LAMP assay to detect *Elsinoë necatrix*, an important *Eucalyptus* shoot and leaf pathogen

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Abstract

Eucalyptus scab and shoot malformation caused by *Elsinoë necatrix* is an emerging disease and a serious threat to the global commercial forestry industry. The disease was first discovered in North Sumatra, Indonesia and now requires a simple and effective method for early pathogen detection. In this study, a rapid and sensitive Loop-mediated isothermal amplification (LAMP) assay was developed for *E. necatrix*. A unique region in a secondary metabolite gene cluster was used as target for the assay. To test robustness of the assay, LAMP amplification was verified in 15 strains of *E. necatrix*. A specificity test against 23 closely related *Elsinoë* species and three fungal species commonly isolated on *Eucalyptus* showed that the LAMP assay exclusively identified *E. necatrix* isolates. The assay had a high level of sensitivity, able to detect 0.01 ng (approximately 400 target copies) of pure *E. necatrix* DNA. Furthermore, using a simple DNA extraction method, it was possible to use this assay to detect *E. necatrix* in infected *Eucalyptus* leaves.

Key words: Diagnostic assay, Eucalyptus scab and shoot malformation; Loop-mediated isothermal amplification; Plantation forestry; Scab disease

Introduction

Eucalyptus scab and shoot malformation caused by *Elsinoë necatrix* (Ascomycota, Dothideomycetes, Myriangiales, *Elsinoaceae*) is a serious disease affecting planted *Eucalyptus* in Indonesia (Pham *et al.* 2021). The disease was first discovered in North Sumatra in 2014, affecting *Eucalyptus* in the subgenus *Symphyomyrtus* and hybrids of these species (Pham *et al.* 2021). *E. necatrix* infections are typified by water-soaked necrotic spots that initially appear on young leaves and petioles, and that become scab-like lesions over time. The scabs commonly dry and drop from the leaves resulting in a "shot-hole" symptom. In severely affected trees, lesions can lead to girdled and distorted leaves and highly susceptible *Eucalyptus* genotypes produce shoots with small leaves that appear feathered (Pham *et al.* 2021 and the images therein).

Scab and malformation symptoms are typical of infection by *Elsinoë* spp*.,* and results from the production of a fungal secondary metabolite known as elsinochrome (Chung 2011; Jeffress *et al.* 2020). The toxin cluster responsible for elsinochrome production, was initially described by Chung and Liao (2008). A later study by EBERT *et al.* (2019) refuted the findings of Chung and Liao (2008), rather linking the annotated cluster to melanin biosynthesis. This redescribed melanin biosynthetic cluster consisted of a group of co-regulated genes with the main functional gene described as polyketide synthase.

Although scab-like symptoms are easily recognizable as typical of infection by *Elsinoë* spp., isolation and characterization of these fungi is challenging. This is mostly due to the absence of visible fungal fruiting structures during infection (Fan *et al.* 2017). Furthermore, cultures of *Elsinoë* spp. are slow-growing and are commonly over grown

by faster growing opportunistic fungi (Fan *et al.* 2017). These challenges complicate the diagnosis of diseases caused by *Elsinoë* spp., and they have significantly delayed the discovery of the causal agent of Eucalyptus scab and shoot malformation, now known to be *E. necatrix* (Pham *et al.* 2021).

Molecular techniques have become increasingly important to support morphological characterization of important plant pathogens. Such methods can distinguish between different species of *Elsinoë,* regardless of the morphological structures found at different stadiums in the pathogen life cycle (Fan *et al.* 2017). Examples of these methods include DNA sequence-based analyses such as restriction analysis of the amplified internal transcribed spacer (ITS) and random amplified polymorphic DNA (RAPD) analyses (Hyun *et al.* 2007). Other molecular techniques rely on species-specific primers that are used for diagnostic PCR and real-time PCR assays (Hyun *et al.* 2007; Hou *et al.* 2013). Although these techniques provide an accurate means to distinguish between different fungal groups, they usually require sophisticated laboratory equipment, experienced personnel and long execution times that limit their application to the laboratory environment (Buja *et al.* 2021).

Loop-mediated isothermal amplification (LAMP) is an isothermal amplification technology that can replace lab-based PCR due to its simplicity, specificity, high efficiency, and speed (Notomi *et al.* 2015). LAMP relies on a unique strand-displacing DNA polymerase (e.g., Bst polymerase), that amplifies DNA at a constant temperature throughout the reaction process (isothermal amplification) (Panno *et al.* 2020). Several factors including high specificity, high sensitivity, time efficiency, and rapid results (Notomi *et al.* 2015) have made LAMP assays popular for diagnostics. Several

comprehensive reviews of this technique and its applications are available (Le and Vu 2017; Becherer *et al.* 2020; Panno *et al.* 2020; Soroka *et al.* 2021). LAMP assays have been developed for various tree pathogenic fungi including for example *Fusarium circinatum* (Meinecke *et al.* 2023), *Dothistroma septosporum* (Myrholm *et al.* 2021) and *Phytophthora cinnamomi* (Dai *et al.* 2019).

Early detection and identification of fungal pathogens facilitates the management of disease outbreaks and can refine quarantine measures aimed at avoiding accidental introductions into new environments (Buja *et al.* 2021). *E. necatrix* is an important threat to plantations of *Eucalyptus* in Indonesia, and to the global forestry industry. The aim of this study was consequently to develop a LAMP assay for the rapid and sensitive detection of this pathogen. The putative melanin cluster belonging to the polyketide class of fungal secondary metabolites was targeted for the development of this assay. Despite the reclassification of this cluster, a region specific to *E. necatrix* based on the melanin cluster was targeted. . The resulting assay was also screened for specificity to other *Elsinoë* species and other common *Eucalyptus* fungal pathogens.

Materials and methods

LAMP assay development

The unannotated full genome sequence of *E. necatrix* (accession number JANZYH000000000; Wingfield *et al.* 2022) was obtained from the Genbank database (http://www.ncbi.nlm.nih.gov/). This species is haploid, with a genome size of 22.8 Mb (Wingfield *et al.* 2022). An annotated full genome sequence of *E. fawcettii* (accession number ASM1297783v1; Jeffress *et al.* 2020) was also retrieved from the Genbank

database. The genecluster, previously identified as a single-copy cluster in *E. fawcettii* (Chung and Liao 2008) (Table 1), was used as a reference to perform a local tBlastn search in Geneious Prime version 2021.0.3 against the full genome sequence of *E. fawcettii* to identify the gene locations and cluster boundary. The predicted cluster in *E. fawcettii* was extracted and used as query in a Blastn search against the full genome sequence of *E. necatrix* to identify the cluster boundary within this species. The predicted putative cluster of *E. necatrix* and *E. fawcettii* were compared using Easyfig 2.2.5 (Sullivan *et al.* 2011). Any region that lacked synteny between *E. necatrix* and *E. fawcettii* was targeted for LAMP primer design with the aim of minimizing the likelihood of amplification in other *Elsinoë* species. To determine whether the targeted region was present and conserved between different strains of *E. necatrix*, a pair of PCR primers (forward1 and reverse1, Table 2) were designed using Geneious Prime. The primers spanned the complete region that lacked synteny between *E. necatrix* and *E. fawcettii*. The primers were synthesized by Inqaba Biotec, (Pretoria, South Africa).

DNA was extracted from five *E. necatrix* isolates (CMW 56126, CMW 56129, CMW 56132, CMW 56136, CMW 56189, see Table 3) following the protocol described by Pham *et al.* (2021). The DNA was used to amplify the target region following the PCR protocol of Fan *et al.* (2017), but adjusting the annealing temperature to 52 °C. The resulting PCR amplicons were mixed with GelRed (Biotium, Hayward, CA, USA) and visualized using 1% agarose gel electrophoresis. ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific) was used to purify the amplicons, which were then sequenced in both directions using the PCR primers and a BigDye terminator sequencing kit v. 3.1 (Applied Biosystem, Foster City, CA, USA). Sequencing of the

amplification reaction was done with an ABI PRISM 3100 DNA sequencer (Applied Biosystems) at the Sequencing Facility of the Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria, South Africa.

The resulting sequences were aligned using the Geneious Alignment tool in Geneious Prime to determine any intraspecific variation. A consensus sequence was generated and compared to other publicly available *Elsinoë* genomes to screen for potential regions with interspecific variation for LAMP primer design. The genomes included were from *E. ampelina* (SMYM00000000), *E. arachidis* (JAAPAX000000000), *E. australis* (QGIG00000000), *E. batatas* (accession number JAESVG000000000), *E. fawcettii* (accession number SWCR00000000), *E. murrayae* (accession number NKHZ00000000), and *E. perseae* (accession number JARDAA000000000.1). The consensus sequence specific to *E. necatrix* was then used to design primers for a LAMP assay with the Primer Explorer V5 software (Eiken Chemical Company; https://primerexplorer.jp/e/) using default parameters. This involved the design of four standard primers targeting six distinct regions on the target sequence, which were the outer primers (F3 and B3) and the inner primers (FIP and BIP), as well as two loop primers (LF and LB). The primer sets were ranked based on Primer Explorer's recommendations including parameters for primer melting temperature (Tm), free energy in the 3' ends, and GC content (Table S1). The specificity of the primers in the top ranked set was validated using BLASTn searches against the available *Elsinoë* genomes in Geneious Prime (Table 2, Table S2). All selected primers were synthesized at Inqaba Biotec, Pretoria, South Africa.

The position of the *E. necatrix* LAMP primers with respect to the predicted cluster was determined by annotating the full region using GeneMark-ES (Ter-Hovhannisyan *et al.* 2008) and confirming the predictions using ORFfinder from NCBI. Identity was assigned to each predicted gene by subjecting the translated protein sequences to a Blastp search against the nr database at NCBI.

LAMP assay validation

Fungal isolates and DNA extraction

Several fungal cultures were sourced from either the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa or from the culture collection (CBS) of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands (Table 3) to validate the designed LAMP assay. All the isolates were grown on potato dextrose agar (PDA; BD Difco) at 25 °C for 10 days before DNA was extracted using Prepman® Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocols.

LAMP amplification

Five isolates of *E. necatrix* (Table 3) were used to test the designed primers in a LAMP assay. Each primer was prepared in a working solution of 10 µM using PCR grade water. A working stock solution of primer mix was prepared that contained 0.5 µM F3, 0.5 µM B3, 0.4 µM LoopF, 0.4 µM LoopB, 2.4 µM FIP, and 2.4 µM BIP (Myrholm *et al.* 2021). The LAMP reaction was carried out in a 15 µl reaction volume with proportions of 0.6 Optigene Isothermal master mix (ISO-001) (OptiGene, Horsham, West Sussex, UK), 0.24 primer mix and 0.16 template DNA (i.e., for a 15 µL reaction, 9 µL of isothermal master mix, 3.6 µL of primer mix, and 2.4 µL of template DNA was used with a

concentration of approximately 0.5 ng of DNA). The LAMP assay was run on a Genie II instrument (Optigene, Horsham, West Sussex, UK) at 65 °C for up to 60 min. A melting curve analysis was performed at the end of each LAMP reaction by denaturation of the product at 98 °C followed by ramping down of the temperature to 80 °C at a rate of -0.05 °C/s to allow for reannealing. To obtain the results and graphs for the LAMP reactions, GenieExplorer was set at a minimum peak detection threshold of 0.010 per fluorescence ratio and the minimum amplification height at 25000 per fluorescence derivative.

LAMP specificity and sensitivity

Specificity of the LAMP assay was tested using the genomic DNA extracted from an additional 10 isolates of *E. necatrix* obtained from infected *Eucalyptus* plant material in North Sumatra, Indonesia (Pham *et al.* 2021), as well as 23 *Elsinoë* spp. that are either phylogenetically related to *E. necatrix* or can be found on *Eucalyptus* trees (Table 3). The tests were also performed on a single isolate each of *Teratosphaeria destructans, T. epicoccoides* and *Calonectria reteaudii*, all common foliar pathogens frequently found on *Eucalyptus* leaves.

A LAMP sensitivity assay was performed using pure genomic DNA extracted from *E. necatrix* isolate CMW 56129, available from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa. The assay was tested using ten-fold serial dilutions prepared in sterile deionized water ranging from 10 ng/µl to $1x10^{-3}$ ng/µl. The total DNA concentration was first quantified using a Qubit™ 4 Fluorometer (Invitrogen, Waltham, MA, USA) and then serial dilutions were performed from the known concentrations. To obtain lower concentrations for testing, a standardized value of 1 ng/µl (as determined by the Qubit™dsDNA HS assay kit) was

prepared and serial dilutions were made to obtain the target concentration range of 1 ng/µl to $1x10^{-3}$ ng/µl. This dilution range was prepared in triplicate (triplicate 1, 2 and 3) with each triplicate containing concentrations of 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001 ng/µl of target DNA. The different DNA dilutions from each triplicate was used for three rounds of LAMP amplification, producing a total of 9 reactions for each target concentration. A non-template control containing nuclease-free water rather than DNA was always included. The same DNA concentration ranges were used to test the sensitivity of a standard PCR reaction with the outer primers (F3/B3). The PCR conditions described by Fan *et al.* (2017) were used, with an annealing temperature of 50 °C. The minimum detection limit was set as 8 of the 9 reactions being positive for the entire test run. Any test runs at concentrations lower than the minimum detection limit were scored as positive but variable if the test run showed positive amplification for at least 4 of the 9 total reactions.

