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Identification and Characterization of *Ceratocystis fimbriata* Causing Lethal Wilt on the *Lansium* Tree in Indonesia

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Bark canker, wood discoloration, and wilting of the duku tree (*Lansium domesticum*) along the watershed of Komering River, South Sumatra Province, Indonesia first appeared in 2013. The incidence of tree mortality was 100% within 3 years in badly infected orchards. A *Ceratocystis* species was consistently isolated from the diseased tissue and identified by morphological and sequence analyses of the internal transcribed spacer (ITS) and β-tubulin regions. Pathogenicity tests were conducted and Koch's postulates were confirmed. The fungus was also pathogenic on *Acacia mangium*, but was less pathogenic on mango. Partial flooding was unfavourable for disease development. Two described isolates (WRC and WBC) had minor variation in morphology and DNA sequences, but the former exhibited a more pathogenic on both duku and acacia. The ITS phylogenies grouped the most pathogenic isolate (WRC) causing wilting of the duku tree within the aggressive and widely distributed ITS5 haplotype of *C. fimbriata*.

Keywords **:** *Acacia mangium*, *Ceratocystis* canker and wilt, *Ceratocystis fimbriata*, *Lansium* tree

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The duku (*Lansium domesticum* Corr.), also known as the langsat and the kokosan is a tropical lowland fruit tree native to western Southeast Asia, from Borneo in the east (Indonesia) to peninsular Thailand in the west. It occurs wild and cultivated in its native countries and is one of the most widely cultivated fruits (Techavuthiporn, 2018; Yaacob and Bamroongrugsa, 1991). Duku is among the most popular local fruits in Indonesia. In 2017, the total number of harvested duku trees in Indonesia was 2.4 million trees, with a total yield of 138.4 metric tons (Badan Pusat Statistik–Statistics Indonesia, 2018). The most famous cultivars are grown in South Sumatra (duku Palembang and duku Komering) due to their sweet flavour combined with a subacid taste and having few seeds, or even being seedless. In South Sumatra, duku is mainly grown as a backyard or garden tree in combination with other native fruit trees along the watershed of the Musi, Komering, Ogan, Lematang, and Rawas rivers.

Lethal disease has rarely been evident on duku trees growing in the wild or cultivated orchard areas. Anthracnose caused by *Colletotrichum gloeosporioides*, appearing as brownish spots on the fruit bunch and often resulting in premature fruit drop and post-harvest losses, is commonly evidenced throughout the tropics (Yaacob and Bamroongrugsa, 1991). Corky bark disease, which makes the bark become rough and corky and flake off, often resulting in little to no fruit production has been reported on dukus in tropical USA (Keith et al., 2013; Whitman, 1980). In Hawaii, a corky bark canker is associated with an Ascomycete fungus, *Dolabra nepheliae*, and insect larvae of *Araecerus* sp. (Coleoptera: Anthribidae) and *Corticeus* sp. (Coleoptera: Tenebrionidae) feeding under the loosened bark (Keith et al., 2013).

During early January 2014, massive mortality of duku trees along the watershed of the Komering River in Ogan Komering Ulu (OKU) District was reported by most local and some national newspapers. In total, more than 2,000

trees of the most popular cultivar, duku Komering, died. The symptoms first appeared during the early rainy season of October 2013. Most of the trees that died were predisposed due to partial flooding to a depth of about 20 cm for about one month from the end of December 2013 to January 2014. However, some affected trees were found growing on non-flooded sites, indicating an infectious disease. In this study, we describe a new bark canker and wilting associated with massive mortality of duku trees in Indonesia, illustrate morphological and molecular-based identification of the pathogen, and describe the pathogenicity of the causal fungus on duku trees and other hosts. Disease progress and spread for 5 years is also discussed.

Materials and Methods

Disease incidence and isolation of the causal agent. Incidence of diseased trees was assessed in 2014 and 2017 at eight duku orchards in OKU District of South Sumatra. In each orchard, five 10 × 10 m plots starting from the centre of the diseased trees were selected. The trees were recorded as infected if any part of the shoot or stem showed disease symptoms. Twenty diseased duku trees were randomly selected from the affected orchards. Sections of the discolored wood from the stem were cut, wrapped in a paper towel and transported to the laboratory for examination. Isolation of the fungal pathogen was performed from discolored wood that had been surface-sterilized with 70% ethanol for 30 s and 1% NaOCl for 2 min. Small sections (5 × 5 mm) from the margin of discoloration were placed on a malt extract agar (MEA) amended with 50 µg/ml streptomycin in Petri dishes. Another subset of surface-sterilized wood sections was wrapped between carrot slices to bait for *Ceratocystis* spp. (Brito et al., 2019; Moller and DeVay, 1968). Baiting was also performed by inserting diseased tissue into freshly harvested cacao pods and cucumber fruit in an attempt to isolate *Phytophthora*.

Initial identification and cultural characteristics. Initial identification was performed based on morphological characteristics of teleomorphs and anamorphs. Isolates were characterized from 2-week-old cultures grown on 2% MEA. One hundred measurements of each teleomorph and anamorph structure from each representative isolate were made with an Olympus microscope and an OptiLab camera system (Yogyakarta, Indonesia). The average (mean) and standard deviation (SD) of measurements were computed and presented as mean±SD. Morphological characteristics were compared with *Ceratocystis* isolates from *A. mangium* (Tarigan et al., 2011) and sweet potato (Engelbrecht and Harrington, 2005).

DNA isolation, PCR, and sequence analyses. Two representative isolates (WRC and WBC), isolated from the diseased duku trees were further used for DNA sequence analysis. DNA was isolated from mycelia cultured at 27°C for 7 days in malt extract broth (Difco Laboratories, Sparks, MD, USA) in plastic Petri dishes. Total DNA was extracted using bead-beating technology (MO BIO Laboratories, Carlsbad, CA, USA) and the silica spin filter method (Geneaid, Taipei, Taiwan) according to the manufacturer's instructions. DNA concentration and purity were measured spectrophotometrically. The ITS1/5.8 S rDNA/ITS2 (internal transcribed spacer, ITS) region of *Ceratocystis* isolates was amplified by PCR, using ITS1 (forward: 5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (reverse: 5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). The β-tubulin gene (TUB) region was amplified by PCR, using βt1a (forward: 5′-TTCCCCCGTCTC-CACTTCTTCATG-3′) and βt1b (5′-GACGAGATC-GTTCATGTTGAACTC-3′) (Glass and Donaldson, 1995). PCR reaction mixtures consisted of 1 µl of each primer (10 mM), 15 µl of REDiant 2× PCR Master Mix (1st BASE, The Gemini, Singapore), 3 µl of DNA template (2-10 ng) and 10 µl nuclease-free water to make up 30 µl total volume reactions. PCR was performed using Thermal Cycler (SureCycler 8800, Agilent, Santa Clara, CA, USA) with a 5-min 95°C denaturation step followed by 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 56°C for ITS and 55°C for *TUB*, and 40 s extension at 72°C, followed by a final extension of 5 min at 72°C. Negative controls (without template DNA) were applied in each assay. The PCR products of ITS and TUB regions were sequenced at 1st BASE, Co., Ltd. (Kuala Lumpur, Malaysia).

