MOLECULAR CHARACTERIZATION OF *PHYTOPHTHORA PALMIVORA* RESPONSIBLE FOR BUD ROT DISEASE OF OIL PALM IN COLOMBIA

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Abstract

Bud rot disease is a damaging disease of oil palm in Colombia. The pathogen responsible for this disease is a species of oomyctes, *Phytophthora palmivora* which is also the causal pathogen of several tropical crop diseases such as fruit rot and stem canker of cocoa, rubber, durian and jackfruit. No outbreaks of bud rot have been reported in oil palm in Malaysia or other Southeast Asian countries, despite this particular species being present in the region. Analysis of the genomic sequences of several genetic markers; the internal transcribe spacer regions (ITS) of the ribosomal RNA gene cluster, *beta-tubulin* gene, *translation elongation factor 1 alpha* gene (EF-1α), *cytochrome c oxidase subunit I & II* (COXI and COXII) gene cluster along with amplified fragment length polymorphism (AFLP) analyses have been carried out to investigate the genetic diversity and variation of *P. palmivora* isolates from around the world and from different hosts in comparison to Colombian oil palm isolates, as one of the steps in understanding why this species of oomycetes causes devastating damage to oil palm in Latin America but not in other regions. Phylogenetic analyses of these regions showed that the Colombian oil palm isolates were not separated from Malaysian isolates. AFLP analysis and a new marker PPHPAV, targeting an unclassified hypothetical protein, was found to be able to differentiate Malaysian and Colombian isolates and showed a clear clade separations. Despite this, pathogenicity studies did not show any significant differences in the level of aggressiveness of different isolates against oil palm in glasshouse tests.

Keywords: bud rot disease, oil palm, oomycetes, *Phytophthora palmivora*,

INTRODUCTION

Phytophthora palmivora belongs to the genus *Phytophthora*, another member of which was responsible for the potato famine in the middle of 19th century (Cooke and Anderson 2013) and is placed in the phylum of oomycota (Pseudofungi), class of oomycetes and a member of the Pythiaceae family (Hawksworth et al. 1995). Most species in the *Phytophthora* genus are plant pathogens responsible for some of the world's most destructive diseases of crops and native vegetation (Brasier 1992; Ho 2018).

Phytophthora palmivora is one of the important *Phytophthora* species in tropical and sub-tropical regions. This species attacks a wide range of plants and can cause diseases in different parts of the plant. It is responsible for various diseases in many tropical perennial crops such as black pod and stem canker of cocoa (*Theobroma cacao*) (Awuah and Frimpong 2002; Turner 1960) and jackfruit (*Artrocarpus heterophyllus*) (Tri et al. 2015), patch canker, black stripe, green pod rot of rubber (*Hevea brasiliensis*) (Chee 1975; Sdoodee 2004), and trunk canker, root rot, fruit rot of durian (*Durio zibethinus*) (Lim and Chan 1986; Pongpisutta and Sangchote 2004) and canker, fruit and root rot in citrus (*Citrus* spp.) (Ahmed et al. 2013; Tashiro et al. 2012). The pathogen is also responsible for various other diseases of cassava (*Manihot esculenta* Crantz) (Sankar et al. 2013), macadamia (*Macadamia integrifolia*) (Aragaki and Uchida 1980), papaya (*Carica papaya*) (Zentmyer 1988), pineapple (*Ananas comosus*), betel vine (*Piper betle*) (Maiti and Sen 1979; Turner 1969b), olive (*Olea* spp.) (Chliyeh et al. 2013), orchid (Uchida and Aragaki 1991), cherry (*Prunus avium* L.) (Türkölmez et al. 2015) and several palms species including coconut (Blaha et al. 1994; Ordoñez et al. 2016) and ornamental palm *Washingonia robusta* (Elliott 2006; Garofalo and McMillan 1999). The pathogen is responsible for the bud rot and premature nutfall diseases of the coconut (*Cocos nucifera*) in the Philippines (Concibido-Manohar 2004), Indonesia (Smith and Flood 2001) and India (Sharadraj and Mohanan 2014).

Recently, *P. palmivora* has also been found to be responsible for a devastating disease of oil palm in Colombia and other oil palm producing countries in South America also known as bud rot disease (Torres et al. 2016). Bud rot disease has been a serious problem in oil palm plantations in South America for more than 50 years with severe outbreaks in Colombia, Brazil, Ecuador, Panama and Surinam and some cases in Costa Rica, Nicaragua, Honduras, Peru and Venezuela (Martínez et al. 2010). The disease is also known by various

other names such as as '*pudricion del cogollo'* (PC) in Spanish speaking countries including Colombia, and '*speerrot*' or '*lethal spear rot*' in Surinam. In Costa Rica, it is known as '*flecha seca*' (Henry et al. 2015). The description of spear rot disease in Surinam by Van de Lande and Zadoks (1999) matched the description in Martinez (2009) and Martínez et al. (2009). Kastelein et al. (1990) and Beuther et al. (1992) described oil palm disease with similar symptoms such as fatal yellowing or '*Amarelecimento Fatal*' (AF) in Brazil. Nevertheless, Boari (2008) believed that fatal yellowing (AF) in Brazil is different to bud rot disease of Colombia. Boari et al. (2012) have listed several studies conducted on AF to understand the epidemiology of the disease but whether these diseases are actually the same as bud rot is still under debate.