Results of the specificity and sensitivity assays were included only if the run was considered as valid. A valid run was defined as one where amplification of the *E. necatrix* genomic DNA positive control (consisting of a LAMP amplification reaction containing approximately 0.5 ng of DNA) was detected (Tm within a range of 25-40 min and annealing derivative peaks at 88.6-88.9 °C) and no amplification was present in the no-template control (no amplification curve or anneal derivative melting temperature (Tp)). If a sample in a valid run did not produce a Tp or Tm value, or only one of these parameters was detected, the sample was considered LAMP negative. In cases where the LAMP reaction was valid, but negative, the quality of the extracted DNA was assessed by conventional PCR amplification of the ITS and TEF1 region using primer

pair ITS1/ITS4 (White *et al.* 1989) and E1F/E1R (Hyun *et al.* 2007) respectively, following the amplification conditions described by Fan *et al.* (2017) with an annealing temperature of 56 °C. This served to exclude the possibility that the LAMP reaction had failed due to inferior DNA quality.

Comparison of DNA extraction methods

A simulated field validation test for the LAMP assay was performed on symptomatic *Eucalyptus* leaves. These leaves were produced by artificial inoculation of spores from *E. necatrix* isolate CMW 56139 onto the leaves of a *Eucalyptus grandis* plant in Indonesia. To prepare the inoculant, distilled H_2O was added to an *E. necatrix* culture containing spores that were gently scraped from the agar surface with a glass rod. The unquantified spore suspension was then sprayed onto the *Eucalyptus* leaves under glasshouse conditions. After 7 days, infected leaf material containing the typical black necrotic scabs were collected from the *Eucalyptus* plants. Leaves were cut into 5x5 mm^2 squares surrounding the necrotic spots to reduce the amount of plant material present. Approximately 100 mg of infected plant material was used per extraction. To test the overall efficacy of each extraction method, pure *E. necatrix* mycelium obtained from growth on PDA was also used as starting material in the DNA extractions.

Different methods to extract DNA from the infected leaf material were evaluated to determine suitability. The Plant Material Lysis Kit (EXT-001; Optigene) and the DNeasy Plant Pro kit (Qiagen) was used following the manufacturer's instructions. A dipstick nucleic acid purification method (renamed as the Tris extraction method) described by Zou *et al.* (2017) was also tested. For the Plant Material Lysis Kit, the DNeasy Plant Pro kit (Qiagen) and the Tris extraction method a vibratory mill (Retch MM301) was used to

grind the tissue during extraction. . Each extraction was repeated 3 times (triplicate 1, 2 and 3). A volume of 2.4 ul of all the extracted DNA samples were subjected to three LAMP runs, resulting in a total of 9 amplifications per extraction method. Both the positive control containing *E. necatrix* genomic DNA from a pure culture and a no-template control containing PCR-grade water in place of DNA was included with each LAMP run.

Results

LAMP assay development

The tBLASTn search of the annotated *E. fawcettii* whole genome sequence using the translated proteins from the cluster (Table 1) previously characterized by Chung and Liao (2008) identified a genomic region with high identity. This region contained annotations for the *EfPKS1, PRF1, ECT1, TSF1, RDT1* and *Efhp1* genes. However, there were no blast matches for the *OXR1, Efhp2, Efhp3,* and *Efhp4* genes in the *E. fawcettii* genome sequence used in this study, in line with the results from Jeffress *et al.* (2020). The genes in the extracted cluster were translated and used in a tBLASTn search of the whole genome sequence of *E. necatrix* which identified a 28462 bp region representing the complete putative cluster. Easyfig analysis between the predicted cluster of *E. fawcettii* and the comparative region of *E. necatrix* showed high sequence similarity across the full region (Figure 1A). However, there was some sequence variation apparent between *E. fawcettii* and *E. necatrix* at a region located around the previously characterized *Efhp1* gene, and this region was targeted for LAMP primer design (Figure 1B).

PCR amplification of the selected region produced a target amplicon of approximately 1000 bp in all five *E. necatrix* isolates used. After sanger sequencing, the sequences were successfully mapped back to the target genomic region. There was 100% sequence identity between the amplified target region, as well as the comparable region from the *E. necatrix* genome sequence. A consensus sequence was generated and submitted to NCBI (GenBank accession number OP856600). This consensus sequence showed a low sequence match to the genomes of other *Elsinoë* spp. This sequence was also submitted to PrimerExplorer which generated five possible LAMP primer sets. Based on the optimal criteria (Table S1), the best-scoring primer set was selected (Table 2, Table S2). A BLASTn search using each primer sequence as query identified a 100% sequence match to all *E. necatrix* isolates in the target region, while some primers also had matches to closely related *Elsinoë* species.

Gene annotation using GeneMarkES and ORFfinder confirmed that the LAMP primers were designed within an exon region of a predicted gene (Figure 2). A BLASTp search with this sequence against the NCBI nt database predicted the closest blast match to be the *E. fawcettii* hypothetical protein 1 (Efhp1) described as part of the putative elsinochrome cluster by Chung and Liao (2008) but later reclassified as the melanin cluster by Ebert *et al.* (2019).

LAMP assay validation

Positive amplification using the LAMP assay was achieved for all 15 *E. necatrix* isolates tested after 30-40 min (Table 3, Figure 3A). The anneal derivative melting temperature peaks ranged between 88.6 °C and 88.9 °C (Figure 3), confirming these as the target product.

No amplification was observed for the 23 non-target *Elsinoë* spp. or for *T. destructans, T. epicoccoides,* and *C. reteaudii* (Table 3, Figure 3B, Figure S1), despite the run being considered valid. The extracted DNA of the 23 non-target *Elsinoë* spp. and the three non *Elsinoë* spp. (*T. destructans, T. epicoccoides, C. reteaudii)* were able to support amplification during a standard PCR reaction, targeting the ITS and TEF1 regions, confirming the integrity of the extracted DNA (Figure S2).

The sensitivity test of the LAMP assay using pure *E. necatrix* genomic DNA showed a minimum detection limit of 0.01 ng per reaction (approximately 400 target copies) (8 of the 9 samples were amplified at 0.01 ng). However, there was still positive amplification but with variable detection below the threshold value of 0.01 ng with 6 of the 9 samples being amplified at 0.005 ng and 4 of the 9 samples being amplified at 0.001 ng (Figure 4, Table 4, Figure S3 A-H, Table S3. The time until amplification detection occurred from 00:28:15 hh:mm:ss up to 01:05:30 hh:mm:ss with an increase in detection time as the DNA concentration decreased. In comparison, conventional PCR amplification with F3/B3 primers had a detection limit of 0.1 ng with 8 of the 9 samples being amplified. However, there was still positive amplification but with variable detection below the threshold value of 0.1 ng with 6 of the 9 samples being amplified at 0.05 ng (Table 4, Table S3, Figure S4).

Comparison of DNA extraction methods

Positive LAMP amplification was possible on DNA extracted from pure *E. necatrix* mycelium grown on PDA, regardless of the extraction method used. All the samples produced detectable amplification within 21:30 - 31:15 minutes (Table 5, Figure S5 A-

D). This confirmed that all three methods were suitable for DNA extraction from this fungus.

The Plant Material Lysis Kit (EXT-001; Optigene), the DNeasy Plant Pro kit (Qiagen) and the Tris-based extraction method (Zou *et al.* 2017) were then tested on infected leaf samples inoculated with *E. necatrix* (Table 5, Figure S6 A-D). Despite the fact that visible symptoms of infection were observed on the *Eucalyptus* leaves, it was not possible to use the LAMP assay to detect the presence of *E. necatrix* in all of the extractions. When DNA extractions were done using the DNeasy Plant Pro kit, positive amplification was observed for 6 of the 9 reactions, while amplification was successful for 5 of the 9 extractions with the Plant Material Lysis Kit. The Tris-based extraction method was less effective, producing DNA that only resulted in amplification in 1 of the 9 reactions. The nature of the DNA extractions (i.e., lesion spots on infected leaves) results in a mixture of both plant and fungal DNA. Although the exact amount of fungal DNA in each sample was not quantified, the time to amplification varied between the different DNA extraction methods indicating variation in total fungal DNA present. Using the Qiagen extraction method resulted in amplification as early as 00:21:15 while the DNA from the Optigene extraction method only resulted in amplification at 00:30:15. All of the positive samples amplified in the LAMP assay had the same annealing temperature (88.8 °C) as the positive control confirming the identity of the samples as *E. necatrix* (Table 5).

Discussion

A LAMP assay was developed to identify the newly discovered *Eucalyptus* pathogen, *E. necatrix*. The assay relied on the amplification of a unique region that was present in the

predicted *Efhp1* gene of *E. necatrix*, making it possible to distinguish the target species from other *Elsinoë* spp. The LAMP assay had a high level of specificity and sensitivity, exclusively detecting *E. necatrix* genomic DNA at amounts as low as 0.01 ng (approximately 400 target copies). Furthermore, *E. necatrix* DNA could be amplified from both pure fungal material and infected plant tissue using two different extraction methods.

The design of the LAMP primers in this study was based on a unique region present in the genome of *E. necatrix.* Previous studies have based the design of a LAMP assay on marker regions that are commonly used to distinguish closely related species, thus exploiting the specificity inherent in these regions (Kikuchi *et al.* 2009; Fukuta *et al.* 2013; Kong *et al.* 2016). Several such genetic markers are found in fungi, including the internal transcribed spacer regions (ITS), Beta-tubulin genes, Calmodulin gene and RNA polymerase II gene (Das *et al.* 2014; Raja *et al.* 2017). However, in *Elsinoë* spp. specifically, it is known that many of the common marker regions (including ITS, transcription elongation factor, the 28S large subunit ribosomal RNA gene and the β-tubulin region) either show low levels of interspecies sequence variation or high intraspecies variability (Fan *et al.* 2017; Ahmed *et al.* 2019). This makes these regions unsuitable for species-specific marker development in *Elsinoë* spp.

The LAMP assay developed in this study targeted a gene cluster initially thought to be responsible for elsinochrome toxin production (Chung and Liao 2008). Elsinochrome production is a trait unique to *Elsinoë* species (Weiss *et al.* 1965; Liao and Chung 2008), making this region useful to putatively exclude other non-*Elsinoë* species. The LAMP primers were then targeted to a region in the cluster that appeared to be unique to *E.*

necatrix. This region was identified through a comparison between the cluster region of this species and the related *E. fawcettii*. The LAMP assay designed was highly specific, distinguishing *E. necatrix* from other *Elsinoë* spp. as well as from fungal pathogens commonly found on *Eucalyptus* leaves. A subsequent study has shown that the gene cluster used for the development of this assay was erroneously identified as the elsinochrome toxin cluster by Chung and Liao (2008). The gene cluster used in this study has subsequently been reclassified as the melanin biosynthesis cluster (Ebert *et al*. 2019). Although the melanin biosynthesis cluster is present in most fungi (Cordero and Casadevall 2017), the LAMP assay described in this study was serendipitously designed in a region of this cluster unique to *E. necatrix*.

The *E. necatrix* LAMP assay had a high level of sensitivity with a minimum detection limit of 0.01 ng of pathogen DNA. This is despite the fact that the target region is present in the genome as a single-copy gene, confirmed by a single 100% BLAST hit when used as a query sequence in a BLASTn search against the *E. necatrix* genome. The LAMP assay was more sensitive than the comparable conventional PCR reaction by a factor of 10, indicating a better potential for pathogen detection. Several described LAMP assays have detection limits ranging from 33 fg to 10 fg of fungal DNA (Fukuta *et al.* 2013: Ghosh *et al.* 2015; Kong *et al.* 2016), significantly lower than those possible in this study. However, many of those assays were based on multi-copy gene regions such as ITS, that could account for the lower detection thresholds. This view is supported by a previous study that demonstrated significant detection limit differences between LAMP assays targeting single-copy and multi-copy gene regions (Schneider *et al.* 2019).

These authors suggested that the differences in sensitivity could be explained by the number of target copies present per genome (Schneider *et al.* 2019).

To make optimal use of the LAMP assay for the management of *E. necatrix*, a rapid DNA extraction method from field samples would be required. In this study, three methods were tested to extract high quality DNA from infected leaf samples. Both the Qiagen and Optigene extraction methods produced DNA that supported successful amplification with the LAMP assay. For the Optigene extraction kit, the manufacturer's instructions suggest manual shaking to homogenize the DNA, although this particular method was not as successful as the vibratory milling device. Optimization of the latter techniques to allow for in-field use without the need of specialized equipment (such as a vibratory milling device) h could be an added benefit. This would minimize any delay between sampling and diagnosis, and it would also improve the accuracy of the diagnosis compared to conventional techniques (Ahmed *et al.* 2019).

