Mating strategy and mating type distribution in six global populations of the *Eucalyptus* foliar pathogen *Teratosphaeria destructans*

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Highlights

- We present two *Teratosphaeria destructans* genomes from Indonesian strains.
- The genomes indicated that this pathogen has a heterothallic mating system.
- The PCR assay identified the mating types of at least six *Teratosphaeria* species.
- An uneven distribution of mating types was noted in all *T. destructans* populations.
- Recombination likely plays a minor role in *T. destructans* reproduction.

Abstract

Teratosphaeria destructans is an aggressive fungal pathogen causing leaf and shoot blight on young *Eucalyptus* trees in plantations. The disease occurs across tropical and subtropical regions of South East Asia and has recently been found in South Africa. Asexual structures of the pathogen are produced on infected tissues, but sexual structures have never been observed. The aim of this study was to investigate the reproductive biology of *T. destructans* by characterising its mating type (*MAT1*) locus and investigating its potential for sexual recombination. We found that *T. destructans* has a heterothallic mating system, with either the *MAT1-1-1* and *MAT1-1-10* genes (*MAT1-1* idiomorph) or the *MAT1-2-1* and *MAT1-2-12*

genes (*MAT1-2* idiomorph) present in a single individual. With a multiplex PCR assay, it was possible to distinguish the two *MAT* idiomorphs in several *Teratosphaeria* species and this approach was applied to six global populations of *T. destructans*. Although both mating types occurred in the South East Asian populations, a single mating type dominated each population. Isolates from the recent disease outbreak in South Africa comprised only a single mating type. Attempts to induce a sexual cycle *in vitro* using strains of opposite mating type were not successful. The uneven distribution of mating types in populations of *T. destructans* and the presence of only an asexual state on infected tissues suggests the absence of or at least a minor role for sexual reproduction where the pathogen occurs on non-native *Eucalyptus* in plantations.

Keywords: heterothallic; asexual reproduction; idiomorph; *Teratosphaeria*; leaf blight; *Eucalyptus*; mating type

1. Introduction

Teratosphaeria destructans (Capnodiales, Teratosphaeriaceae) is one of the most aggressive foliar pathogens of *Eucalyptus*, causing shoot and leaf blight on young trees (Wingfield et al., 1996, Burgess et al., 2006, Andjic et al., 2007, Burgess et al., 2007). The pathogen was first discovered in 1995 when it appeared causing damage to young *Eucalyptus grandis* trees in Northern Sumatra, Indonesia (Wingfield et al., 1996). Since then, it has spread rapidly to tropical and subtropical regions in South East Asia (Andjic et al., 2011), including Thailand, East Timor, Vietnam, China and Lao where *Eucalyptus* trees are grown as non-natives in plantations (Old et al., 2003a, Old et al., 2003b, Burgess et al., 2006, Barber et al., 2012). In 2015, *T. destructans* was found on young plantation trees in the KwaZulu-Natal Province of South Africa (Greyling et al., 2016) and it has subsequently spread to plantations of *E. grandis* and its hybrids in sub-tropical parts of the country (Greyling et al., 2018).

The rapid spread and destructive nature of *T. destructans* is of considerable concern to plantation forestry companies globally (Burgess and Wingfield, 2017). Yet very little is known regarding its biology, including its mode of reproduction. Only asexual reproduction has ever been observed, where pycnidia form beneath the stomata and exude long black cirri of dark septate conidia (mitospores) under moist conditions. (Wingfield et al., 1996, Old et al., 2003b, Burgess et al., 2006). An important question that remains to be resolved is whether sexual reproduction occurs in *T. destructans* and how this might relate to disease impact globally.

Recombination via sexual reproduction can produce genetically unique offspring that are better adapted to new environments and show improved fitness (Ni et al., 2011, Wilson et al., 2015, Nieuwenhuis and James, 2016). In Indonesia, *Eucalyptus* clones previously considered to be resistant to *T. destructans* are now being infected (M.J. Wingfield, unpublished). This may be due to recent adaptations brought about by recombination, highlighting the need to understand the reproductive strategy of important pathogens such as *T. destructans* (Billiard et al., 2012). Host-resistance breeding is generally an effective strategy to combat this pathogen (Andjic et al., 2019), but can only be sustained where the ability of the pathogen to adapt to altered host genotypes is understood (Peever et al., 2002).

Characterisation of mating type genes provides a reliable means to explore the reproductive strategy of ascomycetous fungi (Groenewald et al., 2007). Sexual reproduction, in this case, is controlled by genes present at the mating type locus (*MAT1*) (Kronstad and Staben, 1997). This *MAT1* locus has two mating-type alleles with genes that encode different proteins referred to as "idiomorphs" (*MAT1-1* and *MAT1-2*) rather than alleles (Billiard et al., 2011, Nieuwenhuis and James, 2016). The *MAT1-1* and *MAT1-2* idiomorphs are typically defined by the *MAT1-1-1* and *MAT1-2-1* genes, respectively (Turgeon and Yoder, 2000). These genes contain open reading frames (ORFs) that code for a protein with either an alpha-box (*MAT1-1-1*) or a high mobility group (HMG) box (*MAT1-2-1*) motif (Turgeon and Yoder, 2000, Wilson et al., 2018).

Most filamentous fungi have one of two possible strategies for sexual reproduction (Ni et al., 2011). Heterothallic fungi have a single idiomorph present at the *MAT1* locus of each individual and two partners of opposite mating type are required (Billiard et al., 2011) for sexual outcrossing to occur (Ni et al., 2011, Turgeon and Yoder, 2000). Homothallic fungi have both *MAT* idiomorphs present in one individual, conferring self-fertility (Ni et al., 2011, Wilson et al., 2015). Rare cases of unisexual reproduction are also known, where genes of a single mating type are present, but sexual reproduction can occur in the absence of a partner (Wilson et al., 2018).

Recently, Aylward et al. (2020) analysed the *MAT1* loci in the *Eucalyptus* stem canker pathogens *Teratosphaeria gauchensis* and *Teratosphaeria zuluensis*. Their study showed that both pathogens have a heterothallic mating system, but it was not possible to induce a sexual state when strains of opposite mating type were paired in culture. The only known homothallic *Teratosphaeria* species is *T. nubilosa*, which produces abundant black ascomata on infected leaves, but for which an asexual state is not known (Hunter et al., 2009).

The aim of this study was to use whole genome sequences to investigate the reproductive strategy of *T. destructans*. Our objectives were to identify the *MAT1* locus, characterise the mating type genes and, in so doing, determine whether *T. destructans* has a heterothallic or homothallic reproductive system. Due to the apparent absence of a sexual state on infected *Eucalyptus* tissues, we hypothesized that the pathogen has a heterothallic mating system. Assuming that this were true, secondary aims were to (i) investigate the distribution of the mating types in isolates collected from diseased *Eucalyptus* in various parts of the world and (ii) attempt to induce a sexual state *in vitro* by pairing individuals of opposite mating type.

