Diagnostic markers for *Teratosphaeria destructans* and closely related species

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Abstract

Teratosphaeria foliar pathogens cause leaf and shoot blight on *Eucalyptus* trees in many parts of the world. Among them, *T. destructans* is one of the most aggressive pathogens causing defoliation of young *Eucalyptus* trees in tropic regions. Identification of *T. destructans* to species level is currently not possible based solely on morphological characteristics or ITS sequence data. The aim of this study was to assess *T. destructans* microsatellites and a newly developed *T. epicoccoides* microsatellite as a diagnostic tool to differentiate among *T. destructans* and several closely related foliar pathogens. Based on the number of markers that amplified, the *T. destructans* microsatellites allowed for the differentiation of *T. destructans*, *T. epicoccoides, T. eucalypti, T. nubilosa, T. pseudoeucalypti* and *T. viscidus*. These microsatellites provide a rapid and cost-effective diagnostic tool that will enable the identification of a large number of isolates important in disease surveys and inoculation trials.

Keywords: Population genetics, *Eucalyptus*, Foliar pathogens

1 Introduction

The most prevalent leaf and shoot pathogens of *Eucalyptus* L'Hér. trees reside in the genus *Teratosphaeria* Syd. & P. Syd. Many species are leaf associates or pathogens of minor importance, where they typically affect older leaves and cause limited damage (Andjic et al., 2019). However, a closely related group of species, including *T. destructans* (M.J. Wingf. & Crous) M.J. Wingf. & Crous, are aggressive pathogens that cause defoliation and die-back of young plantation trees, resulting in loss to plantation production (Andjic et al., 2019).

Morphological characteristics and initial disease symptoms of both the aggressive and minor *Teratosphaeria* pathogens are very similar (Hunter et al., 2011). Accurate identification, therefore, relies entirely on DNA-based molecular techniques and rapid identification is critical for effective intervention and disease management. However, a substantial challenge lies in the fact that *Teratosphaeria* species grow slowly in culture, limiting their effective identification.

Currently used identification protocols for *Teratosphaeria* species are based on the commonly applied fungal barcoding genes and regions e.g., ribosomal RNA Internal Transcriber Spacer (ITS), partial β-tubulin (TUB) and elongation factor 1-alpha (EF-1α). In many cases, a combination of these regions is required to differentiate species, such as *T. destructans* and *T. viscidus* (Havenga et al., 2020b). Due to the time and cost involved, large-scale studies typically sequence only a subset of isolates (e.g. Andjic et al., 2019; Havenga et al., 2020b; Hunter et al., 2011), which may lead to inaccuracies where morphologically similar species have been misidentified. For disease surveys and inoculation studies, a diagnostic assay with the potential of being rapid, cost-effective and high-throughput is needed.

Species-specific genetic markers applied in population studies can provide an additional means of verifying species identity. For example, we recently developed 12 polymorphic microsatellite markers to study *T. destructans* (Havenga et al., 2020a). In the process, 41 isolates utilised in the initial dataset were found to have been identified incorrectly. This provides an example where microsatellite markers are ideal for the rapid screening of isolates due to their high amplification success from lower quality or quantity DNA and to the fact that they can be used to amplify loci in closely related species (Hodel et al., 2016).

The aim of this study was to assess whether the previously developed *T. destructans* microsatellite markers could differentiate among *Teratosphaeria* species, including the closely related *T. destructans*, *T. epicoccoides* (Cooke & Massee) Rossman & W.C. Allen, *T. eucalypti* (Cooke & Massee) Crous, *T. nubilosa* (Cooke) Crous & U. Braun (CMW3282), *T. pseudoeucalypti* Andjic & T. Burgess and *T. viscidus* (Andjic, P.A. Barber & T.I. Burgess) Andjic, P.A. Barber & T.I. Burgess.

2 Materials and Methods

Isolates of *T. destructans* and five closely related species were sourced from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria (Table 1). These included *T. epicoccoides* (CMW45681; CMW45690; CMW53049), *T. eucalypti* (CMW53992; CMW53993; CMW53996), *T. nubilosa* (CMW3282), the three *T. pseudoeucalypti* isolates (CMW49159; CMW49161; CMW51515) with sequenced genomes, *T. viscidus* (CMW51323; CMW51324; CMW51325) and two *T. destructans* isolates (CMW44962; CMW45661) for which the genomes have been sequenced. Isolates were cultured at 25°C and purified by single conidial transfer on Malt Extract Agar (MEA, Merck) amended with 3 g of yeast extract (MEA+Y; Oxoid). DNA extraction, sequencing and phylogenetic analyses followed previously described methods (Havenga et al., 2020b). The

Table 1. Amplification of microsatellite markers across six *Teratosphaeria* species