Identification of isolates was accomplished by BLAST searches of the *ITS* and *TUB* sequences on the GenBank database (http://www.ncbi.nlm.nih.gov). BLAST identification suggested that both isolates belonged to the species *Ceratocystis fimbriata*. Phylogenetic analyses were performed to identify the species of *Ceratocystis* most closely related to the *Lansium* isolate from Indonesia. β-tubulin datasets were generated using ex-type and ex-paratype sequences representing species in the Latin American (LAC) and Asian clade of the *C. fimbriata* species complex (Barnes et al., 2018; Fourie et al., 2015; Oliveira et al., 2015). The β-tubulin sequences (Table 1) were aligned using the online software MAFFTv.7 (Katoh et al., 2019) with the best alignment strategy was automatically selected by the software. Sequence alignments were manually edited in MEGA X (Kumar et al., 2018). There were 34 aligned

Table 1. Collection details and GenBank accession number of ITS and β-tubulin sequence for isolates of *Ceratocystis fimbriata* included in this study

Isolate	GenBank accession no.		Species and ITS haplotype	Host	Origin	Reference
	ITS	β-Tubulin				
C1418	AY157956	-	*C. fimbriata* TS1a	*Ipomoea batatas*	USA	Harrington et al. (2014)
C1857	HQ157542	-	*C. fimbriata* ITS1	*Ficus carica*	Brazil	Harrington et al. (2014)
CMW4797	FJ236733	-	*C. fimbriata* ITS1b	*Eucalyptus* sp.	Congo	Harrington et al. (2014)
CMW9998	FJ236721	-	*C. fimbriata* ITSb	*Eucalyptus* sp.	South Africa	Harrington et al. (2014)
C1655	HQ157546	-	*C. fimbriata* ITS2	*Mangifera indica*	Brazil	Harrington et al. (2014)
C1440	HQ157544	-	*C. fimbriata* ITS3	*Eucalyptus* sp.	Brazil	Harrington et al. (2014)
CMW5328	AF395686	-	*C. fimbriata* ITS3	*E. grandis*	Uganda	Harrington et al. (2014)
C1442	HQ157545	-	*C. fimbriata* ITS4	*Eucalyptus* sp.	Brazil	Harrington et al. (2014)
CMW38737	KF878326	KF878335	*C. fimbriata* ITS5	*E. grandis*	Zimbabwe	Jimu et al. (2015)
C1345	AY157966	-	*C. fimbriata* ITS5	*Eucalyptus* sp.	Brazil	Harrington et al. (2014)
A59662	KF650948	-	*C. fimbriata* ITS5	*Camellia sinensis*	China	Xu et al. (2019)
YM061	AM712445	-	*C. fimbriata* ITS5	*Colocasia esculenta*	China	Li et al. (2016)
P20053	AM292204	-	*C. fimbriata* ITS5	*Punica granatum*	China	Li et al. (2016)
C1	MF033455	MF040712	*C. fimbriata* ITS5	*Acacia* sp.	Vietnam	Trang et al. (2018)
CMW22563	EU588656	EU588636	*C. fimbriata* ITS5	*A. mangium*	Indonesia	Tarigan et al. (2011)
WRC	MT229127	MW013766	*C. fimbriata* ITS5	*Lansium domesticum*	Indonesia	Present study
C2055	HQ157548	-	*C. fimbriata* ITS6	*Mangifera* sp.	Brazil	Harrington et al. (2014)
CMW13582	KC261853	-	*C. fimbriata* ITS6z	*Hypocryphalus mangifera*	Oman	Naidoo et al. (2013)
WBC	MT229128	MW013767	*C. fimbriata* ITS6z	*L. domesticum*	Indonesia	Present study
CMW13851	AY953383	EF433308	*C. fimbriata* ITS7b	*M. indica*	Oman	Van Wyk et al. (2005)
CMW23634	EF433302	EF433311	*C. fimbriata* ITS7b	*M. indica*	Pakistan	Van Wyk et al. (2007)
CMW22579	EU588658	-	*C. fimbriata* ITS7b	*A. mangium*	Indonesia	Tarigan et al. (2011)
CMW8856	AY233867	-	*C. fimbriata* ITS8a	*Citrus* sp.	Colombia	Harrington et al. (2014)
CMW17808	EF127990	-	*C. fimbriata* ITS8c	*Eucalyptus* sp.	Colombia	Harrington et al. (2014)
CMW22092	FJ151432	-	*C. fimbriata* ITS8e	*E. deglupta*	Ecuador	Harrington et al. (2014)
C1558	AY157965	-	*C. fimbriata* ITS9	*M. indica*	Brazil	Harrington et al. (2014)
C1914	HQ157540	-	*C. fimbriata* ITS9	*C. esculenta*	Brazil	Harrington et al. (2014)
C994	AY157964	-	*C. fimbriata* ITS10	*M. indica*	Brazil	Harrington et al. (2014)
Cf 4	EF042605	-	*C. fimbriata* ITS10a	*M. indica*	Brazil	Harrington et al. (2014)
C1865	AY526286	-	*C. fimbriata* ITS11	*C. esculenta*	Brazil	Harrington et al. (2014)
C1926	HQ157541	-	*C. fimbriata* ITS12	*C. esculenta*	Brazil	Harrington et al. (2014)
C1688	AY526291	-	*C. fimbriata* ITS14	*M. indica*	Brazil	Harrington et al. (2014)
C925	AY157967	-	*C. fimbriata* ITS15	*Gmelina arborea*	Brazil	Harrington et al. (2014)
C924	HQ157539	-	*C. fimbriata* ITS16	*G. arborea*	Brazil	Harrington et al. (2014)
CMW6569	-	DQ371652	*C. pirilliformis*	*E. nitens*	Australia	Barnes et al. (2018)
CMW6579	-	DQ371653	*C. pirilliformis*	*E. nitens*	Australia	Barnes et al. (2018)
CMW17808	-	EU881898	*C. neglecta*	*E. grandis*	Colombia	Fourie et al. (2015)
CMW18194	-	EU881899	*C. neglecta*	*E. grandis*	Colombia	Fourie et al. (2015)
CMW5751	-	AY177225	*C. colombiana*	*Coffea arabica*	Colombia	Fourie et al. (2015)
CMW5761	-	AY177224	*C. colombiana*	*C. arabica*	Colombia	Fourie et al. (2015)
CMW14803	-	KJ631108	*C. cacaofunesta*	*Theobroma cacao*	Ecuador	Fourie et al. (2015)
CMW15051	-	KJ601510	*C. cacaofunesta*	*T. cacao*	Costa Rica	Fourie et al. (2015)
CMW8850	-	AY233875	*C. papillata*	*Citrus* × *Tangelo* hybrid	Colombia	Van Wyk et al. (2010)
CMW8856	-	AY233874	*C. papillata*	*Citrus limon*	Colombia	Van Wyk et al. (2010)
CMW14797	-	EF433307	*C. fimbriata*	*M. indica*	Brazil	Barnes et al. (2018)
CMW28907	-	FJ200270	*C. fimbriata*	*M. indica*	Brazil	Barnes et al. (2018)

