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Characterization of *Lasiodiplodia theobromae* and *L. pseudotheobromae* causing fruit rot on pre-harvest mango in Malaysia

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Abstract

Lasiodiplodia species are important plant pathogens in mango production countries, infecting plants during both pre- and post-harvest phases. Fruit rot reduces mango production and creates tremendous losses in economy of the involved countries. Fungal isolation was conducted in eight locations throughout Peninsular Malaysia. Species identification was based on morphological characterisation and internal transcribed spacer (ITS) and β-tubulin (BT2) gene sequences analysis. Twenty isolates were obtained and identified as *Lasiodiplodia theobromae* (17 isolates) and *L. pseudotheobromae* (3 isolates). Phylogenetic analysis using maximum-likelihood method demonstrated that all isolates of *L. theobromae* and *L. pseudotheobromae* formed a monophyletic clade. In pathogenicity tests, most fungal isolates inoculated on mango fruit showed fruit rot symptom. The most virulent isolate was *L. theobromae* A1718 with disease severity index (DSI) of 87.5%.

Key words – β-tubulin (BT2) – internal transcribed spacer (ITS) – *Lasiodiplodia* – mango

Introduction

Mango can be attacked by various pathogens that lead to severe pre- and post-harvest diseases. Important diseases include anthracnose, alternaria rot, stem end rot, powdery mildew, black mould rot, gummosis and bacterial black spot (Nasir et al. 2014). According to Barkai-Golan (2001), stem end rot caused by fungi is the most prominent disease reportedly caused by several fungi such as *Dothiorella dominicana*, *D. mangiferae*, *Lasiodiplodia theobromae*, *Pestalotiopsis* and *Alternaria* species. *Lasidioplodia* spp. are considered as the most vital cause of disease on mango (Johnson et al. 1992), and they have a very broad host range damaging almost 500 host plants (Burgess et al. 2006).

In Malaysia, fruit rot is commonly observed in mango orchards during pre-harvest phase. However, there is no comprehensive report on the characterisation of *Lasiodiplodia* species infecting mango production during pre-harvest stage. The presence of fruit rot symptoms in mango fields, together with the emergence of numerous *Lasiodiplodia* species as important mango

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pathogens has increased the necessity to accurately identify, characterise and confirm the pathogenicity of these species, besides improving knowledge of pre-harvest fruit rot disease on mango.

The knowledge on specific characterisation in host-pathogen identification is essential to establish and implement good plant disease management (Ramachandran et al. 2015). Even though post-harvest disease can cause discernible losses, infection occurring in orchard might also spread fungal inoculums leading to a complete loss of mango production. Therefore, in this study, all samples were obtained from the field. Internal transcribed spacer (ITS) region and β-tubulin (BT2) sequence analysis were then utilised to identify the isolated fungi. To verify the causal agent of fruit rot disease in the field, pathogenicity tests were repeatedly conducted to fulfil Koch's postulates.

This study will provide additional knowledge on fungal diversity associated with fruit rot disease in Malaysia, particularly on mango. The objectives of this study were to characterise *Lasiodiplodia* isolates based on ITS and BT2 sequences analysis and to determine whether *Lasiodiplodia* species isolated from infected mango are pathogenic.

Materials & Methods

Sampling and fungal isolation from diseased fruit rot of mango

Sampling locations selected for this study were eight mango orchards in six states (Perlis, Penang, Perak, Selangor, Pahang and Malacca) throughout Peninsular Malaysia. Sampling was conducted from July 2014 to May 2015. In each site, five samples of fruits with the symptoms of fruit rot disease were collected and taken to the laboratory. Three 5 × 5 mm^2 pieces from the margins of infected tissues were cut and surface sterilized in 1% sodium hypochlorite solution by dipping for 3 min and rinsed three times with sterilized distilled water. The tissues were blot dried and placed on the surface of potato dextrose agar (PDA) plates amended with streptomycin to prevent bacterial growth. Fungal mycelia that developed from infected tissues were purified using hyphal tip isolation on 4% water agar (WA) and transferred to a new PDA plate. Cultures were incubated at room temperature (27 ± 1 ºC) with a 12 h photoperiod. All pure cultures obtained were maintained and preserved at -20 ºC using modified filter paper method (Fong et al. 2000).

Morphological characterization

The morphological (macro- and micro-morphological) characteristics of all pure isolates were examined. For macro-morphological characteristics, the colony features and pigmentation of cultures were observed after seven days incubation on PDA, while the growth rate of each isolate was measured in triplicate after 72 h of incubation. The characteristics of conidia (shape, size, colour, longitudinal striations and conidial wall) were observed after three weeks of incubation in 12 h photoperiod on WA with autoclaved mango leaves as substrate with modification of de Oliveira Costa et al. (2010) and observed using a compound microscope (Leica, Microsystems).

Molecular characterisation based on internal transcribed spacer (ITS) and β-tubulin (*BT2*) genes

All isolates were cultured on PDA and incubated for a week with 12 h photoperiod. Genomic DNA was extracted using UltraClean® Microbial DNA isolation kit (MO BIO, Carlsbad, CA, USA) following the instruction manual.