The LAMP assay developed in this study is the first diagnostic technique for any *Elsinoë* spp. that relies on this isothermal amplification technique. Other diagnostic techniques for this group of fungi primarily rely on PCR amplification methods such as real-time PCR (Hyun *et al.* 2007; Hou *et al.* 2013; Ahmed *et al.* 2019; Elliott *et al.* 2022). However, some of these PCR-based methods are unable to allow for differentiation between different *Elsinoë* species (Elliott *et al.* 2022). It has also been shown from studies with other fungal pathogens that PCR-based methods lack the sensitivity inherent in LAMP based diagnostic assays (Khan *et al.* 2018; Foo *et al.* 2020).

A portable thermocycler such as the Genie II amplification system or a comparable commercially available unit should allow for the rapid detection of *E. necatrix* using the

assay developed here. Devices such as the Genie II operating system is fully portable with an internal rechargeable battery lasting an entire day that can be used directly in the field. Based on our results, the entire diagnostic process could be completed in approximately 90 minutes. The possibility of an in-field DNA extraction method combined with the use of a Genie II portable thermocycler adds to the attractiveness of this assay as a rapid diagnostic technique for the identification of *E. necatrix* in future.

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Literature cited

Ahmed, Y., Hubert, J., Fourrier-Jeandel, C., Dewdney, M. M., J. Aguayo and Ioos, R. 2019 A set of conventional and multiplex real-time PCR assays for direct detection of *Elsinoë fawcettii*, *E. australis*, and *Pseudocercospora angolensis* in citrus fruits. Plant Dis. 103: 345-356.

Becherer, L., Borst, N., Bakheit, M., Frischmann, S., Zengerle, R., and Von Stetten, F. 2020 Loop-mediated isothermal amplification (LAMP) – review and classification of methods for sequence-specific detection. Anal. Methods 12: 717-746.

Buja, I., Sabella, E., Monteduro, A. G., Chiriacò, M. S., De Bellis, L., Luvisi, A., and Maruccio, G. 2021 Advances in plant disease detection and monitoring: from traditional assays to in-field diagnostics. Sensors (Basel) 21(6):2129.

Chung, K. R. 2011 *Elsinoë fawcettii* and *Elsinoë australis*: The fungal pathogens causing citrus scab. Mol. Plant Pathol. 12: 123-135.

Chung, K. R., and Liao, H. L. 2008 Determination of a transcriptional regulator-like gene involved in biosynthesis of elsinochrome phytotoxin by the citrus scab fungus, *Elsinoë fawcettii*. Microbiology 154(11): 3556-3566.

Cordero, R. J., and Casadevall, A. 2017 Functions of fungal melanin beyond virulence. Fungal Biol. Rev. 31(2): 99-112

Dai, T., Yang, X., Hu, T., Li, Z., Xu, Y., and Lu, C. 2019 A novel LAMP assay for the detection of *Phytophthora cinnamomi* utilizing a new target gene identified from genome sequences. Plant Dis. 103: 3101-3107.

Das, S., Dash, H. R., Mangwani, N., Chakraborty, J., and Kumari, S. 2014 Understanding molecular identification and polyphasic taxonomic approaches for genetic relatedness and phylogenetic relationships of microorganisms. J. Microbiol. Methods 103: 80-100.

Ebert, M. K., Spanner, R. E., de Jonge, R., Smith, D. J., Holthusen J., Secor, G. A., Thomma, B. P. H. J., and Bolton, M. D. 2019 Gene cluster conservation identifies melanin and perylenequinone biosynthesis pathways in multiple plant pathogenic fungi. Environ. Microbiol. 21: 913-927.

Elliott, A. J., Van Raak, M. M. J. P., Barnes, A. V., Field, C. J., Van Duijnhoven, A. A. L. A. M., Webb, K. M., and Van de Vossenberg, B. T. L. H. 2022 Real-time PCR detection of *Elsinoë* spp. on Citrus. PhytoFrontiers. 0 0:0

Fan, X. L., Barreto, R. W., Groenewald, J. Z., Bezerra, J. D., Pereira, O. L., Cheewangkoon, R., Mostert, L., Tian, C.M., and Crous, P.W. 2017 Phylogeny and taxonomy of the scab and spot anthracnose fungus *Elsinoë* (*Myriangiales, Dothideomycetes*). Stud. Mycol. 87: 1-41.

Fukuta, S., Takahashi, R., Kuroyanagi, S., Miyake, N., Nagai, H., Suzuki, H., Hashizume, F., Tsuji, T., Taguchi, H., Watanabe, H., and Kageyama, K., 2013 Detection of *Pythium aphanidermatum* in tomato using loop-mediated isothermal amplification (LAMP) with species-specific primers. Eur. J. Plant Pathol. 136: 689-701.

Foo, P. C., Nurul Najian, A. B., Muhamad, N. A, Ahamad, M., Mohamed, M., Yean Yean, C., and Huat Lim, B. 2020 Loop-mediated isothermal amplification (LAMP) reaction as viable PCR substitute for diagnostic applications: a comparative analysis study of LAMP, conventional PCR, nested PCR (nPCR) and real-time PCR (qPCR) based on *Entamoeba histolytica* DNA derived from faecal sample. BMC Biotechnol. 20: 34.

Ghosh, R., Nagavardhini, A., Sengupta A., and Sharma, M. 2015 Development of Loop-Mediated Isothermal Amplification (LAMP) assay for rapid detection of *Fusarium oxysporum* f. sp. *ciceris* - wilt pathogen of chickpea. BMC Res. Notes 8: 40.

Hou, X., Huang, F., Zhang, T. Y., and Li, H. Y. 2013 Detection of *Elsinoë fawcettii* in planta through real-time PCR. J. Food Agric. Environ. 11: 1085-1087.

Hyun, J. W., Peres, N. A., Yi, S.-Y., Timmer, L. W., Kim, K. S., Kwon, H.-M. and Lim, H.-C. 2007 Development of PCR Assays for the Identification of Species and Pathotypes of *Elsinoë* Causing Scab on Citrus. Plant Dis. 91: 865-870.

Jeffress, S., K., Arun-Chinnappa, K., Stodart, B., Vaghefi, N., Tan, Y.P., and Ash, G. 2020 Genome mining of the citrus pathogen *Elsinoë fawcettii*; prediction and prioritisation of candidate effectors, cell wall degrading enzymes and secondary metabolite gene clusters. PLoS One 15: e0227396.

Khan, M., Wang, R., Li, B., Liu, P., Weng, Q., and Chen, Q. 2018 Comparative Evaluation of the LAMP Assay and PCR-Based Assays for the Rapid Detection of *Alternaria solani*. Front. Microbiol. 9: 2089.

Kikuchi, T., Aikawa, T., Oeda, Y., Karim, N., and Kanzaki, N. 2009 A rapid and precise diagnostic method for detecting the Pinewood nematode *Bursaphelenchus xylophilus* by loop-mediated isothermal amplification. Phytopathology 99: 1365-1369.

Kong, X., Qin, W., Huang, X., Kong, F., Schoen, C. D., Feng, J., Wang, Z., and Zhang, H. 2016 Development and application of loop-mediated isothermal amplification (LAMP) for detection of *Plasmopara viticola*. Sci. Rep. 6: 28935.

Le, D. T., and Vu, N. T. 2017 Progress of loop-mediated isothermal amplification technique in molecular diagnosis of plant diseases. Appl. Biol. Chem. 60: 169-180.

Liao, H. L., and Chung, K. R. 2008 Cellular toxicity of elsinochrome phytotoxins produced by the pathogenic fungus, *Elsinoë* fawcettii causing citrus scab. New Phytol. 177(1): 239-250.

Meinecke, C. D., De Vos, L., Yilmaz, N., Steenkamp, E. T., Wingfield, M. J., Wingfield, B. D., and Villari, C. 2023 A LAMP assay for rapid detection of the pitch canker pathogen *Fusarium circinatum*. Plant Dis. 107(10): 2916-2923.

Myrholm, C. L., Tomm, B.D., Heinzelmann, R., Feau, N., Hamelin, R.C., McDougal, R., Winkworth, R.C., and Ramsfield, T.D. 2021 Development of a Rapid Loop-Mediated Isothermal Amplification Assay for the Detection of *Dothistroma septosporum*. Forests 12(3): 362.

Notomi, T., Mori, Y., Tomita, N., and Kanda, H. 2015 Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. J. Microbiol. 53: 1-5.

Panno, S., Matić, S., Tiberini, A., Caruso, A. G., Bella, P., Torta, L., Stassi, R., and Davino, A. S. 2020 Loop Mediated Isothermal Amplification. Principles and applications in plant virology. Plants (Basel, Switzerland), 9(4), 461.

Pham, N. Q., Marincowitz, S., Solís, M., Duong, T. A., Wingfield, B. D., Barnes, I., Slippers, B., Abad, J. I. M., Durán, A., and Wingfield, M. J. 2021 Eucalyptus scab and shoot malformation: A new and serious foliar disease of *Eucalyptus* caused by *Elsinoë necatrix* sp. nov. Plant Pathol. 70: 1230-1242.

Raja, H. A., Miller, A. N., Pearce, C. J., and Oberlies, N. H. 2017 Fungal identification using molecular tools: a primer for the natural products research community. J. Nat. Prod. 80: 756-770.

Schneider, S., Jung, E., Queloz, V., Meyer, J. B., and Rigling, D. 2019 Detection of pine needle diseases caused by *Dothistroma septosporum*, *Dothistroma pini* and *Lecanosticta acicola* using different methodologies. For. Pathol. 49: e12495.

Soroka, M., B. Wasowicz and A. Rymaszewska, 2021 Loop-Mediated Isothermal Amplification (LAMP): The Better Sibling of PCR? Cells 10: 1931.

Smith, S., and Tsai, S. C. 2007 The type I fatty acid and polyketide synthases: a tale of two megasynthases. Nat. Prod. Rep. 24: 1041-1072.

Staunton, J., and Weissman, K. J. 2001 Polyketide biosynthesis: a millennium review. Nat. Prod. Rep. 18: 380-416.

Sullivan, M. J., Petty N. K. and Beatson, S. A. 2011 Easyfig: a genome comparison visualizer. Bioinformatics 27(7): 1009-1010.

Ter-hovhannisyan, V., Lomsadze, A., Chernoff Y. O., and Borodovsky, M. 2008 Gene prediction in novel fungal genomes using an ab initio algorithm with unsupervised training. Genome Res. 18(12): 1979-1990.

Weiss, U., Ziffer, H., Batterham, T. J., Blumer, M., Hackeng W. H. L., *et al.*, 1965 Pigments of *Elsinoë* species: i. pigment production by *Elsinoë* species; isolation of pure elsinochromes a, b, and c. Can. J. Microbiol. 11: 57-66.

White, T. J., Bruns, T., Lee S., and Taylor, J. 1989 Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, In: PCR - Protocols and Applications - A Laboratory Manual (pp.315-322). Academic press

Wingfield, B.D., Berger, D.K., Coetzee, M.P.A., Duong, T.A., Martin, A., Pham, N.Q., Van den Berg, N., Wilken, P.M., Arun-Chinnappa, K.S., Barnes, I., Buthelezi, S., Dahanayaka, B.A., Durán, A., Engelbrecht, J., Feurtey, A., Fourie, A., Fourie, G., Hartley, J., Kabwe, E.N.K., Maphosa, M., Narh Mensah, D.L., Nsibo, D.L., Potgieter, L., Poudel, B., Stukenbrock, E.H., Thomas, C., Vaghefi, N., Welgemoed, T., and Wingfield, M.J. 2022 IMA genome-F17. 13:19.

Zou, Y., Mason, M. G., Wang, Y., Wee, E., Turni, C., Blackall, P. J., Trau, M., and Botella, J. R. 2017 Nucleic acid purification from plants, animals and microbes in under 30 seconds. PLOS Biol. 15: e2003916.

Tables and Figures

Table 1. The elsinochrome toxin cluster genes predicted in *E. fawcettii* (Chung and Liao 2008).

Gene name	Protein function	Protein/gene accession number
Core biosynthetic genes		
EfPKS1	polyketide synthase protein	KAF4556316
TSF1	transcription factor-like protein	KAF4556314
RDT1	ESC reductase protein	KAF4556314
PRF1	prefoldin subunit 3 protein	KAF4556317
ECT1	ECT1 transporter protein	KAF4556318
Accessory cluster genes		
OXR1	Oxidoreductase	EU401706
EfHP1	Hypothetical protein 1	ABZ82009.1
EfHP2	Hypothetical protein 2	EU414200
EfHP3	Hypothetical protein 3	EU414201
EfHP4	Hypothetical protein 4	EU414202

Table 2. Primers used for the initial PCR amplification and final LAMP assay.