Symptoms of bud rot disease in oil palm are similar to the bud rot disease of coconut with the initial symptom being chlorosis of the young unopened frond or spear leaf (Darus 2000; De Franqueville 2003; Kastelein et al. 1990; Navia et al. 2014). The infected tissue becomes desiccated and dies, leaving necrotic patches. Browning of internal tissues can be observed when the unopened spear is unfolded. If the infection stops, the small desiccated, necrotic patches damage the frond formation creating various external symptoms such as the '*shark bite'* look on the frond, desiccated and necrotic leaflets and loss of some part of the fronds depending on the level of infection during the initial stages. If the infection continues, the whole spear leaf might become infected, necrotic and turn brown, and then the infection continues to the other fronds in the centre of the palm crown. In the advanced stage, the fronds snap, followed by collapse of the upper crown, but the mature leaves (lower crown) remain green for several months, because they are not affected by the pathogen, although the palms cease production. Usually at this stage, the basal tissue rots, as indicated by the presence of dark brown tissue internally. The palm can recover if the infections have not yet gone too deep into the apical meristem and the rotting stops as indicated by production of new leaves. The first new frond is usually smaller, shorter, more erect and slightly more chlorotic than normal fronds and the growth is slower causing the stunted appearance of the new crown. It has been suggested that bud rot disease should be classified into two forms, and the form when the palm can recover is a non-lethal form. The bud rot found in the eastern region of Colombia (Llanos) is believed to be the non-lethal form, whilst in the southwestern region it is the lethal form. The lethal form is aggressive and can cause total destruction and palm death. Turner (1981) suggested that the non-lethal form of bud rot should be called '*bud rot little leaf'*, due to the formation of the malformed fronds during recovery, and the lethal form as '*lethal bud rot'*. In the lethal form, the infection and

rotting does not stop and advances to the heart of the palm (*cogollo*) and eventually affects the leaf primordia and apical meristem. If the apical meristem, which is the growing point of the palm, is destroyed, the palm will not produce leaves and fruits, and eventually die. It is not known why there are lethal and non-lethal forms of bud rot; current hypotheses are that it may be because of pathogenicity factors, physiology of the palm or other biotic and abiotic reasons.

In Malaysia and South East Asia, the current status of bud rot disease incidence in oil palm plantations is unclear. According to Albertazzi-Leandro et al. (2005) as cited by Turner (1981), symptoms similar to '*pudrición del cogollo'* are not new in Asia. Sharples (1928) and Bunting et al. (1934) have described the disease based on observations of collapse of unopened spear leaves which might be the non-lethal form of bud rot disease of oil palm. Until now, no reports of the lethal form and outbreaks of the disease have been reported in Malaysia or other Southeast Asian countries despite the fact that *P. palmivora* is a common pathogen to this region on other plant species. It is not known why this species causes devastating damage to oil palm in Colombia and other Latin American countries. One possibility is that the *P. palmivora* pathogenic to oil palm in Colombia is genetically distinct from *P. palmivora* in Malaysia, and one of the steps for addressing this question is to identify the phylogenetic relationship and genetic variation of the *P. palmivora* species from both regions and also other regions around the world.

The advances in molecular techniques, particularly PCR and DNA sequencing, have fuelled bioinformatics studies of DNA data of organisms. DNA nucleotide sequence analysis has contributed to the understanding of the phylogenetic and molecular diversity of organisms including in the *Phytophthora* genus (Scibetta et al. 2012). Sequencing of specific target regions (single and multiple) has been widely used to study the diversity of *Phytophthora* (Hu et al. 2013; Rahman et al. 2015), *Pythium* (Arcate et al. 2006) and other microbes such as fungi (Korabecna 2007), phytoplasmas (Jović et al. 2011) and plants (Ritland et al. 1993). Molecular analysis of DNA sequences by Crawford et al. (1996), Cooke and Duncan (1997), Cooke et al. (2000) and Förster et al. (2000) have increased the understanding of the phylogenetic relationships between *Phytophthora* species. Their work has been based mainly on the nucleotide sequence data of a single DNA region, the rDNA internal transcribed spacer (ITS). Earlier work on analysis of sequences to investigate genetic diversity, phylogenetics and genetic variation of *Phytophthora* and fungi were also based on this rDNA and ITS region (Bruns et al. 1992); however, other regions and genes of nuclear or mitochondrial DNA have more

recently been explored extensively, such as *beta-tubulin* (*β-tubulin), translation elongation factor 1 alpha* (*EF-1α*), *NADH dehydrogenase subunit I, cytochrome c oxidase subunit I* (*CoxI*) and *subunit II* (*CoxII*) either being analyzed individually or as multi-locus/multi-gene combinations (Blair et al. 2008; Kroon et al. 2004; Martin and Tooley 2003b; Villa et al. 2006). Phylogenetic analysis based on multiple genes has also been reported for many fungal species such as *Fusarium* (Nalim et al. 2009) and *Corynespora* (Shimomoto et al. 2011).

Apart from the analysis of DNA sequences using selected regions as molecular markers, DNA fingerprinting methods such as amplified fragment length polymorphism (AFLP) have also been widely used to study genetic variation, phylogenetic relationships, population evolution, and diversity without knowing the DNA sequences of the studied organism including oomycetes (Abu-El Samen et al. 2003; Ivors et al. 2004). AFLP is a PCR-based fingerprinting technique that is similar to the random amplified polymorphic DNA (RAPD) but offers higher stringency while retaining time efficiency (Mueller et al. 1996) and has proven useful for investigating genetic variation among individuals (Mueller and Wolfenbarger 1999).

In this study, we have adopted DNA sequence analysis and the AFLP fingerprinting techniques to molecularly characterize *P. palmivora* isolates from Colombia and Malaysia, particularly focusing on the study of the genetic variations between these isolates.

MATERIALS AND METHODS

Isolation of Phytophthora from oil palm, cocoa and durian

Phytophthora palmivora isolate PPC280574 was isolated from infected young unopened spear leaves of oil palm in Colombia. The isolation was carried out using a baiting technique using pear as described by Torres et al. (2010). Isolation of *P. palmivora* from cocoa and durian in Malaysia was carried out using direct plating of the diseased tissue onto P_{10}VP agar (CMA; 16 g, distilled water; 1000 ml, pentachloronitrobenzene (PCNB); 100 µg ml^{-1}, pimaricin; 10 µg ml^{-1}, vancomycin; 200 µg ml^{-1} (Tsao and Ocana 1969). The plates were incubated at 25°C +/- 2°C in the dark and examined daily under microscope for initial identification of *Phytophthora* based on morphological characteristics described by Waterhouse (1963) and Gallegly and Hong (2008). The outgrown culture was transferred onto fresh P_{10}VP agar before sub-culturing onto carrot agar as

described by Drenth and Sendall (2001)(CA; 15g agar, fresh carrot; 200 g, distilled water; 1000 ml). All cultures were maintained in the UK, where this work was undertaken due to restrictions on importing new isolates to Malaysia for biosafety reasons, on carrot agar with or without antibiotic supplements at temperatures of 25°C +/- 2°C.