2. Materials and Methods

2.1 Genome sequencing

Genomes were sequenced for two *T. destructans* isolates collected in July 2015 from the location in Indonesia where the original disease outbreaks were first reported by Wingfield et al. (1996). Both isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria. Isolate CMW45982 was made from diseased leaves of a *Eucalyptus pellita* x *E. grandis* hybrid in North Sumatra and isolate CMW45661 was from *E. pellita* in South Sumatra. Cultures derived from single conidia were grown on 2% Malt Extract Agar (MEA, Merck, South Africa) amended with 150 mg/L streptomycin (Biolab, Merck) (MEA-S) and incubated in the dark at 25°C. Mycelium was harvested from eight-week-old cultures and genomic DNA was extracted from lyophilized mycelium using the DNA isolation protocol for *T. destructans* described by Wingfield et al. (2018).

The genomic DNA samples were submitted to Macrogen Inc. (Korea) for whole genome sequencing. The TruSeq DNA PCR-free and Nextera mate pair library preparation kits (Illumina, California, USA) were used for the paired-end and mate pair library preparation, respectively. A paired-end library with an insert size of 350 bp was generated for both isolates and an additional mate pair library with an insert size of 5 kb was generated for isolate CMW45982. Sequencing was carried out on Illumina HiSeq X Platform (California, USA), with target read lengths of 150 bp. Resulting reads were trimmed with TRIMMOMATIC 0.38 (Bolger et al., 2014) and TrimGalore 0.5.0 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), retaining any unpaired reads.

Genomes were assembled with ABYSS 2.1.0 (Simpson et al., 2009), testing multiple k-values. The final assemblies were based on k-values of 42 for isolate CMW45661 and 54 for isolate CMW45982, which resulted in the most contiguous and complete genomes. Gaps in the assembly were minimised using GapCloser 1.12 (Luo et al., 2012). The completeness of

the genome assemblies was estimated with the Benchmarking Universal Single-Copy Orthologs (BUSCO 2.0.1) tool in conjunction with the *ascomycetes_odb9* dataset (Simão et al. 2015). Bowtie 2 1.1.2 and SAMtools 1.5 were used to estimate the average base coverage across the 50 largest scaffolds (Li et al. 2009, Langmead and Salzberg 2012).

2.2 Characterisation of the *T. destructans MAT1* locus

The two sequenced genomes of the Indonesian isolates (WBMM00000000: WBMN00000000), as well as a previously published genome of a South African *T. destructans* isolate CMW44962 (RIBY01000000; Wingfield et al., 2018) were used to identify and characterise the *MAT* locus of this species. The *MAT1-1-1* (MN119556; *MAT1-1* accession) protein sequences of *T. zuluensis* and the *MAT1-2-1* (MN119559; *MAT1-2* accession) protein sequences of *T. gauchensis*, were used for local BLASTx (Basic Local Alignment Search Tool for protein vs. translated nucleotide) searches in Geneious R11 (Biomatters Ltd., Auckland, New Zealand), applying a maximum e-value of 1.0^{-5} with default settings, to locate the putative *MAT* locus in each of the three genomes. The sequence of the putative *MAT* locus, as well as 7 kb upstream and downstream flanking regions, were extracted from each genome.

Open reading frames (ORFs) were predicted with WebAUGUSTUS (Hoff and Stanke, 2013), using *Aspergillus fumigatus* gene models. Gene predictions were confirmed with FGENESH (Solovyev et al., 2006) using the gene finding parameters of generic *Mycosphaerella.* Predicted ORFs were annotated by conducting BLASTp searches with their protein sequences against the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) protein database.

The *MAT* loci of the three *T. destructans* isolates were compared with each other and with the *MAT* loci of *T. gauchensis and T. zuluensis*. The nucleotide sequences of the *MAT* locus, as well as available flanking regions, were aligned with MAFFT 7.388 (Katoh and Standley, 2013) using the L-INS-I method in Geneious. This also made it possible to define the boundaries between the *MAT1* locus and flanking regions of the two *T. destructans* idiomorphs. The MAT1-1-1 and MAT1-2-1 proteins of *T. destructans* were also compared to commonly characterized MAT proteins of closely related (Aylward et al., 2020) and more distant species (Martin et al., 2010) with a MAFFT alignment.

2.2.1 Fungal isolates and DNA extraction

For population analysis, *T. destructans* isolates were sourced from the culture collection (CMW) at FABI (Table 1). These isolates were collected between 1996 and 2018 from six distinct populations globally. In Indonesia, 35 North Sumatran isolates were collected from four different locations, whereas one population (n=25) was available from South Sumatra.

In South Africa, two populations of 29 and 31 isolates each were collected from two regions within one province and two isolates from an additional province. Lastly, 44 isolates were available from China, 29 isolates from Thailand and 3 isolates Vietnam on the Indochina Peninsula.

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

Population[1]	Country	Province	Number of isolates
1	China	GuangDong	44
2	Indonesia	Northern Sumatra	35
3	Indonesia	Southern Sumatra	25
4	South Africa	KwaZulu-Natal	60
	South Africa	Mpumalanga	2
5	Thailand	Unknown	29
6	Vietnam	Unknown	3[2]
		Total	198

[1] Isolates collected from the same country are considered to be in the same population

[2] The only isolates available in the CMW

Isolates were purified by single conidial transfer and maintained at 25°C on MEA amended with 3 g of yeast extract (MEA+Y; Oxoid, France). DNA was extracted from five-week-old cultures using the protocol described by Damm et al. (2008), with some modifications. Fungal mycelium (~30 mg) was shaken for 5 min at 30 1/s in a MM301 TissueLyser (Retsch, Inc., Germany) with 0.5 mg glass beads and 600 µl acetyl trimethylammonium bromide (CTAB) extraction buffer (2% CTAB, 100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA, 0.2% mercaptoethanol). After an incubation period of 15 min at 65°C, 400 µl chloroform:isoamylalcohol (24:1) was added and centrifuged for 15 min at 16 300 x *g*. The supernatant was extracted and added to 600 µl ice-cold isopropanol and 200 µl 5 M potassium acetate and centrifuged for a further 15 min at 16 300 x *g*. The supernatant was discarded and the DNA pellet washed twice with 500 µl 70% ethanol. The dry DNA pellets were dissolved in 200 µl low TE (Tris-EDTA) buffer (Thermo Fisher Scientific, Wilmington, USA). The quality and quantity of the extracted DNA was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

