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High-resolution melting curve analysis: A detection assay for *Ceratocystis eucalypticola* and *C. manginecans* in infected *Eucalyptus*

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ABSTRACT

Eucalyptus spp. in plantations are negatively affected by canker and wilt diseases caused by several species of *Ceratocystis*, particularly those in the Latin American Clade (LAC). *Ceratocystis eucalypticola* and *Ceratocystis manginecans* are of particular concern where disease epidemics are reported globally, with recent outbreaks emerging in South African and Indonesian *Eucalyptus* plantations. Consequently, a rapid screening protocol is required for these pathogens. In this study, a high-resolution melting curve analysis (HRMA) was developed to detect *C. eucalypticola* and *C. manginecans* that bypasses time-consuming isolation and post-PCR procedures. Primers targeting a 172 bp region of the cerato-platanin (CP) gene were designed. Using these primers, the accuracy of HRMA to detect and distinguish between these two LAC species was assessed using pure fungal DNA, and DNA extracted directly from *Eucalyptus* samples naturally infected with *C. eucalypticola*. The assay accurately detected the presence of *C. eucalypticola* and *C. manginecans* and quantifies their DNA, both from cultures, and directly from wood samples. HRMA further differentiated these two species from all other tested LAC individuals. This assay was also able to detect the presence of all the tested LAC species and distinguish seven of these, including *C. fimbriata,* to species level. *Ceratocystis polyconidia* was the only non-LAC off-target species detected. Based on these results, the developed assay can be used to rapidly identify *C. eucalypticola* and *C. manginecans* directly from infected plant material or fungal cultures, with the potential to also screen for several other LAC species.

1. Introduction

As the land area planted to *Eucalyptus* species has increased to meet the global commercial demand for a variety of wood and paper products, there has been a concomitant increase in pathogens and pests affecting these trees (Ennos, 2015; Hurley et al., 2016; Wingfield, 1999). One such group of pathogens are species of *Ceratocystis* (Harrington et al., 2023; Roux and Wingfield, 2009). Symptoms caused by *Ceratocystis* species on *Eucalyptus* include root rot, cankers, vascular staining and wilt, and severe infections can result in tree mortality (Ferreira et al., 2010; Roux et al., 2020; Wingfield et al., 2023).

Of the ten species of *Ceratocystis* reported from various *Eucalyptus* spp. globally, seven reside in the Latin American Clade (LAC) (Barnes et al., 2003a, 2003b; Harrington et al., 2023; Liu et al., 2015, 2021; Rodas et al., 2008; van Wyk et al., 2007, 2009, 2011, 2012). *Ceratocystis manginecans* and *Ceratocystis eucalypicola* are two phylogenetically closely related species in the LAC that are of particular concern due to new outbreaks of Ceratocystis wilt and canker disease (CWCD) reported in South Africa and Indonesia (Hlongwane 2021; Indrayadi et al., 2023; Roux et al., 2020). These new disease problems have driven the need for rapid screening assays to effectively identify *Ceratocystis* species, including those that infect *Eucalyptus*.

No rapid screening assays that are specific for *C. manginecans* and *C. eucalypticola* are available. Several species-specific probe-based PCR assays have, however, been developed to detect other pathogenic *Ceratocystis* spp. such as *C. platani* (Luchi et al., 2013; Lumia et al., 2018; Pilotti et al., 2012), and *C. lukuohia* and *C. huliohia* (Heller et al., 2023; Heller and Keith, 2018). In addition, Dharmaraj et al. (2022) developed a probe-based TaqMan qPCR assay using the ITS gene region for the broad detection of *Ceratocystis* spp. from environmental samples.

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Following their generic qPCR assay, a species-level identification can be achieved through amplification with additional PCR primers and sequence-based methods (Dharmaraj et al., 2022).

To circumvent a need for downstream PCR assays to identify the species and reduce the cost of probe-based PCR assays, High-Resolution Melting Analysis (HRMA) performed on primer-based PCR assay amplicons can be conducted (Gundry et al., 2003; Wittwer et al., 2003; Zambounis et al., 2015). HRMA is a post-PCR method that exploits the differences in the sequence-related melting profiles of PCR products (Gundry et al., 2003; Wittwer et al., 2003; Zambounis et al., 2015). HRMA monitors the release of intercalated fluorescent dye as the double-stranded DNA dissociates during an applied temperature gradient, and can distinguish single nucleotide differences (Gundry et al., 2003; Wittwer et al., 2003). HRMA has been used effectively to diagnose several fungal plant diseases; for example to differentiate *Diplodia sapinea* from other closely related species in Austrian pine (Luchi et al., 2011) and *Fusarium formae speciales* in the *Fusarium oxysporum* species complex (Ganopoulos et al., 2012; Zambounis et al., 2015).

The efficacy of HRMA to distinguish between variants or species depends on the selected gene region used for PCR amplification. The ITS gene region, particularly in *Ceratocystis* species in the LAC, is suboptimal due to the presence of multiple ITS Types in some single isolates, leading to varied melt curve profiles. For instance, *C. manginecans* ITS Types 1 and 2 were initially treated as separate species before being reduced to synonymy using the genealogical concordance phylogenetic species recognition (GCPSR) approach (Fourie et al., 2015; Tarigan et al., 2011; Taylor et al., 2000; van Wyk et al., 2007). As a result, the ITS gene region alone cannot accurately resolve several species in the LAC (Fourie et al., 2015; Kanzi et al., 2020; Naidoo et al., 2013). An alternative gene region that has shown promise in probe-based PCR assays to detect *Ceratocystis* species is the cerato-platanin (cp) gene, which occurs exclusively in filamentous fungi (Heller and Keith, 2018; Luchi et al., 2013; Lumia et al., 2018; Pilotti et al., 2012). The presence of the cp gene in all LAC species, coupled with its distinct nucleotide sequences among species, makes it an ideal target for HRMA amplification.

The aims of this study were to develop a rapid, cost-effective primer-based qPCR-HRMA assay to detect *C. manginecans* and *C. eucalypticola* infecting *Eucalyptus* spp. A second aim was to test its potential to detect several other *Ceratocystis* spp. in the LAC. To assess the reliability of the assay, the following samples were tested to determine the efficacy of this assay to detect *Ceratocystis* directly from infected plant material collected in the field: i) DNA extracted from pure cultures of *C. manginecans* and *C. eucalypticola,* DNA from another 14 of the 21 available species in the LAC, and DNA from several other phylogenetically closely related fungi; ii) a DNA mixture of *Eucalyptus* DNA spiked with DNA of *C. manginecans* at a known concentration, and iii) DNA obtained from *Eucalyptus* naturally infected with *C. eucalypticola*.

