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Morphological, Pathogenic, and Molecular Characterization of *Phytophthora palmivora* Isolates Causing Black Pod Disease on Cocoa in Peninsular Malaysia

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Abstract

Black pod caused by *Phytophthora palmivora* causes major losses on cocoa (*Theobroma cacao*) in Malaysia and worldwide. In this study, morphological and molecular characterization as well as pathogenicity of 14 isolates of *P. palmivora*, obtained from main cocoa producing states of Peninsular Malaysia including Perak and Pahang, were determined. In general, the results indicated that *P. palmivora* isolates were divided into two groups based on their geographical origins of the isolates. According to morphological characteristics, although some overlap was observed among the isolates obtained from the two states, isolates from Pahang grew faster on different agar media, and various shapes of sporangia including, globose, limoniform, ovoid and sporangia with two papilla were recorded for these isolates. Pathogenicity tests indicated that the isolates obtained from Pahang are more aggressive compared to the isolates from Perak. Phylogenetic analysis of a combined dataset of the ITS, *EF-1α*, and COX1 indicated that all 14 isolates belonged to *P. palmivora* with no difference among the isolates. These results suggest that morphological and pathogenic diversity of Malaysian isolates of *P. palmivora* is related to their geographical origins.

Keywords

Black pod disease, PCR, Phylogeny, *Phytophthora palmivora*, Virulence

1. Introduction

Cocoa (*Theobroma cacao*), a small evergreen tree, is a major beverage crop after coffee and tea in the world. This crop is grown in Africa, Asia, Latin America and Oceania in about 50 countries on an estimated total area of 10 million hectares [1]. Farmers that hold between 1 to 10 hectares cultivate more than 90% of the total cocoa produced in the world [2]. Cocoa is known as the third plantation crop after oil palm and rubber in Malaysia [3]. Plant diseases are the limiting factors for cocoa production in Malaysia and worldwide. Cocoa crop is affected by many diseases caused by fungi, viruses and bacteria, besides nutritional disorders with estimated losses ranging between 30 to 40% of global production [4]. In Malaysia, cocoa production had been reduced significantly from accounting for 9% of the world

production in the years 1990-1991 to only below 2% of the world production until the year 2000 [5]. Four *Phytophthora* species have been reported to associate with black pod rot, namely *P. megakarya*, *P. capsici*, *P. citrophthora* and *P. palmivora*. *Phytophthora megakarya* was found only in west Africa while *P. palmivora* seems to be the principal pathogen in Asia such as Malaysia and Indonesia [5]. *Phytophthora citrophthora* and *P. capsici* are the main causal agents of pod rot in the Americas [6].

Morphological characteristics are the basis for the identification of plant pathogenic fungi. Cultural and morphological characteristics have been used for the identification of *Phytophthora* spp. associated with cocoa [7]. Traditionally *Phytophthora* species have been distinguished by morphological characteristics such as shape of zoosporangia and the presence of oospores. Erwin and Ribeiro (1996) provided morphological descriptions and pathological aspects of many *Phytophthora* spp. Describing *Phytophthora* spp. based on morphology has taken into account the structure of the sporangium and sporangiophore, presence of chlamydospores; caducity and antheridial attachment, and the type of sexual reproduction (i.e. homothallic or heterothallic). As opposite to homothallic species, heterothallic species generally require two mating types. *Phytophthora* spp. also produces asexual structures, including zoosporangia, zoospores, cysts and chlamydospores [6].

PCR-detection technique has several distinguishing features as compared with the conventional diagnostic methods for oomycetes pathogens. This technique has more sensitivity and versatility [8]. Sequences of the internal transcribed spacer (ITS) rDNA region have been widely employed to identify *Phytophthora* species [9, 10] and elucidate their taxonomic relationships [11, 12]. In addition, the ITS rDNA region has proven particularly useful for fungal taxa separation at the species level [9, 13]. However, a previous study indicated that the combination of nDNA genes and mtDNA genes are powerful and reliable for identification of *Phytophthora* at species level [14]. To our knowledge, no study has been done to examine the effect of the three combined regions including ITS, *EF-1α*, and COX1 in *Phytophthora* identification. So far, little information is only available on morphology of *P. palmivora* isolates causing pod rot on cocoa plants in Peninsular Malaysia. Therefore, the main objective of this study was to examine the morphological and molecular characteristics as well as pathogenicity of *P. palmivora* isolates obtained from the two main cocoa plantation states in Peninsular Malaysia. This information will be useful for proper identification of the pathogen as well as the establishment of future management programs.

2. Materials and methods

2.1 Isolation

Collection sites in Perak and Pahang states were chosen based on the recommendations by Malaysian Cocoa Board (MCB). Cocoa pods showing typical symptoms of brownish lesions were randomly collected from abandoned cocoa farms at Teluk Intan, Hilir Perak and Perak Tengah (Perak state) and Raub, Triang, and Damak Jerantut (Pahang state) in 2013 (Figure 1). Pods were then taken to the laboratory at the department of plant protection, Universiti Putra Malaysia for isolation. For this purpose, cocoa pods were surface sterilized with 70% ethanol for one minute and rinsed several times in sterile distilled water. A 0.5 cm^2 section was cut from each end of the sterilized pieces and transferred onto corn meal agar (CMA). Subsequently, subcultures derived from pure colonies were maintained on CMA slants at 25 ± 2°C and used in further experiments. To store cultures of *Phytophthora*, 8-10 small blocks from an actively growing plate cultures were cut and placed in small screw capped glass bottles containing autoclaved CMA. The *Phytophthora* cultures were revitalized once a year.

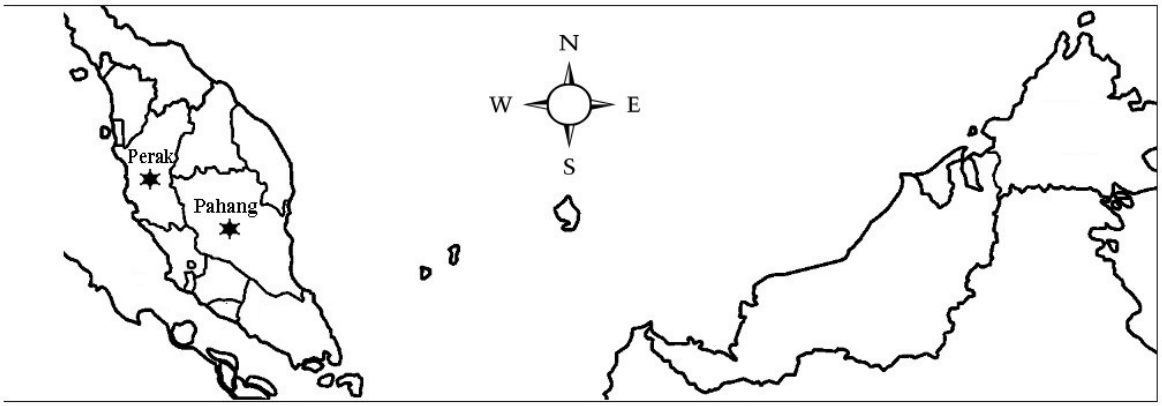

Figure 1. Sites sampled for cocoa black pod disease in Perak and Pahang states.