2.2.2 Confirmation of species identity

The ribosomal RNA Internal Transcribed Spacer gene region (ITS), partial β-tubulin gene (TUB) and elongation factor 1-alpha gene (EF-1α) were extracted from each *T. destructans* genome. PCR products of these same gene regions from a subset of isolates from each country (Table 2) were also sequenced to confirm their identity. The ITS, TUB and EF-1α genes amplified with the Ampliqon Taq DNA Polymerase Master Mix RED (Biomol, Germany). In a total reaction volume of 20 µl, the polymerase chain reaction (PCR) mixture contained 150 ng DNA, 10 µl Ampliqon master mix, 0.4 pmol/µl of each primer and 2 mM $MgCl_2$. Primers ITS1F (Gardes and Bruns, 1992) and ITS4 (White et al., 1990) were used for ITS reactions. PCR amplification conditions, as well as TUB and EF-1α primers used, followed the protocol described by Havenga et al. (2019). The PCR products were visualised on a 1% agarose gel and sequenced at the Central Analytical Facility (CAF), Stellenbosch University, South Africa.

Amplicon identities were confirmed with BLASTn searches against the NCBI nucleotide database and by performing a phylogenetic analysis in Geneious. Sequenced gene regions from each isolate, together with reference sequences (Table 2), were aligned in MAFFT as described earlier and concatenated. Maximum likelihood (ML) analyses were performed with PhyML 2.2.4 (Guindon et al., 2010) under the general time reversible (GTR) model. Both the gamma distribution parameter and proportion of non-variable sites were estimated. Bootstrap support values were calculated from 1000 replicates. Clades with bootstrap support values of ≥ 70% were considered significant (Hillis and Bull, 1993).

Table 2. *Teratosphaeria* isolates and their sequences used for phylogenetic analyses

Species	Strain number	Host	Location	Genbank Accession Number			Source
				ITS	EF	β-tubulin	
Teratosphaeria blakelyi	CBS120089	*Eucalyptus blakelyi*	Australia, New South Wales	KF901565	KF903288	KF902988	Quaedvlieg et al. (2014)
Teratosphaeria corymbiae	CBS124988	*Corymbia henryi*	Australia, New South Wales	KF901569	KF903293	KF902992	Quaedvlieg et al. (2014)
Teratosphaeria destructans	CBS111370	*Eucalyptus grandis*	Indonesia	KF901574	KF903301	KF903000	Quaedvlieg et al. (2014)
Teratosphaeria destructans	CMW17919	*Eucalyptus urophylla*	China	DQ632701	DQ632729	DQ632622	Andjic et al. (2007)
Teratosphaeria destructans	CMW47566	*Eucalyptus grandis*	China, GuangDong	MN483370	MN494106	MN494125	This study
Teratosphaeria destructans	CMW47583	*Eucalyptus grandis*	China, GuangDong	MN483366	MN494105	MN494124	This study
Teratosphaeria destructans	CMW47597	*Eucalyptus grandis*	China, GuangDong	MN483369	MN494104	MN494123	This study
Teratosphaeria destructans	CMW45696	*Eucalyptus pellita* x *E. grandis*	Indonesia, Northern Sumatra	MN483365	MN494109	MN494128	This study
Teratosphaeria destructans	CMW45977	*Eucalyptus pellita* x *E. grandis*	Indonesia, Northern Sumatra	MN483364	MN494108	MN494127	This study
Teratosphaeria destructans	CMW45982	*Eucalyptus grandis* x *E. urophylla*	Indonesia, Northern Sumatra	MN483363	MN494107	MN494126	This study, genome
Teratosphaeria destructans	CMW48675	*Eucalyptus grandis* x *E. urophylla*	Indonesia, Northern Sumatra	MN483362	MN494103	MN494122	This study
Teratosphaeria destructans	CMW45661	*Eucalyptus pellita*	Indonesia, Southern Sumatra	MN483371	MN494110	MN494129	This study, genome
Teratosphaeria destructans	CMW45649	*Eucalyptus pellita*	Indonesia, Southern Sumatra	MN483353	MN494111	MN494130	This study
Teratosphaeria destructans	CMW44958	*Eucalyptus grandis* x *E. urophylla*	South Africa, KwaZulu-Natal, Zululand	MN483361	MN494113	MN494132	This study
Teratosphaeria destructans	CMW44962	*Eucalyptus grandis* x *E. urophylla*	South Africa, KwaZulu-Natal, Zululand	MN483360	MN494112	MN494131	This study, genome
Teratosphaeria destructans	CMW53047	*Eucalyptus grandis* x *E. urophylla*	South Africa, KwaZulu-Natal, Zululand	MN483356	MN494102	MN494121	This study
Teratosphaeria destructans	CMW53082	*Eucalyptus grandis* x *E. urophylla*	South Africa, KwaZulu-Natal, Midlands	MN483355	MN494101	MN494120	This study
Teratosphaeria destructans	CMW13346	*Eucalyptus camaldulensis*	Thailand	MN483368	MN494119	MN494138	This study
Teratosphaeria destructans	CMW13349	*Eucalyptus camaldulensis*	Thailand	MN483367	MN494118	MN494137	This study
Teratosphaeria destructans	CMW13705	*Eucalyptus camaldulensis*	Thailand	MN483359	MN494117	MN494136	This study
Teratosphaeria destructans	CMW15089	*Eucalyptus urophylla*	Vietnam	MN483354	MN494116	MN494135	This study
Teratosphaeria destructans	CMW15090	*Eucalyptus urophylla*	Vietnam	MN483358	MN494115	MN494134	This study
Teratosphaeria destructans	CMW15092	*Eucalyptus urophylla*	Vietnam	MN483357	MN494114	MN494133	This study
Teratosphaeria epicoccoides	CBS110499	*Eucalyptus globulus*	Australia, Western Australia	KF901586	KF903330	KF903027	Quaedvlieg et al. (2014)
Teratosphaeria epicoccoides	CBS117927	*Eucalyptus globulus*	Australia, Tasmania	KF901767	KF903333	KF903030	Quaedvlieg et al. (2014)
Teratosphaeria eucalypti	CMW19461	*Eucalyptus nitens*	New Zealand	FJ793232	EU101583	EU101527	Andjic et al. (2010)
Teratosphaeria eucalypti	CMW19470	*Eucalyptus nitens*	New Zealand	FJ793238	EU101589	EU101533	Andjic et al. (2010)
Teratosphaeria gauchensis	CBS119465	*Eucalyptus grandis*	Uruguay	KF901787	KF903312	KF903010	Quaedvlieg et al. (2014)
Teratosphaeria gauchensis	CBS119468	*Eucalyptus grandis*	Uruguay	KF901788	KF903313	KF903011	Quaedvlieg et al. (2014)