2. Material and methods

2.1. Fungal isolates and DNA extractions/acquisition

Isolates representing both *C. manginecans* ITS Types, *C. eucalypticola,* and fourteen *Ceratocystis* species within the Latin American Clade (LAC) were retrieved from the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa (Table 1). Isolates were grown on 2 % Malt Extract Agar (MEA: 20 g/L malt extract from Biolab and 20 g/L agar from Difco) for 10 days at 25 °C. Genomic DNA was extracted from the mycelia of all fungal isolates using the Zymo Quick-DNA Fungal/Bacterial Kit (Qiagen, Germany), following the manufacturer's instructions. In addition, DNA from 31 isolates representing species in three of the four *Ceratocystis* clades and 12 of the 15 genera of the Ceratocystidaceae (de Beer et al., 2014; Mayers et al., 2020), were sourced from collaborators at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa (Table 1). A Nanodrop ND_1000 (Nanodrop, Wilmington, DE) and Qubit®2.0 Fluorometer (ThermoFisher Scientific) was used to assess DNA quality and quantity, respectively. An aliquot of the extracted DNA was also subjected to AGE to assess the integrity of the extracted DNA.

2.2. qPCR and HRMA development

2.2.1. Primer design

The single copy cerato-platanin gene (cp) (562bp) was amplified with a primer set CP-F (5′ CTTCAATGCCAGACTACT 3′) and CP-R (5′ TCTCTTTTCATACTTCCACC 3′) (Fourie 2014), in sixteen isolates available in the LAC (Table 1). The PCR reaction mixture consisted of 2.5 μL 5 × MyTaq buffer (Bioline, London, UK), 0.25 μL MyTaq DNA polymerases (Bioline), 1 μL DNA template, 0.5 μL of each primer (10 mM), and 8.25 μL of sterile deionized water, for a 13-μL total reaction mixture. The PCR cycler program consisted of 95 °C for 5 min, 10 cycles of 95 °C for 30 s, 51 °C for 45 s, 72 °C for 90 s, another 30 cycles of 95 °C for 30 s, 56 °C for 45 s and 72 °C for 90 s (5 s increase per cycle at 72 °C) with a final step at 72 °C for 10 min. PCR amplicons were visualised using AGE and purified using ExoSAP-IT PCR Product Clean-up Reagent (Thermo Fisher Scientific). Cleaned amplicons were sequenced in both directions using the same primers used for the PCR amplification, using the BigDye terminator sequencing kit 3.1 (Applied Biosystems, Forster City, California). The thermal cycling conditions included 25 cycles of 10 s at 96 °C, 5 s at 51 °C, and 4 min at 60 °C. Sequences were obtained by running samples on an ABI PRISM 3100 DNA sequencer (Applied Biosystems).

The forward and reverse sequencing reads were assembled into contigs using CLC Bio Main Workbench 6 (CLC Bio, www.clcbio.com), aligned, and then manually inspected in MEGA v. 7 (Kumar et al., 2016), using the inbuilt MUSCLE alignment software (Edgar, 2004). The alignment was screened for potential regions that were 200bp or less and varied among the *Ceratocystis* species in the LAC. A primer pair targeting a 172bp shorter region of the cp gene was designed using Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). This region was then tested *in silico* for specificity with Primer Blast analysis on GenBank (http://www. ncbi.nlm.nih.gov), and for secondary structures using the online Multiple Primer Analyzer (Thermo Fisher Scientific, https://www.thermofisher.com/id/en/home.html). The primers were screened against the *C. manginecans* (CMW46461) genome (Fourie et al., 2019) for off-target amplification.

The efficacy of the designed primers, and the validation of the targeted amplicon to possibly differentiate between species in the LAC using the High Resolution Melting curve Analysis (HRMA), was tested *in silico* using the online Melting Curve Predictions Software, uMELT Quartz (https://dna-utah.org/umelt/quartz/), with default settings (Dwight et al., 2011).

2.2.2. Phylogenetic analysis

The designed primer pair (cerato-platanin region (CP.RE)) targeting a shorter region of the cp gene was synthesized by WhiteSci (https://www.whitesci.co.za/IDT/20portal.html). Using this primer pair, the shorter targeted CP.RE gene region was tested for amplification in *C. maginecans* (both ITS Types), *C. eucalypticola* and the other 14 LAC species (Table 1). PCR and sequencing reactions were performed as described above, with the annealing temperatures adjusted to 57 °C. The forward and reverse sequencing reads were assembled into contigs using CLC Bio Main Workbench 6, and the consensus sequences were aligned using the inbuilt MUSCLE alignment software in MEGA v. 7. The resulting alignment was used to construct a maximum likelihood (ML) phylogenetic tree using raxmlGUI 2.0 (Edler et al., 2021), with 1000 bootstrap replicates providing statistical support for the branches. Midpoint rooting of the trees were done.

Table 1
Isolates in the Ceratocystidaceae used in this study for qPCR and HRMA setup.