Name	Type	Length	Sequence (5'-3')
Conventional PCR Primers			
Forward1	Forward	20	GTCGCCCAATTTACTCGCAG
Reverse1	Reverse	22	ACATTCTGCTTGCATTCATGGC
LAMP Specific Primers			
F3	Forward outer	19	CGCATCTGCTTGACCTCTG
B3	Backward outer	19	CCACGCTTCTGTGTTTTGC
FIP	Forward inner	40	GCTGGCAGCATTCCTCGTCT-GCGAACGCATACAAGACAGT[1]
BIP	Backward inner	39	GTTGGGTTCGACCGCCTTCC-GCCTCCATGTTGCACTACA[1]
LF	Loop forward	19	CTGCTTGGGTGGTTTGGGC

[1] The hyphen indicateds the two regions of the FIP and BIP primers

LB	Loop backward	25	ACGAAGAGATCAGAAGAGTTCA AAG

Table 3. List of *Elsinoë* species and other *Eucalyptus* fungal pathogens used in this study, summarizing the LAMP results based on the specificity of the LAMP assay towards *E. necatrix.*

Species	Isolates[2]	Host	Country	Collector	LAMP assay
Elsinoë necatrix	CMW 56128	*Eucalyptus* sp.	Indonesia	N.Q. Pham	+
"	CMW 56130	*Eucalyptus* sp.	Indonesia	N.Q. Pham	+
"	CMW 56132	*Eucalyptus* sp.	Indonesia	N.Q. Pham	+

[2] CMW is the culture collection of the Forestry and Agricultural Biotechnology institute, University of Pretoria, South Africa; CBS is the Culture collection (CBS) of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands.

“	CMW 56134	*Eucalyptus* sp.	Indonesia	N.Q. Pham	+
“	CMW 56137	*Eucalyptus* sp.	Indonesia	N.Q. Pham	+
“	CMW 59240	*Eucalyptus* sp.	Indonesia	N.Q. Pham	+
“	CMW 59241	*Eucalyptus* sp.	Indonesia	N.Q. Pham	+
“	CMW 59242	*Eucalyptus* sp.	Indonesia	N.Q. Pham	+
“	CMW 59243	*Eucalyptus* sp.	Indonesia	N.Q. Pham	+
“	CMW 59244	*Eucalyptus* sp.	Indonesia	N.Q. Pham	+
“	CMW 59245	*Eucalyptus* sp.	Indonesia	N.Q. Pham	+
“	CMW 59246	*Eucalyptus* sp.	Indonesia	N.Q. Pham	+
“	CMW 59247	*Eucalyptus* sp.	Indonesia	N.Q. Pham	+
“	CMW 59248	*Eucalyptus* sp.	Indonesia	N.Q. Pham	+

"	CMW 59249	*Eucalyptus* sp.	Indonesia	N.Q. Pham	+
Elsinoë ampelina	CBS 208.25 CMW 56943	*Vitis vinifera*	Brazil	A.E. Jenkins	-
Elsinoë australis	CBS 314.32 CMW 56947	*Citrus aurantium*	Brazil	A.E. Jenkins	-
Elsinoë eucalypticola	CBS 124765 CMW 56948	*Eucalyptus* sp.	Australia	P.W. Crous	-
Elsinoë eucalyptorum	CBS 120084 CMW 56949	*Eucalyptus propinqua*	Australia	B.A. Summerell	-
Elsinoë euphorbiae	CBS 401.63 CMW 56950	*Euphorbia parviflora*	India	M.J. Thirumalachar	-
Elsinoë fawcettii	CBS 139.25 CMW 56951	*Citrus* sp.	USA	A.E. Jenkins	-
Elsinoë freylinae	CBS 128204 CMW 56955	*Freylinia lanceolata*	South Africa	P.W. Crous	-
Elsinoë genipae	CBS 342.39 CMW 56956	*Genipa americana*	Brazil	A.A. Bitancourt	-

Elsinoë glycines	CBS 389.64 CMW 56957	*Glycine soja*	Japan	H. Kurata	-
Elsinoë jasminicola	CBS 212.63 CMW 56959	*Jasminum malabaricum*	India	M.J. Thirumalachar	-
Elsinoë leucospermi	CBS 111207 CMW 56960	*Leucospermum* sp.	South Africa	P.W. Crous	-
Elsinoë perseae	CBS 288.64 CMW 56964	*Persea americana*	Brazil	C.A. Salemink	-
Elsinoë phaseoli	CBS 149.95 CMW 56966	*Phaseolus vulgaris*	South Africa	A. Liebenberg	-
Elsinoë piperitae	CBS 144615 CMW 56969	*Eucalyptus piperita*	Australia	P.W. Crous	-
Elsinoë poinsettiae	CBS 109333 CMW 56970	*Euphorbia pulcherrima*	Guatemala	M.E. Palm	-
Elsinoë populi	CBS 289.64 CMW 56972	*Populus serotina*	Argentina	C.A. Salemink	-

Elsinoë preissianae	CBS 142129 CMW 56974	*Eucalyptus preissiana*	Australia		-
Elsinoë protearum	CBS 113618 CMW 56975	*Protea* sp.	Zimbabwe	L. Swart	-
Elsinoë salignae	CBS 145552 CMW 56976	*Eucalyptus saligna*	Australia	A.J. Carnegie	-
Elsinoë quercus-ilicis	CBS 232.61 CMW 56977	*Quercus ilex*	Italy	G.P. Martelli and C. Laviola	-
Elsinoë randii	CBS 170.38 CMW 56978	*Cary* sp.	Brazil	A.S. Costa & J.M. Salles	-
Elsinoë rosarum	CBS 212.33 CMW 56980	*Rosa* sp.	USA	A.E. Jenkins	-
Elsinoë tectificae	CBS 124777 CMW 56983	*Eucalyptus tectifera*	Australia	B.A. Summerell	-
Calonectria retaudii	CMW 50789	*Eucalyptus* sp.	Malaysia	M.J. Wingfield	-
Teratosphaeria destructans	CMW 56797	*Eucalyptus* sp.	South Africa	M. Solís	-
Teratosphaeria epicoccoides	CMW 56798	*Eucalyptus* sp.	South Africa	M. Solís	-

Table 4. Summary of the sensitivity of the LAMP assay by using serial dilutions of *E. necatrix* DNA.

isolate CMW 56129		
Ng of DNA in 15µl sample	**Overall (+) PCR runs**	**Overall (+) LAMP runs**
1	9/9	9/9
0.5	9/9	9/9
0.1	8/9	9/9
0.05	6/9	7/9
0.01	0/9	8/9
0.005	0/9	6/9
0.001	0/9	4/9
Triplicate 1_Run 1 to Run 3		
Ng of DNA in 15µl sample	**PCR reaction**	**LAMP reaction**

	(+) PCR	(+) LAMP	Amplification time (min)	Anneal derivative (°C)
1	3/3	3/3	0:28:30 - 0:30:00	88,8
0.5	3/3	3/3	0:29:45 - 0:30:30	88,8
0.1	3/3	3/3	0:31:45 - 0:34:00	88,9
0.05	2/3	2/3	0:34:00 - 0:36:45	88,8
0.01	1/3	2/3	0:41:15 - 0: 45:45	88,8
0.005	0/3	0/3	-	88,8
0.001	0/3	1/3	0:43:45	88,8
Triplicate 2_Run 1 to Run 3				
Ng of DNA in 15µl sample	PCR reaction	LAMP reaction		
	(+) PCR	(+) LAMP	Amplification time (min)	Anneal derivative (°C)
1	3/3	3/3	0:29:15 - 0:34:00	88,8
0.5	3/3	3/3	0:29:15 - 0:30:30	88,9
0.1	2/3	3/3	0:32:45 - 0:37:00	88,8
0.05	3/3	2/3	0:35:45 - 0:36:15	88,8
0.01	0/3	3/3	0:36:30 - 0:59:15	88,9
0.005	0/3	3/3	0:40:15 - 0:45:45	88,8
0.001	0/3	2/3	0:36:45 - 1:05:30	88,8

Triplicate 3_Run 1 to Run 3				
Ng of DNA in 15µl sample	PCR reaction	LAMP reaction		
	(+) PCR	(+) LAMP	Amplification time (min)	Anneal derivative (°C)
1	3/3	3/3	0:28:15 - 0:30:45	88,8
0.5	3/3	3/3	0:30:00 - 0:32:15	88,8
0.1	3/3	3/3	0:32:15 - 0:33:30	88,8
0.05	1/3	3/3	0:34:00 - 0:35:15	88,8
0.01	0/3	3/3	0:37:15 - 0:39:30	88,8
0.005	0/3	3/3	0:41:30 - 0:58:30	88,8
0.001	0/3	1/3	1:00:30	88,8

Table 5. Summary LAMP results based on amplification of template DNA extracted from infected leave samples and pure cultures. Leaf sample/Cultures_Qiagen indicates results for DNA extracted using the DNeasy Plant Pro kit from Qiagen. Leaf sample/Cultures_Optigene indicates the results for DNA extracted using the Plant Material Lysis Kit from Optigene. Leaf sample/Cultures_Tris indicates results for DNA extracted using the plant extraction method described by Zou *et al.* (2017).

Origin and DNA extraction method	No. of positive amplifications/ No. of samples tested		Average annealing temp (°C)	Time (min)
	No. of overall positive runs	No. of positive runs/ duplicate		
Culture_Qiagen	9/9	3/3	88.8	21:45-23:15
		3/3		
		3/3		
Culture_Optigene	9/9	3/3	88.8	21:30-24:45

		3/3 3/3		
Culture_Tris	9/9	3/3 3/3 3/3	88.8	25:45-31:15
Leaf sample_Qiagen	6/9	3/3 3/3 0/3	88.8	21:15-41:00
Leaf sample_Optigene	5/9	3/3 0/3 2/3	88.8	33:51-40:45
Leaf sample_Tris	1/9	1/3 0/3 0/3	88.8	30:40

Figure 1. Schematic representation of the Blast comparison between the previously described gene cluster of *E. fawcettii* (top; 30,427 bp) and the corresponding genomic region of *E. necatrix* (bottom; 28,956 bp). The orange arrows represent the orientation and exon/intron regions of the predicted genes. The red bars represent the blast matches between *E. fawcettii* and *E. necatrix*. **A)** The green square represents a selected region that show low blast match between *E. fawcettii* and *E. necatrix* for potential LAMP primer design. **B)** Schematic representation of the Blast comparison of the enlarged region identified by the green square in figure A. With the top sequence representing the extracted region in *E. fawcettii* and the bottom sequence representing the extracted region in *E. nexatrix*. The forward and reverse primer indicated in blue was designed to amplify the region with no blast match between *E. fawcettii* and *E. necatrix*.

Figure 2. A) Schematic representation of the Easyfig blast comparison between the annotated target region of *E. necatrix* predicted by GeneMarkES and ORFfinder to the

cluster region in *E. fawcettii*. The purple arrows indicate the region where the selected LAMP primers are positioned indicating that the LAMP primers are designed in an exon region. **B)** Close up schematic representation of the design of the LAMP primers with the sequences listed in Table 2.

Figure 3. Amplification curves demonstrating a summary of the results of the LAMP assay specificity test. **A)** Positive results for all *E. necatrix* strains used in this study (labelled as CMW 59240 to CMW 59249 and summarized in table 3) with no amplification for the negative control (distilled water). **B)** Positive amplification for the positive controls (multiple *E. necatrix* strains) with no amplification for any of the closely related *Elsinoë* species or the negative control (distilled water). Species names are summarized in table 3.

Figure 4. LAMP assay sensitivity test for *E. necatrix* genomic DNA (isolate CMW 56129) diluted from 5 ng up until 0.001 ng. The colors on the graph corresponds to the concentration of genomic DNA measured in ng/ µl. Triplicate 1, run 1 indicated positive detection from 5 ng/ µl up to 0.005 ng/ µl with no detection at 0.001 ng/ µl. The results revealed an increase in time until detectable amplification with a decrease in DNA concentration.

Figure S1 A-C. Amplification curves demonstrating a summary of the results of the LAMP assay specificity test tested on non-target *Elsinoë* species. Positive amplification

is seen for the positive control (*E. necatrix*) with no amplification for any of the non-target *Elsinoë* species summarized in Table 3 or the negative control (distilled water).

Figure S2. A) Gel image of the amplified ITS regions in the 23 non-target *Elsinoë* sp. numbered according to the order in Table 3 from *Elsinoë ampelina* to *Elsinoë tectificae* and the three non *Elsinoë* spp. (*T. destructans, T. epicoccoides, C. reteaudii*) confirming the integrity of the extracted DNA in Figure 3B and figure S1. B) Gel images of the amplified TEF1 regions in the 23 non-target *Elsinoë* sp. numbered according to the order in Table 3 from *Elsinoë ampelina* to *Elsinoë tectificae* confirming the integrity of the extracted DNA in Figure 3B and figure S1.

Figure S3 A-H. LAMP assay sensitivity test for *E. necatrix* genomic DNA (isolate CMW 56129) diluted from 5 ng up until 0.001 ng. The colors on the graph corresponds to the concentration of genomic DNA measured in ng/ µl. The results revealed that there was an increase in time until amplification with a decrease in DNA concentration. A) Triplicate 1, run 1; B) Triplicate 1, run 2; C) Triplicate 1, run 3; D) Triplicate 2, run 1; E) Triplicate 2, run 2; F) Triplicate 2, run 3; G) Triplicate 3, run 2; H) Triplicate 3, run 3.