DNA isolation and PCR amplification

Between 100-120 mg of *Phytophthora* mycelium was scraped from the surface of 7 to 10 day old colonies grown on carrot agar, placed into a sterile 2 ml screw-capped tube and homogenized using glass beads and a miller. DNA extractions of the cultures were then carried out using DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. Primers to amplify the regions of the internal transcribed spacer (ITS) regions, *translation elongation factor 1 alpha* gene (*EF-1α*), *beta-tubulin* gene (*β-tubulin*), *cytochrome oxidase II* (*CoxII*), *cytochrome oxidase I* (*CoxI*) genes of the mitochondrial DNA and PpHPAV marker from this study are detailed in Table 1. All oligonucleotides were synthesized by Sigma-Aldrich, UK. PCR amplification was performed in 25 µl volumes consisting of 12.5 µl of master mix (MangoTaq™ DNA Polymerase), 1 µl each of forward and reverse primers (10 pmol/µl), 9.5 µl sterile distilled water and 1 µl of template DNA. Amplification was set at 95°C for 2 min for initial denaturation, followed by 30 cycles of denaturation at 95°C for 1 min. Annealing was set for 1 min at 41°C for *CoxI*, 55°C (ITS, *CoxII*), and 64°C for *β-tubulin* and *EF-1α*), followed by the extension/elongation at 72°C for 1 min 30 sec and final extension at 72°C for 10 min. The amplicons were run in 1.2% agarose gels stained with ethidium bromide in Tris-borate-EDTA (TBE) buffer at 100 volts for 25-40 min alongside with 1kb DNA marker ladder. The presence of single clear bands was checked for successful amplification using a gel imager. The amplified products were then purified with the QIAquick® PCR Purification Kit (QIAGEN), following manufacturer's instructions.

Table 1 Primers used for PCR amplification

Marker	Oligo-nucleotide	Sequence 5' to 3'	Reference
ITS	ITS1	TCC GTA GGTGAA CCTGCG G	White et al. (1990)
regions	ITS4	TCCTCCGCTTAT TGATATGC	Crawford et al. (1996)

EF-1α	EF1AF	TCACGATCGACATTGCCCTG	Kroon et al. (2004)
	EF1AR	ACGGCTCGAGGATGACCATG	
CoxII	FM82	TTGGCAATTAGGTTTTCAAGATCC	Martin and Tooley (2003b)
	FM78	ACAAATTTCACTACATTGTCC	
CoxI	OomCoxILevup	TCAWCWMGATGGCTTTTTTCAAC	Ginetti et al. (2014)
	Fm85mod	RRHWACKTGACTDATRATACCAAA	Robideau et al. (2011)
β-tubulin	BT5	GTATCATGTGCACGTACTCGG	Villa et al. (2006).
	BT6	CAAGAAAGCCTTACGACGGA	
PpHPAV	AV1F	AATGACGGCTTCTGCGTTTG	This study
	AV1R	GGCGTGACTACAGAGTGTCC	

Cloning and sequencing

Cloning of PCR amplicons was conducted using the cloning kit, pGem®-T Easy Vector System I (Promega) using competent cells of *Escherichia coli* cells strain DH5α and transformation was conducted using heat shock treatment. PCR amplification of targeted using primer M13 forward (5'-GTAAAACGACGGCCAGT-3') and M13 reverse (5'-CAGGAAACAGCTATGAC-3'). Purified amplicons were sent for sequencing at Eurofin MWG Operon, UK. Sequencing data was checked, cleaned and edited using GAP4 software package (Staden-package, USA) by removing the vector sequences, correcting the base errors and generating contig sequences from forward and reverse sequences of each individual clone. For identification of the isolates, ITS sequences were subjected to nucleotide-nucleotide searches with the Basic Local Alignment Search Tool - BLASTn algorithm at the NCBI website (*http://www.ncbi.nlm.nih.gov/BLAST/*). The outputs from the BLAST searches were sorted based on the maximum identity. Identification of each isolate was based on the maximum scoring of identity value and query coverage.

Sequence analysis and phylogenetic analyses

Sequence alignments were performed using ClustalW (Thompson et al. 1994) using default settings. The phylogenetic trees were constructed using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993); both were conducted using *MEGA 6.06* (Tamura et al. 2013) using the data from the sequences obtained in this study, combined with additional sequences obtained from GenBank®, indicated by the presence of accession numbers in the brackets. The evolutionary distances were compared nucleotide-by-nucleotide using the nucleotide substitution model of Maximum Composite Likelihood with rate uniformity and homogeneity pattern as implemented in *MEGA version 6.06* with bootstrap tests of 1000 replicates to estimate error (Felsenstein 1985; Tamura et al. 2004). All alignment gaps and missing data were deleted before the calculation using the complete-deletion option. Phylogenetic trees were constructed using individual datasets of each marker. Nucleotide sequences of all markers were concatenated using *SequenceMatrix* (Vaidya et al. 2011) but the dataset was limited with nucleotide data only available for all five markers of the ITS regions, *EF-1α, β-tubulin, CoxI and CoxII* genes. All external gaps were manually inspected and deleted before alignment. Alignment and phylogenetic analyses of concatenated datasets were carried out with the same method as individual datasets.