Species in Ceratocystidaceae	CMW	qPCR detection	HRMc lustering	NCBI accession No.	Country	Host	Reference
Ceratocystis: Latin American Clade (LAC)[c]							
C. adelpha	14809	+	1	PP297258	Ecuador	*Theobroma cacao*	Crous et al. (2015)
C. alfenasii[b]	–	–	–	–	Brazil	*Actinidia deliciosa*	Harrington et al. (2023)
C. atlantica[b]	–	–	–	–	Brazil	*Colocasia esculenta*	Harrington et al. (2023)
C. cacaofunesta	26375	+	5	PP297265	Brazil	*Theobromae cacao*	Engelbrecht and Harrington (2005)
C. colombiana	5751	+	6	PP297267	Colombia	*Coffea arabica*	van Wyk et al. (2010)
C. costaricensis[b]	–	–	–	–	Costa Rica	*Coffea arabica*	Harrington et al. (2023)
C. cubensis[b]	–	–	–	–	Cuba	*Spathodea campanulata*	Harrington et al. (2023)
C. curvata	22442	+	1	PP297261	Ecuador	*Eucalyptus deglupta*	van Wyk et al. (2011a)
C. diversiconidia[a]	22445	+	8	PP297262	Ecuador	*Terminalia ivorensis*	van Wyk et al. (2011a)
C. ecuadoriana[a]	22092	+	9	PP297260	Ecuador	*Eucalyptus deglupta*	van Wyk et al. (2011a)
C. eucalypticola	11536	+	2	PP297253	South Africa	*Eucalyptus* sp.	van Wyk et al. (2012)
C. fimbriata	14799	+	4	PP297256	USA	*Ipomoea batatas*	Halsted (1890)
C. fimbriatomima	24174	+	1	PP297264	Venezuela	*Eucalyptus* hybrid	van Wyk et al. (2009b)
C. lukuohia	44102	+	7	PP297266	USA	*Metrosideros polymorpha*	Barnes et al. (2018)
C. mangicola[a]	14797	+	1	PP297255	Brazil	*Mangifera indica*	van Wyk et al. (2011b)
***C. manginecans* (ITS Type 1)**	13851	+	1	PP297254	Oman & Pakistan	*Mangifera indica*	van Wyk et al. (2007a); Fourie et al. (2015)
***C. manginecans* (ITS Type 2)**	22563	+	2	PP297263	Indonesia	*Acacia mangium*	Tarigan et al. (2011); Fourie et al. (2016)
***C. manginecans* (ITS Type 2)**	56757	+	2	–	Malaysia	*Eucalyptus* sp.	Current study
***C. manginecans* (ITS Type 2)**	56758	+	2	–	Malaysia	*Eucalyptus* sp.	Current study
C. mangivora	15052	+	1	PP297259	Brazil	*Mangifera indica*	van Wyk et al. (2011b)
C. neglecta[a]	11284	+	1	PP297252	Colombia	*Eucalyptus grandis*	Rodas et al. (2008)
C. papillata	10844	+	3	PP297251	Colombia	*Citrus* × *Tangelo* hybrid	van Wyk et al. (2010)
C. platani	14802	+	1	PP297257	USA	*Platanus occidentalis*	Engelbrecht and Harrington (2005)
C. xanthosomatis[b]	–	–	–	–	USA	*Xanthosoma sagittifolium*	Harrington et al. (2023)
Ceratocystis: Asian-Australian Clade (AAC)							
C. atrox	19385	–	N/A	–	Australia	*Eucalyptus grandis*	van Wyk et al. (2007b)
C. corymbiicola	29120	–	N/A	–	Australia	*Corymbia variegata*	Kamgan et al. (2008)
C. huliohia	47149	–	N/A	–	USA	*Metrosideros polymorpha*	Barnes et al. (2018)
C. larium	25434	–	N/A	–	Indonesia	*Styrax benzoin*	van Wyk et al. (2007ab)
C. obpyriformis	23806	–	N/A	–	South Africa	*Acacia mearnsii*	Heath et al. (2009a)
C. pirillifomis	6579	–	N/A	–	Australia	*Eucalyptus nitens*	Barnes et al. (2003a); Heath et al. (2009a); Nkuekam et al. (2013)
C. polyconidia	23809	+	N/A	–	South Africa	*Acacia mearnsii*	Heath et al. (2009a)
Ceratocystis: North American Clade (NAC)							
C. harringtonii (=*C. populicola*)	27004	–	N/A	–	Canada	*Populus tremuloides*	Johnson et al. (2005); de Beer et al. (2014)
C. smalleyi	14800	–	N/A	–	USA	*Carya cordiformis*	Johnson et al. (2005)
Huntiella							
Huntiella ani	44684	–	N/A		China	*Eucalyptus* sp.	Liu et al. (2020)
H. bellula	49312	–	N/A	–	China	*Eucalyptus* sp.	Liu et al. (2020)
H. eucalypti	44692	–	N/A	–	China	*Eucalyptus* sp.	Liu et al. (2020)
H. fabiensis	44370	–	N/A	–	China	*Eucalyptus* sp.	Liu et al. (2020)
H. fecunda	49301	–	N/A	–	China	*Eucalyptus* sp.	Liu et al. (2020)
H. glaber	43436	–	N/A	–	China	*Eucalyptus* sp.	Liu et al. (2020)
H. inaequabilis	44372	–	N/A	–	China	*Eucalyptus* sp.	Liu et al. (2020)
H. meiensis	44374	–	N/A	–	China	*Eucalyptus* sp.	Liu et al. (2020)
H. moniliformis	10134	–	N/A	–	Ecuador	*Shizolobium parahyba*	van Wyk et al. (2006)
H. moniliformopsis	9986	–	N/A	–	Australia	*Eucalyptus* sp.	Yuan and Mohammed (2002)
H. tyalla	28920	–	N/A	–	Australia	*Eucalyptus grandis*	Kamgan et al. (2012)
H. abstrusa	21092	–	N/A	–	Indonesia	*Eucalyptus* sp.	Marin-Felix et al. (2019)
H. savannah	17300	–	N/A	–	South Africa	*Acacia nigrescens*	Kamgan et al. (2008)
Davidsoniella							
Davidsoniella eucalypti	3254	–	N/A	–	Australia	*Eucalyptus sieberi*	de Beer et al. (2014)
Endoconidiophora							
Endoconidiophora polonica	CMW7750	–	N/A	–	Norway	*Picea abies*	Christiansen and Solheim (1990)
Berkeleyomyces							
Berkeleyomyces basicola	49352	–	N/A	–	Netherlands	*Betula* sp.	Nel et al. (2018)
B. rouxiae	7625	–	N/A	–	South Africa	*Cichorium intybus*	Nel et al. (2018)
Chalaropsis							
Chalaropsis thielavioides	22738	–	N/A	–	Italy	*Lupinus albus*	Nag Raj and Kendrick (1975)
Bretziella							

(*continued on next page*)

Table 1 (*continued*)

Species in Ceratocystidaceae	CMW	qPCR detection	HRMc lustering	NCBI accession No.	Country	Host	Reference
Bretziella fagacearum	2656	–	N/A	–	USA	*Quercus rubra*	de Beer et al. (2017)
Ambrosiella							
Ambrosiella roeperi	55800	–	N/A	–	South Africa	*Xylosandrus crassiusculus*	Harrington et al. (2014)
Meredithiella							
Meredithiella norris	139737	–	N/A	–	USA	*Corthylus punctatissimus*	Mayers et al. (2018)
Toshionella							
Toshionella taiwanesis	141494	–	N/A	–	Taiwan	*Scolytoplatypus pubescens*	Mayers et al. (2020)

[a] Latin American Clade (LAC) species in which cloning was not possible.
[b] LAC species not available for testing at the time of the study.
[c] LAC species in bold used for HRMA testing.

2.3. qPCR and HRMA validation

2.3.1. Cloning

For downstream absolute quantification of the amount of *Ceratocystis* present in unknown samples, using target amplicon copy number, cleaned DNA fragments of the shorter targeted CP.RE gene region from 12 *Ceratocystis* spp. in the LAC were cloned into the pGEM-T easy vector (Promega) according to the manufacturer's instructions. Cloning of four of the LAC species (namely *C. mangicola, C. neglecta, C. diversiconidia* and *C. ecuadoriana*) was not performed due to limited DNA availability. Cloned products were sequenced as described above using primers M13F and M13R to confirm that the detected fragment was from the targeted *Ceratocystis* species. Plasmid DNA with correct inserts was isolated with plasmid DNA purification kits (Macherey-Nagel, Düren, Germany). Plasmid DNA (pDNA) quality and quantity was evaluated as described above for genomic DNA and diluted to a working concentration of approximately 1 ng/μL for downstream analysis.

2.3.2. qPCR: optimization, efficiency, sensitivity, and specificity

qPCR amplifications were carried out on a CFX96 Real-Time PCR Detection System (BIO-RAD) using a KAPA HRM FAST qPCR Kit (Kapa Biosystems). Reactions were performed in 10 μL volumes containing 5 μL of KAPA HRM FAST Master Mix, 0.4 μL of a 10 uM solution of both forward (CPF.RE) and reverse primers (CPR.RE), 1 μL of 25 mM MgCl2 and 1 μL of ddH_20. To find the optimal annealing temperature a gradient experiment using six different temperatures (x = 55, 56, 57, 58, 59, 60 °C) were simultaneously tested, on three isolates (*C. cacaofunesta* CMW 26375; *C. curvata* CMW 22442 and *C. manginecans* CMW 56757) using 2 ng DNA. Cycling conditions were 3 min at 95 °C, followed by 35 cycles of 5 s at 95 °C, 20 s at x °C (x = 55, 56, 57, 58, 59, 60 °C) and 5 s at 72 °C. The amount of fluorescence for each sample, due to the intercalation of EvaGreen® dye into dsDNA, was measured at the annealing stage of each cycle and analysed via CFX-Manager Software v1.6 (Bio-Rad Laboratories, Inc.). For all assays, controls included no-template reactions, and a *C. manginecans* isolate (CWM 56757) as a positive control. Each sample was amplified in triplicate reactions within the same PCR run and PCR runs were repeated. One replicate of each experiment was visualized using AGE to make sure only one amplicon was present, and sequenced as described above to determine if the correct amplicon was amplified. Real-time PCR results were analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The instrument default cycle threshold setting was used throughout.