Isolate number[a]	*Teratosphaeria* species	Locus[b]												
		A	B	C	D	E	Epi[c]	F	G	H	I	J	K	L
		Panel 1						Panel 2						
CMW44962	*T. destructans*	195	387	235	298	312	-	321	413	320	432	372	441	402
CMW45661	*T. destructans*	198	384	233	298	315	-	324	415	323	435	374	438	399
CMW48704	*T. epicoccoides*	-	-	-	-	-	240	-	-	-	-	-	-	-
CMW45689	*T. epicoccoides*	-	-	-	-	-	240	-	-	-	-	-	-	-
CMW53049	*T. epicoccoides*	-	-	-	-	-	240	-	-	-	-	-	-	-
CMW53992	*T. eucalypti*	222	375	221	298	297	-	309	431	309	-	376	420	378
CMW53994	*T. eucalypti*	222	375	221	298	297	-	309	431	309	-	376	420	378
CMW53996	*T. eucalypti*	222	375	221	298	297	-	309	431	309	-	376	420	378
CMW3282	*T. nubilosa*[d]	-	-	215	-	-	-	-	-	-	-	-	-	381
CMW49159	*T. pseudoeucalypti*	225	354	223	298	297	-	309	439	-	411	374	420	375
CMW49161	*T. pseudoeucalypti*	225	354	223	298	297	-	309	439	-	411	374	420	375
CMW51515	*T. pseudoeucalypti*	225	354	223	298	297	-	309	439	-	411	374	420	375
CMW51323	*T. viscidus*	192	393	231	316	315	-	327	417	318	441	362	-	393
CMW51324	*T. viscidus*	192	393	231	316	315	-	327	417	318	441	362	-	393
CMW51325	*T. viscidus*	192	393	231	316	315	-	327	417	318	441	362	-	393

[a] CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria.

[b] Loci indicated according to amplicon sizes in bp.

[c] *Teratosphaeria epicoccoides* microsatellite marker developed in this study.

[d] The only isolate available.

species identity of all isolates was confirmed by constructing a Maximum Likelihood (ML) phylogeny based on sequences of the ITS and β-tubulin genes. GenBank accession numbers are presented in Supplementary Table S1.

All *Teratosphaeria* isolates were genotyped with 12 microsatellite markers in two panels, following the methods developed for *T. destructans* (Havenga et al., 2020a). An additional microsatellite marker (MW045219) had to be designed using the preliminary assembled *T. epicoccoides* CMW31933 genome (BioProject: PRJNA665608) following the methods described by Havenga et al., (2020a). Microsatellite markers and panel designs used to genotype isolates are presented in Table S2. The two *T. destructans* isolates (CMW44962; CMW45661) with sequenced genomes were used as positive controls and ddH_2O as a negative control. Data scoring and allele calling were performed in Geneious R11 (Biomatters Ltd.). The *T. destructans* microsatellite markers (MN991185-MN991196) were identified in the *T. epicoccoides*, *T. nubilosa* (JAAEJT000000000; isolate CBS116005) and *T. pseudoeucalypti* (JABASB000000000, JABBMY000000000 and JABBMZ000000000; isolates CMW49159, CMW49161 and CMW51515) genomes using local BLASTn searches in Geneious.

3 Results

The combined ITS and β-tubulin phylogeny confirmed the identity of all species (Fig. 1). None of the 12 *T. destructans* microsatellite markers produced amplicons for *T. epicoccoides*, two produced amplicons for the single available *T. nubilosa* isolate and all but one produced amplicons for *T. eucalypti*, *T. pseudoeucalypti* and *T. viscidus* (Table 1). The *T. epicoccoides* microsatellite marker only produced amplicons in *T. epicoccoides* isolates and the *T. destructans* loci were not identified in the *T. epicoccoides* genome. Analysis of the *T. nubilosa* genome sequence (Haridas et al., 2020) showed differences in the primer-binding sites of the two loci that produced amplicons as well as six other loci. The microsatellite repeat region

was also missing in these loci. The remaining four loci and the *T. epicoccoides* locus could not be identified in the genome (Table S3).

