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ORIGINAL ARTICLE

Quambalaria eucalypti found on *Eucalyptus* in Indonesia

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Abstract

The *Eucalyptus* plantation industry in Indonesia has expanded rapidly during the last few decades. During routine nursery disease surveys, symptoms of a leaf and shoot blight disease were detected on *Eucalyptus* mother plants. Isolates were obtained from symptomatic tissues and identified using DNA sequence analyses. Phylogenetic analyses showed that the isolates were those of *Quambalaria eucalypti*. Pathogenicity tests were conducted with isolates of *Q. eucalypti* on clones of *E. pellita* and *E. grandis* × *E. pellita* hybrids. These resulted in symptoms similar to those observed on naturally infected plants. *Eucalyptus* genotypes tested showed variation in their susceptibility, highlighting the potential to select and breed for resistance and thus to manage future outbreaks of the disease. This is the first report of the pathogen in Indonesia as well as in Southeast Asia.

KEYWORDS
Eucalyptus hybrids, fungal pathogen, leaf and shoot blight, mother plants, nursery diseases

1 | INTRODUCTION

Plantation forestry especially utilizing non-native tree species has grown rapidly in many parts of the world during the course of the last three decades, including in Southeast Asia (Payn et al., 2015). Due to pest and pathogen problems, *Acacia mangium* plantations in Indonesia, Malaysia and Vietnam have declined in relevance, leading to a rise in planting of *Eucalyptus* spp. in these regions (Harwood & Nambiar, 2014; Nambiar et al., 2018; Tarigan et al., 2011). *Eucalyptus pellita* and its hybrids with *E. grandis*, *E. brassiana* and *E. urophylla* are now most widely planted in this region (Hardiyanto et al., 2021). Similar to other non-native trees grown in new environments, the emergence of insect pests and pathogens poses a threat to the sustainability of these planted forests (Burgess & Wingfield, 2017; Coetzee et al., 2011; Crous et al., 1998; McTaggart et al., 2016; Pham et al., 2021; Wingfield, 2003; Wingfield et al., 1996, 2008).

Diseases that have affected eucalypt plantation forestry in various parts of the world include those caused by species of *Quambalaria*. The genus *Quambalaria* (Quambalariaceae, Microstromatales, Basidiomycota) includes several important pathogens of eucalypts in the tropics and southern hemisphere (Braun, 1998; Cheewangkoon et al., 2009; Crous et al., 2019; Wingfield et al., 1993). Some of these species cause severe leaf and shoot die-back as well as stem cankers (Alfenas et al., 2004; Chen et al., 2017; Pegg et al., 2008; Roux et al., 2006; Santos et al., 2020). These include *Q. eucalypti* that has caused significant damage to plantations and nurseries in various parts of the world (Alfenas et al., 2001; Bettucci et al., 1999; Pegg et al., 2008; Roux et al., 2006; Simpson, 2000; Wingfield et al., 1993).

Quambalaria eucalypti was first described causing a disease of *E. grandis* in South Africa and where the causal agent was named *Sporothrix eucalypti* (Wingfield et al., 1993). The fungus was later transferred to *Quambalaria* and where it was first recognized to be a

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basidiomycete. Disease problems caused by the pathogen were subsequently reported from Brazil (Alfenas et al., 2001), Uruguay (Bettucci et al., 1999), Australia (Pegg et al., 2008), Portugal (Bragança et al., 2016) and China (Chen et al., 2017). The disease is characterized by the appearance of powdery white fungal spore masses on the surface of infected shoots and leaves (de Beer et al., 2006; Pegg, Carnegie, et al., 2009; Roux et al., 2006; Simpson, 2000; Wingfield et al., 1993). *Quambalaria eucalypti* most commonly infects *Eucalyptus* plants in nurseries (Simpson, 2000; Wingfield et al., 1993), but it is also known to infect established trees in plantations (Alfenas et al., 2004; Chen et al., 2017; Pegg et al., 2008; Roux et al., 2006; Santos et al., 2020).

Beginning in 2018, symptoms of a leaf and shoot blight disease resembling infection by a *Quambalaria* species were observed in nurseries in North Sumatra, Riau and in North Kalimantan (Indonesia). These infections were mostly on clonal mother plants used to vegetatively propagate *Eucalyptus* for plantation establishment. The objectives of this study were to identify the causal agent of this disease and to test the pathogenicity of the putative pathogen on different *Eucalyptus* genotypes.