Figure S4. PCR sensitivity test for *E. necatrix* genomic DNA (isolate CMW 56129) diluted from 5 ng up until 0.001 ng. The same diluted genomic DNA used for the LAMP sensitivity test was used for the PCR sensitivity test. The letter (L) represents the 1kb ladder and the letter (B) represents a blank. The labelled wells represent the triplicate

number, run number and concentration of DNA (ng/ µl) etc. 11-1_triplicate 1, Run 1, 1 ng/ µl. Red arrows indicate a positive amplification with a clear visible band while the white arrows indicate a possible positive amplification with a faded band. The overall detection limit was seen to be 0.1 ng with positive but variable detection at 0.05 ng.

Figure S5. Amplification curves demonstrating a summary of the results of the LAMP assay for the *E. necatrix* mycelia grown on PDA extracted by the DNeasy Plant Pro kit from Qiagen, the Plant Material Lysis Kit from Optigene and the Tris extraction method described by Zou et al. (2017). *E. necatrix* strains used in this study labelled as 244, 245 and 246 represents isolates CMW 59244, CMW 59245 and CMW 59246 summarized in Table 3).

A) LAMP assay for DNA extracted from CMW 59244, CMW 59245 and CMW 59246 using the Qiagen extraction method. All 9 samples were amplified with no amplification for the negative control (distilled water).

B/C) LAMP assay for DNA extracted from CMW 59244, CMW 59245 and CMW 59246 using the Optigene extraction method. All 9 samples were amplified with no amplification for the negative control (distilled water).

D) LAMP assay for DNA extracted from CMW 59244, CMW 59245 and CMW 59246 using the Tris extraction method. All 9 samples were amplified with no amplification for the negative control (distilled water).

Figure S6. Amplification curves demonstrating a summary of the results of the LAMP assay for the *E. necatrix* (isolate CMW 56139) infected leaf samples extracted by the DNeasy Plant Pro kit from Qiagen, the Plant Material Lysis Kit from Optigene and the Tris extraction method described by Zou *et al.* (2017).

A) Q-1-1 represents the Qiagen extraction method, duplicate 1, replicate 1 etc. 6/9 samples were amplified with no amplification for the negative control (distilled water).

B/C) O-1-1 represents the Optigene extraction method, duplicate 1, replicate 1 etc. 5/9 samples were amplified with no amplification for the negative control (distilled water).

D) T-1-1 represents the Tris extraction method, duplicate 1, replicate 1 etc. 1/9 samples were amplified with no amplification for the negative control (distilled water).

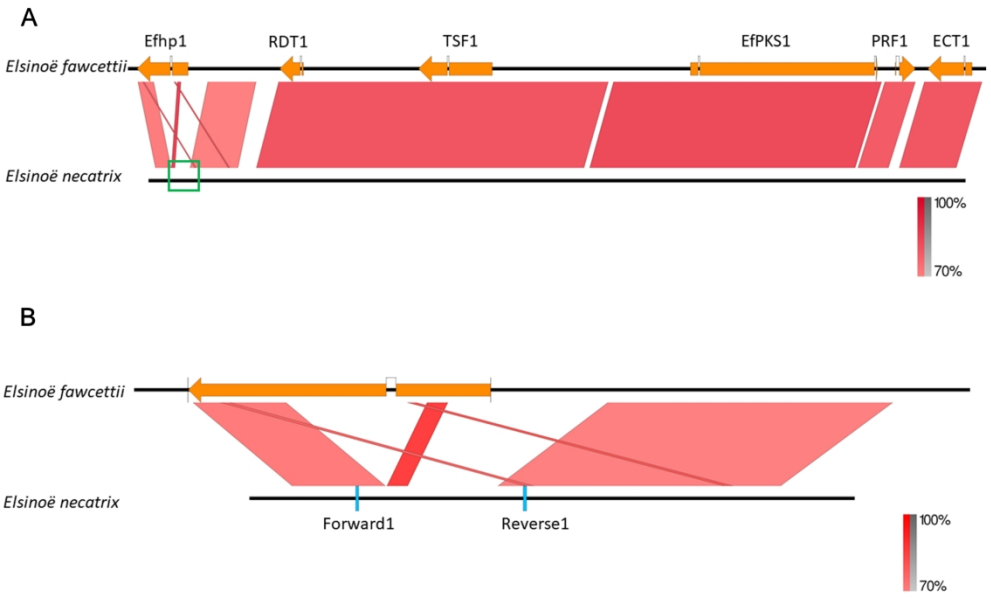

Figure 1. Schematic representation of the Blast comparison between the previously described gene cluster of E. fawcettii (top; 30,427 bp) and the corresponding genomic region of E. necatrix (bottom; 28,956 bp). The orange arrows represent the orientation and exon/intron regions of the predicted genes. The red bars represent the blast matches between E. fawcettii and E. necatrix. A) The green square represents a selected region that show low blast match between E. fawcettii and E. necatrix for potential LAMP primer design. B) Schematic representation of the Blast comparison of the enlarged region identified by the green square in figure A. With the top sequence representing the extracted region in E. fawcettii and the bottom sequence representing the extracted region in E. nexatrix. The forward and reverse primer indicated in blue was designed to amplify the region with no blast match between E. fawcettii and E. necatrix.

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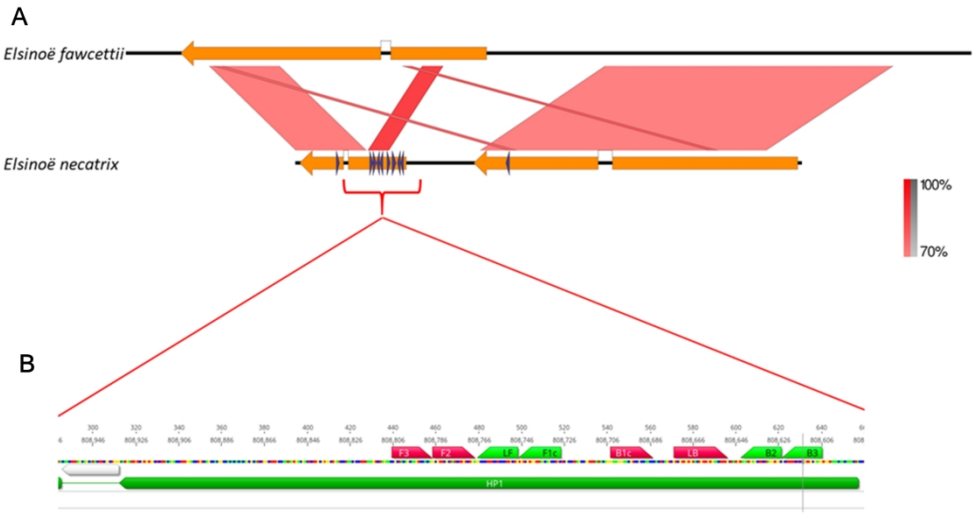


Figure 2. A) Schematic representation of the Easyfig blast comparison between the annotated target region of E. necatrix predicted by GeneMarkES and ORFfinder to the cluster region in E. fawcettii. The purple arrows indicate the region where the selected LAMP primers are positioned indicating that the LAMP primers are designed in an exon region. B) Close up schematic representation of the design of the LAMP primers with the sequences listed in Table 2.

169x101mm (330 x 330 DPI)

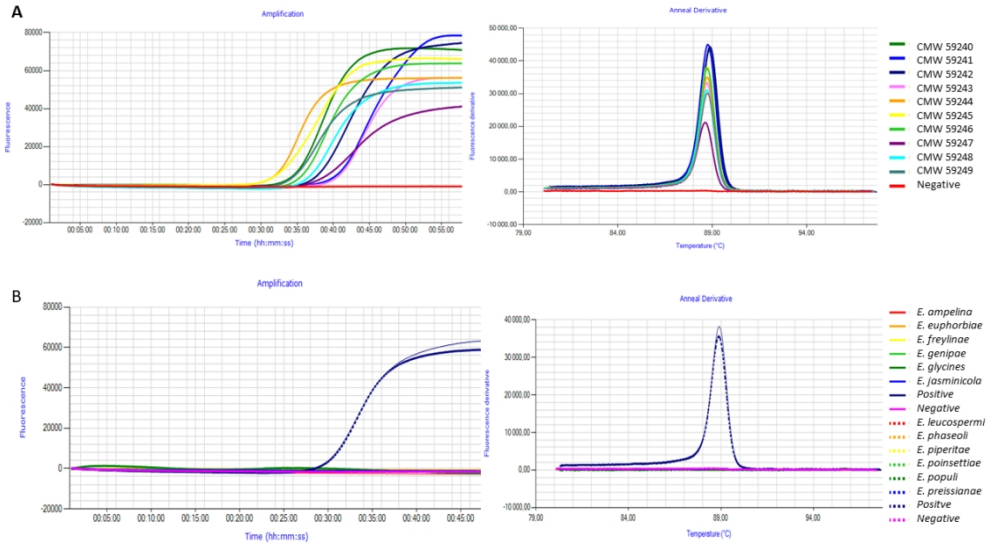


Figure 3. Amplification curves demonstrating a summary of the results of the LAMP assay specificity test. A) Positive results for all E. necatrix strains used in this study (labelled as CMW 59240 to CMW 59249 and summarized in table 3) with no amplification for the negative control (distilled water). B) Positive amplification for the positive controls (multiple E. necatrix strains) with no amplification for any of the closely related Elsinoë species or the negative control (distilled water). Species names are summarized in table 3.

338x190mm (96 x 96 DPI)

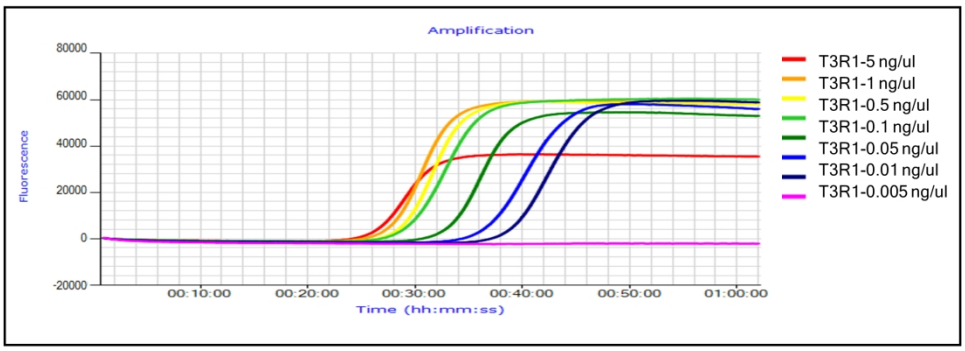


Figure 4. LAMP assay sensitivity test for E. necatrix genomic DNA (isolate CMW 56129) diluted from 5 ng up until 0.001 ng. The colors on the graph corresponds to the concentration of genomic DNA measured in ng/ µl. Triplicate 1, run 1 indicated positive detection from 5 ng/ µl up to 0.005 ng/ µl with no detection at 0.001 ng/ µl. The results revealed an increase in time until detectable amplification with a decrease in DNA concentration.

169x60mm (330 x 330 DPI)

Supplementary material

Table S1. PrimerExplorer parameters for LAMP primers.

Label	Tm range (°C)	3' change in free energy	5' change in free energy	Range of GC content
F3	59-61	ΔG ≤ -4 kcal/mol	-	50% - 60%
B3	59-61	ΔG ≤ -4 kcal/mol	-	50% - 60%
F1c	64-66	-	ΔG ≤ -4 kcal/mol	50% - 60%
B1c	64-66	-	ΔG ≤ -4 kcal/mol	50% - 60%
F2	59-61	ΔG ≤ -4 kcal/mol	-	50% - 60%
B2	59-61	ΔG ≤ -4 kcal/mol	-	50% - 60%
LF	64-66	ΔG ≤ -4 kcal/mol	-	50% - 60%
LB	64-66	ΔG ≤ -4 kcal/mol	-	50% - 60%

Table S2. PrimerExlorer parameters for Efhp1 LAMP primer set.

label	5'pos	3'pos	length	Tm (°C)	5'dG	3'dG	GCrate	Sequence
F3	156	174	19	60.02	-6.08	-5.19	0.58	CGCATCTGCTTGACCTCTG
B3	338	356	19	59.41	-7.18	-4.68	0.53	CCACGCTTCTGTGTTTTGC
FIP			40					GCTGGCAGCATTCCTCGTCT-GCGAACGCATACAAGACAGT
BIP			39					GTTGGGTTCGACCGCCTTCC-GCCTCCATGTTGCACTACA
F2	175	194	20	60.30	-6.19	-4.90	0.50	GCGAACGCATACAAGACAGT
F1c	215	234	20	64.56	-7.09	-5.43	0.60	GCTGGCAGCATTCCTCGTCT
B2	319	337	19	59.40	-6.54	-4.13	0.53	GCCTCCATGTTGCACTACA
B1c	258	277	20	65.27	-5.61	-5.30	0.65	GTTGGGTTCGACCGCCTTCC

Table S3. Summary of the sensitivity of the LAMP assay by using serial dilutions of *E. necatrix* DNA.