Teratosphaeria majorizuluensis	CBS120040	*Eucalyptus botryoides*	Australia, New South Wales	KF901581	KF903319	KF903017	Quaedvlieg et al. (2014)
Teratosphaeria nubilosa	CBS116005	*Eucalyptus globulus*	Australia, Victoria	KF901686	KF903336	KF903033	Quaedvlieg et al. (2014)
Teratosphaeria nubilosa	CPC11879	*Eucalyptus* sp.	Portugal	KF901694	KF903337	KF903034	Quaedvlieg et al. (2014)
Teratosphaeria pseudoeucalypti	MUCC598	*Eucalyptus globulus x E. camaldulensis*	Australia	FJ793215	EU101592	EU101536	Andjic et al. (2010)
Teratosphaeria pseudoeucalypti	MUCC613	*Eucalyptus* sp.	Australia	FJ793229	EU101611	EU101554	Andjic et al. (2010)
Teratosphaeria pseudoeucalypti	CBS124577	*Eucalyptus grandis* x *E. camaldulensis*	Australia, Queensland	NR137817	KF903349	KF252757	Quaedvlieg et al. (2014)
Teratosphaeria pseudonubilosa	CPC13831	*Eucalyptus globulus*	Australia	KF901594	KF903350	KF903047	Quaedvlieg et al. (2014)
Teratosphaeria stellenboschiana	CBS124989	*Eucalyptus punctata*	South Africa	KF901732	KF903355	KF903052	Quaedvlieg et al. (2014)
Teratosphaeria stellenboschiana	CBS125215	*Eucalyptus punctata*	South Africa	KF901733	KF903356	KF903053	Quaedvlieg et al. (2014)
Teratosphaeria toledana	CBS113313	*Eucalyptus* sp.	Spain	KF901734	KF903361	KF903058	Quaedvlieg et al. (2014)
Teratosphaeria toledana	CBS115513	*Eucalyptus* sp.	Spain	KF901600	KF903362	KF903059	Quaedvlieg et al. (2014)
Teratosphaeria viscidus	CBS121156	*Eucalyptus grandis*	Australia	KT972309	KT972373	KT972341	Andjic et al. (2016)
Teratosphaeria viscidus	CBS124992	*Eucalyptus* sp.	Australia, Queensland	KF901602	KF903366	KF903063	Quaedvlieg et al. (2014)
Teratosphaeria zuluensis	CBS120301	*Eucalyptus grandis*	South Africa	KF901735	KF903368	KF903064	Quaedvlieg et al. (2014)
Teratosphaeria zuluensis	CBS120302	*Eucalyptus grandis*	South Africa	KF901736	KF903369	KF903065	Quaedvlieg et al. (2014)
Zasmidium eucalypti	CBS121101	*Eucalyptus tereticornis*	Australia, Queensland	KF901606	KF903389	KF903083	Quaedvlieg et al. (2014)

2.2.3 Distribution of MAT idiomorphs

Since a single *MAT1* idiomorph was identified in each genome (see results 3.2), the conserved regions in the *MAT1-1-1* and *MAT1-2-1* genes were amplified to enable rapid mating type identification in *T. destructans* isolates. The *MAT1-1* primer set (T_Ma1-F and T_Ma1-R) designed by Aylward et al. (2020) was used to amplify the *MAT1-1-1* alpha-box domain and a new primer set was developed using PRIMER3 2.3.7 (Rozen and Skaletsky, 2000) to amplify the *MAT1-2-1* HMG-box.

The mating type of each isolate (Table 1) was determined with a single multiplex PCR containing primers of both the *MAT1-1-1* or *MAT1-2-1* gene regions. In a total reaction volume of 20 µl, the multiplex PCR reaction contained 150 ng DNA, 10 µl Ampliqon Taq DNA Polymerase Master Mix RED (Ampliqon, Denmark) and 0.4 pmol/µl of each of the four primers. Reaction conditions included an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 30 s at 94°C, 30 s at 61°C and 45 s at 72°C, and a final extension step at 72°C for 10 min. The identity of the *MAT* idiomorph (*MAT1-1* or *MAT1-2*) in each isolate was determined by visualizing the size of the PCR amplicons on a 1% agarose gel. Five of each putative *MAT1-1-1* and *MAT1-2-1* amplicons were sequenced at CAF to confirm amplification of the correct gene regions. The ratio of *MAT1-1*:*MAT1-2* for each population, as well as for the entire collection of isolates, was determined (Table 1). The null hypothesis was a 1:1 ratio of mating types and Pearson's Chi-Square test was calculated in R 3.6.0 (R Core Team, 2013) to determine whether the ratios significantly deviated from the null hypothesis. P-values were adjusted with the Benjamini-Hochberg (BH) correction method (Benjamini and Hochberg, 1995) for multiple testing.

The two described primer sets were also tested for the ability to amplify the targeted *MAT1* genes from four other economically important *Teratosphaeria* species including *T. eucalypti* (CMW54002; CMW54011), *T. gauchensis* (CBS117261; CBS117256), *T. pseudoeucalypti* (CMW51515; CMW51517), *T. viscidus* (CMW51323) and *T. zuluensis* (CBS119470; CMW7449). PCR reaction conditions were the same as those described above.

2.3 Attempt to induce a sexual state in culture

An attempt was made to induce the sexual state of *T. destructans* by pairing cultures of opposite mating type (*MAT1-1*: CMW13321 and CMW45686; *MAT1-2*: CMW45977 and CMW47615). Six different MEA-based growth media were used in these tests. These were MEA amended with (i) 3 g/L yeast extract (Oxiod, France), (ii) 100 g/L of dry, crushed *Eucalyptus* leaves, (iii) yeast extract and 100 g/L of dry, crushed *Eucalyptus* leaves, (iv) 6 g/L oak tannin (Enartis Tan Rouge, USA), (v) 6 g/L grape seed extract (Procudin, Value Added Life Health Products, South Africa) and (vi) 6 ml/L *Eucalyptus* oil (Alpha, South Africa). Additionally, a basal medium (Sati and Bisht, 2006) amended with 15 g of

bacteriological agar (Merck, South Africa) and either 10 g glucose (Merck, South Africa) or 3.3 g NH_4SO_4 (Sigma Aldrich, USA) was used to create nitrogen-deficient and carbon-deficient media, respectively.