2 | MATERIALS AND METHODS

2.1 | Sample collection and fungal isolation

Symptoms of a leaf and shoot blight disease were observed on the leaves of *Eucalyptus* mother plants in nurseries in three regions, including North Sumatra, Riau and North Kalimantan. The disease was characterized by powdery white fungal spore masses on young leaves, shoots and stems (Figure 1). The symptoms were mainly observed on mother plants of *E. pellita* and its hybrids, including *E. pellita* × *E. grandis*, *E. pellita* × *E. brassiana* and *E. pellita* × *E. urophylla*.

Symptomatic leaf and shoot samples (Figure 1) were collected from *Eucalyptus* clones in five nurseries in different regions including one in North Sumatra, three in Riau and one nursery in North Kalimantan (Figure 2). Each sample was placed in a separate brown paper bag and then transferred to the laboratory for isolation in culture.

White spore masses on the surface of the leaves and shoots were scraped from the samples with a sterile needle and transferred to the surface of potato dextrose agar (PDA Acumedia®: 40 g/L) in Petri dishes. The culture plates were then incubated at 27°C for 7 days, and single hyphal tips from primary isolations were subcultured on clean PDA to obtain pure isolates. The resulting isolates were stored in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

2.2 | DNA extraction, PCR amplification and sequencing

DNA was extracted from mycelium of 7-day-old cultures using Prepman® Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, MA, USA). The internal transcribed spacer (ITS) regions 1 and 2, including the 5.8S rRNA region and the large subunit (LSU) of the rRNA, were amplified using primers ITS1F/ITS4 (Gardes & Bruns, 1993; White et al., 1990) and LR0R/LR5 (Rehner & Samuels, 1994; Vilgalys & Hester, 1990), respectively. Polymerase chain reaction (PCR) amplifications were performed in 13 μL reactions containing 1 μL of genomic DNA, 2.5 μL of 5× MyTaq buffer (Bioline, London, UK), 0.25 μL MyTaq DNA polymerases (Bioline), 0.5 μL of each primer (10 μM) and 8.25 μL sterile deionized water. Thermal cycling included an initial denaturation at 95°C for 5 min, followed by 10 primary amplification cycles of 30 s at 95°C, 30 s at 55°C and 60 s at 72°C, then 30 additional cycles of the same reaction sequence, with the annealing step increasing by 5 s per cycle. Reactions were completed with a final extension at 72°C for 10 min. Polymerase chain reaction products were purified using ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced using BigDye terminator sequencing kit 3.1 (Applied Biosystems, Forster City, CA, USA) in both the forward and reverse primers. Sequencing was performed on an ABI PRISM 3100 DNA sequencer (Applied Biosystems, Forster City, CA, USA). Geneious Prime 2023.0.3 (https://www.geneious.com) was used to assemble and edit the raw sequences. All the sequences resulting from this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/) (Table 1).

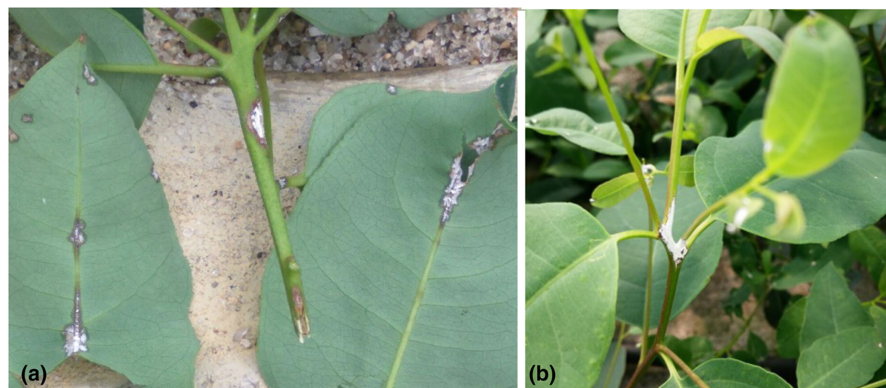

FIGURE 1 Symptoms of *Q. eucalypti* infection on leaves and stems.