CMW 56129_Triplicate 1_Run 1				
Ng of DNA in 15µl sample	**PCR**	**LAMP**	**Amplification time (min)**	**Anneal derivative (°C)**
1,00	(+)	(+)	0:30:00	88,80
0.5	(+)	(+)	0:30:30	88,80
0.1	(+)	(+)	0:33:30	88,90
0.05	(+)	(+)	0:36:45	88,80
0.01	(-)	(+)	0:41:15	88,80
0.005	(-)	(-)		
0.001	(-)	(+)	0:43:45	88,80
CMW 56129_ Triplicate 1_Run 2				
Ng of DNA in 15µl sample	**PCR**	**LAMP**	**Amplification time (min)**	**Anneal derivative (°C)**
1,00	(+)	(+)	0:28:30	88,80
0.5	(+)	(+)	0:30:15	88,90
0.1	(+)	(+)	0:34:00	88,80
0.05	(-)	(+)	0:34:00	88,80
0.01	(+)	(+)	0:45:45	88,90
0.005	(-)	(-)		
0.001	(-)	(-)		
CMW 56129_ Triplicate 1_Run 3				

Ng of DNA in 15µl sample	PCR	LAMP	Amplification time (min)	Anneal derivative (°C)
1,00	(+)	(+)	0:29:30	88,80
0.5	(+)	(+)	0:29:45	88,80
0.1	(+)	(+)	0:31:45	88,80
0.05	(+)	(-)		
0.01	(-)	(-)		
0.005	(-)	(-)		
0.001	(-)	(-)		
CMW 56129_ Triplicate 2_Run 1				
Ng of DNA in 15µl sample	PCR	LAMP	Amplification time (min)	Anneal derivative (°C)
1,00	(+)	(+)	0:29:15	88,70
0.5	(+)	(+)	0:29:15	88,80
0.1	(+)	(+)	0:32:45	88,70
0.05	(+)	(-)		
0.01	(-)	(+)	0:36:30	88,80
0.005	(-)	(+)	0:40:15	88,80
0.001	(-)	(+)	1:05:30	88,80
CMW 56129_ Triplicate 2_Run 2				
Ng of DNA in 15µl sample	PCR	LAMP	Amplification time (min)	Anneal derivative (°C)
1,00	(+)	(+)	0:34:00	88,70

0.5	(+)	(+)	0:30:30	88,80
0.1	(+)	(+)	0:34:00	88,90
0.05	(+)	(+)	0:36:15	88,80
0.01	(-)	(+)	0:38:45	88,90
0.005	(-)	(+)	0:45:45	88,80
0.001	(-)	(+)	0:36:45	88,80
CMW 56129_ Triplicate 2_Run 3				
Ng of DNA in 15µl sample	**PCR**	**LAMP**	**Amplification time (min)**	**Anneal derivative (°C)**
1,00	(+)	(+)	0:30:00	88,70
0.5	(+)	(+)	0:30:00	88,80
0.1	(-)	(+)	0:37:00	88,80
0.05	(+)	(+)	0:35:45	88,80
0.01	(-)	(+)	59:15	88,60
0.005	(-)	(+)	0:44:00	88,80
0.001	(-)	(-)		
CMW 56129_ Triplicate 3_Run 1				
Ng of DNA in 15µl sample	**PCR**	**LAMP**	**Amplification time (min)**	**Anneal derivative (°C)**
1,00	(+)	(+)	0:30:00	88,80
0.5	(+)	(+)	0:31:00	88,80
0.1	(+)	(+)	0:32:15	88,90
0.05	(+)	(+)	0:35:15	88,80

0.01	(-)	(+)	0:39:30	88,90
0.005	(-)	(+)	0:41:30	88,90
0.001	(-)	(-)		
CMW 56129_ Triplicate 3_Run 2				
Ng of DNA in 15µl sample	PCR	LAMP	Amplification time (min)	Anneal derivative (°C)
1,00	(+)	(+)	0:28:15	88,80
0.5	(+)	(+)	0:30:00	88,80
0.1	(+)	(+)	0:33:00	88,70
0.05	(+)	(+)	0:34:00	88,70
0.01	(-)	(+)	0:37:15	88,90
0.005	(-)	(+)	0:46:30	88,70
0.001	(-)	(-)		
CMW 56129_ Triplicate 3_Run 3				
Ng of DNA in 15µl sample	PCR	LAMP	Amplification time (min)	Anneal derivative (°C)
1,00	(+)	(+)	0:30:45	88,80
0.5	(+)	(+)	0:32:15	88,70
0.1	(+)	(+)	0:33:30	88,80
0.05	(-)	(+)	0:35:00	88,70
0.01	(-)	(+)	0:38:45	88,90
0.005	(-)	(+)	0:58:30	88,70
0.001	(-)	(+)	1:00:30	88,70

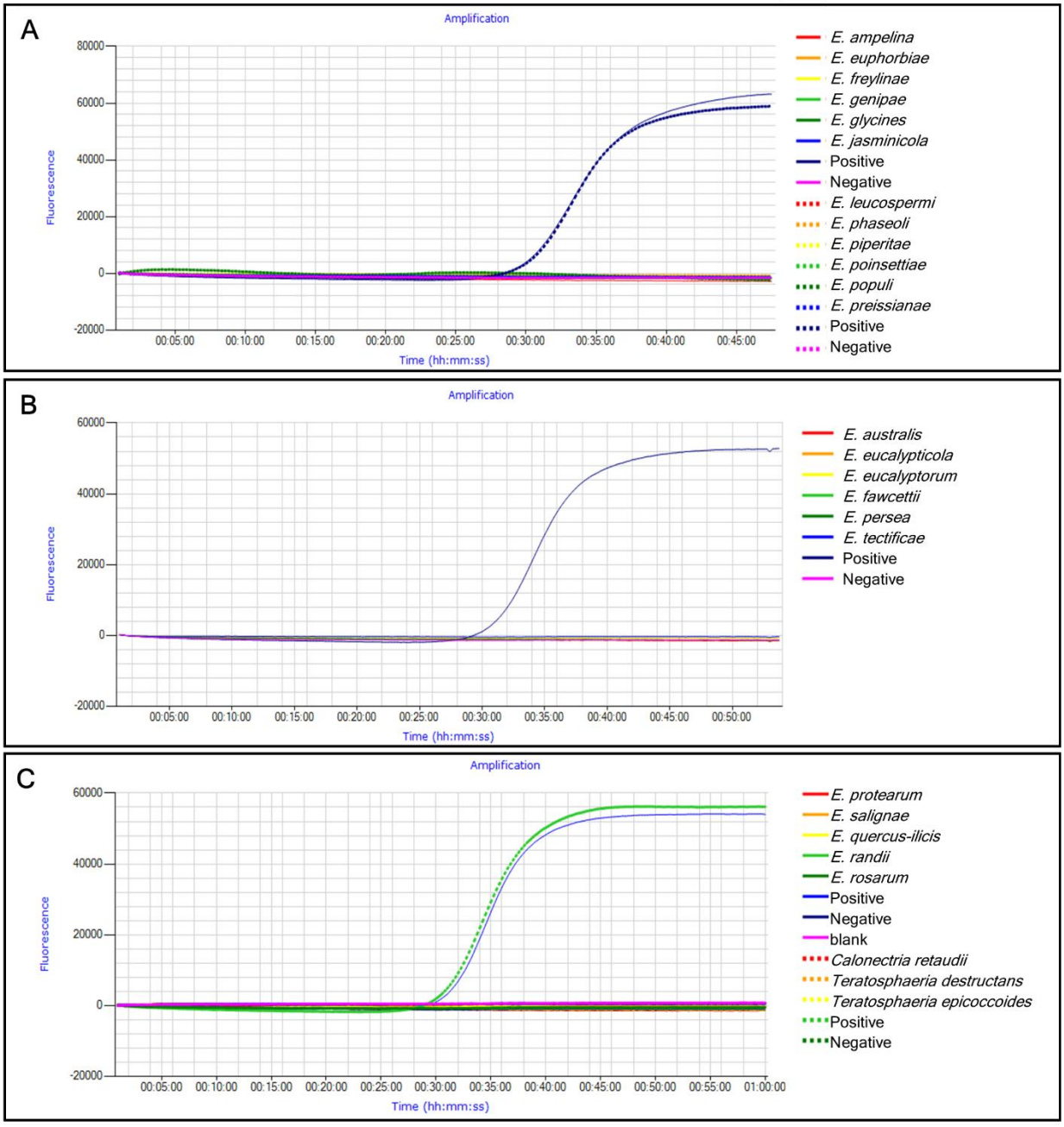
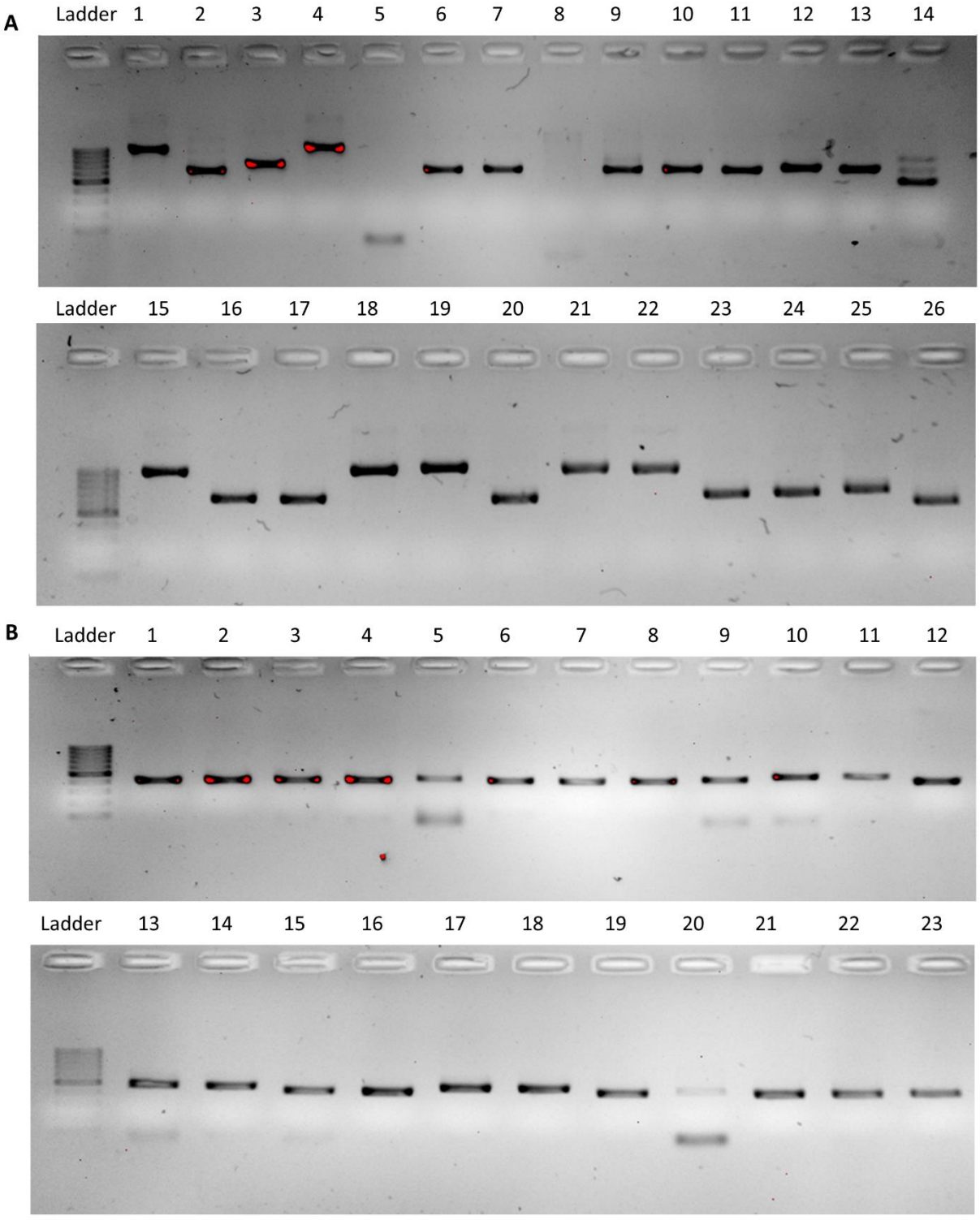

A
Amplification
Fluorescence
80000
60000
40000
20000
0
-20000
00:05:00
00:10:00
00:15:00
00:20:00
00:25:00
00:30:00
00:35:00
00:40:00
00:45:00
Time (hh:mm:ss)
E. ampelina
E. euphorbiae
E. freylinae
E. genipae
E. glycines
E. jasminicola
Positive
Negative
E. leucospermi
E. phaseoli
E. piperitae
E. poinsettiae
E. populi
E. preissianae
Positive
Negative
B
Amplification
Fluorescence
60000
40000
20000
0
-20000
00:05:00
00:10:00
00:15:00
00:20:00
00:25:00
00:30:00
00:35:00
00:40:00
00:45:00
00:50:00
Time (hh:mm:ss)
E. australis
E. eucalypticola
E. eucalyptorum
E. fawcettii
E. persea
E. tectificae
Positive
Negative
C
Amplification
Fluorescence
60000
40000
20000
0
-20000
00:05:00
00:10:00
00:15:00
00:20:00
00:25:00
00:30:00
00:35:00
00:40:00
00:45:00
00:50:00
00:55:00
01:00:00
Time (hh:mm:ss)
E. protearum
E. salignae
E. quercus-ilicis
E. randii
E. rosarum
Positive
Negative
blank
Calonectria retaudii
Teratosphaeria destructans
Teratosphaeria epicoccoides
Positive
Negative

Figure S1 A-C. Amplification curves demonstrating a summary of the results of the LAMP assay specificity test tested on non-target *Elsinoë* species. Positive amplification is seen for the positive control (*E. necatrix*) with no amplification for any of the non-target *Elsinoë* species summarized in Table 3 or the negative control (distilled water).