Amplified fragment length polymorphism (AFLP)

Digestion of 400-500 ng genomic DNA was done with 10 U *Eco*RI and 5 U *Mse*I in 2x *Eco*RI restriction enzyme buffer Tango™ in a total volume of 25 µl at 37°C for 3 hours. Enzymes were deactivated at 65°C for 5 min. *Eco*RI adapters (5'-CTCGTAGACTGCGTACC-3' and 5'-AATTGGTACGCAGTCTAC-3') and *Mse*I adapters (5'-GACGATGAGTCCTGAG-3' and 5'-TACTCAGGACTCAT-3') were prepared by mixing 20 µl each of forward and reverse adapters (100 pmol/µl) with 160 µl distilled sterile water and then incubated at 65°C for 10 min and allowed to cool slowly to room temperature. Ligation was carried out by adding 1 µl 10 pmol/µl *Eco*RI adapter, 1 µl 10 pmol/µl *Mse*I adapter, 1 µl 1U/µl T4 DNA ligase enzyme, 8 µl 10x T4 DNA ligase buffer and 11 µl of sterile distilled water to the ligation mixture tube and incubated at 4°C overnight. The digestion-ligation solution was diluted with TBE buffer at 1:10 ratio and kept at -20°C. Pre-amplification was carried out using 5 µl of diluted ligation mixture, 1 µl 10 pmol/µl *Eco*RI-universal primer (5'-CGTAGACTGCGTACCAATTC-3') and 1 µl 10 pmol/µl *Mse*I-universal primer (5'-

GACGATGAGTCCTGAGTAA-3'), 18 µl sterile distilled water and Illustra™ puReTaq Ready-To-Go™ PCR Beads (GE Healthcare, UK). Amplification conditions were 94°C for 1 min followed by 10 cycles at 94°C for 40 sec, 65°C for 1 min and 72°C for 1 min and then 25 additional cycles at 94°C for 40 sec, 56°C for 1 min and 72°C for 1 min. Selective amplification was done using a mixture of 5 µl of diluted pre-amplification product (1:20 dilution), 18 µl of sterile distilled water and Illustra™ puReTaq Ready-To-Go™ PCR Beads (GE Healthcare, UK) together with combination of 1 µl 10 pmol/µl EcoRI selective primer with different nucleotide tails (n) (5'-CGTAGACTGCGTACCAATTC-n-3') labelled with WellRED® fluorescence, D3 or D4 dye and 1 µl 10 pmol/µl MseI selective primers with different nucleotide tails (n) (5'-GACGATGAGTCCTGAGTAA-n-3') and each assay were carried out in two replicates. Based on previous primers selection, three informative primers were used for analysis: *Eco*RI - A / *Mse*I-AG, *Eco*RI - AC / *Mse*I-AG and *Eco*RI - TA / *Mse*I-AG. Amplification products were separated using 2% w/v agarose gels run at 120 volts for 60 min and sent for automated capillary electrophoresis using the CEQ™ 8000 System. Data from the CEQ Genetic Analysis System was exported to *MS Excel* and manually examined, cleaned up and edited before being transformed to binary coding. The absence of a peak/band is denoted with '0' and the presence of a band is denoted with '1'. Monomorphic fragment peaks were not scored. The phylogenetic analysis of the AFLP data was done using *FreeTree* software using UPGMA (Hampl et al. 2001; Pavlicek et al. 1999). The distance matrix was calculated using Nei and Li distance (Nei and Li 1979). Resampling was done by bootstrapping with 1000 replicates. The phylogenetic tree derived from *FreeTree* was viewed using *Treeview* and *MEGA 6.0*.6.

Pathogenicity evaluations

Zoospore production: Zoospore inoculum was prepared as described by Dick et al. (2014) and Chee (1975) with modification. The isolates were grown on carrot agar at 25°C +/- 2°C for 7 days. Old stock cultures were reactivated by using the fruit bait technique prior to culturing. Four to five mycelial plugs cut from the actively growing region of agar culture plates were immersed in sterilized carrot juice in a 90 mm Petri dish and incubated at room temperature with illumination for 7-10 days. The zoospore release was induced by incubating the culture in the dark at 4°C for approximately 20 to 45 min followed by exposure to room temperature (25°C to 28°C). The zoospore suspension was then collected in a sterile beaker. The concentration

of zoospores was determined microscopically using a *Neubauer* haemocytometer. Zoospore suspensions were used within two hours of preparation. Detached-leaf assay: The assay was conducted using mature leaves, green unopened spear leaves and white unopened spear leaves taken from 12 month old oil palms grown in the glasshouse. The leaves were cut into small piece about 14 cm in length. The white unopened spears were divided into two parts, the upper older (greenish) part and lower younger (whitish) part. The leaf/spear pieces were washed with tap water and surface sterilized using 2% v/v sodium hypochlorite (NaOCl) by dipping the whole leaf into the solution for 60 seconds followed by rinsing with sterile distilled water twice and then left to completely dry on clean tissue towels. Each end of the piece was cut approximately 0.5 cm from the margin. The spear leaves were pricked/wounded twice using a sterile sharp pointed blade (no. 11) approximately 4 cm from the end on both sides. The clean 5 mm x 5 mm cotton plugs were put on top of the wounded sites. One hundred microliters (µl) of zoospore suspension (10,000 zoospore/ml) was dropped onto the cotton pad. Distilled water was used in control assays. The inoculation was also done using mycelial plugs. The chambers were covered and incubated at room temperature with illumination for 7 days. Presence of lesions was observed and diseased leaf tissue samples were cut into small pieces, soaked in 3% KOH for five minutes and observed microscopically using a compound microscope.