2.2 Molecular characterization

2.2.1 DNA extraction

All isolates were grown in 50 ml of 10% CV8 broth medium in the dark at 25 ± 2°C for five days. After that, mycelial mats were harvested by filtration through a double layer of sterile muslin and washed with sterile distilled water twice. All mycelia were grounded in liquid nitrogen using pestle and mortar. Genomic DNA was extracted from 300 mg of grounded mycelia using the modified Hexadecy1trimethylammonium bromide (CTAB) method described by [15].

2.2.2 PCR amplification and sequencing

PCR amplification of the ITS-rDNA region was performed using the primers ITS1 (5′-TCCGTAGGTGAACCTG CGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3) as described by [16]. COX 1 gene was amplified using the primers COXF4N (5′-GTATTTCTTCTTTATTAGGTGC 3′) and COXR4N (5′ CGTGAACTAATGTTACATATAC-3′) as described by [17]. *EF-1α* gene was amplified using the primers ELONGF1 (5′ TCACGATCGACATTGCCCTG 3′) and ELONGR1 (5′ ACGGCTCGAGGATGACCATG-3′) as described by [18]. All primers were purchased from 1st BASE, Malaysia (supplier of IDT, USA oligos). Amplification reactions were prepared in a total volume of 25 µl containing 12.5 µl master mixture (1st BASE, Serdang, Malaysia), 1.5 µl each of forward and reverse primers (10 µM), 2 µl of template DNA, and PCR reaction mix was adjusted to a final volume of 25 µl with nuclease-free water. Thermal cycling consisted of initial denaturation for 4 min at 95°C, followed by 29 cycles of denaturation at 95°C for 40 sec, annealing temperatures were 58.8 °C for ITS-rDNA, 57.3 °C for *EF-1α* and 51.7 °C for COX1, an extension at 72°C for 1 min; and final extension of 72°C for 10 min (Table 1).

Table 1. Biometra Thermocycler for ITS rDNA, *tef-1 α* and cox1 regions

PCR amplification program for ITS rDNA					
Initial denaturation	Denaturation	Annealing	Extension	Final extension	Hold (optional)
94 °C 2 min 1 cycle	94 °C 1 min 40 cycle	58.8 °C 40 sec	72 °C 1 min	72 °C 10 min 1 cycle	∞ at 4 °C
PCR amplification program for *tef-1 α*					
Initial denaturation	Denaturation	Annealing	Extension	Final extension	Hold (optional)
94 °C 2 min 1 cycle	94 °C 1 min 40 cycle	57.3 °C 40 sec	72 °C 1 min	72 °C 10 min 1 cycle	∞ at 4 °C
PCR amplification program for cox1					
Initial denaturation	Denaturation	Annealing	Extension	Final extension	Hold (optional)
94 °C 2 min 1 cycle	94 °C 1 min 40 cycle	51.7 °C 40 sec	72 °C 1 min	72 °C 10 min 1 cycle	∞ at 4 °C

PCR products of the ITS, *EF-1α* and COX1 gene regions were purified and sequenced with the same forward and reverse primers using commercial sequencing service provider (1st Base laboratories Sdn Bhd). Sequencing results of each isolate were manually edited using BioEdit Sequence Alignment Editor Software. The consensus sequence from the assembled contigs for each individual isolate was subjected to NCBI BLAST search (http://www.ncbi.lm.nih.gov/BLAST) in the GenBank database prior to phylogenetic analyses. Clustal W (version 1.8) was used to perform multiple sequence alignment. DNA sequencing data were submitted to GenBank to obtain accession numbers.

2.2.3 Phylogenetic analysis

Phylogenetic analysis was conducted using combined dataset of the ITS-rDNA, COX1 and *EF-1α* gene regions. Maximum likelihood method was applied in Mega 7 with the substitution model of Tamura 3-parameter [19]. Branch support of the trees was assessed through bootstrapping with 1000 replications. All position containing gaps or missing data were treated as missing data. *Phytophthora* species sequences were chosen from the NCBI GenBank database. Nucleotide sequence of *Pythium* sp. (CHE-287) was treated as an outgroup for elongation factor and COX1 loci, while no sequence was available for the ITS DNA region for (CHE-287).

2.3 Morphological characterization

Morphological characteristics including sporangiophore morphology, papillation, hyphal swellings, the breeding system, caducity, sporangium shape, presence of chlamydospores and antheridial attachment have been used to characterize the *Phytophthora* isolates [20].

2.3.1 Colony morphology and growth rates on culture media

Colony morphology of all *Phytophthora* isolates were assessed by culturing 5-mm^3 of mycelial discs on four different types of media including Corn Meal Agar (CMA), Carrot Agar (CA), Vegetable Juice Agar (VJA) and Potato Dextrose Agar (PDA). Plates were incubated for seven days in the dark at room temperature (28 ± 2°C). Colony characteristics were recorded after seven days. Growth rate diameter measurements were taken daily for seven days on different agar media by recording two diameter measurements at right angles on each colony. The experiment was carried out with four replications and repeated twice.

2.3.2 Sporangial and chlamydospore morphology and measurements

All *Phytophthora* isolates were cultured on VJA medium in the dark for 14 days at 25 ± 2°C. The volume of 20 ml of sterile distilled water were added on the culture plates prior to harvesting zoosporangia and chlamydospore using a bent glass rod in order to dislodge them. A drop of suspension was then mounted on a clean glass slide in half-strength lactophenol blue/safranin O dye to facilitate observation of morphology and their measurements under light microscope. A minimum of 25 sporangia, pedicel length, exit pore and chlamydospore for each isolate were measured with the aid of an ocular micrometer.

2.3.3 Sexual compatibility and oospore production

Phytophthora palmivora mating type A1 22G8 (ATCC® MYA-4039™) (*Citrus* sp., Florida, USA) and A2 22G9 (ATCC® MYA-4038™) (*Theobroma cocoa*, Costa Rica) were obtained from the American Type Culture Collection (ATCC). They were paired separately with all *Phytophthora* isolates obtained from cocoa by placing an agar disk of an unknown isolate, 2-3 cm apart from a tester isolate on clarified V8 (CV8) agar. Then, the cultures were incubated at 20°C in dark and observed weekly for three weeks. Different isolates were also paired among themselves. All compatibility testing was made in four replications and after two weeks, plates were examined for oospore production. A minimum of 25 oospore for each isolate were measured with the aid of an ocular micrometer.

2.3.4 Zoospores production

Zoospore's production was induced based on the methods described by [20] by transferring and floating four mycelial discs (5 mm^3) grown on CMA, V8 (CV8) and CA agar cultured for three days on sterile distilled water and incubated at 25 ± 2°C in fluorescent light for three days. The plates were subsequently chilled for 15 min at 5ºC, and then transferred into falcon Eppendorf tube 50 ml and vortexed for one min. Release of zoospores was regularly examined by checking under the light microscope. The experiment was repeated twice.