Using the qPCR cycling conditions stated above with an annealing temperature of 57 °C, standard curves using 10-fold serial dilutions (1 ng–0.002 ng DNA) of the plasmid (p) containing the targeted CP.RE region from 12 *Ceratocystis* species (Supplementary Fig. 1) in the LAC in circular form, were generated to determine the efficiency and linearity of the KAPA HRM FAST qPCR assays and for later quantification of *Ceratocystis* samples. To determine the detection limit, an analysis of a 10-fold dilution series (1 ng–0.002 ng DNA) of fungal gDNA for five *Ceratocystis* species (*C. cacaofunesta* CMW 26375; *C. curvata* CMW 22442; *C. platani* CMW 14802, *C. fimbriata* CMW 14799 and *C. manginecans* CMW 13851) and for all 12 CP.RE region-containing plasmids available, was performed.

The specificity of the assay to *C. maginecans* (both ITS Types), *C. eucalypticola,* and to the other species in the LAC was tested using 1 ng of DNA from 16 isolates representing the on-target LAC fungal species, and 31 isolates representing non-target fungal species within the Ceratocystidaceae (Table 1). The CP.RE primers were screened against isolates included in this study using both a standard PCR cycler with non-fluorescent detection, as well as by applying the EvaGreen qPCR method.

2.3.3. HRMA validation

To obtain melt curves for the PCR amplicons, a dissociation temperature gradient was performed directly after the qPCR amplification cycle, with a temperature gradient starting at 60 °C – 95 °C. Data acquisition was performed for every 0.2 °C increase in temperature, for 10 s. HRMA was performed using the Precision Melt Analysis™ software (BioRad). For HRMA, the following settings were used: the "Auto detect melt regions" was selected, the "Temperature shift bar height" was set at the recommended 0.20, the "Melt Curve Shape Sensitivity" was set to the default 50 % and the "Tm Difference Threshold" was set to the recommended 0.15 °C.

HRMA was performed on PCR products of the 12 cloned LAC species using 1 ng of pDNA and on 2 ng gDNA from all 16 on-target LAC species and all positive reactions of the 31 non-target fungal species within the Ceratocystidaceae to determine the efficacy of the identified primers to differentiate between species in the LAC. HRMA was also performed on the standard curve reactions described above. This was to determine the validity of species calling by the HRMA at different concentrations of pure fungal DNA. All the samples were run in triplicate and reactions were repeated. To verify the HRMA results, sequencing analysis was performed on all positively detected samples as described above. The resulting sequences were aligned and used to construct a maximum likelihood (ML) phylogenetic tree, as previously described. Midpoint rooting of the trees was done.

2.4. Protocol testing in-vivo and in-vitro

2.4.1. Sample collection

Wood samples from *Eucalyptus* clones showing typical symptoms of Ceratocystis wilt including wilting, tip die back, vascular staining and host mortality, were collected in KwaZulu-Natal, South Africa (Table 2). A wood sample from a healthy *Eucalyptus* tree showing no disease symptoms was also collected as a control (Q_Neg1: Table 2). Using a sterilized scalpel, thin shavings of discoloured wood were taken from samples, after which approximately 50 g were placed between two 1 cm carrot discs that had been soaked overnight in ddH20 amended with 0.001 g/vol of streptomycin sulphate (SIGMA, Steinheim, Germany).

Table 2
List of wood samples screened using the qPCR-HRMA assay.

Samples	Country of origin	*Ceratocystis* detection		Average Cq Value	Copy number[a]	HRMA cluster	Sequence confirmation[b]
Eucalyptus wood		Culture based methods	QPCR (2 μL)				
QM9.1	South Africa	+	+	24,24	$1,25 \times 10^5$	1	Yes
QM9.2	South Africa	+	+	25,62	$5,06 \times 10^4$	1	Yes
QM9.3	South Africa	+	+	24,80	$8,67 \times 10^4$	1	Yes
QM9.4	South Africa	+	+	24,15	$1,32 \times 10^5$	1	Yes
QM10.1	South Africa	+	+	25,46	$5,63 \times 10^4$	1	Yes
QM10.2	South Africa	+	–	30,94	N/A	N/A	N/A
QM10.3	South Africa	+	+	21,75	$6,29 \times 10^5$	1	Yes
QM10.4	South Africa	+	+	25,89	$4,27 \times 10^4$	1	Yes
QM10.5	South Africa	+	+	24,09	$1,37 \times 10^5$	1	Yes
QM10.6	South Africa	+	+	22,88	3×10^5	1	Yes
QM7.1	South Africa	+	+	24,57	$1,01 \times 10^5$	1	Yes
QM7.2	South Africa	+	+	22,80	$3,17 \times 10^5$	1	Yes
QM7.3	South Africa	+	–	31,57	N/A	N/A	N/A
QM7.4	South Africa	+	–	35,81	N/A	N/A	N/A
Q_Neg1	South Africa	–	–	35,13	N/A	N/A	N/A
Corresponding culture representative							
QM9_F (CMW61085)	South Africa	N/A	+	N/A	N/A	1	Yes
QM10_F (CMW61084)	South Africa	N/A	+	N/A	N/A	1	Yes
QM7_F (CMW61086)	South Africa	N/A	+	N/A	N/A	1	Yes
Positive controls							
C. manginecans (ITS Type 1) CMW13851	Oman & Pakistan	N/A	+	25,42	$5,02 \times 10^5$	2	N/A
C. manginecans (ITS Type 2) CMW22563	Indonesia	N/A	+	24,93	$6,77 \times 10^5$	3	N/A
C. fimbriata CMW14799	USA	N/A	+	26,13	$2,16 \times 10^4$	4	N/A
C. eucalypticola CMW11536	South Africa	N/A	+	25,56	$5,28 \times 10^4$	1	N/A

[a] Calculated linear equation generated by the standard curve of the corresponding species.
[b] Based on CP gene region and on the CP and MS204 gene regions of their corresponding fungal isolates.

Carrot baits were then placed in moist chambers and incubated at 28 °C for up to 14 days to stimulate *Ceratocystis* sporulation (Moller and De Vay, 1968). Where present, single ascospore drops, characteristic of *Ceratocystis* spp., were isolated from the carrot baits and placed onto 2 % Malt Extract Agar (MEA: 20 g/L malt extract from Biolab and 20 g/L agar from Difco) for 10 days at 25 °C. Pure cultures were obtained from single spore isolations and used for DNA extractions as stated above. Thin fresh shavings (100 mg) taken from the same healthy and infected wood samples were used to extract genomic DNA (gDNA) using a DNeasy Plant Mini Kit (Qiagen, Germany), following the manufacturer's instructions. The quality and quantity of the extracted DNA was assessed using a Nanodrop ND_1000 (Nanodrop, Wilmington, DE) and Qubit®2.0 Fluorometer (ThermoFisher Scientific) respectively. An aliquot of the extracted DNA was also subjected to AGE to assess the integrity of the extracted DNA.