Table 1. Continued

Isolate	GenBank accession no.		Species and ITS haplotype	Host	Origin	Reference
	ITS	β-Tubulin				
CMW1547	-	EF070443	*C. fimbriata*	*I. batatas*	Papua New Guinea	Barnes et al. (2018)
C1421	-	KF302689	*C. fimbriata*	*I. batatas*	USA	Barnes et al. (2018)
CMW24174	-	EF190951	*C. fimbriatomima*	*Eucalyptus* hybrid	Venezuela	Fourie et al. (2015)
CMW24176	-	EF190952	*C. fimbriatomima*	*Eucalyptus* hybrid	Venezuela	Fourie et al. (2015)
CMW21127	-	EU588643	*C. fimbriata*	*A. crassicarpa*	Indonesia	Oliveira et al. (2015)
CMW24664	-	JQ862720	*C. fimbriata*	*Eucalyptus* hybrid	China	Chen et al. (2013)
CBS115173	-	KF302700	*C. fimbriata*	*Gmelina arborea*	Brazil	Luchi et al. (2013)
CBS14653	-	KF302702	*C. fimbriata*	*C. arabica*	Suriname	Luchi et al. (2013)
CMW14802	-	EF070425	*C. platani*	*Platanus occidentalis*	USA	Barnes et al. (2018)
CMW23450	-	KJ601513	*C. platani*	*P. occidentalis*	Greece	Barnes et al. (2018)
CMW11424	-	AY528966	*C. polychroma*	*Syzygium aromaticum*	Indonesia	Barnes et al. (2018)
CMW11436	-	AY528967	*C. polychroma*	*S. aromaticum*	Indonesia	Barnes et al. (2018)
CMW19383	-	EF070430	*C. atrox*	*E. grandis*	Australia	Barnes et al. (2018)
CMW19385	-	EF070431	*C. atrox*	*E. grandis*	Australia	Barnes et al. (2018)

datasets (Supplementary Fig. 1) and the sequences were used for phylogenetic tree construction using a maximum parsimony (MP) analysis under PAUP 4.0b10 (Swofford, 2002). To determine relatedness of isolates from duku with known *C. fimbriata* populations, the ITS sequence was manually aligned with known ITS haplotypes as designated by Harrington et al. (2014) (Supplementary Fig. 2) and phylogenetic analyses were performed. Representative sequences of ITS haplotypes of *C. fimbriata* as designated by Harrington et al. (2014) and ITS sequences of accession numbers KF878326, KF650948, AM712445, AM292204, MF033455, EU588656, KC261853, which most closely matched with isolates from duku, were used in the analyses. *C. variospora* (accessions AF395683) was used as the outgroup taxon. There were 35 ITS sequences in the dataset (Table 1) and the sequences were initially aligned using MAFFTv.7 (Katoh et al., 2019) and then manually adjusted and trimmed in MEGA X (Kumar et al., 2018) (Supplementary Fig. 3). The relationships between ITS sequences of isolates from *L. domesticum* and other representative genotypes of the *C. fimbriata* sensu stricto (Harrington et al., 2014; Oliveira et al., 2015) were analysed using genetic distance matrices, unweighted pair group method with arithmetic means (UPGMA), and 1,000 bootstrap replications under PAUP 4.0b10 (Swofford, 2002).

Pathogenicity tests. Two isolates identified using DNA sequence data were used to test for pathogenicity. Pathogenicity tests were conducted on 1-year-old duku (*Lansium domesticum* var. *domesticum*) seedlings grown in a partially flooded and in a non-flooded nursery. Seedlings were grown in 20 cm diameter plastic pots containing a mixture of topsoil and compost under a 25% shading net. The pots from the flooded nursery were placed in a tray filled with tap water, which was maintained to a depth of 2-3 cm. Pathogenicity was also tested on 3-month-old acacia (*A. mangium*) and 6-month-old mango (*Mangifera indica* cv. Arumanis) seedlings.

Preliminary tests showed that stem inoculations with a mycelial plug were ineffective unless the bark was wounded. Therefore, wound inoculation was used throughout the experiments. Wounds were made by puncturing three points on the bark to a 3-mm depth using a sterile 28 g needle, and a 2 × 2 mm agar plug taken from an actively growing colony on 2% MEA was placed in the wound with the mycelium downward. This was covered with a section (10 × 10 mm) of wetted tissue paper and wrapped with clear tape to reduce contamination and desiccation. The inoculum along with the wrapping plastic was removed at 3 days post-inoculation. Each isolate was injected into 10 seedlings for each flooded and non-flooded group of seedlings. For uninoculated controls, wounded bark was wrapped with sterile MEA plugs. Whole experiments were repeated twice and data were pooled after verifying the variance homogeneity using the Levene test.

Disease severity was assessed 20 days post-inoculation based on the length of wood discoloration. Sections were cut from the margins of lesions, surface-sterilized, and plated on MEA or inserted into a carrot dish to re-isolate the inoculated fungus to complete Koch's postulates. Fungal identity was verified by colony, anamorph, and teleomorph morphology.