Nursery Inoculation: Oil palm germinated seeds (*Dura* x *Pisifera*) were sown into trays filled with a mixture of soil (Levington F2 Seed & Modular Compost) and perlite at the ratio of 8:1 in a glasshouse at 28°C (day) and 22°C (night) with a photoperiod of 14-16h. After 3 months, the seedlings were transferred into larger pots (5 litre) filled with a soil mixture of sand based soil (John Innes No.3), perlite and vermiculite with the ratio of 8:1:1. The seedlings were watered every day during summer and on alternate days during winter and fed with liquid fertilizer (10% solution) containing N, P, K in the ratio of 4:2:2 and trace elements. The humidity in the glasshouse was maintained by wetting the floor of the glasshouse every morning. Inoculation was carried out based on the methods by Sarria et al. (2016) with modification where three point wounding was introduced prior to inoculation at the base of the seedlings using a sterile hyperdermic needle (21G × 1 1⁄2″ /0.8 × 40 mm). Volume of inoculum was at 2 ml zoospore suspension (10^3-10^4 spore/ml). The whole plant was covered with a clean plastic bag for 30 days to retain humidity. Inoculations were carried out in the same glasshouse where the seedlings were grown and the conditions were maintained throughout the experiment and were done at

least in triplicate. The inoculated seedlings were observed for any development of lesion or any physical symptoms of bud rot disease as described by Sarria et al. (2016). Diseased samples were collected and re-isolated by direct plating onto selective media and confirmed by microscopic evaluation.

RESULTS

Oomycete Isolates

Thirty one isolates of *P. palmivora* from different hosts and geographical origins worldwide, eleven isolates of other *Phytophthora* species and a *Pythium* spp. were analysed in this study. Details of isolates collected and used in this study are as presented in Table 2. Isolates P19537 and P19538 identified as *P. palmivora* by WOGRC were re-identified as *P. parasitica* and isolate CBS358.59 from the CBS-KNAW, were re-identified as *P. colocasiae* in this study.

DNA amplifications

PCR amplification of ITS regions of *Phytophthora* and *Pythium* produced approximately 900 bp PCR fragment. Fragments amplified for *CoxII* and *CoxI* genes using primer pairs FM82/FM78 and OomCoxILevup/Fm85mod were 600 bp and 800 bp respectively. Primer pair BT5/BT6 amplified the beta tubulin region of 750 bp. For primer EF1AF/EF1AR, only the *Phytophthora* elongation factor was amplified at 1000 bp. Primers AV1F and AV1R only specifically amplified the PpHPAV '*region*' of *P. palmivora* with the PCR amplicon of 1000 bp.

Sequence and phylogenetic analyses of multigenes

Low intraspecific variation was observed in the ITS1 and ITS2 sequence data for all 24 isolates of *P. palmivora*. Isolates of *P. palmivora* originating from oil palm in Colombia (PPC280574) showed a high similarity (97% to 100% identity, based on BLAST report) with other isolates obtained from various hosts and regions, including all six Malaysian isolates. Further assessments using phylogenetic analysis showed similar results. The evolutionary history inferred using the Maximum Likelihood method based on the Tamura-Nei model grouped all *P. palmivora* isolates into one clade (Clade 1) with a strong bootstrap value regardless of the host and demographic origin of the isolates and other species in Clade 2 (Figure 1). The tree with the highest log

likelihood likelihood is shown. The tree was constructed using 37 nucleotide sequences and involved 801 nucleotides in the final dataset. Heuristic searches of the initial tree(s) were automatically calculated by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Sub-branching of clade 1 was observed (bootstrap value 84%), which consisted of two isolates originating from Malaysian durian (PPM4 and PPM5) and isolates from betel palm (Guam) (P11007), cocoa (Ghana) (PPG8) and bamboo palm, USA (*P. arecea=P. palmivora*) (CBS148.88). There was no consistent pattern for the origin/host of these isolates except that two isolates were from Malaysia. Interestingly, one isolate of *P. palmivora* (PPM3) isolated from cocoa in Malaysia was separated from the large *P. palmivora* clade with a high bootstrap value.

Initial tree(s) for the heuristic search using partial nucleotide sequences of *EF-1α*, *β-tubulin*, *CoxI* and *CoxII* were obtained by applying the Neighbour-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach (Saitou and Nei 1987; Tamura et al. 2004). The analyses involved 43 sequences with a total of 870 positions in the final dataset for *EF-1α* (Figure 2) and 41 sequences with a total of 648 positions in the final dataset for *β-tubulin* (Figure 3). For both *CoxI* and *CoxII*, the analysis involved a total of 38 sequences which incorporated 773 and 621 nucleotides, respectively (Figures 4 & 5). All the *P. palmivora* isolates (including *P. arecae*) were clustered in one clade (Clade 1) with bootstrap values of more than 97% for all trees. Sub-branching of Clade 1 was observed for condensed trees with 50% bootstrap value cut off for all datasets except *β-tubulin*. In the *EF-1α* tree, isolates PPM4 and PPM5 were grouped together in a sub-clade 2, branched out from Clade 1, similar to the ITS tree. The other Malaysian isolates were distributed randomly in Clade 1. Some Colombian isolates were grouped in sub-clade 2 and sub-clade 3 with other isolates from Ghana and Sri Lanka also randomly distributed in Clade 1. Sub-clades were also observed with the *β-tubulin* tree but with low bootstrap values (<50%) (Figure 3). In the *CoxI* tree, two isolates from Malaysia, PPM1 and PPM2, were grouped in a sub-clade (Figure 4). There were other sub-clades but with lower than 50% bootstrap values. The *CoxII* tree also grouped all 27 isolates of *P. palmivora* in one clade, but the clade was sub-branched into another sub-clade of 26 isolates with one isolate (P11007) separated (Figure 5). All trees were drawn to scale, with branch lengths measured in the number of substitutions per site.

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches when ≥ 50. The labeled sub-clade is with ≥50% bootstrap value.

Interspecific variation among other species of *Phytophthora* was clearly observed using all nucleotide datasets. *Phytophthora palmivora* is clearly distinguished from other species included in this study. Some species with more than one isolate were grouped together into the same clade such as for *P. megakarya*. Isolate CBS358.59, originally identified as *P. colocasiae* in this study, was always grouped with isolate CBS581.69 from Malaysia in all trees. Both original hosts of these isolates are rubber.