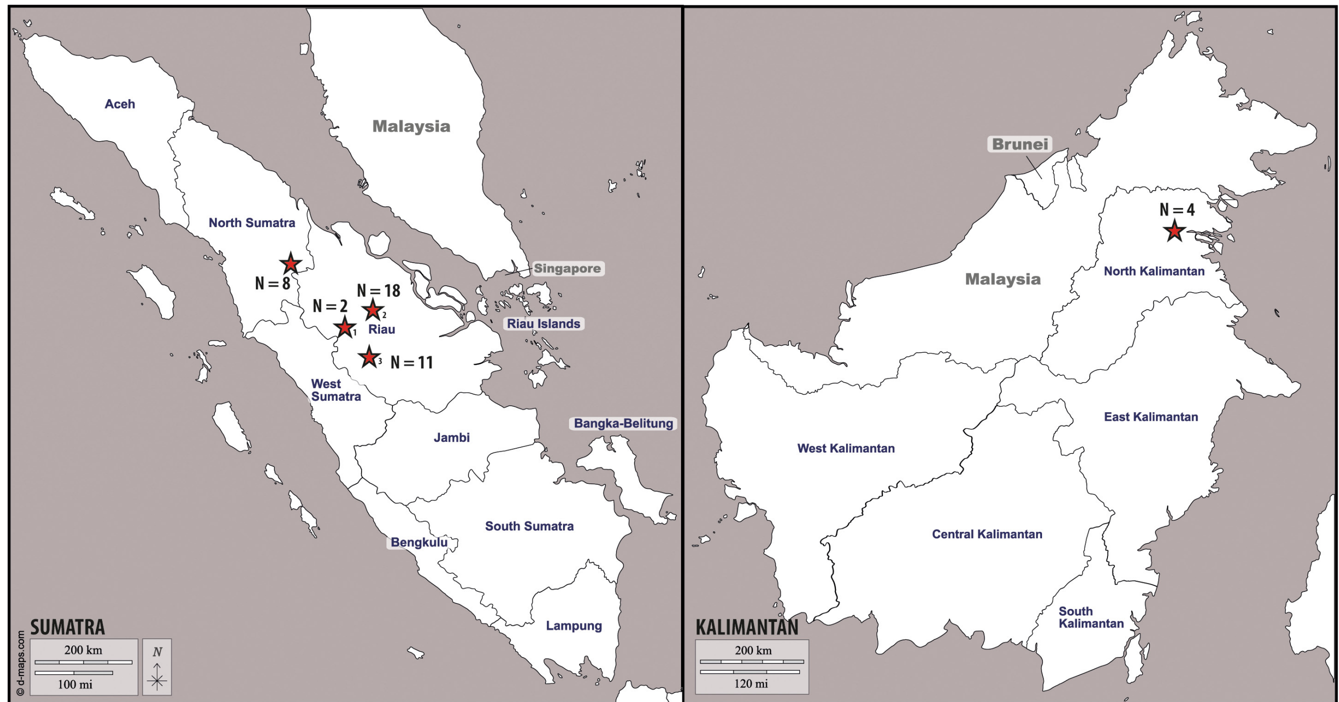

FIGURE 2 Geographic location of the sampling sites in Sumatra and Kalimantan, Indonesia. Number of samples collected from each site was indicated on the map.

2.3 | Phylogenetic analyses

Reference sequences for species closely related to those found in this study were downloaded from the GenBank database (Table 1). All sequences were aligned using MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server/) (Katoh & Standley, 2013), and then manually confirmed in MEGA v. 7 (Kumar et al., 2016) where necessary. Maximum likelihood (ML) analyses were performed on the individual regions as well as on the combined ITS and LSU data sets, using RaxML v. 8.2.4 on the CIPRES Science Gateway v. 3.3 (Stamatakis, 2014) with the default GTR substitution matrix and 1000 rapid bootstraps. Sequences for *Microstroma juglandis* (RB2042) were used as the outgroup. The resulting trees were viewed using MEGA v. 7 (Kumar et al., 2016).

2.4 | Pathogenicity tests

2.4.1 | Relative aggressiveness of isolates

Initially, each isolate was subcultured on clean PDA and incubated at 27°C for 21 days. To induce the spore production, 10 mL of sterilized distilled water (SDW) was spread over the surface of the cultures. The water was then removed from the plates, and the cultures were further incubated at 27°C for 48 h, after which this technique was repeated three times. The spore suspension was then harvested and adjusted to 1×10^6 spore/mL using a haemocytometer. A drop of Tween 20 (Sigma–Aldrich) was added to the spore suspension prior to the inoculation to facilitate dispersion of the spores.

An initial inoculation trial was conducted using an *E. pellita* clone (clone ECL04) known to be susceptible to infection in the nursery, following the methods described by Mafia et al. (2009) and Bragança et al. (2016) with some modifications. Eight-week-old plants were used in this test that included nine different isolates chosen to represent the range of collection sites (Table 1). Five plants were inoculated with each isolate and where the leaves had either been wounded or not. Wounds were induced using a sterile hypodermic needle (disposable needle 21G, duraSurge) and four 10-mm-long scratches on four expanding leaves were made on each plant. The spore suspension was sprayed onto the surface of leaf until run-off. For the controls, an equal number of plants were sprayed with sterilized distilled water. Each plant was then covered with a clear plastic bag to maintain a high relative humidity, and these were incubated at 30°C for 48 h. The plastic bags were then removed and the plants maintained at 30°C for 14 days.