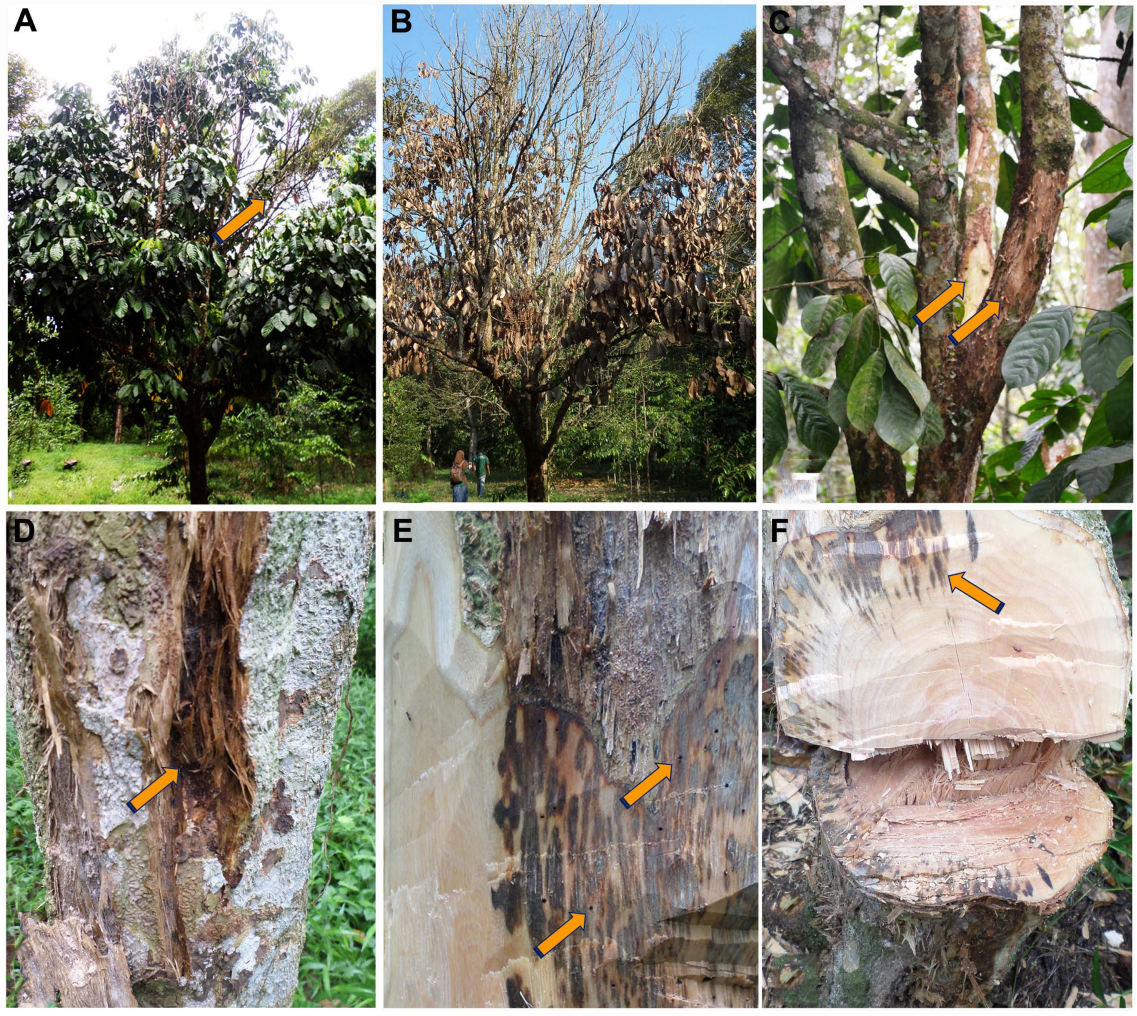

Fig. 1. Symptoms of *Ceratocystis* wilt on duku trees (*Lansium domesticum* var. *domesticum*). (A) Partial wilting and fast dieback of upper twigs and branches. (B) Total plant wilt and dieback after 6 months of partial wilting. (C) Peeled-off bark of branches due to squirrel attacks on diseased tree. (D) Bark canker on heavily infected trunk. (E) The discolored wood beneath the outermost layers of sapwood and a beetle entry/exit hole on affected wood. (F) The discolored wood extended to the heartwood of the basal stem. Arrows indicate the sites of described symptoms.

Results

Field observations and symptom development. Diseased trees were characterized by wilting of some twigs or branches, followed by defoliation and dieback. In most cases, total plant wilt or death was observed within 6 months from the first appearance of wilt (Fig. 1A and B). Bark canker was eventually found on heavily infected trunks or dead trees (Fig. 1D). Scraping the bark down to the wood along the wilted side of the trunk up to the branch revealed extensive areas of discolored tissue (Fig. 1E and F). The discolored wood typically had a streaked appearance, turning a uniform dark brown with age and could be found beneath the outermost layers of sapwood (Fig. 1E) and in some cases, discoloration extended to the heartwood (Fig. 1F). All diseased trees had been attacked by squirrels (Fig. 1C) and lesions appeared to originate from surrounding beetle entry/exit holes (Fig. 1E) on the peeled-off bark, indicating the involvement of a wound pathogen.

The disease was observed along the watershed of the Komering River, including Lubuk Batang (OKU District) and Rasuan (OKU Timur District), all in South Sumatra Province of Sumatra. Affected trees ranged from young (<5 years) to old (>50 years) in age. Disease incidence and severity were highest in Lubuk Batang Lama, where the disease first appeared. The disease progress both in term of incidence and severity was fast. All trees (100%) from

Table 2. Incidence of *Ceratocystis* wilt in duku orchards of Ogan Komering Ulu District, South Sumatra

Location (trees/location)	Incidence (%)		
	February 2014	August 2014	November 2017
Belatung (n = 66)	36	86	100
Lubuk Batang Baru (n = 85)	38	55	100
Lubuk Batang Lama (n = 69)	63	100	100

eight sampled duku orchards in OKU District of South Sumatra where the disease originated had wilted and died in the November 2017 survey (Table 2). In the 2019 field observation, the disease was found to have sporadically killed duku trees in Ogan Komering Ulu Timur (OKUT) District (within 100 km of the disease origin). Squirrel attacks were not found on the recently infected trees. Disease was not found in other duku orchards of South Sumatra in OKI, PALI, and Muara Enim districts. There was no appearance of squirrel scratches in those disease-free orchards.

Culture characteristics and morphology. Fungi typical of genus *Ceratocystis* were consistently isolated from direct plating of diseased wood on to both MEA and carrot slices.