Concatenated tree of ITS and other housekeeping genes

The concatenated tree was constructed from sequences of five different markers (Figure 6). The reconstruction of the tree was done using the same methods as previous trees. The tree involved 35 sequences from this study and from GenBank® marked with an asterisk (*). There was a total of 3773 nucleotide positions in the final concatenated dataset. As in other trees, all *P. palmivora* isolates were grouped in one clade, Clade 1. There are three sub-clades branching out from Clade 1 with more than 50% bootstrap value. Observation of the members of each sub-clade show no relationship in terms of host and demographic origin of the isolates involved.

Phylogenetic analysis of the PpHPAV marker

The initial tree(s) for the heuristic search from datasets of PpHPAV sequences were obtained by using the Neighbour-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The analysis involved 31 sequences with a total of 958 positions in the final dataset (Figure 7). The final tree consists of several major clades. *Phytophthora palmivora* Colombian isolates and Malaysian isolates were clearly separated in different clades. All the Colombian isolates were strongly grouped (bootstrap value of 98%) in Clade 1 together with isolates from the USA, Ghana, Trinidad & Tobago, Guam, Sri Lanka and India including *P. arecae* from the USA which later separated as an outgroup from the rest of the Clade 1 members which were further grouped in sub-clade 1. Isolates from Malaysia and Indonesia from different hosts of cocoa, durian, coconut and rubber clustered in several clades. Clade 2 consists of three Malaysian isolates obtained from cocoa (PPM1, PPM2 and PPM3). Clade 3 consist of a mixture of Malaysian and Indonesian isolates form coconut, cocoa and durian. Clade 4 also contains a mixture of Malaysian and

Indonesian isolates from various hosts. One isolate from South Korea (CBS111146) was also included in this clade.

Phylogenetic analyses of AFLP data

Phylogenetic tree (s) constructed from individual AFLP datasets of marker *Eco*RI-A/*Mse*I-AG, *Eco*RI-AC/*Mse*I-AG and *Eco*RI-TA/*Mse*I-AG which each involved 75, 121 and 149 random markers of polymorphic bands were able to separate Colombian and Malaysian isolates into two distinct clades (Figure 8) which was further shown in the concatenated tree of the three datasets involved 345 random markers (Figure 9) where all three Colombian isolates were grouped together in Clade 1, whilst the two Malaysian isolates were clustered in Clade 2. Other species were clearly distinguished as outgroups.

Pathogenicity Tests

Detached leaf assay: No lesions were observed in the initial trials using green mature leaves and green unopened spear leaves of 12 month old oil palms inoculated with zoospore suspensions (approximately 10^4 zoospores ml^{-1}) and mycelial plugs of oil palm pathogenic isolate PPC280574, both with and without wounding, by the 5^{th} day after inoculation. Brown lesions were observed at 4 days after inoculation using white unopened spears on the lower part (whitish) nearer to the crown/growing point but not the with the upper greenish part, but only with wounded leaves (Figure 10). The presence of *P. palmivora* in the diseased tissue was confirmed by microscopic evaluation of the diseased tissue and re-isolation using selective media. *Phytophthora palmivora* was not observed in control assays and there was no mycelial growth on the selective media.

Glasshouse inoculation: No lesions were observed on any of the inoculated seedlings conducted in the winter. Brown lesions was firstly observed on some of the inoculated seedlings at the end of May on the 6^{th}-7^{th} day after inoculation using isolates P16385, CBS111346 and PPG1 (Figure 11). Subsequent inoculations with 12 isolates showed that all isolates can cause brown lesions at the inoculation sites but not all inoculated seedlings formed lesions (Table 3). Similar findings were shown with the seedlings in another trial repeated with only four isolates (Table 4). Lesions appeared to be localized on the wounded site and no further infection was

observed after two weeks of inoculation at the infection site. The size of the lesions was mostly small (approximately 3 mm-15 mm) and did not expand or grow. Nevertheless, there were three seedlings (inoculated with PPM4, PPM1 and CBS111346) that had larger infection areas, where half of the young spear leaf become brown and infected. On all infected leaves, the diseased tissue become necrotic and dried out. After some time, the necrotic tissue fell out leaving a hole in the leaf, but the rest of the leaf (the healthy tissue) kept on growing, including the new healthy shoot. No recurrent infections were observed on any inoculated seedlings.

DISCUSSION

Studies on diversity, phylogenetics and polymorphisms among oomycetes, particularly *Phytophthora*, have been carried out using various molecular tools including analysis of DNA sequences of target regions or genes. The ITS, which is the non-coding spacer region between the 28S and 18S rDNA, has been shown to be particularly useful such as in the work of Lee and Taylor (1992) and Cooke and Duncan (1997) where high resolution of interspecific levels were achieved. However, intraspecific variations using this region are rather limited and rarely encountered (Sorensen et al. 1998), although not impossible. For example, Cohen et al. (2003) demonstrated some intraspecies variations and phylogenetic separation of *P. citrophthora*, whilst Vinuesa et al. (2001) showed up to 16% variation for *Mycocalicium substantial* but only 1% for *M. albonigrum*. In this current study, variation at the intraspecific level within 26 isolates of *P. palmivora* from various hosts and demographic origins was not clearly observed in the DNA sequences of the ITS regions. The small percentage of DNA nucleotide variations (0-3%) between some isolates might be due to errors during PCR and sequencing even though effort was taken to minimise such errors. Analyses with 32 additional ITS sequences of *P. palmivora* obtained from GenBank® also showed similar findings. Since the evolution of one gene may not represent the entire genome (Villa et al. 2006) phylogenetic analyses using other genes; *CoxI*, *CoxII*, *β-tubulin* and *EF-1α* genes were included in this study.

