Wingfield, M. J. (2011). A new wilt and die-back disease of *Acacia mangium* associated with *Ceratocystis*

manginecans and *C. acaciivora* sp. nov. in Indonesia. *South African Journal of Botany*, 77, 292– 304. https://doi.org/10.1016/j.sajb.2010.08.006.

- Wingfield, M. J., Crous, P. W., & Boden, D. (1996). *Kirramyces destructans* sp. nov., a serious leaf pathogen of *Eucalyptus* in Indonesia. *South African Journal of Botany*, 62(6), 325– 327. https://doi.org/10.1016/s0254-6299(15)30673-6.
- Yahya, A. Z. (2020). Planting of *Eucalyptus* in Malaysia. *Acta Scientific Agriculture*, 4(2), 0 1–0 2. https://doi.org/10.31080/ASAG.2020.04.0785.
- Zaiton, S., Paridah, M. T., Hazandy, A. H., & Azim, R. A. R. A. (2018). Potential of *Eucalyptus* plantations in Malaysia. *The Malaysian Forester*, 81(1), 64– 72, ISSN 0302–2935.