Polymerase chain reaction (PCR) amplification of ITS region was carried out using the primers ITS1 (5'-TCCGTA GGTGAACCTGCGG-3'), ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990) and of β-tub gene using Bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and Bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) (Glass & Donaldson 1995). PCR mixtures for both reactions were performed using GoTaq® Flexi DNA Polymerase (Promega, USA). Each 20 µl PCR mixture comprised 20 ng DNA template, 1X Green buffer, 0.2 mM dNTP mix and 2.5 mM $MgCl_2$, 0.1 mM primer, 0.125 U Go *Taq* Polymerase and sterile distilled water.

Amplification of ITS region followed White et al. (1990) in which the initial denaturation was started with 95 ºC for 30 s, followed by 36 cycles of denaturation for 10 s at 95 ºC, annealing for 15 s at 59 ºC and extension at 72 ºC for 30 s before a single cycle of 5 min of final extension step at 72 ºC was carried out. β-tubulin (BT2) amplification protocol was performed according to Prihastuti et al. (2009) with slight modification; initial denaturation was completed at 95 ºC in 3 min, 34 cycles of denaturation for 1 min at 95 ºC were continued with annealing step at 59 ºC in 30 s and extension at 72 ºC for 60 s before proceed with a single cycle of the final extension step at 72 ºC for 10 min. Amplifications of DNA using ITS and *BT2* markers were performed using Biometra (T Professional) to verify the absence of any non-specific reaction and contaminants, one control reaction with no DNA replaced with distilled water was used. The amplicons of ITS and *BT2* were observed between 500–600 bp and 400–500 bp, respectively.

Gel purification and gene sequencing: The amplicons were separated by electrophoresis using 1.5% agarose gels in 1.0×Tris Borate-acid EDTA (TBE) buffer amended with FloroSafe DNA stain according to manufacturer's instructions (1st BASE, Asia). The gel electrophoresis was done at 90 V for 33 min, employing 100 bp DNA ladder (Thermo Scientific) as a DNA marker. The gel was viewed and analysed using Syngene software by a gel documentation system under UV light visualisation (Syngene, Germany). PCR products were purified using the Gel Purification Kit according to manufacturer Qiagen's instruction. The purified ITS and *BT2* products were sequenced in both directions using an Applied Biosystem 3730xl DNA Analyser at MyTACG Bioscience Company, Malaysia.

Forward and reverse ITS and *BT2* sequences were assembled and aligned by molecular evolutionary genetics analysis (MEGA 6.0, Tamura et al. 2013). The DNA sequences of ITS and *BT2* genes obtained from this research were deposited in the GenBank database using Sequin software. Phylogenetic tree was constructed using maximum likelihood (ML) method with 1000 bootstrap replication value in the MEGA 6.0 software (Tamura et al. 2013). Several ex-type sequences (*L. theobromae* (AY640255), *L. pseudotheobromae* (EF622077), *L. gonubiensis* (DQ458892), *L. crassispora* (DQ103552) and *L. rubropurpurea* (DQ103554)) from GenBank were obtained and included in the tree as the representative of each species. *Colletotrichum gloeosporioides* (EU371022) was used as outgroup for ITS and *BT2*.

Pathogenicity test of fruit rot disease on mango

Matured and healthy mango fruits (cv Chok Anan, MA224) with uniform size and age harvested from an orchard in Malacca (2°13'26.205", 102°16' 47.388") were used for pathogenicity test. The fruit were washed in running water and surface disinfected using 1% sodium hypochlorite (NaOCl) for 5 min, before being rinsed twice with sterile distilled water (Than et al. 2008). The fruit was then air-dried, before placing in surface sterilised plastic containers (30 × 20 × 10 cm), ready for inoculation process. The pathogenicity test utilised all 20 obtained isolates of *Lasiodiplodia* species. These isolates were cultured on PDA and incubated with a 12 h photoperiod at 27 ± 1 ºC. Each fruit was inoculated using non-wounded method by directly placing a 5 mm diameter mycelial plug on the mango surface (Kouame et al. 2010, Marques et al. 2013). The mycelial plug was taken from the edge of 5-day-old fungal cultures. Mango fruit used as controls were inoculated with a sterile non-colonised PDA plug. All the inoculated fruits were incubated in the covered containers under the same condition, 27 ± 1 °C in dark. All treatments and control were repeated twice and four fruits were used for each fungal isolate. After eight days of inoculation, the fungal colonies from lesions were re-isolated onto PDA and incubated for a week. The isolated fungi were re-identified and compared with the original isolates to fulfill Koch's postulates.

Pathogenicity of the isolates was evaluated based on a disease scale from 0 to 4 described by Amadi et al. (2009) with a modification for mango. All parameters were kept similar, however, disease scores were characterised using a specific symptom of fruit rot disease on mango as shown in Table 1 and Fig 1.

Table 1 Disease severity scale (Amadi et al. 2009 with modification).