A total of 20 leaves (5 plants × 4 leaves) were evaluated for each treatment. Disease severity of the leaves on each plant was assessed using the rating scale from 0 to 4 (Table 2). Isolations were made from inoculated tissue, and the resulting isolates were identified based on morphology. Data were analysed using Kruskal–Wallis tests to determine whether there were statistically significant differences between the treatments. Pairwise comparisons were then performed using the Wilcoxon rank sum test with continuity correction. All statistical analyses were performed in the R statistical software, version 3.2.0 (R Core Team, 2020).

TABLE 1 Collection details and GenBank accession numbers of isolates included in the phylogenetic analyses.

Species	Isolate	Host	Locality	GenBank accession		Reference
				ITS	LSU	
Q. coyrecup	WAC12947	*Corymbia calophylla*	Western Australia, Australia	DQ823431	DQ823444	Paap et al. (2008)
	WAC12949	*Corymbia calophylla*	Western Australia, Australia	DQ823432	DQ823445	Paap et al. (2008)
	WAC12948	*Corymbia calophylla*	Western Australia, Australia	DQ823433	DQ823446	Paap et al. (2008)
Q. cyanescens	CBS 357.73 = CMW 5583	Human skin	Netherlands	DQ317622	DQ317615	de Beer et al. (2006)
	CBS 876.73 = CMW 5584	*Eucalyptus pauciflora*	New South Wales, Australia	DQ317623	DQ317616	de Beer et al. (2006)
	WAC12952	*Corymbia calophylla*	Western Australia, Australia	DQ823419	DQ823440	Paap et al. (2008)
Q. eucalypti	CBS 118844 = CMW 1101	*Eucalyptus grandis*	South Africa	DQ317625	DQ317618	de Beer et al. (2006)
	CBS 119680 = CMW 11678	*Eucalyptus grandis*	South Africa	DQ317626	DQ317619	de Beer et al. (2006)
	CERC8479	*Eucalyptus urophylla* × *Eucalyptus grandis*	Guangdong, China	KY615012	KY615050	Chen et al. (2017)
	CMW 57583	*Eucalyptus pellita* × *Eucalyptus grandis*	Pangkalan Kerinci, Riau, Indonesia	OR345269	OR345278	This study
	CMW 57589	*Eucalyptus pellita* × *Eucalyptus grandis*	Pangkalan Kerinci, Riau, Indonesia	OR345270	OR345279	This study
	CMW 57591	*Eucalyptus pellita*	Baserah, Riau, Indonesia	OR345271	OR345280	This study
	CMW 57592	*Eucalyptus grandis* × *Eucalyptus pellita*	North Sumatera, Indonesia	OR345272	OR345281	This study
	CMW 57602	*Eucalyptus pellita* × *Eucalyptus brassiana*	Baserah, Riau, Indonesia	OR345273	OR345282	This study
	CMW 57605	*Eucalyptus pellita* × *Eucalyptus brassiana*	North Sumatera, Indonesia	OR345274	OR345283	This study
	CMW 57609	*Eucalyptus pellita* × *Eucalyptus grandis*	Pangkalan Kerinci, Riau, Indonesia	OR345275	OR345284	This study
	CMW 57616	*Eucalyptus grandis* × *Eucalyptus pellita*	North Kalimantan, Indonesia	OR345276	OR345285	This study
	CMW 57618	*Eucalyptus grandis* × *Eucalyptus pellita*	North Kalimantan, Indonesia	OR345277	OR345286	This study
Q. pitereka	DAR19773	*Corymbia eximia*	New South Wales, Australia	DQ823423	DQ823438	Paap et al. (2008)
	CMW 6707	*Corymbia maculata*	New South Wales, Australia	DQ317627	DQ317620	de Beer et al. (2006)
	CBS 118828 = CMW 5318	*Corymbia citriodora* subsp. *variegata*	New South Wales, Australia	DQ317628	DQ317621	de Beer et al. (2006)
Q. simpsonii	CBS 124772	*Eucalyptus tintinnans*	Edith Falls, Australia	GQ303290	GQ303321	Cheewangkoon et al. (2009)
	CBS 124773	*Eucalyptus* sp.	Lamphoon, Thailand	GQ303291	GQ303322	Cheewangkoon et al. (2009)
	CERC8507	*Eucalyptus urophylla* × *Eucalyptus grandis*	Guangdong, China	KY615037	KY615058	Chen et al. (2017)

Note: Isolates obtained in this study are indicated in bold. CBS—The culture collection of Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; CERC—China Eucalypt Research Centre (CERC), Chinese Academy of Forestry (CAF), ZhanJiang, GuangDong, China; CMW—culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; DAR—the plant pathology herbarium for the Department of Agriculture in NSW, Australia; WAC—Department of Agriculture Western Australia Plant Pathogen Collection, Perth, Australia.