Using two methods, isolates were paired in six possible combinations; four *MAT1-1*x*MAT1-2* and two negative control pairings (one *MAT1-1*x*MAT1-1* and one *MAT1-2*x*MAT1-2*). In the first method, two five mm diameter mycelial plugs were cut from the actively growing margins of three-week-old cultures on MEA+Y and placed adjacent to each other at the centre of a Petri dish. In the second method, approximately 15 mg of mycelium was scraped from the surface of cultures of the two test isolates, placed into a 2 mL Eppendorf tube containing 1.5 mL ddH_2O, briefly agitated with a vortex mixer and streaked out evenly over the surface of the agar in a Petri dish. Pairings were repeated three times on each of the growth media described above. All Petri dishes were incubated in the dark at 25°C for three months and monitored for a further two months at room temperature. Microscope slide mounts were made after three- and five months from mycelium at the zone between the paired cultures in order to determine whether sexual structures (ascomata and ascospores) had formed.

3. Results

3.1 Genome sequencing

The 27.15 Mb genome assembly for isolate CMW45661 consisted of 341 scaffolds (>500 bp) with an estimated base coverage of ~1 200X and a GC content of 54.9%. The largest scaffold was 962 915 bp; N50 = 330 664 bp and L50 = 28 scaffolds. The assembled genome of isolate CMW45982 was 26.71 Mb in size with ~370X average base coverage and a GC content of 51.7%. The largest of the 244 scaffolds (>500 bp) was 1.67 Mb; N50 = 615 530 bp and L50 = 14 scaffolds. Of the 1315 Ascomycota BUSCOs, 1284 single-copy and complete BUSCOs were identified in isolate CMW45661 and 1289 in isolate CMW45982. The two genomes were, therefore, estimated to be 97.8% and 98.1% complete, respectively.

3.2 Characterisation of the *T. destructans MAT1* locus

Multiple BLASTx results were obtained from all genomes, but only one region in each genome was sufficiently large (>300 bp) to be the *MAT1* locus. For isolate CMW45661, the reference *MAT1-1-1* protein sequence of *T. zuluensis* had a BLAST result of 359 bp on scaffold 37, with a pairwise identity of 78.8% (MN531144; *MAT1-1* accession of CMW45661). The *MAT1-2-1* reference sequence of *T. gauchensis* had BLAST results of 309 bp on scaffold 33 of isolate CMW45982 (77.1% identity) (MN531145; *MAT1-2* accession of CMW45982) and 376 bp on scaffold 374 of isolate CMW44962 (74.9% identity) (MN531146; *MAT1-2* accession of CMW44962). Alignment of each idiomorph against the respective *T.*

gauchensis and *T. zuluensis* idiomorphs indicated that the newly described *MAT1-1-10* and *MAT1-2-12* genes (Aylward et al., 2020) were also present in *T. destructans* (Fig. 1), with a pairwise identity of 65.9% and 61.8%, respectively.

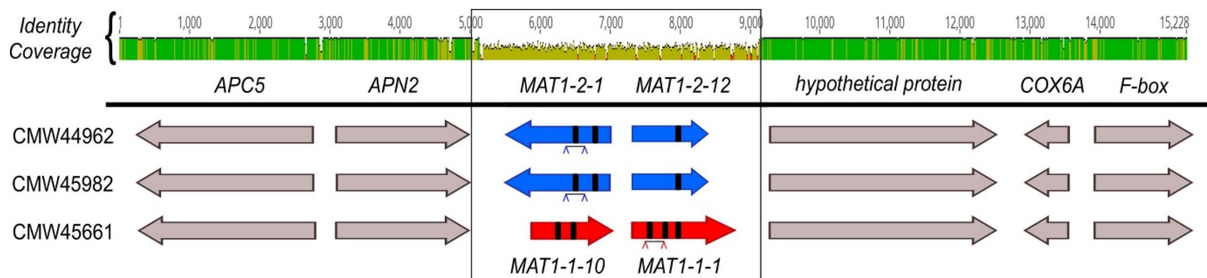

Figure 1. *MAT1* locus (boxed) and flanking regions of *Teratosphaeria destructans*. Alignment of the *MAT1-1* idiomorph of isolate CMW45661 (red arrows) and the *MAT1-2* idiomorphs of isolate CMW44962 and CMW45982 (blue arrows) with identity coverage between isolates. Primer pairs are indicated below (^----^) and introns indicated on top (■) of the genes of the *MAT1* locus. Primer pairs T_Ma1-F and T_Ma1-R (red brackets) bind within the *MAT1-1-1* gene and TdMAT2_1 and TdMAT2_2 (blue brackets) in the *MAT1-2-1* gene.

WebAUGUSTUS predicted an unusually large *MAT1-1-1* gene, with a coding sequence (CDS) of 2316 bp, in the genome of isolate CMW45661. This is similar to the initial prediction made for the *MAT1-1* idiomorph of *T. zuluensis* in which the *MAT1-1-1* and *MAT1-1-10* genes were merged (Aylward et al., 2020). A comparison between the *MAT1-1* idiomorphs of *T. destructans* and *T. zuluensis* confirmed that the large *MAT1-1-1* prediction represented two separate genes. The *MAT1-1* idiomorph of *T. destructans* thus consisted of *MAT1-1-1* and *MAT1-1-10* genes (Fig. 1). The predicted CDS of *MAT1-1-1* was 1404 bp, encoding a 362 amino acid protein and that of *MAT1-1-10* was 1140 bp, encoding a 312 amino acid protein. The *MAT1-2* locus consisted of the *MAT1-2-1* gene, containing an HMG-box domain, and a hypothetical *MAT1-2-12* gene (Fig. 1). *MAT1-2-1* had a 1302 bp predicted CDS, coding for a 433 amino acid protein. The 663 bp CDS predicted for *MAT1-2-12* codes for a 220 amino acid protein.

The MAT1-1-1 and MAT1-2-1 proteins of *T. destructans* were very similar to those of *T. zuluensis* and *T. gauchensis* (Fig. S1, Supplementary File 1) and homologous to the MAT proteins of distantly related ascomycete fungi (Supplementary Dataset 1). The homology of the *T. zuluensis* and *T. gauchensis MAT* genes to species within the Capnodiales was established by Aylward et al. (2020). The number and position of the exons predicted for

each gene and a comparison with the predictions of *T. gauchensis* and *T. zuluensis* are presented in Supplementary File 1.