[a] Description	Disease score
No visible symptoms on fruit	0
1–25% of fruit surface area covered with slight necrotic lesion	1
26–50% of fruit surface area covered with necrotic lesion	2
51–75% of fruit surface area covered with necrotic lesion	3
> 76% of fruit surface area covered with necrotic lesion and decay soft tissue	4

[a]100% area equal to 16 cm² and above

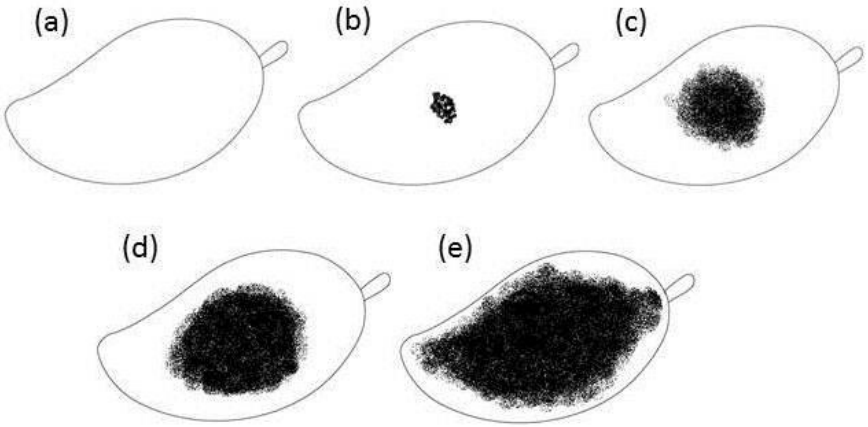

Fig 1 – Severity scale used for the assessment of mango fruit rot. a, No visible symptoms on fruit. b, 1–25% of inoculated area covered with slight necrotic lesion. c, 26–50% of inoculated area covered with necrotic lesion. d, 51–75% of inoculated area covered with necrotic lesion. e, more than 76% of inoculated area covered with necrotic lesion and decay soft tissue.

The score obtained for each isolate was then used to calculate the disease severity index (DSI) by the following formula (Abu Bakar et al. 2013):

$$\text{DSI (\%)} = \frac{\text{Sum of individual ratings}}{\text{Number of mango fruit assessed}} \times \frac{100}{\text{Maximum disease score}}$$

The test was conducted in a completely randomised design with four replicates per treatment (isolate) with one mango per replicate. To compare the variation of the disease severity index (DSI) among isolates, data were analysed using the Friedman Test from the non-parametric test in the SPSS programme at $p < 0.05$ (IBM Corp. Armonk, NY).

Results

Morphological characteristics of fungal isolates associated with fruit rot

Fruit rot symptoms were observed in the field on infected mature green mango fruit. The symptoms were characterized as black spots with brown edges and distinct shapes. Circular spots that were initially observed to be scattered at the middle of the fruit were derived from the end of stem.

Twenty isolates of *Lasiodiplodia* species were obtained from pre-harvest fruit rots in eight locations throughout Peninsular Malaysia (Table 2). These isolates were recovered from different varieties of mango displaying the symptoms of fruit rot in the field. The isolates were identified as *L. theobromae* and *L. pseudotheobromae.* Both species were characterised with similar colony features, the presence of abundant woolly mycelium and colonies from olivaceous-grey to dark

grey. The colonies were initially white to pale grey becoming dark grey with age and with fluffy aerial mycelia and black pigment on the reverse side of plate.

Table 2 *Lasiodiplodia* species associated with fruit rot disease on mango.

Isolate no.	Mango variety	Location (State, City)	Species identification (ITS and β-tubulin)	Sequence length (bp)		GenBank accession no.	
				ITS	β-tubulin	ITS	β-tubulin
M1560	Melaka Delight	Telok Mas, Melaka	*L. theobromae*	523	436	KT968468	KX034536
M1564	Melaka Delight	Telok Mas, Melaka	*L. theobromae*	528	439	KT968480	KX034537
R1835	Perlis sunshine	Chuping, Perlis	*L. theobromae*	527	434	KT968479	KX034524
B1475	Epel (MA 194)	Meru, Selangor	*L. theobromae*	524	432	KT968462	KX034535
B1523	Telur	Meru, Selangor	*L. theobromae*	526	435	KT968464	KX034534
B1512	Telur	Meru, Selangor	*L. theobromae*	524	432	KT968465	KX034533
P1769	Chokanan (MA 224)	Seberang Perai, Penang	*L. theobromae*	524	434	KT968466	KX034532
P1772	Chokanan (MA 224)	Seberang Perai, Penang	*L. theobromae*	530	434	KT968467	KX034531
A1700	Chokanan (MA 224)	Bota Kiri, Perak	*L. theobromae*	524	436	KT968469	KX034530
A1718	Chokanan (MA 224)	Bota Kiri, Perak	*L. theobromae*	525	436	KT968471	KX034529
A1719	Chokanan (MA 224)	Bota Kiri, Perak	*L. theobromae*	522	439	KT968472	KX034528
R1738	Chokanan (MA 224)	Arau, Perlis	*L. theobromae*	518	437	KT968473	KX034527
R1743	Chokanan (MA 224)	Arau, Perlis	*L. theobromae*	521	438	KT968474	KX034526
R1736	Chokanan (MA 224)	Arau, Perlis	*L. theobromae*	522	437	KT968475	KX034525
R1839	Chokanan (MA 224)	Chuping, Perlis	*L. theobromae*	526	437	KT968478	KX034523
R2251	Lemak manis	Beseri, Perlis	*L. theobromae*	521	436	KT968460	KX034521
R2252	Lemak manis	Beseri, Perlis	*L. theobromae*	455	436	KT968461	KX034522
B1494	Epel	Meru, Selangor	*L. pseudotheobromae*	524	432	KT968463	KX034518
R1757	Harum manis (MA 128)	Beseri, Perlis	*L. pseudotheobromae*	522	437	KT968476	KX034519
R1761	Harum manis (MA 128)	Beseri, Perlis	*L. pseudotheobromae*	520	436	KT968477	KX034520