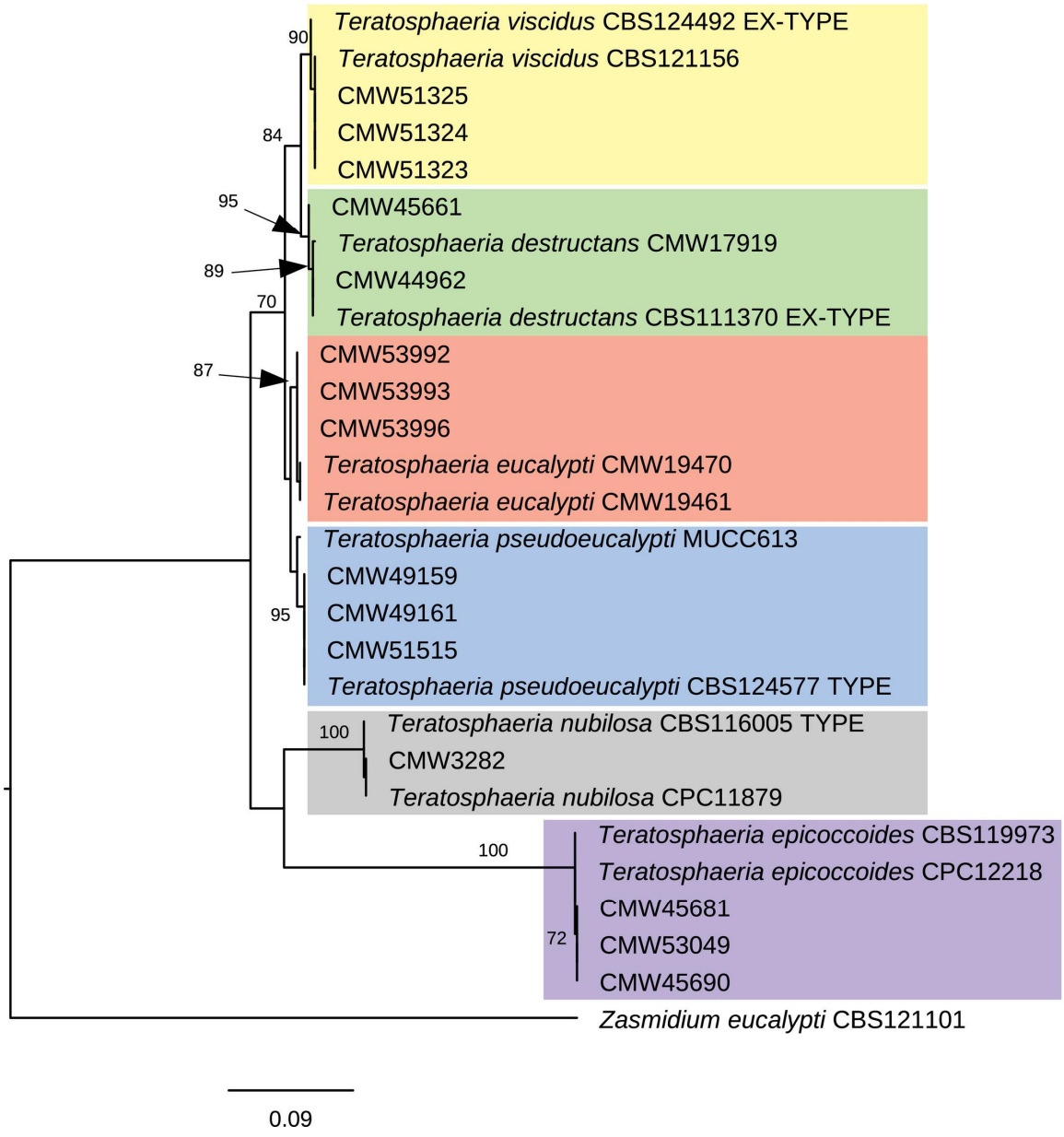

FIGURE 1. Maximum likelihood phylogenetic tree of *Teratosphaeria* species, based on ITS and β-tubulin sequence data. Bootstrap support of ≥ 70% is shown. Outgroup = *Zasmidium eucalypti*.

Five of the markers that produced amplicons for the *T. pseudoeucalypti* isolates had sequence differences in their primer-binding sites, whereas the primer binding sites of six loci were

identical to that observed in *T. destructans*. The primer sequences of the remaining locus H, that also did not produce an amplicon, were situated on different scaffolds in all three genomes. The *T. epicoccoides* locus was not identified in the *T. pseudoeucalypti* genomes (Table S3). Microsatellite loci were monomorphic among isolates of *T. epicoccoides, T. eucalypti, T. pseudoeucalypti* and *T. viscidus*.

4 Discussion

Accurate and rapid identification of *T. destructans* isolates is currently not possible based on morphology or ITS data (Andjic et al., 2019; Havenga et al., 2020b; Hunter et al., 2011). In this study, microsatellite markers have been identified and provided as an alternative high-throughput and cost-effective diagnostic tool. The *T. destructans* microsatellites can accurately distinguish between *T. destructans*, the minor pathogens including *T. epicoccoides* and *T. viscidus* as well as among the aggressive pathogens *T. eucalypti*, *T. pseudoeucalypti* and *T. nubilosa*.

Identification of *T. nubilosa* was based only on a single isolate and a greater number of isolates would strengthen our results. However, *T. nubilosa* occurs in temperate regions and produces only sexual ascospores in the field (Hunter, Crous, Carnegie, & Wingfield, 2009). It can thus reliably be distinguished based on morphology from the other *Teratosphaeria* species that have dark septated asexual conidia.

Compared to traditional sequencing, the microsatellite-based assay developed in this study provides a rapid and cost-effective means to verify the identity of *Teratosphaeria* species. Another advantage of these microsatellite markers is that analysis can be performed with very small amounts of DNA (~20 ng), as illustrated by Hodel et al. (2016), accelerating the process because less fungal material is needed.

In countries where *T. destructans* already occurs (Andjic et al., 2019), the microsatellite markers developed in this study will be useful to verify the identity of isolates collected during field surveys. Furthermore, in clonal *Eucalyptus* forestry, such as is practiced in many parts of the world, these markers can be applied in population biology studies to understand the disease tolerance of different commercial *Eucalyptus* clones to prevalent pathogen genotypes in the area. They will also be important in monitoring for new *T. destructans* introductions and quarantine procedures.

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