3.2.1 Flanking regions

The assembly of the previously sequenced *T. destructans* (isolate CMW44962) genome was not as complete as that for isolate CMW45982. A section of the upstream flanking region in isolate CMW44962 was at the edge of a different scaffold and was identified by comparing the *MAT1* locus and flanking region in isolate CMW45982 with that in isolate CMW44962. In the flanking regions of the *MAT1* locus, WebAUGUSTUS consistently identified the same five ORFs in all of the *T. destructans* genomes. BLAST results identified ORFs commonly associated with the *MAT1* locus in the flanking regions, specifically *APC5* (anaphase-promoting complex) and *APN2* (DNA, apurinic or apyrimidinic, site lyase 2-like) upstream and *COX6a* (cytochrome c oxidase subunit 6A) downstream (Fig. 1). Both a hypothetical protein without a conserved domain as well as an ORF with an F-box domain were identified downstream (Fig. 1). Since *SLA2* (cytoskeleton assembly control) was absent from the flanking regions of the *MAT1* locus, a local BLASTx search was conducted for all three genomes against the *SLA2* protein sequence of *Zymoseptoria tritici* (XP_003847543). The *SLA2* gene was found on a different scaffold to the *MAT1* locus in all three *T. destructans* genomes.

3.2.2 Confirmation of species identity

Identification of isolates to species level was not possible based on ITS sequence data alone. Consequently, phylogenetic analyses were conducted using concatenated sequences of the ITS, TUB and EF-1α gene regions. The representative isolates formed a monophyletic clade including the ex-type isolate of *T. destructans*, with 78% bootstrap support (Fig. 2). They were consequently treated as *T. destructans* in all subsequent analyses.

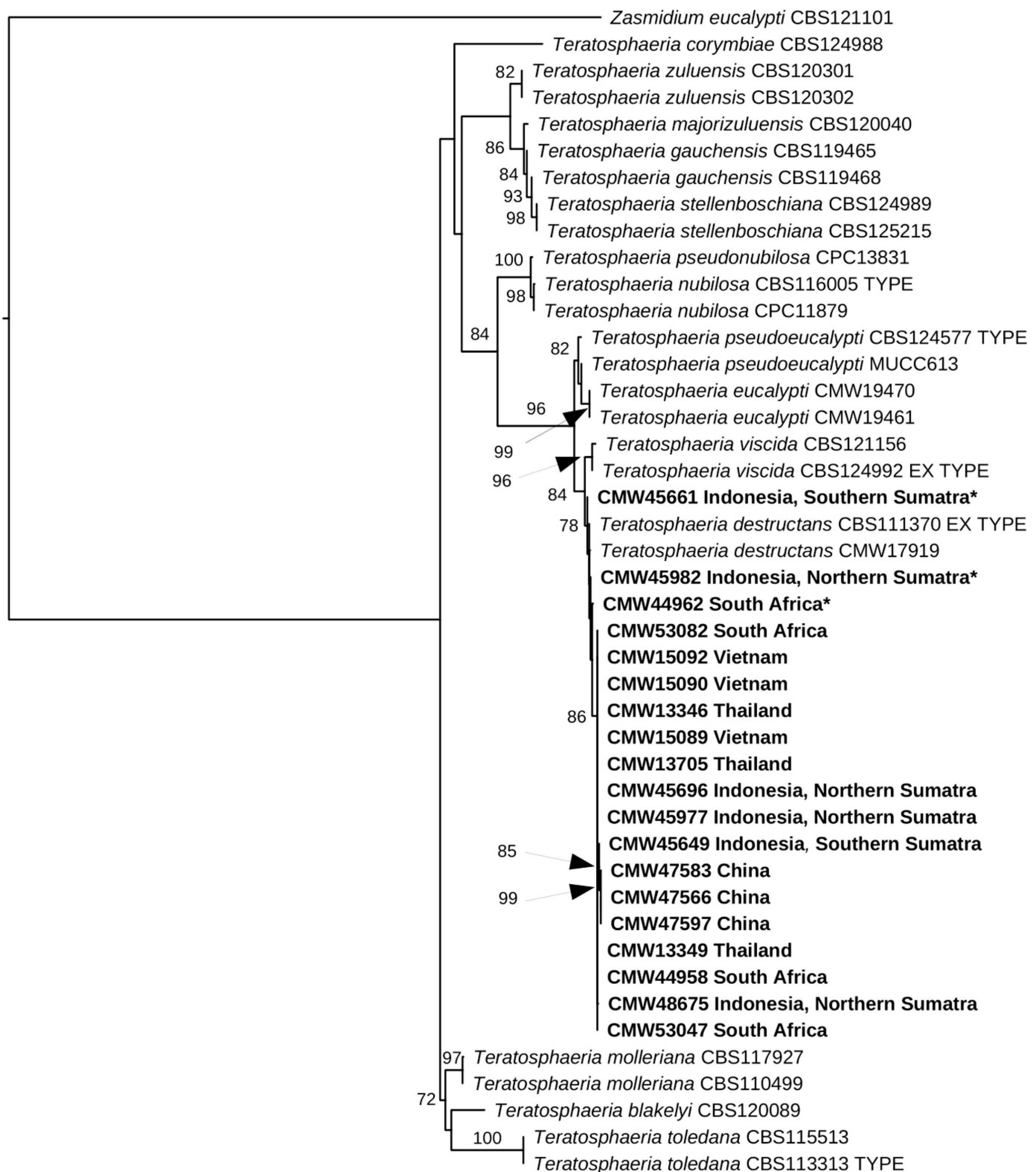

Figure 2. Maximum likelihood phylogenetic tree of *Teratosphaeria* species, based on concatenated ITS, β-tubulin and EF-1α sequence data. Bootstrap support of ≥70% is shown. Outgroup = *Zasmidium eucalypti*. Isolates sequenced in this study are indicated in bold and genome sequences are indicated with an asterisk (*).

3.2.3 Distribution of MAT idiomorphs

Aylward et al. (2020) developed two primer sets (T_Ma1-F; T_Ma1-R and T_Ma2_2 and T_Ma2_1) to identify the *MAT1* idiomorphs of *T. gauchensis* and *T. zuluensis*. The *MAT1-2* primer set (T_Ma2_2 and T_Ma2_1) (Aylward et al., 2020) did not amplify consistently in *T. destructans*, which necessitated the development of primers TdMAT2_2 (5′-CGAACGCCTTCATCATCTACAG-3′) and TdMAT2_1 (5′-GAGCTTGATCATCCTGGCTATC-3′). The new multiplex PCR (containing primers T_Ma1-F; T_Ma1-R T; TdMAT2_2; TdMAT2_1) amplified fragments of ~449 bp and ~247 bp for *MAT1-1* and *MAT1-2* isolates, respectively. Using these primers, it was possible to accurately identify the *MAT1* idiomorphs present in isolates of *T. destructans*, *T. eucalypti*, *T. gauchensis*, *T. pseudoeucalypti*, *T. zuluensis* as well as the *MAT1-1* idiomorph of *T. visidus* (no *MAT1-2* isolate is currently available) (Fig. 3). The sequences of the amplified *T. destructans MAT1* bands aligned with the associated *MAT1-1-1* alpha-box domain and *MAT1-2-1* HMG-box domain, respectively, with 100% identity.