A
Ladder 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Ladder 15 16 17 18 19 20 21 22 23 24 25 26
B
Ladder 1 2 3 4 5 6 7 8 9 10 11 12
Ladder 13 14 15 16 17 18 19 20 21 22 23

32

33

Figure S2: A) Gel image of the amplified ITS regions in the 23 non-target *Elsinoë* sp. numbered according to the order in Table 3 from *Elsinoë ampelina* to *Elsinoë tectificae* and the three non *Elsinoë* spp. (*T. destructans, T. epicoccoides, C. reteaudii*) confirming the integrity of the extracted DNA in Figure 3B and figure S1. B) Gel images of the amplified TEF1 regions in the 23 non-target *Elsinoë* sp. numbered according to the order in Table 3 from *Elsinoë ampelina* to *Elsinoë tectificae* confirming the integrity of the extracted DNA in Figure 3B and figure S1.

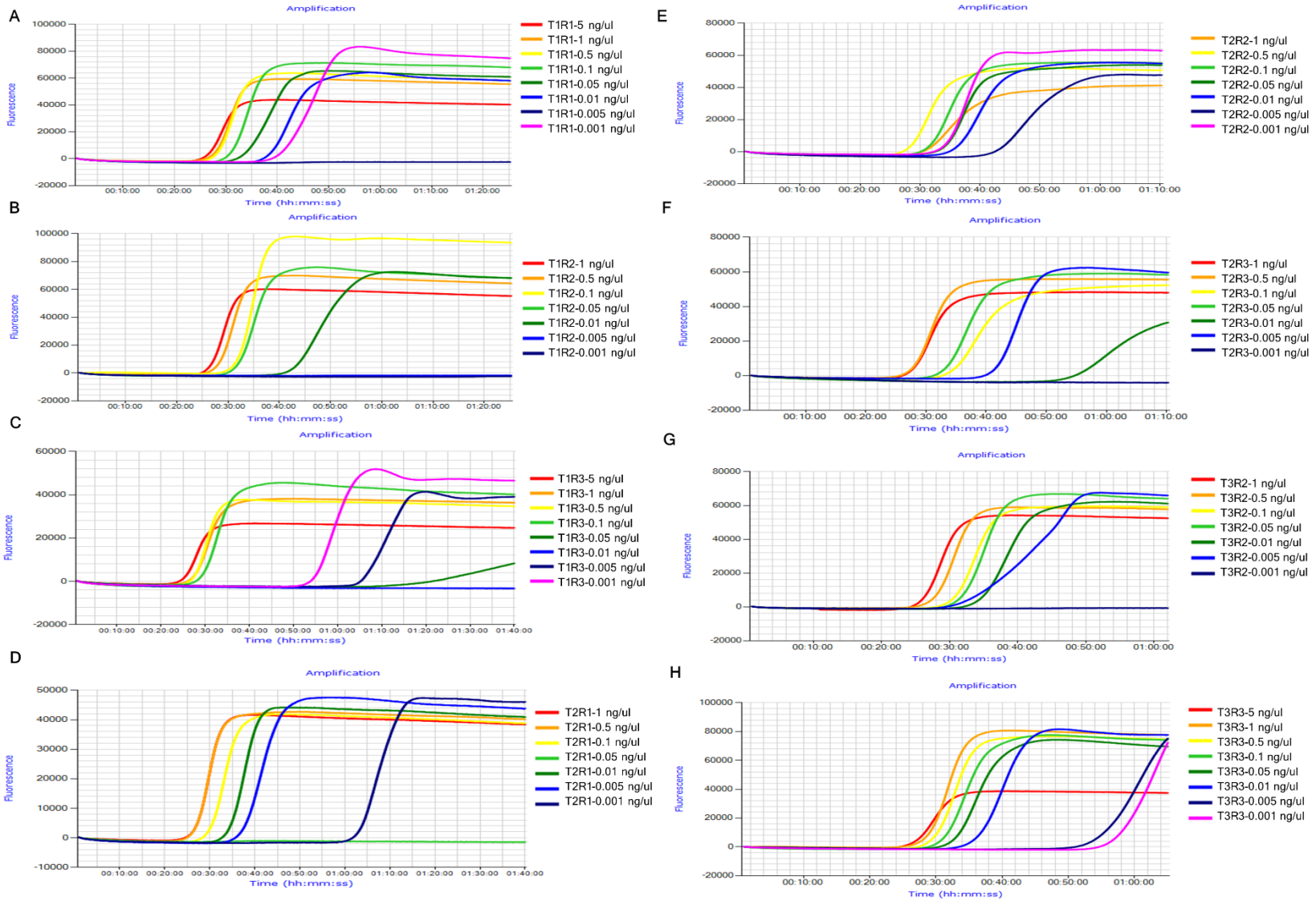
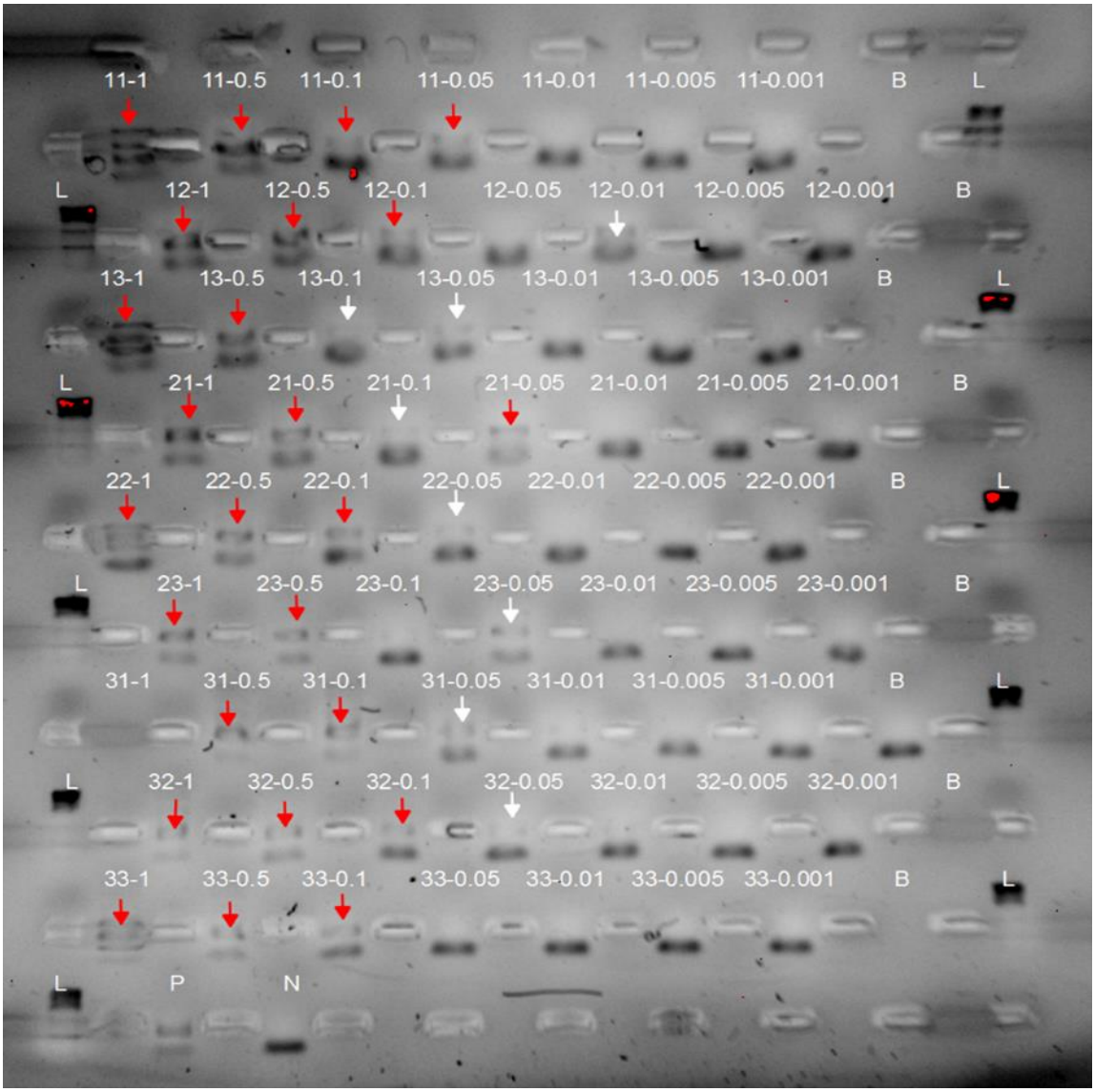

A
Amplification
Fluorescence
Time (hh:mm:ss)
T1R1-5 ng/ul
T1R1-1 ng/ul
T1R1-0.5 ng/ul
T1R1-0.1 ng/ul
T1R1-0.05 ng/ul
T1R1-0.01 ng/ul
T1R1-0.005 ng/ul
T1R1-0.001 ng/ul
B
Amplification
Fluorescence
Time (hh:mm:ss)
T1R2-1 ng/ul
T1R2-0.5 ng/ul
T1R2-0.1 ng/ul
T1R2-0.05 ng/ul
T1R2-0.01 ng/ul
T1R2-0.005 ng/ul
T1R2-0.001 ng/ul
C
Amplification
Fluorescence
Time (hh:mm:ss)
T1R3-5 ng/ul
T1R3-1 ng/ul
T1R3-0.5 ng/ul
T1R3-0.1 ng/ul
T1R3-0.05 ng/ul
T1R3-0.01 ng/ul
T1R3-0.005 ng/ul
T1R3-0.001 ng/ul
D
Amplification
Fluorescence
Time (hh:mm:ss)
T2R1-1 ng/ul
T2R1-0.5 ng/ul
T2R1-0.1 ng/ul
T2R1-0.05 ng/ul
T2R1-0.01 ng/ul
T2R1-0.005 ng/ul
T2R1-0.001 ng/ul
E
Amplification
Fluorescence
Time (hh:mm:ss)
T2R2-1 ng/ul
T2R2-0.5 ng/ul
T2R2-0.1 ng/ul
T2R2-0.05 ng/ul
T2R2-0.01 ng/ul
T2R2-0.005 ng/ul
T2R2-0.001 ng/ul
F
Amplification
Fluorescence
Time (hh:mm:ss)
T2R3-1 ng/ul
T2R3-0.5 ng/ul
T2R3-0.1 ng/ul
T2R3-0.05 ng/ul
T2R3-0.01 ng/ul
T2R3-0.005 ng/ul
T2R3-0.001 ng/ul
G
Amplification
Fluorescence
Time (hh:mm:ss)
T3R2-1 ng/ul
T3R2-0.5 ng/ul
T3R2-0.1 ng/ul
T3R2-0.05 ng/ul
T3R2-0.01 ng/ul
T3R2-0.005 ng/ul
T3R2-0.001 ng/ul
H
Amplification
Fluorescence
Time (hh:mm:ss)
T3R3-5 ng/ul
T3R3-1 ng/ul
T3R3-0.5 ng/ul
T3R3-0.1 ng/ul
T3R3-0.05 ng/ul
T3R3-0.01 ng/ul
T3R3-0.005 ng/ul
T3R3-0.001 ng/ul

Figure S3 A-H. LAMP assay sensitivity test for *E. necatrix* genomic DNA (isolate CMW 56129) diluted from 5 ng up until 0.001 ng. The colors on the graph corresponds to the concentration of genomic DNA measured in ng/ µl. The results revealed that there was an increase in time until amplification with a decrease in DNA concentration. A) Triplicate 1, run 1; B) Triplicate 1, run 2; C) Triplicate 1, run 3; D) Triplicate 2, run 1; E) Triplicate 2, run 2; F) Triplicate 2, run 3; G) Triplicate 3, run 2; H) Triplicate 3, run 3.