TABLE 2 Disease severity scale used to score infections on leaves inoculated with *Q. eucalypti*, determined based on the presence of spore masses typical of *Q. eucalypti*.

Score	Infection rate	Observation
0	No infection	No typical white spore masses present
1	Low infection	25% of the total wounds infected
2	Moderate infection	50% of the total wounds infected
3	High infection	75% of the total wounds infected
4	Very high infection	All wounds on inoculated leaves infected

2.4.2 | Relative susceptibility of different *Eucalyptus* clones

Six *Eucalyptus* clones, including four of *E. pellita* (ECL01, ECL02, ECL03 and ECL04) and two of a *E. grandis* × *E. pellita* (ECL05 and ECL06), were selected to test for susceptibility to the most aggressive isolates arising from the prior test. Inoculation was conducted using only the wounding method. Eight-week-old *Eucalyptus* plants generated from cuttings were inoculated with 10 plants per treatment and an equal number as controls. A total of 40 leaves (10 plants ×4 leaves) were tested per clone. Inoculations, disease severity assessment and re-isolation were carried out following the protocol described above. These data were analysed in the same way as the initial inoculation trial.

3 | RESULTS

3.1 | Isolates

A total of 43 isolates were obtained from infected leaf and stem samples, morphologically resembling a *Quamlabaria* species. Of these, eight isolates were obtained from North Sumatera, 31 isolates were obtained from Riau, including Riau 1 (2 isolates), Riau 2 (18 isolates) and Riau 3 (11 isolates), while four isolates were obtained from North Kalimantan.

3.2 | Phylogenetic analyses

Nine isolates collected across the various sampling regions were selected for further studies. Amplicons of approximately 680 bp for the ITS and 870 bp for the LSU were generated. The ITS, LSU and combined sequence data sets used for phylogenetic analyses included 24 in-group taxa and contained 632, 561 and 1193 characters, respectively. Both the individual tree and combined trees were found to be congruent, having similar topologies. All isolates sequenced in this study were grouped in monophyletic clades with the ex-type and representative isolates of *Q. eucalypti* in all the analyses (Figure 3). These isolates were thus identified as *Q. eucalypti*.

3.3 | Pathogenicity tests

3.3.1 | Relative aggressiveness of isolates

No symptoms of *Quambalaria* infection were found on plants inoculated without wounding, and there were also no symptoms on the control plants. In the case of the wounded leaves, white spore masses typical of *Q. eucalypti* were found on most of the inoculated plants 12 days after inoculation (Figure 4). The aggressiveness varied between isolates, with disease severity ranging from 0 to 4 (Figure 5). Isolate CMW 57605 was the most aggressive followed by CMW 57602 and CMW 57618 where the disease severity ratings were 4, 3.6 and 3.6, respectively. Some isolates produced less infection on the wounds with severity of 1, 0.8, 0.7 and 0.6 for CMW 57616, CMW 57589, CMW 57583 and CMW 57609, respectively, while two isolates (CMW 57591 and CMW 57592) failed to induce infection [Kruskal–Wallis test, $H = 589.42$ $df = 9$ and $p < 2.2e\text{-}16$]. *Quambalaria eucalypti* was easily re-isolated from the spore masses on the infected but never the control plants.

3.3.2 | Relative susceptibility of different *Eucalyptus* clones

Isolate CMW 57605, found to be the most aggressive in the test where isolates were compared, was used in the clone screening test. Twelve days after inoculation, all inoculated *Eucalyptus* clones showed white spore masses typical of *Q. eucalypti* (Figure 4). Disease severity ranged from 1.4 to 3.7 (Figure 6), with clone ECL02 (*E. pellita*) as the most tolerant with a severity of 1.4 compared with other clones of ECL04 (*E. pellita*), ECL06 (*E. grandis* × *E. pellita*), ECL01 (*E. pellita*), ECL03 (*E. pellita*) and ECL05 (*E. grandis* × *E. pellita*) with disease severities of 3.7, 3.5, 3.2, 3.2 and 2.9, respectively (Figure 6). Based on the results of the Kruskal–Wallis test, disease tolerance in clone ECL02 was statistically different from the other five clones tested ($H = 1659.7$, $df = 11$, and $p < 2.2e\text{-}16$). Isolates morphologically typical of *Q. eucalypti* were easily recovered from all inoculated plants. No symptoms were observed on the control plants.