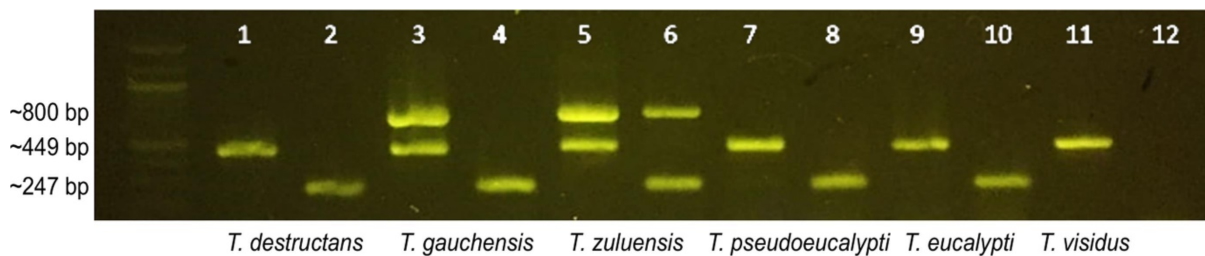

Figure 3. Agarose gel (1%) showing the PCR amplicons produced by a multiplex PCR using the *MAT1-1* (T_Ma1-F and T_Ma1-R) and *MAT1-2* (TdMAT2_2 and TdMAT2_1) primers pairs. Conserved regions were amplified in the *MAT1-1-1* (1, 3, 5, 7, 9, 11) and *MAT1-2-1* genes (2, 4, 6, 8, 10) of *Teratosphaeria destructans*, five related species and a negative control (12). The *MAT1-1* fragment is ~449 bp and the *MAT1-2* fragment ~247 bp in size.

An unexpected ~800 bp band was amplified in some of the multiplex PCR reactions, specifically in lanes 3 (*T. gauchensis*) as well as lanes 5 and 6 (*T. zuluensis*) (Fig. 3). This band was also observed in amplifications of some *T. destructans* isolates (data not shown). The ~800bp band from the multiplex PCR was extracted from the gels for the *T. destructans* isolates of both mating types (CMW45661 – *MAT1-1*; CMW44962 – *MAT1-2*) and sequenced with the TdMAT2_2 primer. The ~800 bp sequence aligned to the same area in all three *T. destructans* genomes, situated on a different scaffold than the *MAT1* locus. The binding site in the genome differed by one bp and two bp from primers TdMAT2_1 and TdMAT2_2, respectively, and explains why the additional band amplified in only in some *T. destructans* isolates. Irrespective of the ~800 bp band, these primer sets can be used to

accurately differentiate between the two *MAT1* idiomorphs in at least five *Teratosphaeria* species.

Isolates of both mating types were identified in most of the six distinct populations of *T. destructans* isolates, with a single dominant idiomorph occurring in each population (Fig. 4). The global population was skewed towards the presence of the *MAT1-2* idiomorph, with 64 isolates having the *MAT1-1* idiomorph and 135 having the *MAT1-2* idiomorph (Fig. 4). Populations from Thailand and North Sumatra (Indonesia) were dominated by the *MAT1-1* idiomorph, whereas populations from South Sumatra and China had more *MAT1-2* individuals (Fig. 4). The Vietnam isolates included only *MAT1-1* individuals, but this population contained only three isolates (Table 1). Only the *MAT1-2* idiomorph was found among isolates from South Africa, even though these were from two distinct geographic localities (Table 1).

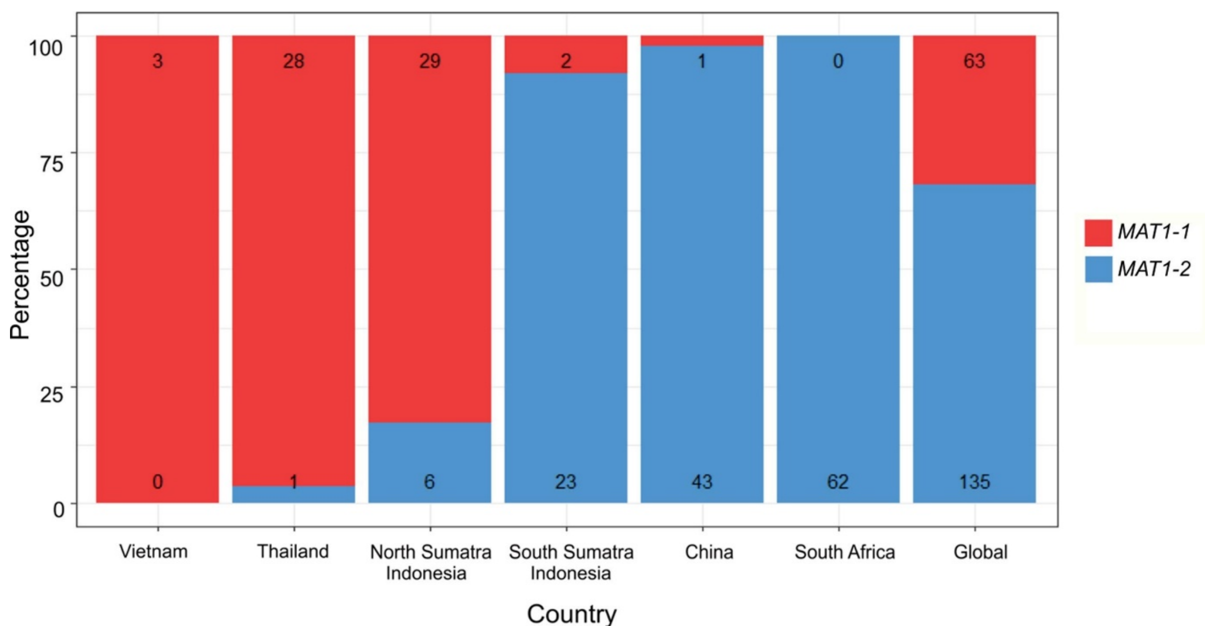

Figure 4. Bar graph showing the distribution of mating type idiomorphs (*MAT1-1*: *MAT1-2*) in six populations of *Teratosphaeria destructans*. All populations show a significant deviation ($P < 0.05$) from the expected 1:1 mating type distribution.

3.3 Attempt to induce a sexual state in culture

Isolates of *T. destructans* with different mating types and paired in culture grew well on all media other than on MEA amended with *Eucalyptus* oil (Supplementary File 2). However, sexual structures were not found on any of the media tested after three or five months of incubation.