Characteristics of conidia and paraphyses were used to differentiate between the two species. Salient features are summarized in Table 4, along with results obtained by other authors. *Lasiodiplodia theobromae* (17 isolates) were induced to sporulate using sterile mango leaves. Pycnidia formed with septate paraphyses between the conidiogenous cells. The conidia measured 20–21.8 × 9.1–10.9 µm. They were initially hyaline, thin-walled and aseptate, cylindrical to subovoid in shape. They later turned dark brown, formed a single medium septum and became thick-walled with longitudinal striations on the inner surface. Mature conidia were ovoid with a broad and rounded apex and tapered at the base (Fig 2).

For *L. pseudotheobromae* (3 isolates), the conidia measured 25.5–27.3 × 12.7–14.6 µm, and were cylindrical to ellipsoidal with both apex and base having a rounded shape. The young conidia were hyaline becoming dark brown with a medium septum and longitudinal striation as they matured. The paraphyses formed in this species were septate (Fig 3).

Molecular identification based on internal transcribed spacer (ITS) and β-tubulin (BT2) gene

The sequences of ITS and *BT2* of all 20 isolates of *Lasiodiplodia* species were successfully amplified using the primers ITS1/ ITS4 and Bt2a/Bt2b, respectively. The amplified target band of ITS region and *BT2* (Fig 4) were observed with the approximate size ranging from 500–600 bp and 400–500 bp, respectively. For both gene sequences analyses, 17 isolates were identified as *L. theobromae* and 3 isolates as *L. pseudotheobromae* with percentage of similarity between obtained isolates and established species ranging from 98–100%. The identity of all isolates based on ITS and *BT2* are shown in Table 2. The sequences of isolates obtained were deposited into GenBank (http://www.ncbi.nlm.gov) with the accession numbers also listed in Table 2.

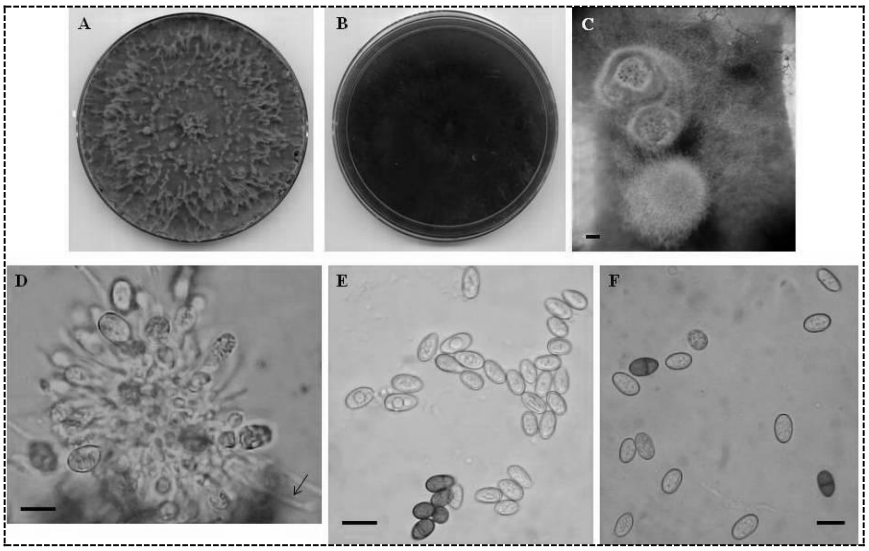

Fig 2 – Morphological characteristics of *Lasiodiplodia theobromae*. A, Dark grey colony with woolly aerial mycelium on PDA. B, Black pigmentations on reverse plate of PDA. C, Pycnidia formed on mango leaf. D, Conidiogenous cells and septate paraphyses. E, Hyaline, immature conidia. F, Dark bovoid mature conidia with middle septum and longitudinal striation. – Bars = 20 µm.