2.4.2. Efficiency of Eva Green assay on field samples

To evaluate the background effect of *Eucalyptus* DNA on the detection efficiency of target DNA, standard curves generated using a dilution series (1 ng–0.002 ng DNA) of fungal gDNA from *C. manginecans* (CWM56757), and from fungal gDNA (1 ng) from *C. manginecans* (CWM56757) spiked with 60 ng of *Eucalyptus* DNA, were compared. To assess the reliability and sensitivity of this assay to detect small amounts of target DNA, standard curves were generated using 10-fold serial dilutions of 4 ng–0.006 ng of wood-extracted DNA derived from three *Eucalyptus* trees infected with *Ceratocystis*. HRMA was also performed on these standard curve reactions to determine the validity of HRMA species calling at different concentrations for fungal DNA mixed with plant host DNA. All the samples were run in triplicate and reactions were visualized using AGE.

2.4.3. Detection, quantification, and identification of samples

Using the same protocol described above, qPCR amplification was performed on 4 ng of gDNA extracted from wood samples. No template reactions and reactions containing *C. fimbriata* (CMW14799), two *C. manginecans* isolates representing each ITS Type (Type 1 CMW 22563; Type 2: CMW13851) and *C. eucalypticola* (CMW11536) were used as controls in all reactions. These LAC species were selected as positive controls as the hosts and geographical data of the samples collected from South Africa suggested they would most likely be the target species. gDNA from three representative pure fungal cultures (CMW61084-61086) isolated from the *Eucalyptus* samples infected with *Ceratocystis* from South Africa, were also included as controls in the assays screening the wood samples. All the samples were analysed in triplicate and reactions were visualized using AGE. The assay was repeated.

Absolute quantification of *Ceratocystis* for the unknown samples was achieved by calculating the pDNA quantity via the interpolation of their generated Ct values using the linear equations of the previously generated pDNA standard curves. The resulting pDNA quantities were then used to determine target amplicon copy number using the "Calculator for determining the number of copies of a template" as described in Pilotti et al. (2012). HRMA was performed on all positively detected samples.

2.4.4. HRMA results validation

To verify the HRMA results, all positively detected samples were sequenced using the CP.RE primer set. In addition, the guanine nucleotide-binding protein subunit beta-like protein (ms204) using primers MS204F.cerato and MS204R.cerato, were also amplified and sequenced as described above and by Fourie et al. (2015), and the results compared to that of the HRMA clustering. The resulting sequences were aligned, as described above, and used to construct a ML phylogenetic tree. *Ceratocystis fimbriata* CMW14799 was used to root the ML tree.

3. Results

3.1. qPCR and HRMA development

3.1.1. Primer design

The cerato-platanin gene (cp) (562 bp) was amplified for all 16 LAC species tested. The resulting alignment of the 16 LAC species was 499 bp (Supplementary Fig. 2). The primer pair designed from this alignment - CPF.RE (5′-CCATGGCTGTCTCGAGTATG-3′) and CPR.RE (5′-ACCCAGTGTAGGGTATTGSTC-3′) - targeted a 172 bp region. In the LAC, four species, namely *C. cacaofunesta, C. platani*, *C. lukuohia* and *C. papillata* had a single base mismatch that was present in either the forward or reverse primer binding regions (Supplementary Fig. 2). This, however, did not affect the downstream PCR amplification or sequencing of these species and did not result in informative sequence variations in the resulting consensus sequences. Primers passed all quality control standards. Screening of these primers against the *C. manginecans* (CMW46461) genome and Primer Blast databases showed that they were specific, as no off-target hits were recovered. *In-silico* testing of the designed primer pair and the targeted region it would amplify, using uMELT Quartz, revealed double melt curve peak profiles (e.g. see Supplementary Fig. 3), for all 16 LAC species screened.

3.1.2. Phylogenetic analysis

The CP.RE primer pair successfully amplified 16 species in the LAC screened in this study. Visualization of the amplicons confirmed that a single product of the expected size had been amplified. Sequencing of the product confirmed the targeted regions for each species had been amplified and that they were generally GC-poor in the first half and GC-rich in the second half of the sequence. Due to the non-homogenous GC content of the 16 LAC targeted amplicons, the double peak melt curve profiles from a single PCR product observed in the *in-silico* testing were expected.

Sequencing of these amplified regions differentiated between eight of the species using phylogenetic analysis (Fig. 1a) and seven species using *in-silico* HRMA. Using these primers, *C. curvata, C. mangivora, C. manginecans* (Type 2), *C. platani*, *C. fimbriatomima, C. mangicola* and *C. neglecta* were grouped as a single phylogenetic clade. *Ceratocystis manginecans* (Type 1) and *C. eucalypticola* also grouped together in a separate phylogenetic clade (Fig. 1a).

3.2. qPCR and HRMA validation

3.2.1. qPCR: optimization, efficiency, sensitivity, and specificity

qPCR amplification using the CP.RE primers was observed at all six temperatures tested (55, 56, 57, 58, 59, 60 °C) for all three isolates (*C. cacaofunesta*, CMW 26375; *C. curvata,* CMW 22442 and *C. manginecans* CMW 56757) tested. On average, samples that amplified at 57 °C produced the lowest Ct values and this was selected as the optimal run temperature. No amplification was detected for the no-template reactions, confirming that no primer-dimers were formed.

PCR efficiencies calculated from serial dilutions of the target genes cloned into pGEM-T easy vector were high for nine out of the 12 LAC species tested (efficiency = 90–105 % and R > 0.98) and within an acceptable range for the remaining three species tested (86.6–88.9 %), namely *C. manginecans* (ITS Type 1), *C. cacaofunesta* and *C. colombiana* (Supplementary Fig. 1). Overall, the standard curves generated using the cloned target genes, showed the repeatability of the assays, high amplification efficiency, and linearity of the data. Analysis of reactions on agarose gels confirmed that a single product of the expected size had been amplified. Sequencing of the product confirmed target regions had been amplified (see GenBank numbers in Table 1). Double melt curve peak profiles for all 12 LAC species screened were observed as predicted in the *in-silico* analysis (Supplementary Fig. 3).

Using the 10-fold dilution series of fungal gDNA for five *Ceratocystis* isolates a Ct value of <34 was established as the positive detection limit for the assay. However, a Ct value of 30 was established as the highest useable value for accurate downstream HRMA. Based on this parameter, all 12 LAC target-containing plasmids were well within the detection limit with an average Ct value of 23.75 at the lowest DNA quantity tested (0.0039 ng = 1.71×10^5 number of copies). The detection limit for the fungal gDNA tested on five isolates was averaged as 0.088 ng (copy number = $2{,}95 \times 10^3$).