2.3.5 Pathogenicity test

For pathogenicity, healthy green cocoa pods of the cultivar KKM22, known as a good quality of cocoa bean in Malaysia, were harvested from a cocoa plantation in Perak. All *Phytophthora* isolates with four replicates (pods) were for the test. Control pods (four replicates) were inoculated with distilled water. Each pod was cleaned in running tap water and surface-disinfected for 1 min in 75% ethanol. Inoculation was conducted under a laminar flow chamber where a 12 mm^3 plug of 5 days old culture of *Phytophthora* isolates on CMA was placed over the intact skin at the center of each cocoa pod by making a 1 cm^2-deep wound on the pod skin to put the mycelial disc using a cork borer. The pods were incubated at 25 ± 2ºC in the dark and kept in plastic trays lined by moist paper. The trays containing the inoculated pods were covered with black polythene to maintain the moisture. Measurements of lesions were recorded at three and five days after inoculation. The size of lesions on each cocoa pod was scaled with the aid of a sheet of one-millimeter-scaled tracing graphic paper and to assess the internal growth of lesions into the pods, slices were cut out and the brown lesions measured with a hand-held caliper.

2.3.6 Statistical Analysis of Data

Statistical analysis of data was carried out by the standard analysis of variance (ANOVA) test procedure using the SAS software (9.2).

3. Results

3.1 Isolation

A total of 33 infected pods were randomly collected from the abandoned cocoa plantations holding at Perak and Pahang states in Peninsular Malaysia (Figure 2). Fourteen isolates were obtained from all infected pods and from five cultivated varieties (QH 22, PBC 123, QH 1306, QH 95 and KKM22) of cocoa (Table 2). Eight isolates were obtained from Perak (TI01, TI02, TI03, TI04, TI05, TI06, TI07 and TI08) and 6 isolates from Pahang (RB01, RB02, RB03, RB04, RB05 and RB06). Examination of *Phytophthora* cultures under light microscope showed that sporangia are typical of *Phytophthora palmivora* according to [19].

3.2 Molecular characterization

3.2.1 PCR amplification and sequencing

PCR amplification of the ITS, *EF-1α* and COX1 gene regions of 14 *Phytophthora* isolates from cocoa produced amplicon sizes of approximately 700, 1000, and 1000 bp respectively on 1% agarose gel electrophoresis.

Figure 2. Symptoms of black pod disease in a field in Raub, Pahang.

Table 2. Number of pods sampled from two locations.

Origin	Variety of pod	No. of infected pods sampled
Perak	QH 22, PBC 123, QH 1306, QH 95, KKM22	19
Pahang	QH 22, PBC 123, QH 1306, QH 95, KKM22	14
	Total	33

3.2.2 Sequence analysis

Sequence analysis of the amplicons identified all isolates as *P. palmivora* with nucleotide similarity ranging from 99-100% to reference isolates from NCBI (Table 3). All sequences obtained in this study were submitted to the National Center for Biotechnology Information (NCBI). Accession numbers for isolates from this study are shown in Table 3. Phylogenetic analysis of the combined dataset of the ITS rDNA, *EF-1α*, and COX1 indicated that the 14 isolates obtained in this study clustered into the same clade with the reference *P. palmivora* species with 100% bootstrap value (Figure 3). Therefore, these results confirmed that all isolates belonged to *P. palmivora. P. megakarya* is the nearest species to *P. palmivira* based on the three genes sequenced.

3.3 Morphological characterization

According to morphological characteristics, some overlap was observed among isolates obtained from the two states

whereas isolates obtained from Pahang state grew faster on different agar media, and different sporangia shapes such as globose, limoniform and sporangia with two papilla were only recorded from Pahang. Sporangia size from Perak state were bigger than Pahang state.

3.3.1 Colony morphology and growth rates on culture media

Phytophthora isolates from diseased cocoa pods grown on different agar media demonstrated different appearances (Figure 4). All isolates from both states did not show specific colony pattern on all four types of media. Each medium demonstrated its own morphological appearance. On CMA, colonies were appressed with regular margin; on CA, colonies were dense and fluffy with uniform margin; on VJA, colonies were slightly dense with regular margin; and on PDA, colonies were felty with vague or irregular margin. Pahang isolates grew fast on CMA medium with an average growth rate 13.92 mm/day and covered plates within 6 to 7 days while on VJA and CA media, isolates covered plates within 8 to 9 days with an average growth rate of 11.21 and 10.92 mm/day, respectively (Table 4). Perak isolates grew fast on CMA medium with an average growth rate 12.76 mm/day and covered plates within 6 to7 days, while on VJA and CA media, isolates covered plates within 8 to 9 days with an average growth rate of 9.05 and 9.72 mm/day, respectively. A statistical comparison of the overall mean growth of various media showed significant difference between isolates (Table 4).

Table 3. *Phytophthora* isolates identified by molecular method matched with accession numbers and reference strains with their accession numbers obtained from the GenBank and used in this study

Isolate	Species	Origin	Host	Year	Accession Number		
					ITS rDNA	Tef-1 α	CoxI
RB01	*P. palmivora*	Raub, Pahang	Cocoa	2013	KP796413	MH428719	MH428775
RB02	*P. palmivora*	Raub, Pahang	Cocoa	2013	KP796414	MH428720	MH428776
RB03	*P. palmivora*	Raub, Pahang	Cocoa	2013	KP796415	MH428721	MH428777
RB04	*P. palmivora*	Raub, Pahang	Cocoa	2013	KP796416	MH428722	MH428778
RB05	*P. palmivora*	Raub, Pahang	Cocoa	2013	KP796417	MH428723	MH428779
RB06	*P. palmivora*	Raub, Pahang	Cocoa	2013	KP796418	MH428724	MH428780
TI01	*P. palmivora*	Teluk Intan, Perak	Cocoa	2013	KP796405	MH428729	MH428785
TI02	*P. palmivora*	Teluk Intan, Perak	Cocoa	2013	KP796406	MH428730	MH428786
TI03	*P. palmivora*	Teluk Intan, Perak	Cocoa	2013	KP796407	MH428731	MH428787
TI04	*P. palmivora*	Teluk Intan, Perak	Cocoa	2013	KP796408	MH428732	MH428788
TI05	*P. palmivora*	Teluk Intan, Perak	Cocoa	2013	KP796409	MH428733	MH428789
TI06	*P. palmivora*	Teluk Intan, Perak	Cocoa	2013	KP796410	MH428734	MH428790
TI07	*P. palmivora*	Teluk Intan, Perak	Cocoa	2013	KP796411	MH428735	MH428791
TI08	*P. palmivora*	Teluk Intan, Perak	Cocoa	2013	KP796412	MH428736	MH428792
CBS 274.33	***P. citrophthora***	**Cyprus**	**Citrus Limonium**	**NA**	**NA**	**AY564112**	**AY564171**
IMI342898	***P.***	**UK**	**Syringa**	**1990**	**AF266789**	**AY564128**	**AY564187**
IMI337098	***P.***	**Equatorial Guinea**	**Theobroma cacao**	**NA**	**NA**	**AY564134**	**AY564193**
IMI133317	***P.***	**Australia**	**Malus sylvestris**	**1968**	**AF266794**	**AY564135**	**AY564194**
CBS 292.35	***P.***	**USA**	**Beta vulgaris**	**1935**	**KJ744314**	**AY564116**	**AY564175**
CBS 951.87	***P.***	**Australia**	**Solanum tuberosum**	**NA**	**NA**	**AY564117**	**AY564176**
CBS 587.05	***P.***	**Taiwan**	**Soil**	**1979**	**NA**	**AY564131**	**AY564190**
G922	***P.***	**Costa Rica**	**Soil**	**NA**	**KF317086**	**KX251057**	**KF317108**
CHE-287	***Pythium***	**Switzerland**	**NA**	**NA**	**NA**	**EU199087**	**EU199099**