Cox genes of subunit I and II code for enzymes that catalyse the terminal step in the electron transport chain and are encoded in the mitochondria, which is considered generally to be more variable than nuclear DNA and has proven to be good for studying the relationship at the sub-generic level for various taxa (Villa et al. 2006). Phylogenetic relationships of the *Phytophthora* genus based on the *CoxI* and *CoxII* genes has been established by Martin and Tooley (2003a), whilst Villa et al. (2006) used *β-tubulin* data along with *ITS* and

403 *CoxI*. Blair et al. (2008) used seven multi-locus markers (28S rDNA*, 60S ribosomal protein L10, β-tubulin,*
404 *EF-α1, Enolase, heat shock protein 90* and *TigA gene fusion protein*) and found that *β-tubulin* provided the
405 highest level of phylogenetic variation across the *Phytophthora* genus. However, in this study, all the individual
406 phylogenetic trees reconstructed using sequence data for *CoxI*, *CoxII*, *β-tubulin* and *EF-1α,* demonstrated
407 similar findings to the ITS, with low intraspecific variations in DNA sequences. The trees did not exhibit
408 consistent similarities in grouping based on demographic and host origin. A multiple loci sequences data
409 constructed from the combination of all five datasets to enhance the phylogenetic inference as suggested by
410 (Bininda-Emonds et al. 2001; Sanderson et al. 2003) and has been demonstrated in many studies such as
411 Bapteste et al. (2002), Kroon et al. (2004), Martin and Tooley (2003a) and Blair et al. (2008). However, the
412 multi-locus tree constructed from the five loci in this study showed no clear separation of *P. palmivora* isolates
413 from Malaysia, Colombia and other isolates from different demographic origin and hosts. These five molecular
414 markers shown to be suitable for inter-specific studies between species but not intra-specific evaluation within
415 species of *P. palmivora.*

416 This study was expanded by looking at genome level variation using AFLP which has the ability to
417 simultaneously screen many DNA regions distributed randomly throughout the genome rather than looking at
418 specific loci, although these are dominant markers, so they cannot differentiate homologous alleles, making it
419 less useful for studies that involve allelic states such as heterozygosity analyses (Mueller and Wolfenbarger
420 1999). AFLP analysis using some representative isolates of *P. palmivora* from Malaysia and Colombian using
421 three AFLP primer combinations was able to show some variations. The isolates from Colombian and Malaysia
422 were separated into different clades in the phylogenetic tree.

423 The results from the AFLP encouraged the exploration of other loci as molecular markers to study
424 variations among Colombian isolates and Malaysian isolates. One region of interest was the gene clusters or
425 regions encoding effector/avirulence proteins that are involved in the infection process and colonization of
426 plant tissue. The genome sequencing of *Phytophthora* species such as *P. infestans* has revealed a diverse and
427 large class of effectors (Bozkurt et al. 2012) such as *AVR3a* (Armstrong et al. 2005; Bos et al. 2009), *AVR1b*
428 (Shan et al. 2004) and *PiAVR4* (van Poppel 2009; van Poppel et al. 2008). The effector proteins are secreted
429 by the oomycetes to suppress the immune responses of the host plant (such as pathogen associated molecular
430 patterns (PAMPs) trigger immunity (PTI) triggered by their own elicitors. For example, *P. infestans* effector

AVR3a suppresses perception of the PAMP INFI through stabilization of the U-box protein CMPG1 (Fawke et al. 2015). The AVR3a protein is encoded by avirulence gene *Avr3a* and belongs to a large, oomycete-specific family of highly divergent effectors that share a conserved domain named RXLR-dEER (Tyler et al. 2006) which triggers disease resistance and the hypersensitive response (HR) (Armstrong et al. 2005). The corresponding resistance *R* gene of the host plant to *Avr3a* is the *R3a*, and R proteins generally activate resistance responses effector-triggered immunity of the plant host (ETI).

The primers to amplify the unknown region of only *P. palmivora*, named as PpHPAV were designed from the sequences of *P. infestans Avr4* (*PiAvr4*) sequences which encodes a typical oomycete RXLR effector molecule (van Poppel et al. 2008). The PpHPAV sequences did not match closely to DNA or protein sequences in the GenBank® database, probably because whole genome sequencing and studies on effector proteins and avirulence genes of *P. palmivora* have not yet been published and are still on going. Although the nature of the PpHPAV sequences is vague, the locus was shown to have some intraspecific variation within *P. palmivora* species, at least between Colombian and Malaysian isolates. Phylogenetic analyses using PpHPAV sequences separated all the Colombian isolates into one clade along with other isolates except isolates from Malaysia, Indonesia and South Korea, which were clustered in separated clades. However, these three clades did not show any other characteristics based on host and origin, but the phylogenetic observation suggested that they share common ancestry. It will be interesting to further explore isolates from South East Asia such as Thailand, the Philippines and Myanmar to confirm the distinct nature of SE Asian isolates.

Pathogenicity studies using isolates from Malaysia, Colombia and others however, did not show any significant differences in the level of aggressiveness against oil palm. In *in vitro* assays, infection was successfully established on the very young spear leaves using the Colombian isolates PPC280574. The initial symptoms of small brown lesions with water-soaking at the edge were observed at 3-4 days after inoculation, which coincides with the symptoms described in several reviews such as Sarria (2013) and Torres et al. (2016). Similar water-soaked symptoms were also described by Tri et al. (2015) on the jackfruit leaf inoculation using the same species. Turner (1969a) reported that the water-soaked margin was only observed on inoculated immature leaves of *Piper betle* and both upper and lower leaf surfaces can be inoculated. In our study, it was observed that wounding of the spear was required for the infection to occur in contrast with the findings by Sarria et al. (2016), where the infection readily occurred without wounding. However, Sarria et al. (2016) used

459 individual leaflets of the young spear instead of direct inoculation on the un-opened spear. Mohamed Azni et
460 al. (2016) also reported the need for wounding for infection to occur. The re-isolation of the diseased tissue
461 and microscopic evaluation confirmed the presence of *P. palmivora*.