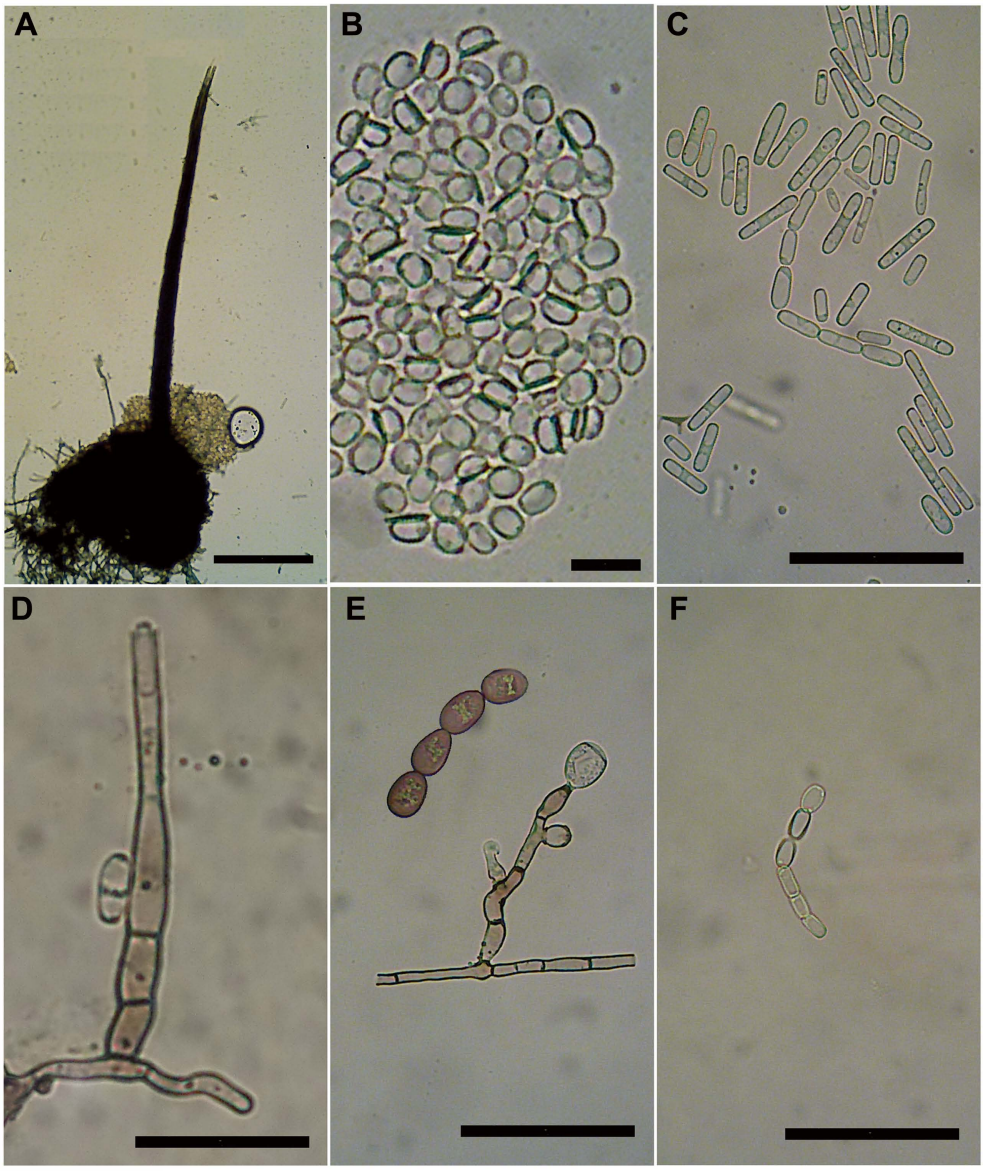

Fig. 2. Morphological characteristics of *Ceratocystis fimbriata* isolate WRC from bark canker of *Lansium domesticum*. (A) Globose ascomata with long neck. (B) Ascospores. (C) Cylindrical conidia. (D) Primary phialidic conidiophore with emerging cylindrical conidia. (E) Chlamydospore. (F) Barrel-shaped conidia in chain. Scale bars = 100 µm (A), 10 µm (B), 50 µm (C-F).

Colonisation of *Phytophthora* on diseased wood was not detected by baiting using cacao pods and cucumber fruit. *Ceratocystis* isolates from *L. domesticum* trees were typical of *Ceratocystis* spp. in the *C. fimbriata* sensu lato species complex, having characteristic olive-green colonies and the typical banana-fruit odour. They had globose to sub-globose ascomata with long necks and typical divergent ostiolar hyphae at their tips (Fig. 2). Teleomorph and anamorph structures were produced within 2 weeks on MEA cultures. Two isolates (WRC and WBC) were described and both had ascospore (4-7 × 3-5 µm), cylindrical conidia (14-25 × 4-5 µm), and aleuroconidia sizes (11-16 × 7-11 µm) within

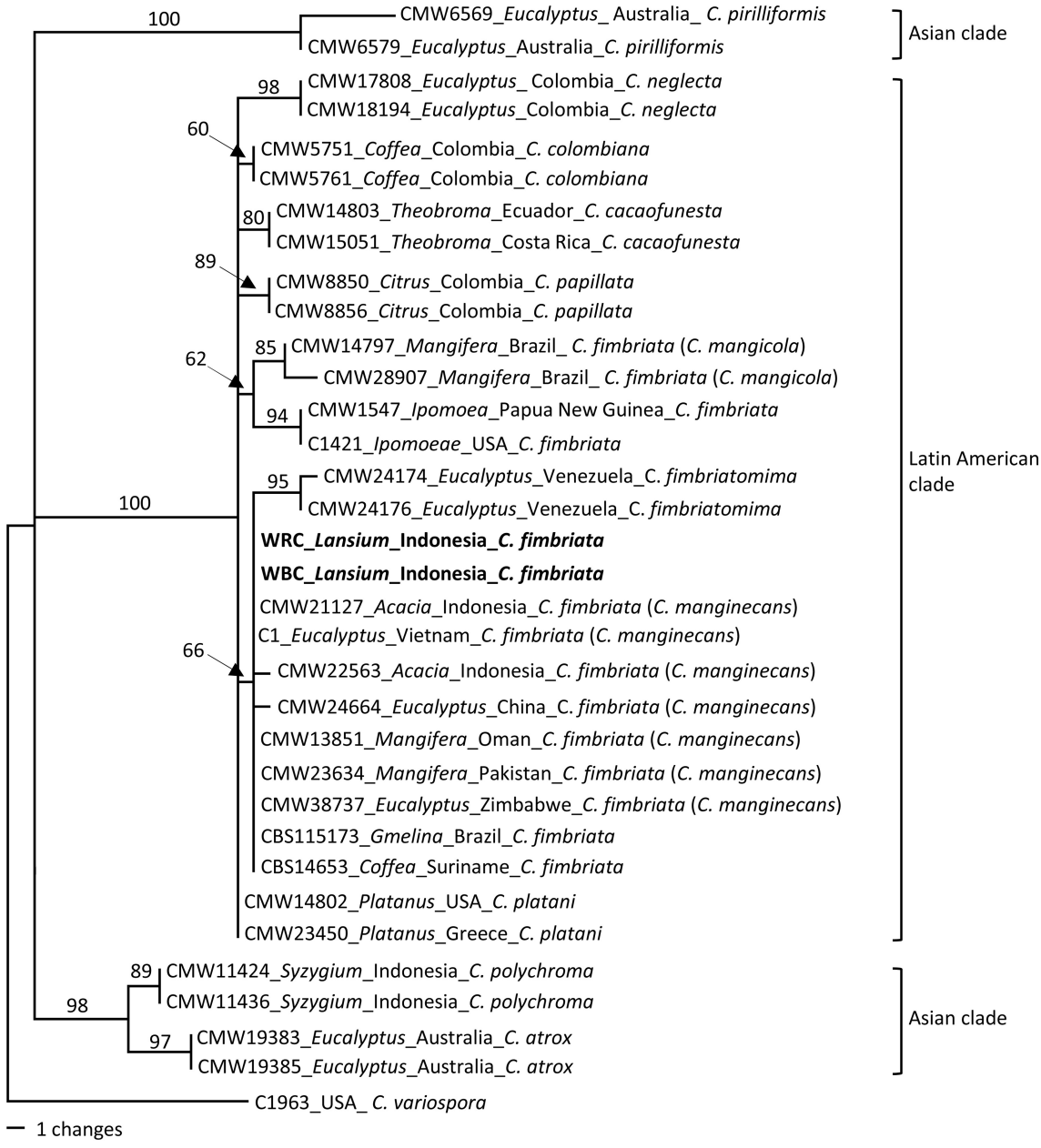

Fig. 3. Phylogenetic tree generated from maximum parsimony analysis of the β-tubulin sequences showing the relationship between *Ceratocystis fimbriata* from *Lansium* tree in Indonesia (marked in bold) and other species in the Latin American and Asian clade of the *C. fimbriata* species complex. The strain numbers, host genera, countries of origin, and species are given for the representatives of each isolate. Species names considered to be synonyms of *C. fimbriata* sensu stricto are in parentheses (Harrington et al., 2014; Oliveira et al., 2015). *C. variospora* was used as the outgroup taxon. Bootstrap values greater than 50% obtained after a bootstrap test with 1,000 replications are indicated on appropriate nodes.

the range of those of *C. fimbriata* sensu stricto neotype BPI 595863 (Engelbrecht and Harrington, 2005). Both isolates produced a barrel-shaped (doliform) conidia (8-10 × 6-8 µm) in chain (Fig. 2).