4 | DISCUSSION

The results of this study showed that a new leaf and shoot disease emerging on *Eucalyptus* nursery plants in three regions of Indonesia was caused by *Q. eucalypti*. This was determined based on isolations made from symptomatic material, identification of the resulting isolates using DNA sequence analyses as well as pathogenicity tests. This is the first report of the pathogen in Indonesia as well as in Southeast Asia.

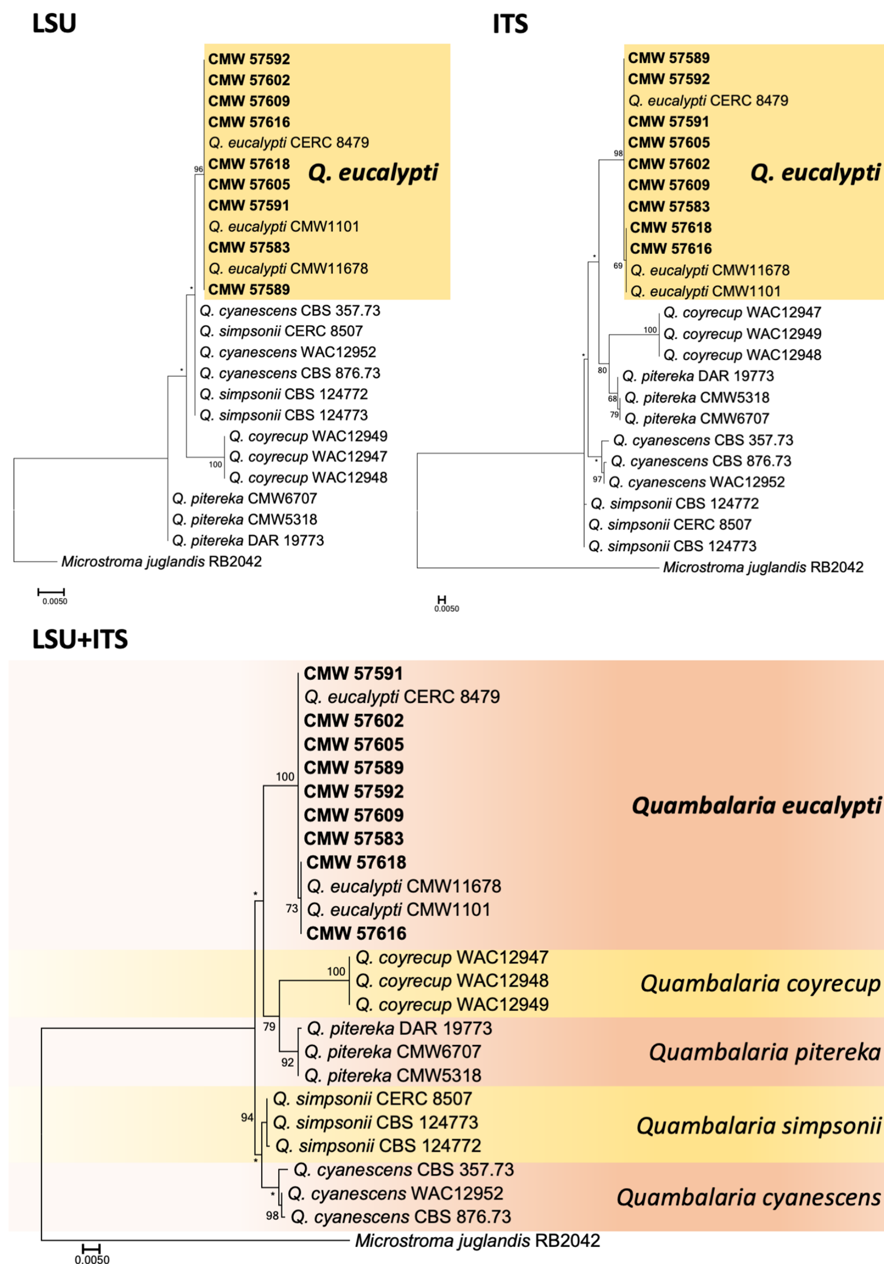

FIGURE 3 Phylogenetic tree based on maximum likelihood (ML) analyses of ITS, LSU and combined sequences for *Quambalaria* spp. Isolates sequenced in this study are presented in boldface. Bootstrap values of ≥60% for ML analyses are indicated at the nodes. Bootstrap values <60% are marked with '*'. *Microstroma juglandis* (isolate RB2042) represents the outgroup.