Using the traditional PCR assay, *C. maginecans* (both ITS Types), *C. eucalypticola*, the other 14 on-target LAC species, and 14 of the 31

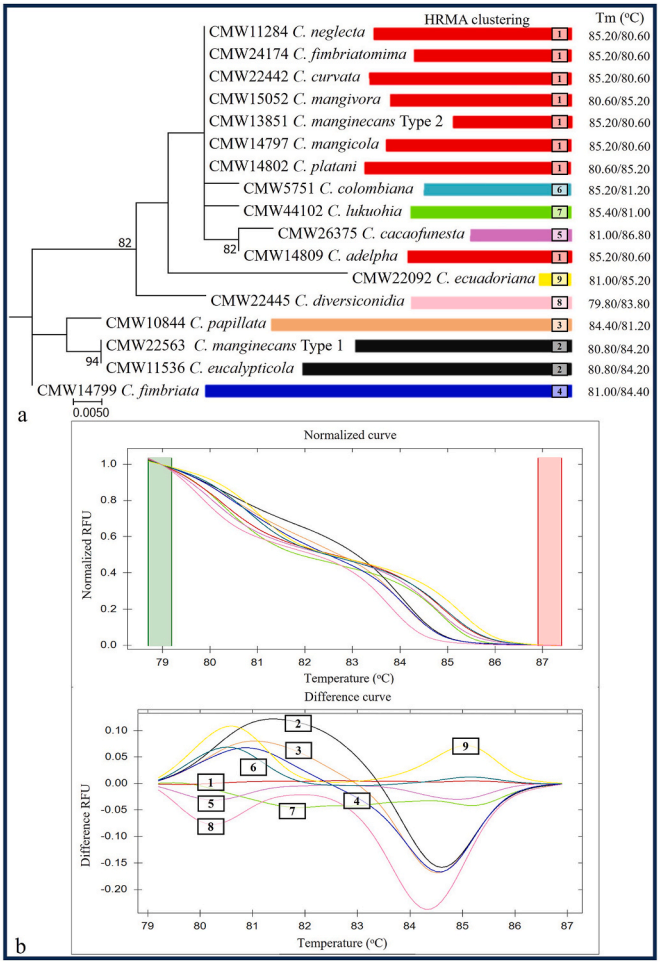

Fig. 1. Phylogenetic and HRMA grouping of *Ceratocystis* species in the Latin American Clade (LAC) using the CP.RE gene region a) Phylogenetic tree based on maximum likelihood (ML) analysis of CP.RE gene sequences for *Ceratocystis* species in the LAC. These primers successfully detected 16 species in the LAC screened in this study and further differentiated between eight of these species. *Ceratocystis curvata, C. mangivora, C. manginecans* (ITS Type 2), *C. platani, C. fimbriatomima*, *C. mangicola* and *C. neglecta* were grouped together as a single phylogenetic clade. *Ceratocystis manginecans* (ITS Type 1) and *C. eucalypticola* were also grouped together as a single but distinct phylogenetic clade. The tree was midpoint rooted 1b) The HRMA clustering and melting temperature (Tm) of each species is depicted on the right of the phylogenetic tree. HRMA results were identical when tested on pDNA and gDNA and distinguished seven out of the 16 LAC species tested, as represented by the different melt curve profiles and colours. The melting temperature (Tm) of each species, as indicated by the HRMA protocol, consistently distinguished *C. papillata*, *C. fimbriata, C. colombiana, C. cacaofunesta, C. lukuohia, C. diversiconidia* and *C. ecuadoriana* which formed their own distinct clusters across all assays. *Ceratocystis manginecans* (ITS Type 1) and *C. eucalypticola* grouped together and formed their own distinct cluster consistently across all assays. *Ceratocystis curvata*, *C. mangivora, C. manginecans* (ITS Type 2), *C. fimbriatomima, C. platani, C. adelpha, C. mangicola* and *C. neglecta* grouped together and formed their own distinct cluster consistently across all assays.

non-target fungal species within the Ceratocystidaceae amplified and produced bands when visualized using AGE. When screened using the EvaGreen qPCR assay, *C. maginecans* (both ITS Types), *C. eucalypticola*, the other 14 on-target LAC species, and only one isolate of a non-target fungal species within the Ceratocystidaceae had an average Ct value below 30 for positive detection: *Ceratocystis polyconidia* (Ct 25.31) (Table 1). Sequencing and phylogenetic analysis of the resulting amplicon of the one non-target species showed that *C. polyconidia* in the Asian-Australian clade (AAC) formed a phylogenetic clade with the LAC species *C. manginecans* (Type 1) and *C. eucalypticola* (Supplementary Fig. 4).

3.2.2. HRMA validation

HRMA results were identical when tested on pDNA and gDNA and distinguished seven out of the 16 LAC species tested (Fig. 1b). The melting temperature (Tm) of each species as indicated by the HRMA protocol consistently separated *C. papillata*, *C. fimbriata, C. colombiana, C. cacaofunesta*, *C. lukuohia, C. diversiconidia* and *C. ecuadoriana* into distinct clusters across all assays (Fig. 1b). *Ceratocystis manginecans* (ITS Type 1) and *C. eucalypticola* grouped together and formed a single cluster consistently across all assays (Fig. 1b). *Ceratocystis curvata*, *C. mangivora, C. manginecans* (ITS Type 2), *C. fimbriatomima, C. platani, C. adelpha, C. mangicola* and *C. neglecta* grouped together and formed a distinct cluster consistently across all assays (Fig. 1b). Sequencing of the amplicons confirmed the HRMA results and showed the expected sequence variations between species where present. The HRMA results were congruent with the phylogenetic analysis except for the HRMA clustering of *C. adelpha.* The melt curve profile of *C. adelpha* was identical to those of *C. curvata, C. mangivora, C. manginecans* (ITS Type 2), *C. fimbriatomima* and *C. platani*. When considering the sequence variation, *C. adelpha* had one base pair difference. This SNP is a C/G base change (SNP class 3), resulting in a small Tm curve shift (0.2–0.5 °C) and could explain why HRMA could not resolve it. HRMA of the only non-target fungal species within the Ceratocystidaceae, clustered *C. polyconidia* with *C. manginecans* (Type 1) and *C. eucalypticola*. These results are congruent with those of the phylogenetic analysis (Supplementary Fig. 4).

HRMA correctly clustered the reactions of pDNA of all 12 LAC species at the 10 different concentrations tested (Supplementary Fig. 5a). HRMA analysis of positively detected reactions of fungal gDNA for the five *Ceratocystis* isolates screened, correctly clustered species with template DNA ranging from 2 ng to 0.06 ng per PCR reaction. HRMA analysis of reaction concentrations lower than the average detection limit (>0.088 ng or Ct < 30) of the fungal gDNA tested, periodically clustered species incorrectly (Supplementary Fig. 5b). However, the shape of the normalized melt curves produced by the reactions of fungal gDNA templates at >0.088 ng were identical to those <0.088 ng (Supplementary Fig. 5b).

3.3. Protocol testing in-vivo and in-vitro

3.3.1. Sample collection

Fourteen samples of discoloured wood from symptomatic *Eucalyptus* clones in South Africa were obtained. Based on culture-dependent techniques, all samples were confirmed to be infected with *Ceratocystis*. Three representative fungal isolates (CMW61084, CMW61085 and CMW61086) from infected wood samples were obtained for further downstream analysis (Table 2). *Ceratocystis* was never isolated from the healthy and asymptomatic *Eucalyptus* tree.