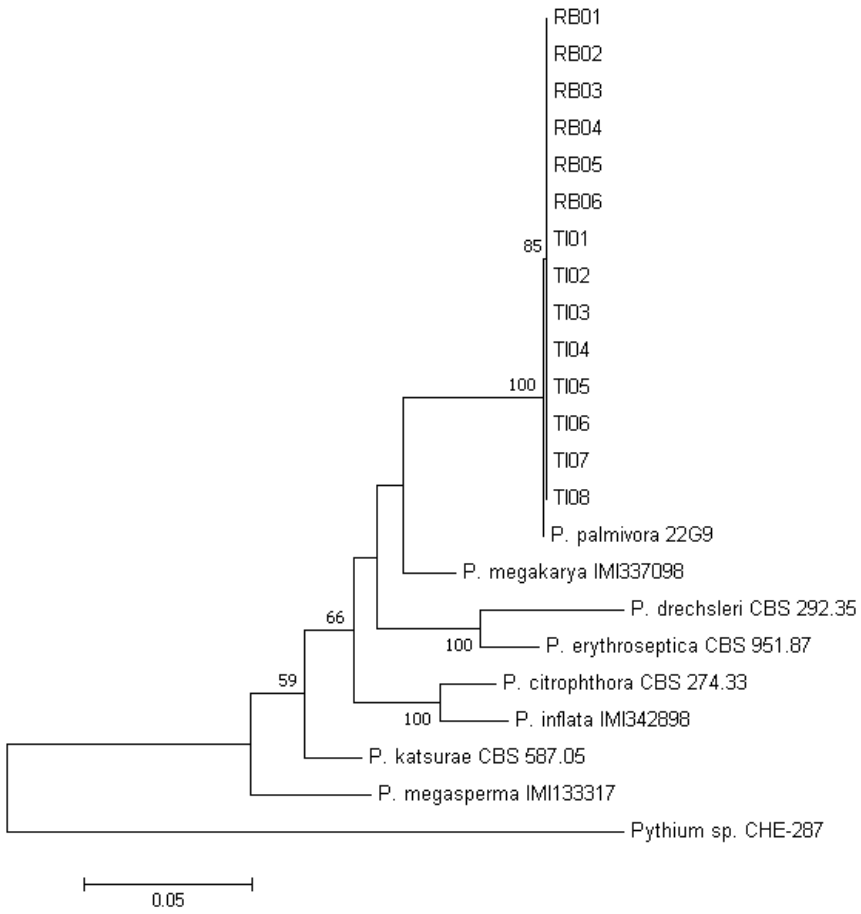

Figure 3. Phylogenetic tree using Maximum likelihood of *Phytophthora* isolates based on the ITS, COX subunit I and EFα1-I combined. The numbers at branch node indicate the confidence values from bootstrap analysis using 1000 replications. Out-group: *Pythium* sp. CHE-287.

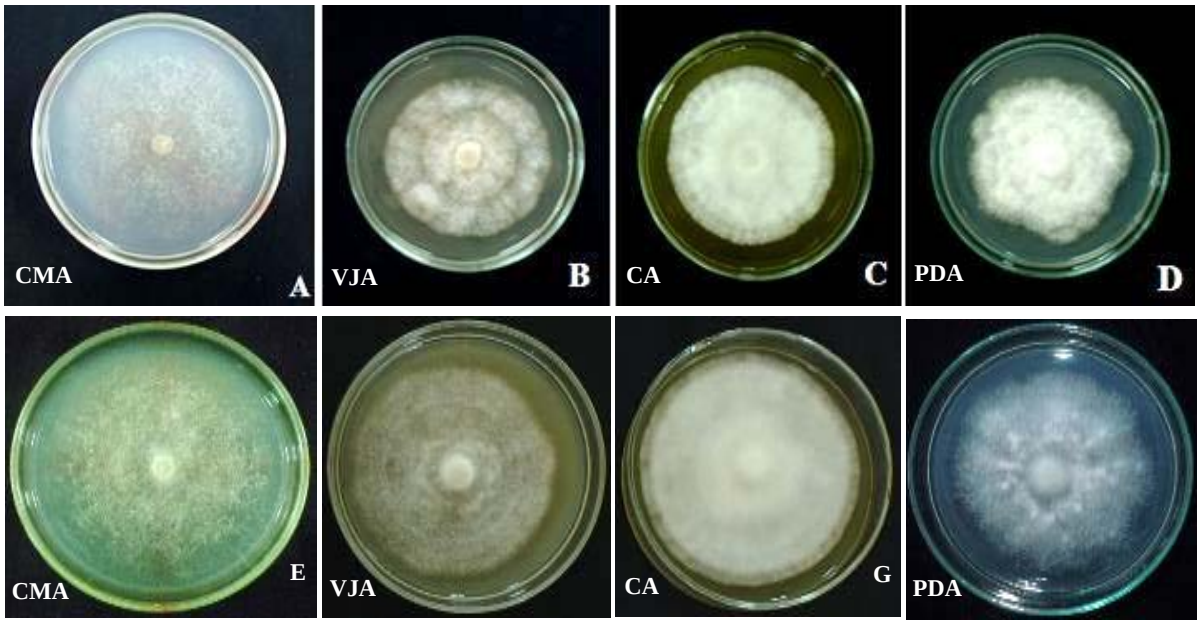

Figure 4. Colony morphology of *P. palmivora* after eight days of incubation on various media. (A-D) TI08 Perak isolates, (E-H) RB03 Pahang isolates.