Several *Eucalyptus* species or genotypes have previously been reported to be affected by *Q. eucalypti* including *E. grandis* and *E. nitens* in South Africa (Roux et al., 2006; Wingfield et al., 1993), *E. globulus* and *E. saligna* × *E. maidenii* hybrids in Brazil (Alfenas et al., 2001), *E. globulus* and *E. grandis* in Uruguay (Bettucci et al., 1999), *E. grandis*, *E. longirostrata*, *E. grandis* × *E. camaldulensis* hybrids, *E. microcorys* and *E. dunnii* in Australia (Pegg et al., 2008), *E. globulus* in Portugal (Bragança et al., 2016), and *E. urophylla* × *E. grandis* hybrids in China (Chen et al., 2017). However, this is the first time that *Q. eucalypti* has been reported on *E. pellita* and its hybrids including hybrids with *E. grandis*, *E. brassiana* and *E. urophylla*. This is of substantial concern given the growing importance of *E. pellita* as a plantation species in the humid tropics (Bristow et al., 2006; Nambiar et al., 2018).

The pathway of entry of *Q. eucalypti* into Indonesia is unknown. This pathogen is likely native to Australia (de Beer et al., 2006; Pegg, Carnegie, et al., 2009; Roux et al., 2006; Wingfield et al., 1993; Zhou et al., 2007) but has been accidentally introduced into countries in Africa, Asia, Europe and South America (Alfenas et al., 2001; Bragança et al., 2016; Chen et al., 2017; Pegg et al., 2008; Wingfield et al., 1993). Based on DNA sequence analysis, two ITS haplotypes were detected in the *Q. eucalypti* collection in this study. The majority of the isolates, collected in North Sumatra and Riau, shared the same haplotype as those known in China, while the isolates from Kalimantan shared the same haplotype as that in South Africa, Portugal and Uruguay (Chen et al., 2017). Future studies at the population genetic level are planned to understand the likely source of origin of the pathogen in Indonesia.

An inoculation trial showed that wounds were necessary for *Q. eucalypti* to infect leaves. This is an interesting result as the pathogen has previously been shown to easily infect young and unwounded leaf and shoot tissue (Pegg, Webb, et al., 2009; Wingfield et al., 1993). In contrast, our results are similar to those of

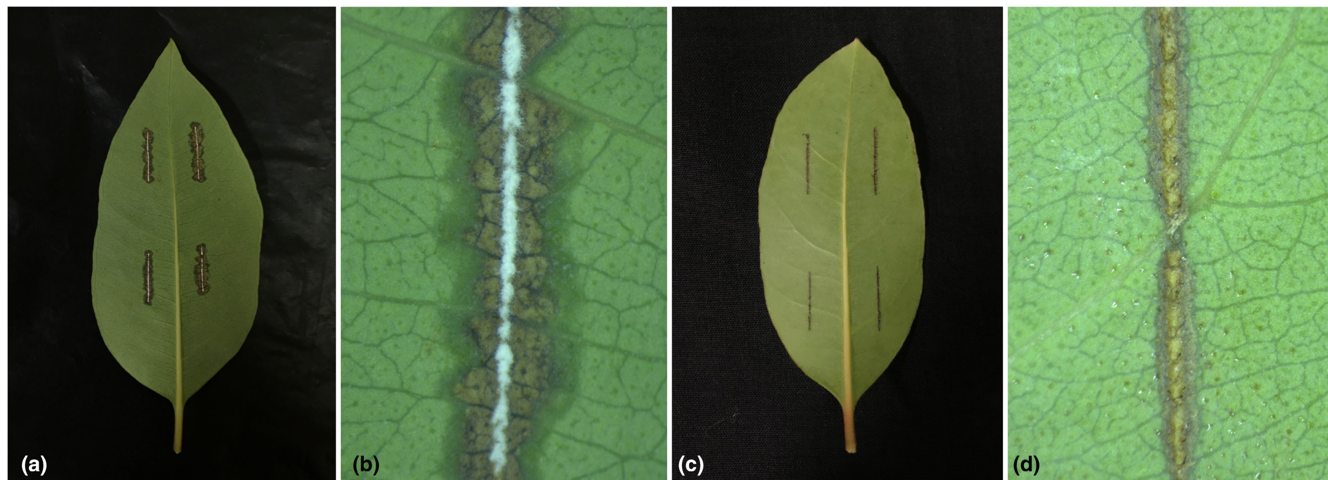

FIGURE 4 Symptoms of *Q. eucalypti* infection on *Eucalyptus* leaves after inoculation showing typical white spore masses of the pathogen (a, b), while control produce no symptom (c, d).