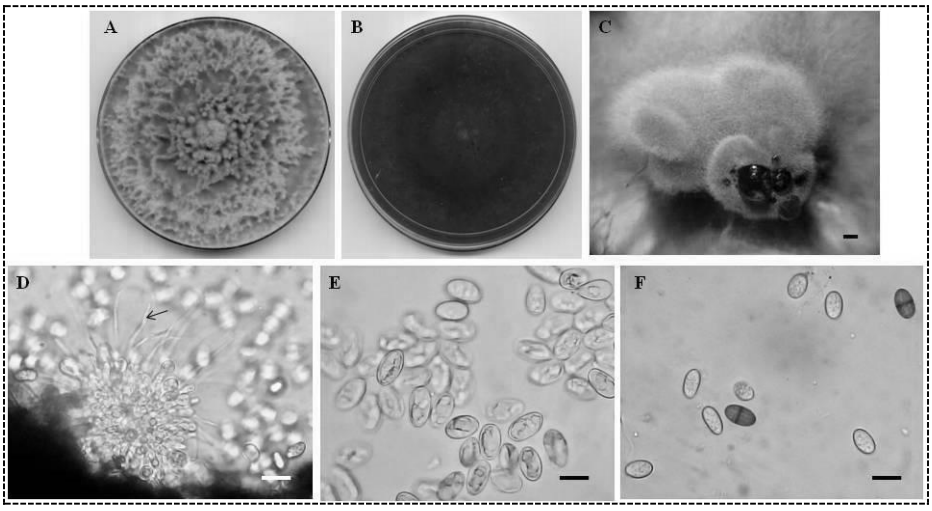

Fig 3 – Morphological characteristics of *Lasiodiplodia pseudotheobromae*. A, Pale grey colony with light grey aerial mycelium on PDA. B, Black pigmentation on the reverse side of PDA. C, Pycnidia formed on mango leaf. D, Conidiogenous cells with aseptate paraphyses. E, Hyaline, ellipsoid immature conidia. F, Dark coloured mature conidia. – Bars = 20 µm.

The phylogenetic tree constructed from combined ITS and *BT2* is shown in Fig 5. The *Lasiodiplodia* isolates obtained in this study were classified into two main clades, Clade 1 and Clade II. Clade 1 comprised the majority of isolates (M1560, M1564, R1835, B1475, B1523, B1512, P1769, P1772, A1700, A1718, A1719, R1738, R1743, R1736, R1839, R2251 and R2252), which were grouped together with a reference sequence of *L. theobromae* (BOT-4; Ismail et al. 2012) supported by a bootstrap value of 69%. For Clade II, three isolates (R1757, R1761 and B1494) were represented as *L. pseudotheobromae* and they grouped together with *L. pseudotheobromae* isolate from *Mangifera indica* (BOT-11; Ismail et al. 2012) supported with a bootstrap value of 80%. Another three species, *Lasiodiplodia gonubiensis*, *L. rubropurpurea* and *L. crassispora*, obtained from GenBank were included in the tree to demonstrate the species variation.

The outgroup control used in the tree is *Colletotrichum gloeosporioides*, ancestral to the group and distinctly separated from the main clade.

Table 3 Disease severity index 1–6 days after inoculation.

Isolates no.	*Disease severity index (DSI) days after inoculation					
	1	**2**	**3**	**4**	**5**	**6**
Lasiodiplodia theobromae						
M1560	0^a	12.50^b	25.00^b	50.00^b	56.25^b	56.25^b
M1564	0^a	12.50^b	18.75^b	31.25^b	50.00^b	56.25^b
R1835	0^a	0^a	12.50^b	25.00^b	31.25^b	50.00^b
B1475	18.75^b	18.75^b	25.00^b	25.00^b	62.50^b	75.00^b
B1523	0^a	0^a	0^a	0^a	12.50^b	12.50^b
B1512	0^a	6.25^b	6.25^b	6.25^b	6.25^b	12.50^b
P1769	18.75^b	18.75^b	31.25^b	56.25^b	62.50^b	66.67^b
P1772	6.25^b	18.75^b	18.75^b	50.00^b	50.00^b	56.25^b
A1700	6.25^b	6.25^b	12.50^b	12.50^b	12.50^b	18.75^b
A1718	6.25^b	31.25^b	50.00^b	56.25^b	87.50^b	87.50^b
A1719	0^a	0^a	12.50^b	18.75^b	25.00^b	31.25^b
R1738	0^a	18.75^b	31.25^b	37.50^b	50.00^b	81.25^b
R1743	0^a	12.50^b	12.50^b	25.00^b	43.75^b	68.75^b
R1736	0^a	0^a	12.50^b	12.50^b	31.25^b	37.50^b
R1839	0^a	18.75^b	37.50^b	68.75^b	81.25^b	81.25^b
R2251	0^a	6.25^b	6.25^b	25.00^b	25.00^b	37.50^b
R2252	0^a	6.25^b	6.25^b	18.75^b	31.25^b	50.00^b
Lasiodiplodia pseudotheobromae						
B1494	0^a	0^a	0^a	0^a	0^a	0^a
R1757	6.25^b	25.00^b	37.50^b	43.75^b	75.00^b	75.00^b
R1761	0^a	12.50^b	18.75^b	25.00^b	43.75^b	50.00^b
Control fruit	0^a	0^a	0^a	0^a	0^a	0^a

* Values are means of two repetitions of DS. Means for respective isolates in same column indicated with letter 'b' were significantly different with control ('a') which $p < 0.05$.