Table 4. Differences in radial growth rates (mm) of *Phytophthora* isolates on different agar media

Location	Isolates	Media			
		CMA	VJA	CA	PDA
Pahang	RB01	13.85±0.12^{a}	10.10±0.21^{c}	8.812±0.20^{c}	6.1±0.11b^{c}
	RB02	14.04±0.05^{a}	13.87±0.04^{a}	10.14±0.23b^{c}	6.77±0.34^{a}
	RB03	13.29±0.40^{b}	8.91±0.13^{d}	11.69±0.84^{ab}	5.85±0.25^{c}
	RB04	12.48±0.03^{c}	11.6±0.35^{b}	11.16±0.66^{ab}	5.37±0.19^{d}
	RB05	12.41±0.10^{c}	11.29±0.07^{b}	12.45±0.61^{ab}	5.89±0.04^{cd}
	RB06	12.60±0.12^{c}	11.47±0.06^{b}	11.27±0.46^{a}	6.54±0.18^{ab}
	Mean	**13.92±0.059^{a}**	**11.21± 0.67^{b}**	**10.92±0.52^{b}**	**6.08±0.20^{c}**
Perak	TI01	12.67±0.24^{b}	8.47±0.21^{d}	9.27±0.26^{b}	5.22±0.20b^{c}
	TI02	12.33±0.12^{b}	8.37±0.21^{d}	9.43±0.19^{b}	4.72±0.07^{d}
	TI03	12.68±0.26^{b}	8.64±0.32^{cd}	8.93±0.60^{bc}	4.98±0.13^{cd}
	TI04	12.53±0.14^{b}	9.13±0.19^{bc}	9.56±0.18^{b}	5.06±0.07^{bcd}
	TI05	13.83±0^{a}	10.87±0.15^{a}	13.39±0.17^{a}	8.72±0.07^{a}
	TI06	12.8±0.19^{b}	9.12±0.10^{bc}	7.98±0.56^{c}	5.021±0.18^{bcd}
	TI07	12.61±0.19^{b}	8.47±0.23^{d}	9.79±0.21^{b}	5.06±0.06^{bc}
	TI08	12.63±0.15^{b}	9.29±0.20^{b}	9.43±0.19^{b}	5.37±0.08^{b}
	Mean	**12.76±0.16^{a}**	**9.05±0.28^{b}**	**9.72±0.55^{b}**	**6.15±0.71^{c}**

3.3.2 Sporangial, chlamydospore morphology and measurements

Different shapes of sporangia were observed for the isolates with the majority being ovoid or ellipsoid (Figures 5, 6). Most of the isolates produced sporangia with eminent papillae from Perak and Pahang states. The sporangia were often symmetrical, usually with a rounded base but sometimes with a slightly tapering base. Occasionally, spherical sporangia and sporangia with two papillae were observed only from Pahang isolates. All the isolates produced sporangia with short, fairly broad pedicels. Measurements of sporangial dimensions, pedicel length and exit pores size of isolates are shown in Table 5. There was significant difference in measurements between isolates from Perak and Pahang states.

All isolates from Pahang and Perak states produced chlamydospores in abundance after two weeks of incubation on VJA medium. Shapes of chlamydospore were usually spherical and were pale to light brown in color with presence of clear thick walls (Figures 5, 6). Occasionally, chlamydospores showed different branching patterns of the hyphae. There were three positions observed from both states which were lateral, terminal and intercalary. Measurements of chlamydospores of Pahang and Perak isolates showed diameter size 40.29 and 39µm, respectively (Table 5). There was no significant difference in size between isolates from Perak and Pahang states.

3.3.3 Sexual compatibility and oospore production

Oospores were produced only when all *P. palmivora* isolates under the present study were crossed with another compatible *Phytophthora* tester (A1, A2). Cocoa isolates formed abundant oospores with amphigynous antheridia when crossed with the slandered A2 *Phytophthora* culture and not with the A1 tester (Figure 7). Oogonia and antheridia were formed within seven days, but oospore formation took longer. Measurements of chlamydospores of Pahang and Perak isolates showed diameter sizes of 30.39 and 29.93µm, respectively (Table 5). There was no significant difference in size between isolates from the two places.

3.3.4 Zoospore production

Zoospores were induced based on the method by [21] on CMA, VJA and CA. The number of zoospores was variable with the highest number of zoospores observed on CA medium from both states. All isolates from Pahang and Perak states produced zoospores with sufficient amount used for inoculation of 104-105 zoospores/ml on CA and VJA. However, the poorest amount was observed on CMA medium from both states, which was not sufficient for inoculation.

3.3.5 Pathogenicity test

All *Phytophthora* isolates from Pahang and Perak states, were artificially inoculated on green healthy pods of cocoa

(cv. KKM22). After three days of incubation, brownish lesions developed on the inoculated pods and were measured at three and five days after inculcation (DAI) (Figure 8). Table 6 summarizes the results of the mean lesion diameters measured. At 3 DAI, it showed that isolate RB03 from Pahang was the most virulent based on lesion size compared to other isolates tested. However, at 5 DAI, isolate RB04 was shown to be the most virulent isolate as compared with others except for RB03 from the same state. Complete brown to black lesions from both states covered all inoculated pods after 7 DAI.