77


11-1 11-0.5 11-0.1 11-0.05 11-0.01 11-0.005 11-0.001 B L
L 12-1 12-0.5 12-0.1 12-0.05 12-0.01 12-0.005 12-0.001 B
13-1 13-0.5 13-0.1 13-0.05 13-0.01 13-0.005 13-0.001 B L
L 21-1 21-0.5 21-0.1 21-0.05 21-0.01 21-0.005 21-0.001 B
22-1 22-0.5 22-0.1 22-0.05 22-0.01 22-0.005 22-0.001 B L
L 23-1 23-0.5 23-0.1 23-0.05 23-0.01 23-0.005 23-0.001 B
31-1 31-0.5 31-0.1 31-0.05 31-0.01 31-0.005 31-0.001 B L
L 32-1 32-0.5 32-0.1 32-0.05 32-0.01 32-0.005 32-0.001 B
33-1 33-0.5 33-0.1 33-0.05 33-0.01 33-0.005 33-0.001 B L
L P N

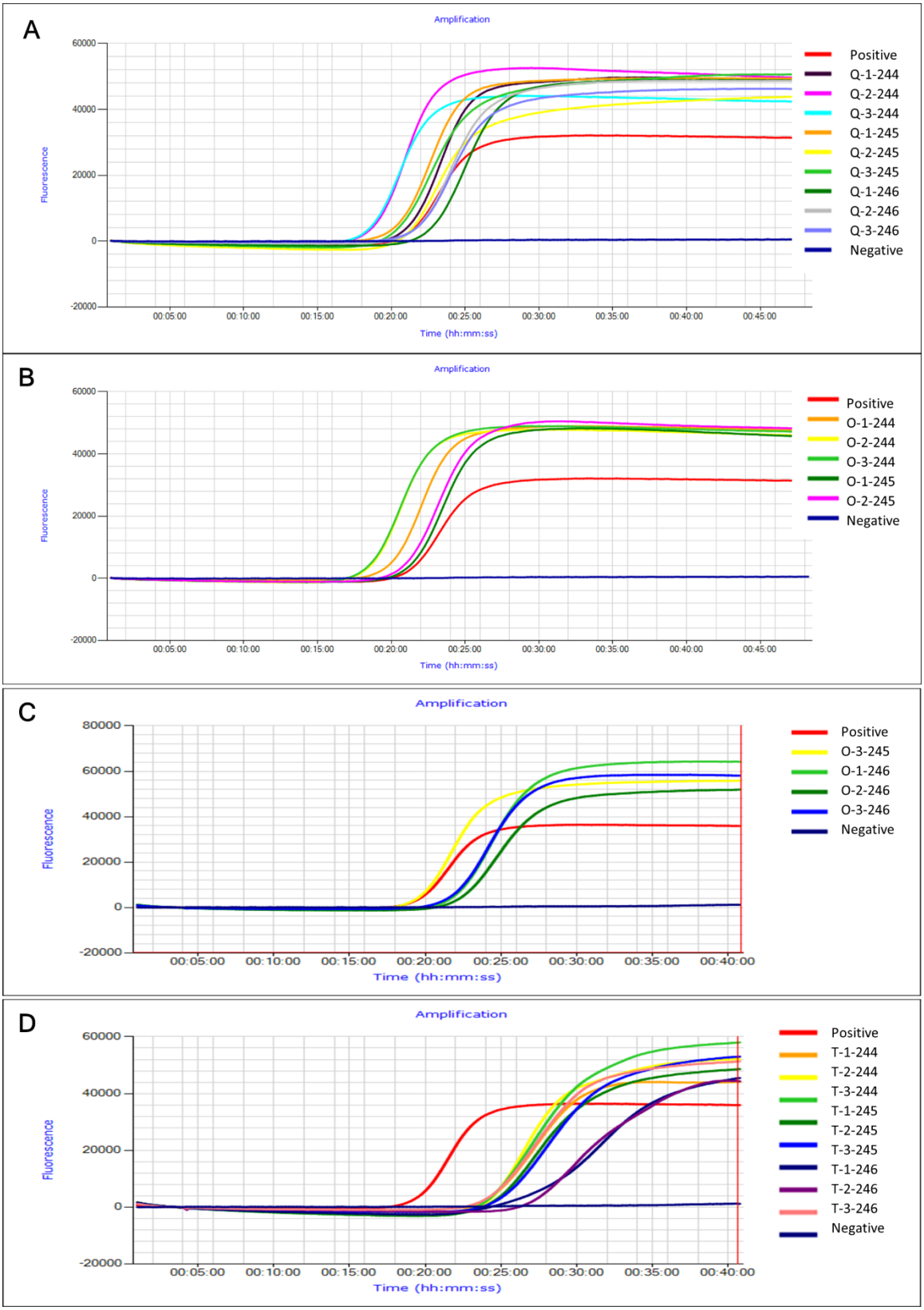
78

79

80

81

Figure S4. PCR sensitivity test for *E. necatrix* genomic DNA (isolate CMW 56129) diluted from 5 ng up until 0.001 ng. The same diluted genomic DNA used for the LAMP sensitivity test was used for the PCR sensitivity test. The letter (L) represents the 1kb ladder and the letter (B) represents a blank. The labelled wells represent the triplicate number, run number and concentration of DNA (ng/ µl) etc. 11-1_triplicate 1, Run 1, 1 ng/ µl. Red arrows indicate a positive amplification with a clear visible band while the white arrows indicate a possible positive amplification with a faded band. The overall detection limit was seen to be 0.1 ng with positive but variable detection at 0.05 ng.


A
Amplification
Fluorescence
Time (hh:mm:ss)
Positive
Q-1-244
Q-2-244
Q-3-244
Q-1-245
Q-2-245
Q-3-245
Q-1-246
Q-2-246
Q-3-246
Negative
B
Amplification
Fluorescence
Time (hh:mm:ss)
Positive
O-1-244
O-2-244
O-3-244
O-1-245
O-2-245
Negative
C
Amplification
Fluorescence
Time (hh:mm:ss)
Positive
O-3-245
O-1-246
O-2-246
O-3-246
Negative
D
Amplification
Fluorescence
Time (hh:mm:ss)
Positive
T-1-244
T-2-244
T-3-244
T-1-245
T-2-245
T-3-245
T-1-246
T-2-246
T-3-246
Negative

102

Figure S5: Amplification curves demonstrating a summary of the results of the LAMP assay for the *E. necatrix* mycelia grown on PDA extracted by the DNeasy Plant Pro kit from Qiagen, the Plant Material Lysis Kit from Optigene and the Tris extraction method described by Zou et al. (2017). *E. necatrix* strains used in this study labelled as 244, 245 and 246 represents isolates CMW 59244, CMW 59245 and CMW 59246 summarized in Table 3).

A) LAMP assay for DNA extracted from CMW 59244, CMW 59245 and CMW 59246 using the Qiagen extraction method. All 9 samples were amplified with no amplification for the negative control (distilled water).

B/C) LAMP assay for DNA extracted from CMW 59244, CMW 59245 and CMW 59246 using the Optigene extraction method. All 9 samples were amplified with no amplification for the negative control (distilled water).

D) LAMP assay for DNA extracted from CMW 59244, CMW 59245 and CMW 59246 using the Tris extraction method. All 9 samples were amplified with no amplification for the negative control (distilled water).

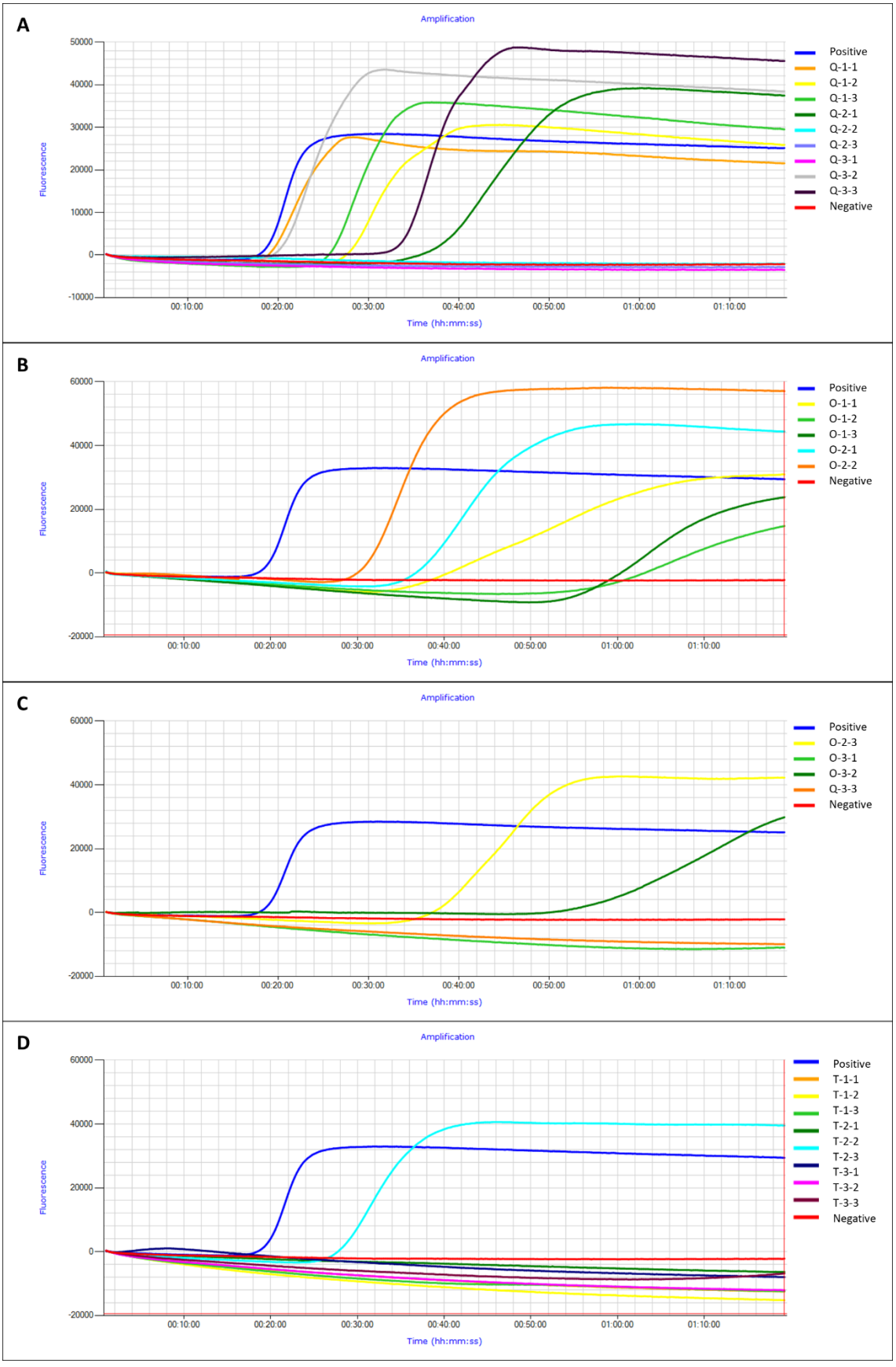

A
Amplification
Fluorescence
Time (hh:mm:ss)
Positive
Q-1-1
Q-1-2
Q-1-3
Q-2-1
Q-2-2
Q-2-3
Q-3-1
Q-3-2
Q-3-3
Negative
B
Amplification
Fluorescence
Time (hh:mm:ss)
Positive
O-1-1
O-1-2
O-1-3
O-2-1
O-2-2
Negative
C
Amplification
Fluorescence
Time (hh:mm:ss)
Positive
O-2-3
O-3-1
O-3-2
Q-3-3
Negative
D
Amplification
Fluorescence
Time (hh:mm:ss)
Positive
T-1-1
T-1-2
T-1-3
T-2-1
T-2-2
T-2-3
T-3-1
T-3-2
T-3-3
Negative

Figure S6: Amplification curves demonstrating a summary of the results of the LAMP assay for the *E. necatrix* (isolate CMW 56139) infected leaf samples extracted by the DNeasy Plant Pro kit from Qiagen, the Plant Material Lysis Kit from Optigene and the Tris extraction method described by Zou *et al*. (2017).

A) Q-1-1 represents the Qiagen extraction method, duplicate 1, replicate 1 etc. 6/9 samples were amplified with no amplification for the negative control (distilled water).

B/C) O-1-1 represents the Optigene extraction method, duplicate 1, replicate 1 etc. 5/9 samples were amplified with no amplification for the negative control (distilled water).

D) T-1-1 represents the Tris extraction method, duplicate 1, replicate 1 etc. 1/9 samples were amplified with no amplification for the negative control (distilled water).

Calculation of the target copy number as described by:
https://bitesizebio.com/20669/how-to-calculate-the-number-of-molecules-in-any-piece-of-dna/

((0.01) x (6.022 x 10e23)) / ((22.8 x 10e6 genome length in bp) x (1 x10e9) x (650)).
=406.34