Pathogenicity test of fruit rot disease on mango fruits

The isolates were inoculated with a fungal plug using non-wounded method and the severity levels were observed from day 1 until day 6. After day 6, the mango fruit were overripe with immeasurable DSI. Among the 20 isolates, only *L. pseudotheobromae* B1494 did not produce disease symptoms and was classified as a non-pathogenic isolate. The other isolates showed symptoms with different levels of severity (Table 3).

Most isolates started to show symptoms of fruit rot two days after inoculation. There was a significant difference in DSI of isolates following inoculation compared to control fruit, which $p < 0.05$. *L. theobromae* A1718 was the most virulent isolate on mango fruit with 87.50% DSI on day 6. This was followed by moderate to severe symptoms from *L. theobromae* R1743 and R1839 that were both measured up to 50% severity index after day 6, while *L. theobromae* A1700 represented the least severe symptom as shown in Fig 6. For *L. pseudotheobromae*, the most severe infection was produced by isolate R1757 with 75% DSI followed by two moderately severe isolates (R1761 and R2251) with 50% DSI and one isolate of the least severe (R2251) as well as a non-pathogenic isolate (B1494).

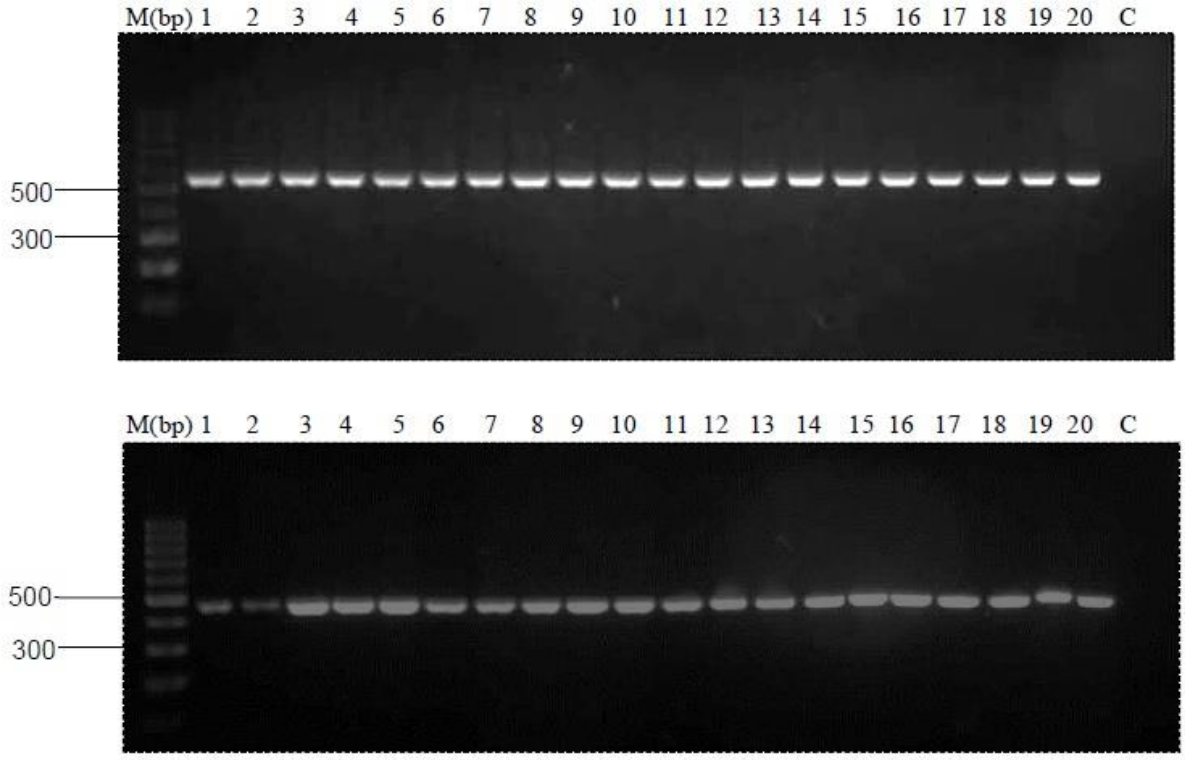

Fig 4 – Banding pattern of ITS and β-tubulin gene amplification, respectively. Expected band sizes ranging from 400–600 bp. Lanes 1–17: *Lasiodiplodia theobromae* (M1560, M1564, R1835, B1475, B1523, B1512, P1769, P1772, A1700, A1718, A1719, R1738, R1743, R1736, R1839, R2251 and R2252). Lanes 15–20: *Lasiodiplodia pseudotheobromae* (R1757, R1761 and B1494). Lane M: Marker 100 bp. Lane C: Control.

Discussion

Symptoms of fruit rot were frequently observed on mango fruit from the field in Peninsular Malaysia. According to Singh et al. (2013), fruit rot symptoms are commonly found in infected fruits and cause serious loss in postharvest stage, characterised by the black spots on fruits before harvest, these symptoms decrease mango yield for the growers. Rahman et al. (2015) reported that the quality of fruits, consumers' confidence and profits of growers are reduced due to poor cosmetic appearance of the fruits caused by this disease.