3.3.2. Efficiency of Eva Green assay on field samples

Reactions containing *C. manginecans* gDNA (CWM56756) and a mixture of fungal gDNA (CWM56756) and gDNA extracted from healthy *Eucalyptus,* both gave equivalent melt curve profiles, but the detection limit went from 0.125 ng in pure fungal gDNA to 0.226 ng when spiked with plant gDNA. The detection limit for the *Ceratocystis* infected wood samples across the three samples tested averaged at 1.08 ng total DNA.

HRMA on pure fungal gDNA from *C. manginecans* (CWM56756) correctly clustered all reactions from 2 ng to 0.03 ng, compared to the mixed reaction in which HRMA correctly clustered all reactions from 2 ng to 0.5 ng. However, the shape of the curves at different concentrations were identical. HRMA analysis of positively detected reactions of DNA extracted from *Eucalyptus* wood infected with *Ceratocystis* from all three samples screened, correctly clustered reactions together as one cluster across the different concentrations tested (Fig. 2).

3.3.3. Detection, quantification, and identification of samples

qPCR analysis confirmed the presence of *Ceratocystis* in 11 out of the 14 wood samples tested (Table 2). The DNA concentration in the three samples that gave a false negative result using the qPCR analysis was near zero or the quality of the DNA was poor. Absolute quantification of *Ceratocystis* for these unknown positive samples indicated low DNA concentrations ($\sim 4{,}1 \times 10^{-5}$) and the subsequent target amplicon copy number in wood samples was calculated (Table 2). No-template reactions and gDNA extracted from a healthy *Eucalyptus* sample did not yield any fluorescence signals above the background threshold.

Ceratocystis infected wood samples that were positively detected by qPCR were screened using HRMA and formed a single cluster, grouping with the positive controls of *C. eucalypticola* (CMW11536) and *C. manginecans* Type 1 (CMW 22563) (Fig. 3a). HRMA of the three representative pure fungal cultures isolated from the *Eucalyptus* samples infected with *Ceratocystis* from South Africa also formed a single cluster, grouping with the positive controls of *C. eucalypticola* (CMW11536) and *C. manginecans* ITS Type 1 (CMW 22563). Therefore, the HRMA clustering of the gDNA extracted from infected wood samples and the gDNA extracted from the pure fungal cultures isolated from the infected wood samples were congruent.

3.3.4. HRMA result verification

The phylogenetic analysis of sequenced CP.RE amplicons of positively detected *Ceratocystis* infected wood samples formed a single cluster, grouping with the positive controls of *C. eucalypticola* (CMW11536) and *C. manginecans* ITS Type 1 (CMW 22563) (Fig. 3b). These results were congruent with the HRMA results (Fig. 3a). The identification of the *Ceratocystis* species infecting the *Eucalyptus* as *C. eucalypticola* based on HRMA, were also confirmed by the phylogenetic analysis of the CP.RE (Fig. 3b) and MS204 (Supplementary Fig. 6) gene regions of the three representative fungal isolates (CMW61084-61086). ITS sequences for the representative fungal isolates could not be used to identify the three isolates due to multiple peaks observed in the resulting sequence reads caused by the presence of multiple ITS Types, and were, therefore, confirmed using sequences for the MS204 region.

4. Discussion

The high-resolution melting curve analysis developed in this study consists of a two-step process where qPCR analysis of the CP.RE gene region was first used to detect *C. eucalypticola* and *C. manginecans* (both ITS Types), followed by HRMA, which allowed for further species identification. The diagnostic assay was able to detect and differentiate *C. eucalypticola* and *C. manginecans* (ITS Type 1) from all other LAC species. Both *C. manginecans* ITS Types could be differentiated from one another, and *C. manginecans* (ITS Type 2) could be differentiated from eight other LAC species. The assay was also able to positively detect *C. eucalypticola* in wood samples taken directly from naturally infected *Eucalyptus* trees. The diagnostic assay showed potential to be used for other species in the LAC, and an HRMA curve reference database was generated for all LAC species evaluated in the study.

The reaction efficiency (E) of the assay developed in this study differed across the LAC species tested. Analysis of the generated standard curves revealed that the E values across the 12 species tested were ideal for nine species (E values = 90–105 %), resulting in a doubling of

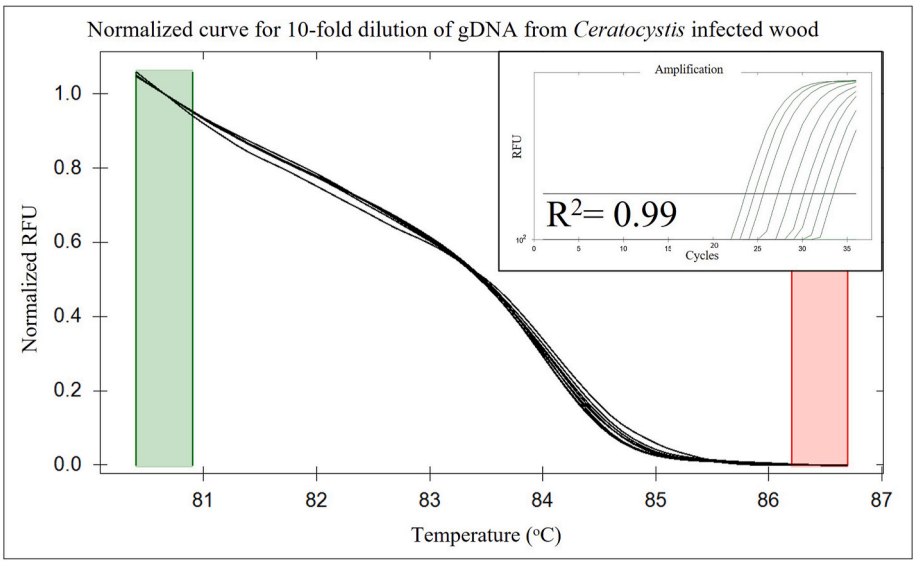

Fig. 2. Amplification curves obtained at different concentrations (2 ng→0,003 ng) of wood-extracted DNA derived from a fresh *Ceratocystis* infected *Eucalyptus* sample. R^2 values depicted an acceptable range. HRMA on positively detected reactions correctly clustered reactions together as one cluster across the different concentrations tested.

the amplicon in the exponential phase of the reaction cycle, and close to ideal for *C. manginecans*, *C. cacaofunesta* and *C. colombiana* (E values 86–88 %). The lower efficiency observed for *C. cacaofunesta* could be due to a base pair mismatch in the reverse primer sequence for this species (Supplementary Fig. 2). The lower reaction efficiencies observed for *C. manginecans* and *C. colombiana* could be related to the reaction conditions employed in this study. While adjusting the reaction conditions might enhance reaction efficiencies, a standard protocol was maintained for all assays. Using this standard protocol, the designed primers amplified and resolved *C. manginecans*, *C. cacaofunesta* and *C. colombiana* across all assays.

The assay developed in this study was able to detect *Ceratocystis* directly from infected wood. The presence of *Eucalyptus* gDNA did, however, have an impact on the reaction efficiency (E). This could be due to the level of potential PCR inhibitors from the necrotic *Eucalyptus* tissue used for DNA extraction (Sidstedt et al., 2020). To overcome this problem, samples with less necrotic tissue were selected for screening, a DNA extraction method that better removes potential PCR inhibitors was used, and input DNA concentrations were standardised (Lehmann et al., 2018). Importantly, R^2 values of the standard curves generated using plasmid DNA and infected *Eucalyptus* gDNA were all in the same range, indicating that the amplification efficiency did not vary among the different template concentrations.