Sequence analyses. WBC and WBC isolates had differences in two bases of ITS sequence (99.6% similarity), but had a 100% similarity in the TUB sequence. BLAST searches of the ITS region of WRC (MT229127) and WBC (MT229128) identified both sequences with the GenBank deposits for *C. fimbriata* with 100% of similarity and query coverage. A similar BLAST result was obtained with the TUB sequence (MW013766 and MW013767 for WBC and WBC, respectively) and confirmed the assignment to *C. fimbriata* with 100% of similarity and query coverage.

MP analyses for the β-tubulin resulted in single most parsimonious tree of 84 steps (Fig. 3), with a homoplasy index = 0.036, consistency index = 0.964, rescaled consistency index = 0.979, and retention index = 0.944. *Ceratocystis* isolates from *Lansium* in Indonesia reside in the LAC of *C. fimbriata* sensu lato and they are phylogenetically clustered closely with ex-type and ex-paratype of *C. manginecans* and *C. fimbriata. C. manginecans* is considered synonym or conspecific of *C. fimbriata* sensu stricto (Harrington et al., 2014; Oliveira et al., 2015).

Manual alignment of the ITS sequences with previously described ITS genotypes (Harrington et al., 2014) grouped the isolates into ITS5 and ITS6z haplotype of *C. fimbriata*

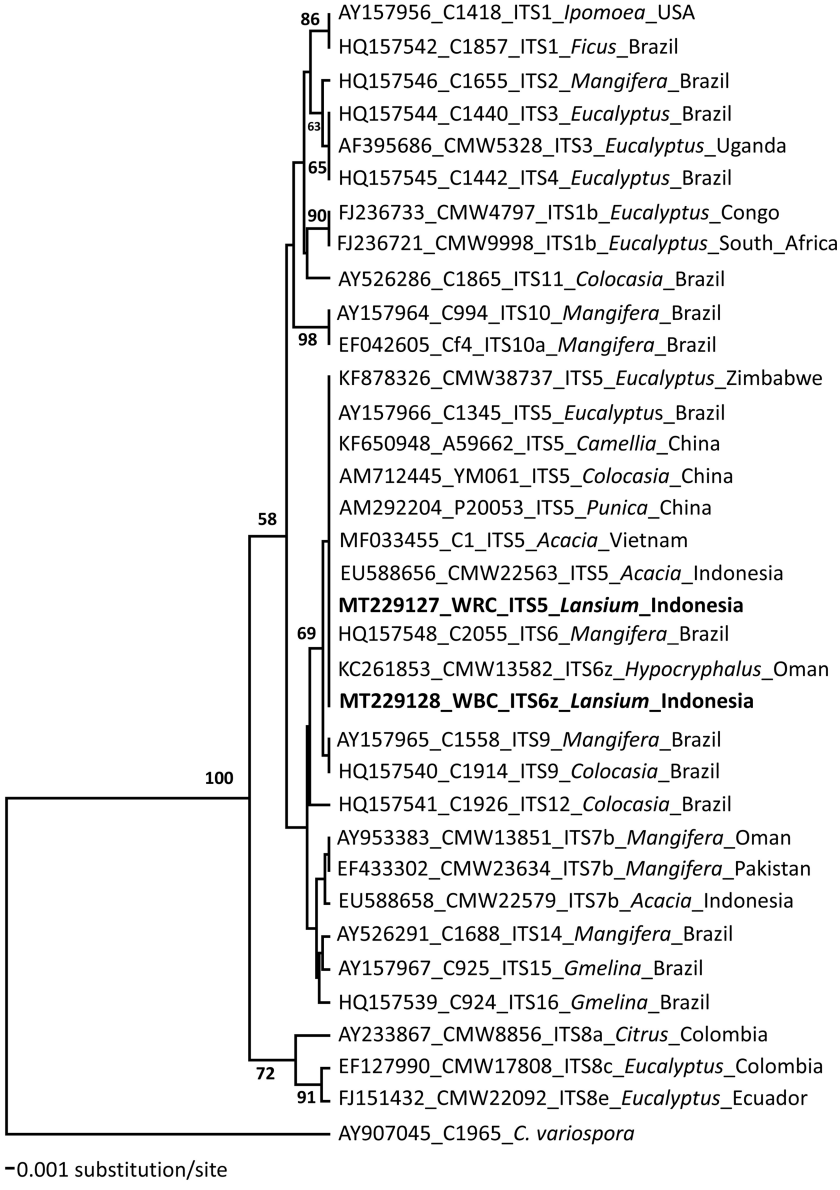

Fig. 4. Dendrogram generated by unweighted pair group method with arithmetic means showing the genetic relatedness of representative the internal transcribed spacer (ITS) rDNA genotypes (sequences) of the *Ceratocystis fimbriata* sensu stricto. The GenBank accession numbers, strain numbers, ITS haplotypes, host genera and countries of origin are given for the representatives of each haplotype. Isolates from *Lansium domesticum* in Indonesia were marked in bold. The ITS haplotypes of *C. fimbriata* are numbered following the numerical designations of Harrington et al. (2014). *C. variospora* was used as the outgroup taxon. Bootstrap values greater than 50% obtained after a bootstrap test with 1,000 replications are indicated on appropriate nodes. Scale bar indicates genetic distance.

for WRC and WBC, respectively. The WRC showed 100% similarity with other ITS5 haplotype of *C. fimbriata* isolated from tea tree (KF650948), taro (AM712445), pomegranate (AM292204) in China; from eucalyptus (KF878326) in Zimbabwe; from acacia (MF033455) in Vietnam; and from acacia (EU588656) in Indonesia. WBC had 100% similarity with member of ITS6z haplotype of *C. fimbriata* isolated from *Hypocryphalus mangiferae* (KC261853) in Oman. UPGMA analysis clustered both isolates from *L. domesticum* within a single group consisted of both ITS5 and ITS6 haplotypes (Fig. 4).