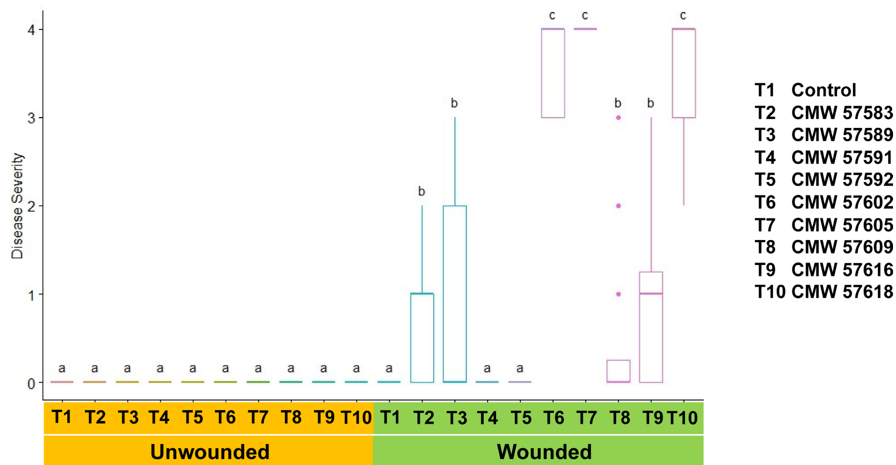

FIGURE 5 Inoculation with unwounded and wounded methods on *Eucalyptus* clone ECL04 using nine *Quambalaria eucalypti* isolates.

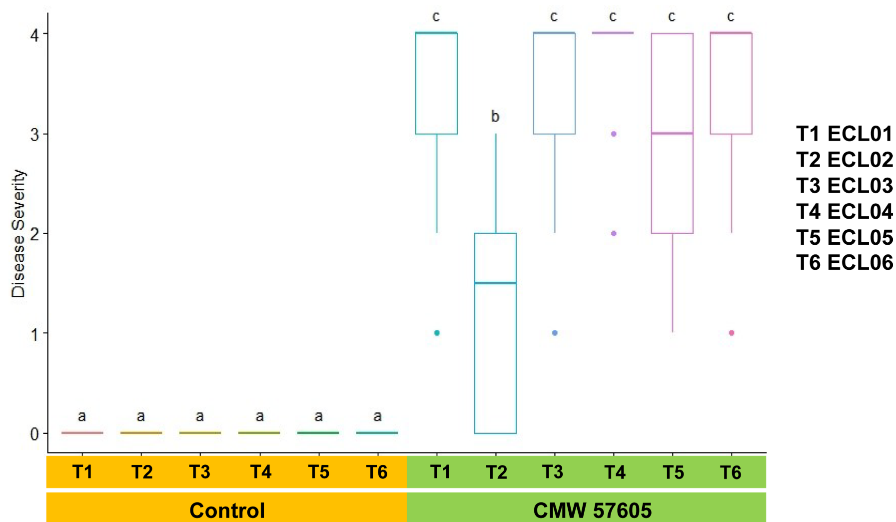

FIGURE 6 Bar chart indicating the severity score resulting from inoculation trials of six *Eucalyptus* genotypes inoculated with *Q. eucalypti* (CMW 57605) and the controls.

Mafia et al. (2009) who showed that wounds favoured infection by *Q. eucalypti*. These results are intriguing given the fact this pathogen is clearly able to infect leaves via the stomata (Pegg, Webb, et al., 2009) and thus typical of a primary pathogen.

Inoculation on *Eucalyptus* leaves of single susceptible genotype with numerous different *Q. eucalypti* isolates showed that these differed markedly in their ability to initiate infections. The most aggressive of the isolates used in an inoculation trial also showed that

different *Eucalyptus* genotypes differ in their susceptibility to infection. It should thus be possible to select clones resistant to infection using a relatively simple screening procedure. This approach has been used previously (Bragança et al., 2016; Pegg et al., 2011; Roux et al., 2006) providing an opportunity to manage the problem. Such screening is particularly relevant in the Indonesia situation where mother plant hedges are needed to mass propagate planting stock and leaf or shoot diseases, such as those caused by *Q. eucalypti* can significantly reduce productivity. The results of this study show the potential to manage *Q. eucalypti* in future by selecting materials tolerant to the disease.

Quambalaria eucalypti appears to be a pathogen of increasing prevalence in many regions of both the northern and southern hemispheres (Alfenas et al., 2001; Bettucci et al., 1999; Bragança et al., 2016; Chen et al., 2017; Pegg et al., 2008; Roux et al., 2006; Simpson, 2000; Wingfield et al., 1993). In Indonesia, its incidence is mainly in the nursery, and there are currently no reports of the pathogen causing problems on established trees. This situation could easily change, as it has in South Africa (Roux et al., 2006), making it important not to establish susceptible clones in plantations. The pathogen could also undergo a host shift to infect commonly occurring native trees and shrubs in the Myrtaceae, as has been found in Uruguay (Pérez et al., 2008).

ACKNOWLEDGEMENTS

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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