Lasiodiplodia species belong in family Botryosphaeriaceae and are classified in Dothideomycete fungi, which have a cosmopolitan distribution (Crous et al. 2006, Phillips et al. 2008). Among the members of Botryosphaeriaceae, *Lasiodiplodia* species are one of the important and opportunistic plant pathogens since species in this genus can cause severe disease symptoms ants (Sakalidis et al. 2011). In the present study, two species of *Lasiodiplodia* were found to be associated with fruit rot symptom on mango fruits during preharvest stage, namely *L. theobromae* and *L. pseudotheobromae*.

Based on morphology and molecular identification, *L. theobromae* (sexual stage: *Botryosphaeria rhodina*) was more frequently isolated than *L. pseudotheobromae* from mango fruit rot samples. *L. theobromae* and *L. pseudotheobromae* are known to have similar features such as the colony appearance, mature conidia with thick walls and longitudinal striations formed due to deposition of melanin on inner wall surface (Alves et al. 2008). Conidia of *Lasiodiplodia* species become dark coloured when mature, produce a septum and have longitudinal striations. The presence of striations on mature conidia was used for genus identification (Sutton 1980, de Oliveira Costa et al. 2010). Although both species resemble each other, size, shape of their conidia and paraphyses can be used in species separation. *Lasiodiplodia theobromae* conidia are smaller in size

with an ovoid shape compared to those of *L. pseudotheobromae*, which are larger and ellipsoid in shape. This is similar to findings of previous studies (Alves et al. 2008, Abdollahzadeh et al. 2010, Ismail et al. 2012) (Table 4).

Both *Lasiodiplodia* species were induced to sporulate using sterile mango leaves as a substrate with modification from the method proposed by Slippers et al. (2004) and Marquest et al. (2013). Formation of pycnidia on mango leaves was observed to be much faster in one to two weeks compared to cultures growing without mango leaves. *Lasiodiplodia theobromae* is a plurivorous plant pathogen that commonly infects tropical and subtropical woody plants and fruit plants (Ismail et al. 2012) such as avocado, banana, barbados cherry, cashew, citrus, coconut palm, custard apple, grapevine, guava, muskmelon, papaya, passion fruit, soursop and watermelon (Marquest et al. 2013). However, among these mango is regarded as the most affected fruit by this fungus with several symptoms such as dieback, stem-end rot, decline, gummosis and canker (Ploetz et al. 1996, Abdollahzadeh et al. 2010, Ismail et al. 2012, Marquest et al. 2013).