Pathogenicity test. In pathogenicity tests, initial symptoms appeared as water-soaked brown lesions on the wound site within 3 days after inoculation. The lesions remained small at inoculation sites on bark, but scraping the bark down to the wood revealed extensive areas of discolored xylem tissue upward and downward from the inoculated site (Fig. 5A). Upward extension of xylem discoloration from the inoculation site was more extensive ($P < 0.0001$) than downward extension on duku seedling inoculated with WRC. However, no significant difference ($P \geq 0.05$) between upward and downward discoloration extension was exhibited by WRC on acacia and mango and by WBC on all hosts (Table 3). This kind of discolored xylem was similar to a typical symptom of diseased trees in the field. The WRC isolate was more pathogenic on duku seedling than WBC as it induced significantly ($P < 0.05$) longer lesions and caused more ($P < 0.05$) plant wilt and death (Fig. 5A). Plant wilt and death was observed within 20 days post-inoculation and later the wilting incidence gradually increased. Regrowth of lateral shoots was observed on wilted plants. The control plants, inoculated with MEA, remained asymptomatic and had only a trace of xylem discolouration (less than 5 mm in length) at the wound site (Table 3). Partial flooding of duku seedling did not significantly ($P = 0.163$) affect extension of the xylem discoloration, but plant mortality by WRC was lower ($P < 0.05$) than on non-flooded seedling (Table 3). Fungus with the same morphological characteristics was re-isolated from diseased wood

Fig. 5. Symptoms reproduced from mycelial plug inoculation with *Ceratocystis fimbriata* isolates (WRC and WBC) from *Lansium domesticum* 20 days after inoculation. (A) Symptoms on 1-year-old duku seedlings (*L. domesticum*) inoculated with malt extract agar plug (control) (I), restricted wood discoloration and non-wilted plant inoculated with WBC (II), partial and total wilting of plant inoculated with WRC (III, IV), upward extensive wood discoloration from inoculated site (red arrow) (V). (B) Symptoms on 3-month-old seedlings of *Acacia mangium* showing extensive wood discoloration by WRC and limited lesions by WBC. Yellow arrows indicate new lateral shoot growth on diseased *Acacia* and red arrows indicate point of inoculation. (C) Symptoms on 6-month-old seedlings of *Mangifera indica* cv. Arumanis showing wood discoloration at site of inoculation (red arrows).

Table 3. Pathogenicity of *Ceratocystis fimbriata* isolates on 1-year-old duku (*Lansium domesticum* var. *domesticum*), 3-month-old *Acacia mangium*, and 6-month-old *Mangifera indica* cv. Arumanis seedlings

Isolate and plant species	Flooding stress	Length (mm) of wood discoloration[a]			Wilting and death at 20 dpi	Wilting and death at 60 dpi[b]
		Downward	Upward	Total		
Lansium domesticum						
WRC	Partial flooding	11.3 ± 1.7*	22.8 ± 6.1	34.1 ± 6.4 ab	1/20	7/20 b
	Without flooding	12.6 ± 1.9*	37.3 ± 11.1	49.9 ± 11.4 a	5/20	15/20 a
WBC	Partial flooding	6.2 ± 0.8	9.6 ± 3.3	15.8 ± 3.4 bc	0/20	0/20 c
	Without flooding	5.0 ± 0.5	5.6 ± 0.8	10.6 ± 1.3 c	0/20	2/20 bc
MEA (control)	Partial flooding	1.9 ± 0.1	2.0 ± 0.1	3.9 ± 0.2 d	0/20	0/20 c
	Without flooding	1.9 ± 0.2	1.9 ± 0.1	3.8 ± 0.3 d	0/20	0/20 c
Acacia mangium						
WRC	Without flooding	42.1 ± 3.5	34.9 ± 7.3	76.9 ± 14.8 a	6/20	17/20 a
WBC	Without flooding	17.8 ± 4.1	18.0 ± 8.4	35.8 ± 6.3 b	1/20	5/20 b
MEA (control)	Without flooding	2.1 ± 0.2	2.1 ± 0.2	4.1 ± 0.4 c	0/20	0/20 c
Mangifera indica cv. Arumanis						
WRC	Without flooding	5.1 ± 1.0	5.6 ± 0.9	9.7 ± 1.7 a	0/20	0/20
WBC	Without flooding	7.1 ± 1.3	7.3 ± 1.1	14.4 ± 1.7 a	0/20	0/20
MEA (control)	Without flooding	1.3 ± 0.1	1.3 ± 0.1	2.6 ± 0.1 b	0/20	0/20

MEA, malt extract agar.

[a]Wood discoloration was measured 20 days post-inoculation (dpi). Means of downward lesion length labelled with asterisks are significantly different from upward lesion according to the Welch two sample *t*-test. Means of total lesion length by different plant species followed by common letter are not significantly different according to the honestly significant difference test.

[b]Number of death plants by different plant species labelled by same letter are not significantly different according to the Fisher's exact test of independence with applying the Bonferroni corrected alpha level.

of inoculated seedlings, but not from any of the control plants.

Ceratocystis isolates also induced xylem discolouration and wilt symptoms on inoculated *A. mangium* seedlings (Fig. 5B), similar to that observed on duku seedlings. Xylem discoloration on acacia developed faster than on duku and was equally extensive ($P \geq 0.05$) for both upward and downward expansion (Table 3). Plant wilt and death was observed earlier on acacia compared to duku with half the WRC-inoculated acacia dying within 20 days post-inoculation. Similar to what was observed on duku seedlings, the WRC isolate caused significantly ($P < 0.05$) longer lesion and more death on acacia and therefore, proved to be more pathogenic than WBC (Table 3). *Ceratocystis* isolates were also pathogenic on mango (*M. indica*), but did not induce wilting symptoms (Fig. 5C). Mycelial plug inoculation on stems of mango resulted in wood discoloration similar to the symptoms on duku and acacia (Fig. 5C), but with less expansive discoloration (Table 3).

Discussion

This study presents the first report of *C. fimbriata* associated with massive mortality of *L. domesticum* trees in South Sumatra, Indonesia. This fungus was shown to be pathogenic by producing expansive wood discoloration and causing lethal wilt on inoculated duku seedlings similar to that found in the field. Fungus with the same morphological characteristics was easily re-isolated from diseased wood of inoculated seedlings, suggesting fulfilment of Koch's postulates. Inoculation experiments on acacia seedlings suggested that the pathogen was also pathogenic there by producing more expansive wood discoloration, bark canker, wilting symptoms, and plant death. *Ceratocystis* isolates from duku proved to be less pathogenic on mango, as less wood discoloration was induced, without wilting and plant death.