To validate and assess the sensitivity of the optimized assay, unknown test samples were screened. qPCR-HRMA correctly detected and identified all fungal and environmental samples from plant material naturally infected with *C. eucalypticola*. The assay demonstrated an average detection capability of 3.56×10^{-5} ng of *Ceratocystis* DNA in a plant host matrix among the unknown test samples. These results were consistent with studies utilizing the multi-copy ITS gene region, where concentrations as low as 2×10^{-4} ng/μL of *Ceratocystis* DNA in a host DNA matrix were successfully detected (Dharmaraj et al., 2022). The assay sensitivity was similar to that in previously designed assays using the multiple copy ITS gene but had the added benefit that the cp gene region proved more reliable for species identification when using HRMA. This was because only single melt curve profiles are generated per sample compared to the multi-copy ITS gene region.

When testing the specificity of the HRMA assay on closely related off-target species, one species outside the LAC was detected. This was *C. polyconidia* that resides in the Asian-Australian clade (AAC) and is known only from *Acacia mearnsii* in South Africa (Heath et al., 2009). Based on the CP.RE region, this species is identical to *C. manginecans* (Type 1) and *C. eucalypticola*. The HRMA of these species confirmed the results from the phylogenetic analysis and thus cannot be used to distinguish this species from the two target LAC species. However, this ambiguity and the taxonomy of *C. polyconidia* requires further investigation as DNA sequences for several gene regions have shown that *C. polyconidia* falls within the LAC (Barnes et al., 2018). The specificity of the developed assay for the five new species in the LAC requires further investigation as they were not available for testing at the time of this study.

The results of this study confirmed that the diagnostic qPCR-HRMA tool developed meets the requirements to detect *Ceratocystis* in *Eucalyptus* hosts. The cost and time-efficient assay can facilitate high-throughput screening that will aid in quarantine practices. Future work designing a qPCR assay for the plant internal control to confirm competent DNA extraction of test samples would further enhance the use of this tool for large-scale testing of plant samples. The sensitivity of the assay can also be used to inform host range or epidemiological studies. Although not validated in other plant hosts, the results of this study show that this diagnostic assay can be used to screen for several other LAC species. A simplified user's protocol can be found as Supplementary data.

5. Conclusions

This study provides a diagnostic assay to rapidly detect and differentiate *C. eucalypticola* and *C. manginecans* using a qPCR-HRMA assay. This assay was shown to be effective when tested on DNA extracted from fungal cultures and from DNA extracted directly from *C. eucalypticola* naturally infected *Eucalyptus* tissue. The diagnostic assay showed potential to be used for other species in the LAC, however further research is needed to determine the efficiency of this assay to detect those species directly from infected plant material. To aid with this future research, a HRMA curve reference database was generated for all LAC species evaluated in the study.

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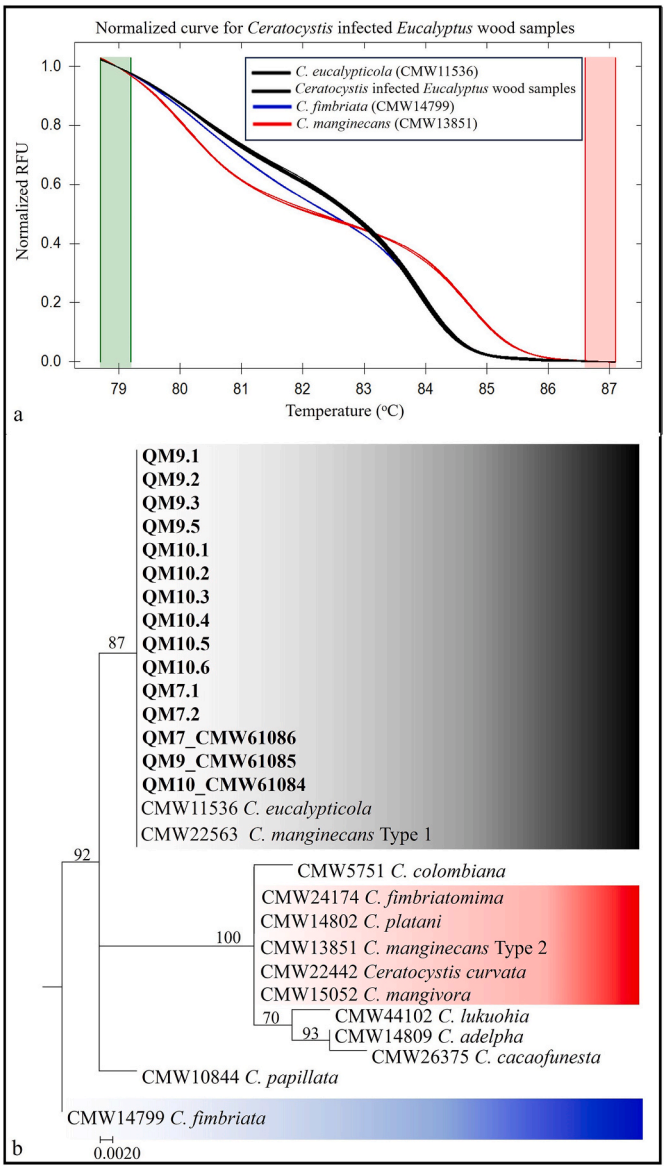

Fig. 3. Screening of *Eucalyptus* wood samples naturally infected with *Ceratocystis*. a) Detection and HRMA of different *Ceratocystis* infected *Eucalyptus* wood samples from South Africa by EvaGreen Real-Time PCR. *Ceratocystis fimbriata* (CMW14799), two *Ceratocystis manginecans* isolates each representing each ITS Type (Type 2: CMW13851; Type 1 CMW 22563), and *Ceratocystis eucalypticola* (CMW11536) were used as controls.
HRMA profiles of the CP.RE region from the DNA obtained from *Ceratocystis*-infected *Eucalyptus* wood, grouped these samples with *Ceratocystis eucalypticola* (CMW11536). 2b) Phylogenetic tree based on maximum likelihood (ML) analysis of the CP.RE gene sequences for 12 *Ceratocystis* species in the LAC and positively detected *Ceratocystis* infected wood samples and their corresponding fungal isolates. *Ceratocystis* infected wood samples and their corresponding fungal isolates all formed a phylogenetic cluster with *Ceratocystis manginecans* ITS Type 1 (CMW 22563) and *Ceratocystis eucalypticola* (CMW11536). Support for bootstrap values above 60 % are presented at the nodes. The tree was midpoint rooted.

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CRediT authorship contribution statement

Kira M.T. Lynn: Conceptualization, Investigation, Methodology, Formal analysis, Validation, Writing - original draft. **Michael J. Wingfield:** Methodology, Funding, Project administration, Writing - review & editing, Supervision. **Almuth Hammerbacher:** Methodology, Resources, Writing - review & editing. **Irene Barnes:** Methodology, Funding, Project administration, Writing - review & editing, Primary supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2024.07.011.

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