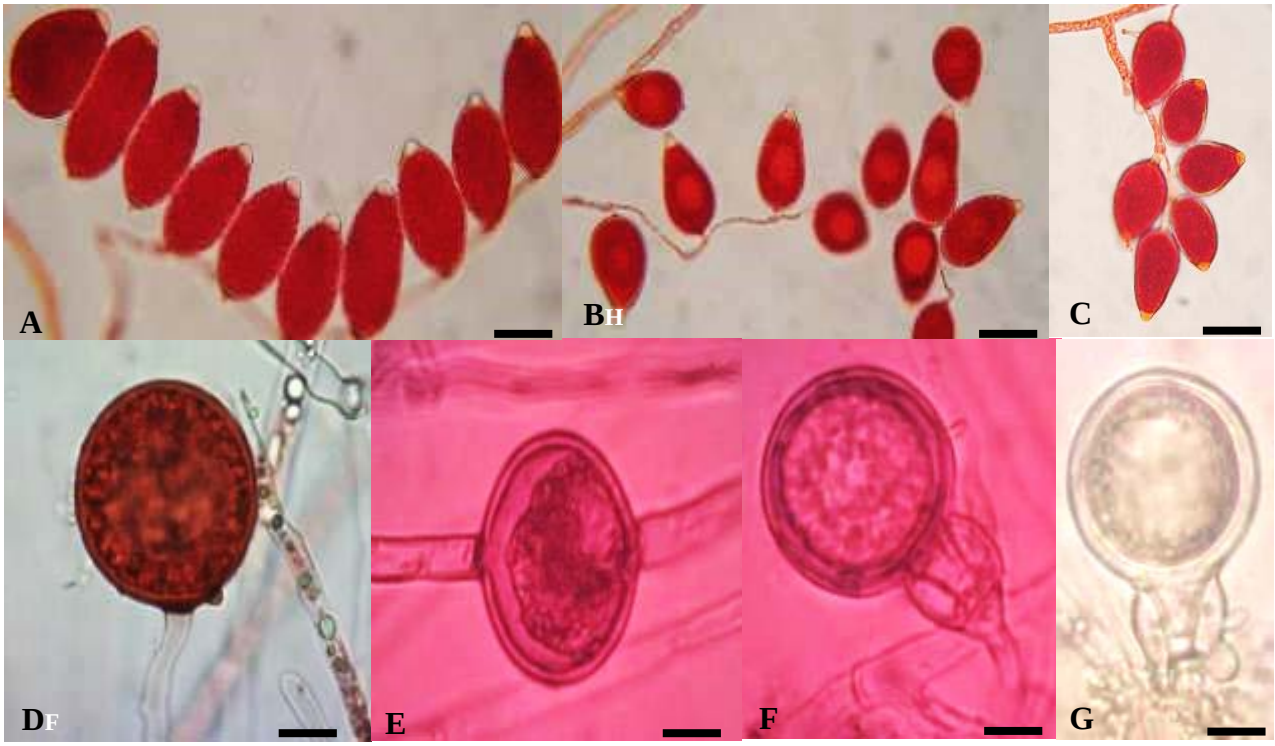

Figure 5. Microscopic features of *Phytophthora palmivora* isolate TI08 from Perak. Sporangia (A, B, C), Chlamydospores (D and E) and Oospores (F and G) on CMA medium. Scale bar =20 µm.

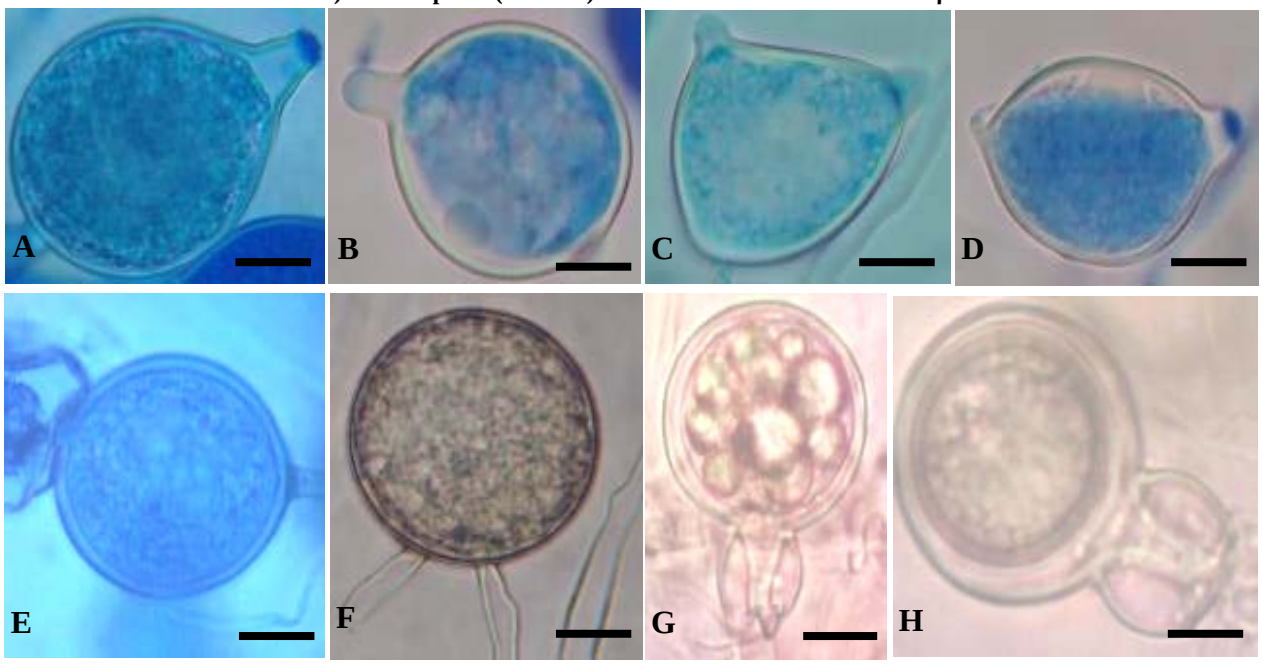

Figure 6. Microscopic features of *Phytophthora palmivora* isolate RB03 from Pahang. Sporangia (A, B, C, D), Chlamydospores (E and F) and Oospores (G and H) on CMA medium. Scale bar =20 µm.

Table 5. Dimension measurements of *Phytophthora* isolates from Pahang and Perak states

Location	Isolates	Sporangia		Pedicel length µm	Exit pore µm	Oospore	Chlamydospore
		Length µm	Width µm				
Pahang	RB01	35.10±1.09	25.58±0.92	4.17±0.23	5.53±0.19	34.05± 0.43	39.05±0.58
	RB02	43.07±1.65	29.25±0.84	3.11±0.20	5.53±0.20	32.65±0.39	38.55±1.03
	RB03	35.46±1.51	26.06±1.02	3.63±0.24	5.33±0.19	30.95±0.70	42.5±1.06
	RB04	44.016±1.43	28.22±0.76	3.25±0.21	5.23±0.17	26.75±0.21	43.25±0.78
	RB05	43.64±0.73	31.66±0.58	3.69±0.28	5.18±0.18	30.15±0.53	39.8±0.93
	RB06	41.85±1.05	30.38±0.69	4.02±0.25	4.40±0.10	27.8±0.39	38.6±0.99
	Mean	**40.52±1.24**	**28.53±0.80**	**3.65±0.24**	**5.20±0.17**	**30.39±0.45**	**40.29±0.90**
Perak	TI01	45.21±1.12	28.2±0.69	3.57±0.23	5.67±0.23	31.2±0.35	40.6±0.70
	TI02	45.59±0.83	31.20±0.65	3.67±0.21	5.48±0.19	30.2±0.47	40.5±0.84
	TI03	48.78±1.48	30.55±0.55	3.23±0.22	5.23±0.18	31.15±0.37	38.25±0.55
	TI04	51.32±1.09	35.34±0.68	3.08±0.19	5.48±0.19	28.15±0.70	40.45±1.29
	TI05	42.39±0.98	29.04±0.72	3.86±0.21	5.87±0.22	29±0.70	37.45±0.69
	TI06	46.62±1.01	29.51±0.71	3.18±0.22	5.14±0.15	29.85±0.57	40.35±0.76
	TI07	45.68±1.41	28.38±0.71	3.33±0.21	5.04±0.14	29.55±0.62	37.45±0.79
	TI08	46.15±1.18	29.61±0.57	3.57±0.21	5.53±0.21	30.3±0.67	36.95±0.61
	Mean	**46.47±1.14**	**30.23±0.66**	**3.44±0.21**	**5.43±0.19**	**29.93±0.57**	**39±0.78**