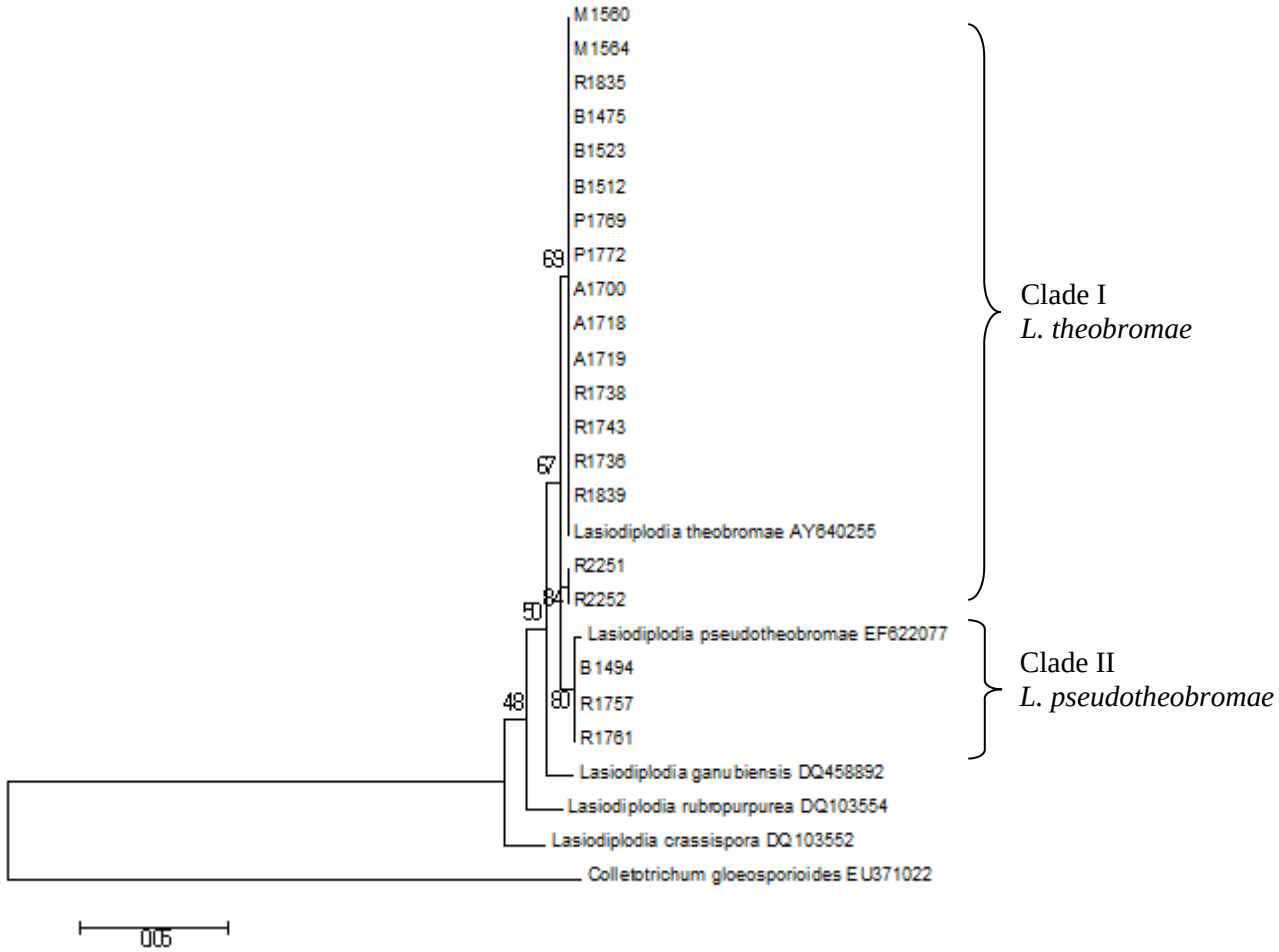

Fig 5 – Maximum likelihood tree demonstrating the relationship of 20 isolates of *Lasiodiplodia* species from fruit rot disease on mango. All isolates generated from the combined analysis of ITS and β-tubulin sequence data were grouped into two subclades (I and II) represented as *L. theobromae* and *L. pseudotheobromae*. The bootstrap values (1000 replication) are shown next to branches. *Colletotrichum gloeosporioides* (EU371022) was used as the outgroup.

Fig 6 – Variation in severity of fruit rot on mango, A–C, inoculated with *L. theobromae*. D–E, inoculated with *L. pseudotheobromae*, F, control. A, Severe symptom inoculated with isolate A1718, with more than 76% of surface area covered by black necrotic lesion in the presence of watery and pale grey mycelium. B, Moderately severe symptom inoculated with isolate R1743, with 50% of surface area covered with necrotic rot in the presence of pale grey mycelium. C, Least severe symptom with less than 25% area covered with brown to black lesion when inoculated with isolate A1700. D, Moderately severe symptom inoculated with isolates R2251 and R1761. E, Colonised agar showing no lesion formation, when inoculated with B1494. F, Non-colonised agar plug.

Table 4 Comparison of conidial and paraphyses characteristics of *Lasiodiplodia* spp.

Fungus	Conidial size (average, µm)	Paraphyses (µm)			References
		Length	Width	Septation	
L. theobromae	26.2 × 14.2	55	3–4	Septate	Alves et al. (2008)
	23.7 × 13.3	44	2–3	Septate	Ismail et al. (2012)
	25.9 × 14.0	-	-	-	Marquest et al. (2013)
	20.9 × 10.0	38.2	2.7	Septate	This study
L. pseudotheobromae	28.0 × 16.0	58	3–4	Aseptate	Alves et al. (2008)
	26.7 × 12.3	52	2–3	Aseptate	Ismail et al. (2012)
	26.6 × 14.5	-	-	-	Marquest et al. (2013)
	26.4 × 13.6	58.2	3.6	Aseptate	This study

Lasiodiplodia pseudotheobromae was separated as a cryptic species from within *L. theobromae* Alves et al. (2008), *L. pseudotheobromae* occurs in Africa, Europe and Latin America (Adetunji et al. 2013). In this study, five isolates of *L. pseudotheobromae* were identified using morphological and phylogenetic analyses, which possessed the microscopic characteristics similar to that described in ex-type isolate CBS116459 by Alves et al. (2008). *L. pseudotheobromae* was originally thought to have a limited host range, but recent studies have revealed the expanded the host range and found it associated with mango diseases in several countries such as Western Australia, China, Egypt and Brazil (Ismail et al. 2012).

Both species produced fruit rot symptoms in the pathogenicity test with different levels of severity. An isolate of *L. theobromae* was the most virulent. However, four isolates of *L. pseudotheobromae* were found to be more severe than *L. theobromae*, as also found by Ismail et al. (2012), Surprisingly, one isolate of *L. pseudotheobromae* was non-pathogenic. This phenomenon might be due to the ability of isolates in the same genus that can act as a pathogen or endophyte on host plant (de Oliveira Costa et al. 2010). Since most pathogenicity studies on *Lasiodiplodia* species have used the wounded method, positive results for non-wounded method performed in this pathogenicity test revealed that both species are capable to naturally penetrate the host and cause lesion by entering through stomata, lenticels or reproductive structures (Sakalidis et al. 2011). Hence, all isolates species obtained were observed to be able to cause disease on mango fruits without the presence of any injuries, therefore increasing the chances of mango being attacked preharvest. To the best of our knowledge, this study represents the first report of *L. theobromae* and *L. pseudotheobromae* on preharvest mango in Peninsular Malaysia associated with fruit rot symptom.

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