4. Discussion

Results of this study have demonstrated that the important *Eucalyptus* leaf pathogen *T. destructans* has a heterothallic reproductive system. This shows that it has the genetic ability to undergo sexual reproduction, even though a sexual state has never been found on naturally infected tissues. This includes careful inspection of infected leaves in areas where strains of both mating types are now known to occur. Likewise, strains of *T. destructans* with opposite mating types were not able to undergo sexual reproduction when paired in culture.

The structure of the *T. destructans MAT1* locus and flanking regions was shown to be identical to that recently found in the *Eucalyptus* stem pathogens *T. gauchensis* and *T. zuluensis* (Aylward et al. 2020). The mating type genes (*MAT1-1-1* and *MAT1-2-1*) of these three species were also very similar. The hypothetical *MAT* proteins, MAT1-1-10 and MAT1-2-12, had low levels of nucleotide identity and varied in the number and position of exons in the different *Teratosphaeria* species considered in this study. This was consistent with the low pairwise nucleotide identity (30% to 40%) reported between *Teratosphaeria* and other species in the Capnodiales (Aylward et al. 2020). The location and orientation of the *MAT1-1-10* and *MAT1-2-12* in the *MAT1* locus were found to be conserved in *T. destructans*, *T. gauchensis* and *T. zuluensis*. These hypothetical proteins also occur in the *MAT1* locus or its flanking regions in other species of the Capnodiales such as *Zymoseptoria tritici*, *Pseudocercospora* spp. and *Cercospora beticola* (Arzanlou et al., 2010, Bolton et al., 2014), but their placement varies and requires further investigation (Aylward et al., 2020).

The flanking regions of the *T. destructans MAT1-1* and *MAT1-2* idiomorphs were almost identical (97.7% nucleotide identity) and contained five genes. Among these, *APN2*, *APC5* and *COX6a* protein coding genes are commonly associated with *MAT* loci in filamentous ascomycete fungi (Vaghefi et al., 2015, Debuchy et al., 2010). The *SLA2 gene*, which is also frequently found upstream of the *MAT1* locus in other filamentous ascomycetes (Debuchy and Turgeon, 2006, Li et al., 2013, Tsui et al., 2013) was absent from the flanking regions of the *T. destructans MAT1* locus. This is consistent with other species residing in the Capnodiales (*e.g.* Arzanlou et al., 2010). Aylward et al. (2020) showed that this gene has moved to a different location on the same chromosome in *T. gauchensis.*

Both mating types occurred in most of the *T. destructans* populations considered. The exceptions were for the South African and Vietnamese populations that harboured only one mating type. The Vietnamese population, however, consisted of a limited number of isolates and accurate conclusions cannot be made for this population. However, within-country populations of *T. destructans* harbouring isolates of both mating types, revealed a distinctly uneven distribution of these mating types. An even distribution would be expected to occur in natural populations undergoing sexual reproduction (Groenewald et al., 2006), but this does

not appear to be the case for the *T. destructans* populations sampled in this study. An uneven distribution of mating types could have resulted from a genetic bottleneck which is usually associated with introduced populations undergoing asexual reproduction (Taylor et al., 1999).

The origin of *T. destructans* remains unknown, but is thought to be an area in which *Eucalyptus* spp. are native, such as Australia or East Timor (Andjic et al., 2019). The populations of isolates examined in this study did not represent either of the hypothesised areas of origin and populations represents alien pathogen invasions. The skewed distribution of mating type in the sampled populations supports the view that these are all introduced populations (Groenewald et al., 2007). The presence of asexual structures of *T. destructans* on heavily diseased trees in plantations (Wingfield et al., 1996, Burgess et al., 2006) and the uneven distribution of mating type also suggests that the asexual cycle represents the primary mode of distribution and reproduction in introduced populations.

In populations where both *T. destructans* mating types occur, such as in Thailand, Indonesia (north and south Sumatra) and China, sexual reproduction would be genetically possible. If recombination does take place, this would lead to the emergence of unique individuals that may be adapted to a wider range of conditions (Billiard et al., 2011) and have the ability to infect newly deployed host genotypes. The apparent ability of *T. destructans* to overcome host resistance (M.J. Wingfield, unpublished) and the co-occurrence of both mating types in some populations suggest that recombination may already be taking place. A detailed population genetics study using polymorphic markers is required to better understand the possible role of a sexual cycle in *T. destructans.* The availability of three genome sequences, will facilitate development of such molecular markers.

The most recent outbreak of *T. destructans* has been in South Africa and this represents the only occurrence of the pathogen found outside South East Asia (Greyling et al., 2016). It was interesting that the South African populations of isolates, which were relatively large and from different geographic regions, represented only the *MAT1-2* mating type. The relatively recent appearance of this pathogen in plantations that are regularly monitored for disease (Greyling et al., 2016), suggests a single or at least a very limited number of introduction of this pathogen into South Africa. But it also implies that *T. destructans* has spread rapidly in the country, most likely from a single source.

Importation of contaminated seed has been shown to be the likely route of introduction for *T. zuluensis* infecting *E. grandis* (Jimu et al., 2016). Likewise, *T. destructans* could have entered South Africa with seed imported into the country as suggested by Greyling et al. (2016). *Eucalyptus* seed is regularly traded internationally, raising the possibility of new

introductions of *T. destructans*, including new genotypes representing the *MAT1-1* mating type.

Our attempts to induce the sexual state of *T. destructans* utilising a wide variety of media and isolates of different mating type were unsuccessful. Similar results commonly emerge from such studies (Bihon et al., 2014, Aylward et al., 2020, Nel et al., 2018) and they most likely reflect the complexity of environmental and biological conditions required for sexual reproduction to occur (Debuchy et al., 2010 O'Gorman et al., 2009). Pairing of isolates on living host tissue or under stressful conditions, such as low levels of oxygen and moisture (Ni et al., 2011), could potentially induce sexual reproduction and such tests might be considered in the future.

5. Conclusions

This study has shown that the important *Eucalyptus* pathogen *T. destructans*, has a heterothallic reproduction system and that it has the genetic means to undergo genetic outcrossing in areas of the world where both mating types co-occur. This has important implications for *Eucalyptus* breeding and selection and it highlights the relevance of quarantine measures based on genetic data (McTaggart et al., 2016, Wingfield et al., 2008) and not simply on pathogen names. A study of the genetic diversity of *T. destructans* representing a global collection of isolates would provide important additional insights into the possibility that cryptic sexual reproduction is occurring in some parts of the world. Such knowledge, including the information derived in the present study, will be valuable to the globally important *Eucalyptus* plantation industry.

Declaration of competing interests

The authors declare that they have no competing interests.

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