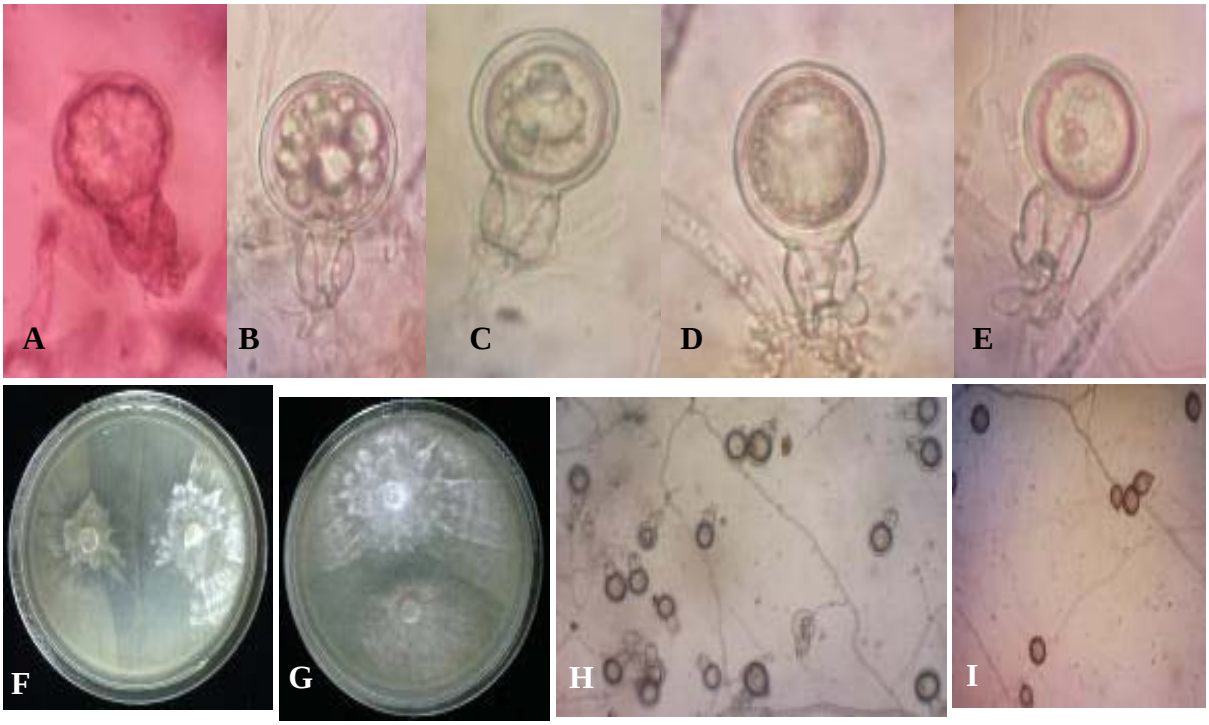

Figure 7. Oospore production using *P. palmivora* A1 and A2 mating type provided by ATCC. (A, B) Immature oospore (Paragynous), (C, D and E) Matured oospore (Amphigynous), (F, D) mating point, (H) Oospore production with mating type 2, (I) Absent of oospore with mating type A1, grow on VJA medium at 14 days at 25 ± 2°C. A calibrated microscope (Olympus, Japan) at magnification of 40x was used to observe the oospore.

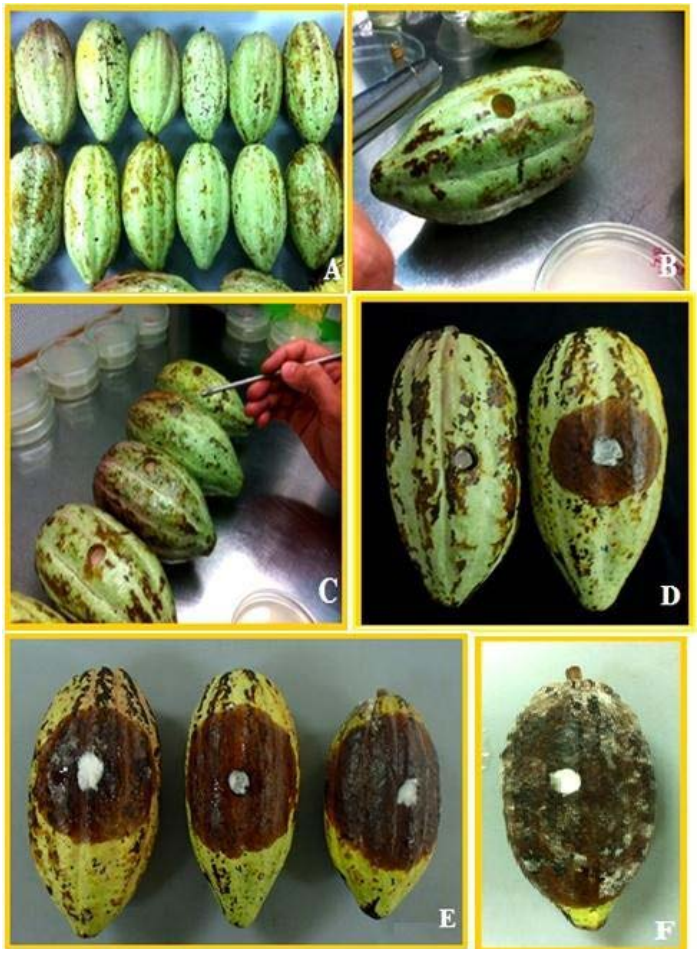

Figure 8. Artificial inoculation of *Phytophthora* isolates on green healthy cocoa pods. (A) Uninoculated (control) pods. (B) 12 mm hole on pod made by a cork borer. (C) Artificial inoculation of pods. (D) Brown lesions at 3 DAI. (E) Brown lesions at 5 DAI. (F) Complete coverage of lesion at 7 DAI.

Table 6. Pathogenicity test results of *Phytophthora* isolates on healthy green pods

Source of isolates	Isolate code	Mean lesion diameter (mm) 3 days after inoculation (DAI)	Mean lesion diameter (mm) 5 days after inoculation (DAI)
Pahang	RB01	26.96^{b}	105^{cb}
	RB02	26.9^{b}	94.25^{c}
	RB03	34.26^{a}	117.75^{ab}
	RB04	28.3^{b}	129.75^{a}
	RB05	28.8^{b}	112.25^{b}
	RB06	26.95^{b}	107.25^{bc}
Perak	TI01	22.55^{cd}	64.75^{d}
	TI02	22.9^{cd}	64.5^{d}
	TI03	23.87^{cd}	67^{d}
	TI04	23.79^{cd}	65^{d}
	TI05	24.42^{c}	64.25^{d}
	TI06	23.55^{cd}	68.25^{d}
	TI07	23.62^{cd}	64^{d}
	TI08	21.95^{cd}	71.25^{d}

* Means in the same column with the same letter in common are not significantly different at P=0.05.