The ITS rDNA sequence of the most pathogenic isolate, WRC (MT229127), had an identical sequence to the isolates of *C. fimbriata* from tea tree (KF650948), taro (AM712445), and pomegranate (AM292204) in China; from eucalyptus (KF878326) from Zimbabwe; from acacia (MF033455) in Vietnam; and from acacia (EU588656) in Indonesia. All these isolates were confirmed belong to ITS5 haplotype of *C. fimbriata* (Harrington et al., 2014; Li et al., 2016). Some of these isolates were previously identified as *C. acaciivora* (Tarigan et al., 2011) and subsequently reconsidered as *C. manginecans* (Fourie et al., 2015), but

Oliveira et al., (2015) considered those cryptic species to be synonyms or conspecifics of *C. fimbriata* sensu stricto. The ITS5 haplotype is an aggressive genotype of *C. fimbriata* causing a lethal wilt disease of economically important plants worldwide. This genotype represented the native *C. fimbriata* populations in Brazilian forest plantations of *Eucalyptus* spp. (Harrington et al., 2014, 2015; Li et al., 2016). This ITS haplotype was also found infecting *Acacia* spp. and its original host, *Eucalyptus* spp. in China, Indonesia, South Africa, Thailand, Uruguay (Harrington et al., 2014), Zimbabwe (Jimu et al., 2015) and Vietnam (Trang et al., 2018). The member of this *Eucalyptus* population of *C. fimbriata* cause the wilt epidemic on kiwifruit in Brazil (Ferreira et al., 2017). In China, the ITS5 genotype has been considered to be introduced from Brazil through *Eucalyptus* cuttings and reported to cause epidemics on pomegranate, loquat, and taro (Harrington et al., 2015; Li et al., 2016), and tea tree (Xu et al., 2019).

The less pathogenic isolate, WBC, is grouped as ITS6z, a minor haplotype derived from a single haploid strain of C2759 (CBS 135868). The C2759 was originated from *Dalbergia sissoo* in Pakistan and its single-ascospore culture yielded many different haplotypes with the ITS7b as the major genotype (Harrington et al., 2014). WBC had 100% similarity with other member of ITS6z haplotype (type Y = KC261853) of *C. fimbriata* isolate CMW13582 originated from the bark beetle, *H. mangiferae* in Oman (Naidoo et al., 2013). The ITS7b is a common ITS genotype of *C. fimbriata* from Oman, Pakistan, and Indonesia that previously described as *C. manginecans* (Harrington et al., 2014; Oliveira et al., 2015). Many isolates in Asia and Oman have mixed ITS sequences due to crosses between the ITS5, ITS6, and ITS7b genotypes (Oliveira et al., 2015). In this study, *Ceratocystis* isolates from Indonesia (ITS5 and ITS6z) and members of ITS7b haplotype (CMW13851 and CMW23634 from Oman and Pakistan, respectively) are grouped into a single phylogenetic cluster of *C. fimbriata* sensu stricto based on partial β-tubulin sequence. It is likely that the population of *C. fimbriata* causing disease on duku and acacia in Sumatra is a combination of ITS5, ITS6, and ITS7b, with the ITS6z a result of crossing of these haplotypes. Morphological characteristics showed that the pathogen belonged to the species *C. fimbriata* (Engelbrecht and Harrington, 2005). Both *Ceratocystis* isolates from duku (WRC and WBC) had a similar morphology to *C. fimbriata* sensu stricto neotype BPI 595863 (Engelbrecht and Harrington, 2005), except for doliform conidia that were absent on BPI 595863. Phylogenetic analyses based on the ITS and β-tubulin regions showed conclusively that *Ceratocystis* isolates causing bark canker and lethal wilt on duku tree in Indonesia is identified as *C. fimbriata* sensu stricto. There were two ITS genotypes of *C. fimbriata* associated with disease on *Lansium* tree in Indonesia, one consistent with that found in Oman and Pakistan on the mango bark beetle and *Dalbergia* (and other hosts) and a second sequence found in China, Indonesia, Vietnam and Brazil on various hosts, including acacia. *C. fimbriata* has been known to infect a wide variety of annual and perennial host plants throughout the world. In Indonesia, diseases caused by *C. fimbriata* are considered to be of minor importance due to non-lethal and sporadic infestation. The fungal infection has long been noted to cause a non-lethal disease known as mouldy rot on the trunk of rubber trees (Tayler and Stephens, 1929). The role of fungal infection as the primary causal agent of the disease has been dismissed since mouldy rot is considered an advanced stage of a physiological disorder induced by excessive tapping and ethylene overstimulation (Putranto et al., 2015) and the disease can be eliminated by treatment with non-fungicidal biostimulants (Suwandi et al., 2018). In the last decade, disease incited by *C. fimbriata* has been one of the most destructive and economically important diseases on acacia plantations in Indonesia, shortly after an outbreak on the industrial forest plantations throughout the world (Roux and Wingfield, 2009). Outbreaks of *Ceratocystis* disease have forced the replacement of thousands of hectares of *A. mangium* plantations in eastern Sabah, Malaysia (Brawner et al., 2015). In Indonesia, *Ceratocystis* infection has contributed to 2% mortality by the fourth rotation of *A. mangium* in Sumatra, Indonesia (Hardie et al., 2018). Pathogens causing lethal wilt of duku belong to ITS haplotype 5, which represented *C. fimbriata* populations from forest plantations of *Acacia* spp. and *Eucalyptus* spp. Pathogenicity tests also confirmed that *A. mangium* is more susceptible than the original host (duku tree), suggesting the establishment of *C. fimbriata* pathogenicity on acacia as the main host. Similar disease symptoms caused by *Ceratocystis* infections were found to be endemic on acacia and eucalyptus plantations located about 30 km away from the site of study. It is likely that population of *C. fimbriata* pathogenic on acacia plantation could extend their host range to native fruit tree such as *Lansium* and cause a serious threat to the neighbouring fruit tree species. The host-range extension by the ITS5 haplotype of *C. fimbriata* to the susceptible neighbouring plants occurred in Brazil, in which the genotype from eucalyptus showed strong aggressiveness on taro (Harrington et al., 2011) and caused epidemic on grapevine (Ferreira et al., 2017). Similar host extension by the ITS5 haplotype also occurred in China, in which the eucalyptus population caused epidemic on

pomegranate, loquat, and taro (Harrington et al., 2015; Li et al., 2016), and tea tree (Xu et al., 2019).

All sampled diseased trees had been previously attacked by squirrels and lesions appeared to originate from surrounding beetle entry/exit holes on peeled-off bark from squirrel scratches, suggesting the involvement of the wild vertebrate as the wound creator and beetles for fungal spore dispersion. Fungal feeding insects, such as *H. mangiferae*, have been suggested to be associated with the rapid distribution of *C. fimbriata* in Oman and Pakistan (Al Adawi et al., 2013). Squirrel attacks on either diseased or healthy duku trees were found only during the disease outbreaks in 2013-2014 and these attacks were likely due to the limitation of squirrel feed sources in the field. All affected orchards had grown duku in a monoculture. Pathogenicity tests supported the idea that partial flooding was not likely to predispose duku trees to *Ceratocystis* infection as the disease did not develop well under partial flooding. Recent field observations in areas near the disease origin suggested that the disease spreads sporadically with limited mortality. Squirrel attacks were not found on recently infected trees, suggesting the possible involvement of the wild vertebrate wounds on the massive disease spread in duku orchards. Vertebrate-incited wounds, such as those from squirrels and monkeys, are considered to contribute to the spread of *Ceratocystis* wilt on *A. mangium* plantations (Brawner et al. 2015; Hardie et al. 2018; Nasution et al., 2019).

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (http://www.ppjonline.org/).

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