4. Discussion

In Malaysia, *Phytophthora* diseases are common on cocoa, durian, rubber and pepper [5]. Previous work on *Phytophthora* focused on identification and control, mostly on guava, jackfruit, citrus, passionfruit, roselle, potato, and orchids [5]. *Phytophthora palmivora* has been identified as the causal agent of black pod of cocoa in Malaysia in 1961 using morphological characterization only [22] and no other *Phytophthora* species have been reported so far. This study was conducted to investigate whether *P. palmivora* is the only causal agent of black pod of cocoa in Malaysia using morphological and molecular characterization. Therefore, morphological and molecular characteristics as well as pathogenicity of *P. palmivora* isolates obtained from main cocoa producing states of Peninsular Malaysia including, Perak and Pahang, were investigated. Fourteen *Phytophthora* isolates were obtained from the diseased cocoa pods; all isolates proved to be *P. palmivora* based on phylogenetic analysis of combined dataset of the ITS rDNA, *EF-1α*, and Cox1 gene regions. The ITS rDNA, mitochondrial cox1 and elongation factor 1α sequences are powerful and reliable regions for the identification of *Phytophthora* species. Phylogeny of ITS, *EF-1α*, and cox1 region or using another gene certainly provided an excellent opportunity to assess the traditional taxonomy of *Phytophthora* spp. and compare it with *Phytophthora* tree [11]. The sequence information was used to study phylogenetic relationships in the species and below the species level [16]. However, most of these studies have involved non-cocoa *Phytophthora* species, and no study used the three combined regions including ITS, *EF-1α*, and cox1.

The three examined genes have been used successfully to differentiate *P. palmivora*. The selected *P. palmivora* isolates were compared with the Malaysian isolates for the investigation of genetic relationship of *P. palmivora* isolates worldwide. Evolutionary history via maximum likelihood tree analysis showed a significant relationship of *P. palmivora* in Malaysia and worldwide. This assumption was supported by [11, 14]. Previous studies of the multilocus phylogenetic tree of *Phytophthora* spp. using different genes such as ITS rDNA, elongation factor b-tubulin and COXI genes clustered 82 *Phytophthora* species into 10 clades without any differences between the species which allows a better understanding of the evolution of this genus [23, 24]. Cytochrome oxidase subunit 1, NADH dehydrogenase subunit 1, translation elongation factor 1 alpha and beta-tubulin showed clustering to eight clades and presented more accurate phylogenetic analysis result. However, the morphological traits did not correlate well with the correct molecular phylogenetic analysis [14]. We sequenced *EF-1α* and COX genes and because there were a few reliable sequences in GenBank, all *EF-1α* and COX sequences analyzed in this study were deposited in GenBank. Our sequence data would contribute to a taxonomic study of *Phytophthora* based on *EF-1α* and COX genes in the near future studies. Furthermore, the accuracy of identification will be higher more trustable and in case of using conventional orientation to classify different genus of *Phytophthora* especially close species.

Morphological characteristics such as sporangium (shape, size, presence of papilla), oospores, chlamydospores (present or not), hyphal swellings, presence of caducity, colony morphology and growth rates were used to examine variation within *P. palmivora* isolates obtained from Pahang and Perak states in Peninsular Malaysia. These results indicated that the isolates obtained from Pahang were different in sporangia shapes as globose, limoniform and sporangia with two papilla were only recorded from Pahang and the isolates from Perak recorded sporangia size bigger than Pahang. According to chlamydospores and oospores, there were no significant difference in size and shape among the isolates. The average length of sporangia was between 35.10-44.016 µm, and average width between 25.58-31.66µm from Pahang state. The average length of sporangia was between 42.39-51.32µm, and average width between 28.2-35.34µm from Perak state. Sporangia were also similarly caducous with short pedicel less than 5 µm from both states [25]. The pedicel length of sporangium had been accepted to be one of most important characteristics to differentiate *Phytophthora* species due to its stability under various environmental conditions [26, 27]. Previous studies demonstrated that *P. palmivora* form variable colonies such as stellate, striate, chrysanthemum and rosette patterns [28, 29]. [28] reported that *P. palmivora* did not produce specific colony types. Radial growth rates were tested on four types of agar media at 25 ± 2°C in the dark for all isolates. All isolates obtained from Pahang state grew faster on different agar media, it grew fastest on CMA medium followed by CA, VJA and PDA media. Our findings on morphological studies are in agreement with results of [30, 31].

Production of chlamydospore is considered a significant identification parameter for *P. palmivora*. Chlamydospores are spherical in shape and used to differentiate *Phytophthora* species. Hyphal swellings are not considered to be a strong character to differentiate between species but could be useful to identify specific species such as *P. cryptogea* and *P. drechsleri* [32]. Induction of zoospores was attempted on different media, with CMA, VJA and CA demonstrating variable number of zoospores. The highest number of zoospores was observed on CA medium followed by VJA and CMA. [33] showed that the wet plate method was the best for differentiating heterothallic species of *Phytophthora*. Overall, they found that 20% clarified V8 broth produced more zoospores compared with lima bean and carrot media.

Production of oospores is considered another significant parameter for identification *P. palmivora*. The mating types A1 and A2 for *P. palmivora* were obtained from American Type Culture Collection during 2018. Production of oospores with amphigynous antheridia was achieved with ease when cocoa isolates were paired with A2 tester isolate.

No oospores were observed when the isolates were paired with themselves. Crossings with standard A1 and A2 testers confirmed that cocoa isolates under the present work were A1 compatibility type. This heterothallic nature of *P. palmivora* was recorded on cocoa [35, 36, 20].

Accurate identification of *Phytophthora* species based on morphological characteristics is very essential to support the preliminary step of identification. However, molecular identification methods are faster, more specific and more sensitive than morphological identification of *Phytophthora* species [22]. With the development of molecular techniques together with the recent comprehensive multigene phylogenetic analysis of the genus [23], sequence databases are available to simplify identification of unknown species. DNA analysis is used as an alternative to the usual morphological characterization of *Phytophthora* species. The ribosomal DNA genes are highly stable and possess characteristics that are suitable for the identification of pathogens at the species level [34]. The ITS rDNA, mitochondrial COX1 and elongation factor 1α regions have been used in classifying many fungal species. Sequencing in the three regions has been shown to be highly variable in *Phytophthora* spp. [32].

Pathogenicity tests of 14 isolates positively confirmed on detached cocoa pods and demonstrated variability in virulence between isolates. Based on the size of the developed disease lesions, isolates from Pahang were found to be significantly more virulent compared with other isolates from Perak after three and five days of artificial inoculation. Most of the Malaysian isolates caused various degrees of black pod disease on all cocoa pods and were found to have high virulence.

5. Conclusion

This study confirmed that only *P. palmivora* is the causal pathogen of black bod of cocoa in Peninsular Malaysia and no other *Phytophthora* species was found. The combined dataset of the ITS rDNA, *EF-1α*, and Cox1 genes was successful for the identification at species level. However, in the future, more informative genes or various markers are required to examine the isolates of other species. Morphological characteristics of colony morphology, growth rates, sporangia shape and size indicated that there were variations among the isolates from Pahang and Perak. Pathogenicity tests indicated that the isolates obtained from Pahang are more virulent compared to the isolates from Perak. Therefore, based on morphological characteristics and pathogenicity, the *P. palmivora* isolates were divided into two groups based on the geographical regions (states) of the isolates. This variation raises a question concerning exclusive clonality in *P. palmivora* in Malaysia.

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