462 Initially, we could not establish infection in the glasshouse through artificial inoculation of *P.*
463 *palmivora* on oil palm seedlings using the isolate originating from oil palm in Colombia believed to be
464 pathogenic to oil palm. Several trials were conducted including trials with modifications of inoculation methods
465 including increasing the inoculum (in term of volume, zoospore counts, combinations of mycelium,
466 sporangium, chlamydospores and zoospores), introduction of wounding at the stem base of the seedlings and
467 waterlogging the seedlings before and after inoculation. In order to avoid loss of virulence during sub-culturing,
468 the isolate was reactivated in the fruit (apple/pear) and re-isolated onto selective media prior to production of
469 zoospores. Positive infection on rubber leaves suggested the continued existence of the pathogenic nature of
470 the isolate. The artificial inoculation was then extended to other isolates originating from oil palm and cocoa
471 in Colombia, cocoa, durian, rubber in Malaysia, *Cymbidium* orchid (South Korea, betel palm (Guam), kentia
472 palm (California) and cocoa (Ghana), regardless of the failure to established infection using the isolate from
473 the oil palm as positive reference. It is believed that the infection of *P. palmivora* to the seedlings is affected
474 by the temperature because eventually infections were observed at the end of May, which was the beginning
475 of spring in the UK, and subsequent inoculations with the same isolates as tested before (with no infection),
476 showed positive infections when retested during the summer months.

477 The initial symptoms of brown lesions with water-soaked margins (observed on the seedlings
478 inoculated with Colombian and other isolates) coincided with the previous detached leaf assay and observations
479 from Sarria et al. (2016). However, the lesions appeared to be localized in our study as reported by Mohamed
480 Azni et al. (2016) with work using Malaysian isolates in Malaysia. The infection did not grow further in most
481 infected seedlings. In other words the Colombian, Malaysian and other isolates from different hosts all caused
482 mild symptoms, potentially equating to the non-lethal form of the disease that has been found in some parts of
483 the world including possibly Malaysia (Sharples (1928); Sharples (1928); Turner (1981), and the disease did
484 not progress to a severe form with the typical aggressive symptoms that had been found to occur in inoculation
485 tests in Colombia (Sarria 2013; Torres et al. 2010). Torres et al. (2010) reported 15% of the seedlings inoculated
486 with 40,000 zoospores developed into typical bud rot symptoms but none in our study even though up to

180,000 zoospores were used per seedling. The disease cycle of *Phytophthora* often involves primary and secondary inoculum. Primary inoculum initiates the infection and upon successful infection, a second generation of secondary inoculum is produced. The rate of propagation of secondary inoculum determines the severity of the next infection (Drenth and Guest 2004). In the case of infection in the glasshouse in the UK, there were some factors affecting the propagation of secondary inoculum including environmental conditions such as temperature, humidity and maybe the presence or absence of other microbes as secondary invaders that are different in the UK and Malaysia compared to Latin America. In terms of disease incidence, not all seedlings inoculated with each isolate were infected. The incidence observed on the inoculated seedlings was variable between and within isolates. Some difficulty was experienced in producing zoospores for each trial, such that the inoculum strength in terms of zoospore could not be exactly standardized and was in the range of 1-9 x 10^4 zoospore ml^{-1} for the first summer inoculation and 5-8 x 10^3 zoospore ml^{-1} for the second round summer inoculation, and this may have affected the incidence scores between tests. However, the incidence data is useful in providing information on the cross pathogenicity between isolates against oil palm seedlings even though it may not be appropriate for showing the aggressiveness levels of each isolate.

Cross pathogenicity assays showed isolates from oil palm could cause infection in rubber and durian leaves. Both inoculum sources of zoospores and mycelial plugs have the potential to infect the leaves and lesion growth varied in each assay using the same isolates which might reflect the influence of many factors such as humidity in the inoculation chamber, age/condition of the leaf and inoculum potential. Cross pathogenicity of isolates from different hosts; coconut, cocoa, durian, rubber, bamboo palm, betel palm and orchid on both durian and rubber leaves suggested that *P. palmivora* does not have specific strains adapted for each host as observed for *Fusarium* sp., supporting the hypothesis of a broad host range for *P. palmivora* (Drenth and Guest 2004). Pongpisutta and Sangchote (2004) showed cross pathogenicity of *P. palmivora* isolates from durian against black pepper and rubber leaves. However, not all *Phytophthora* species have broad host ranges. Different species of *Phytophthora* may have different degrees of host specificity. Some species such as *P. havea* have narrow ranges and *P. colocasiae* is very host specific to taro (*Colocasia esculenta*) (Drenth and Guest 2004). Some species of *Phytophthora* seem to have both non-host and host-specific receptor-based recognition systems for induction of encystment of zoospores by host surface components, therefore enabling general and host specific pathogenicity, which enables them to invade compromised plants in the absence of

515 preferred hosts (Raftoyannis and Dick 2006a). However, there is also the possibility that the specificity of host
516 selection arises during the attempts at penetration and invasion of plant tissue and that the zoospore stage is
517 non-specific (Van West et al. 2002). Several reports with root diseases show that zoospores of *Phytophthora*
518 species are attracted to and encyst similarly on roots of susceptible and resistant seedlings of plants
519 (Raftoyannis and Dick 2006a). Raftoyannis and Dick (2006b) found that the relationship between encystment
520 of zoospores and disease development depends on the oomycete–plant combination.

521 Similar to the inoculation of oil palm young spear leaves, the inoculation on durian and rubber leaves
522 in the cross-pathogenicity assays using several isolates from various hosts including oil palm conducted in this
523 study also failed to established infection without wounding. Introduction of wounding in the artificial
524 inoculation is not new in pathogenicity studies of *Phytophthora* spp. using stems to facilitate infection and has
525 been shown by others especially when working with stem rots. Nevertheless, most studies with leaves usually
526 do not involve wounding and infections on the leaves become established without wounding such as in citrus
527 (Ann 1984), durian (Lim and Chan 1986) and jackfruit (Tri et al. 2015). Meanwhile, other researchers have
528 introduced wounding prior to inoculation such as Pongpisutta and Sangchote (2004). O'Gara et al. (2004a)
529 reported that *P. palmivora* was attracted to fresh wounds on the durian leaf and rapidly colonized the entire
530 leaf lamina when infection happened through the fresh wound and non-wounded durian leaves did not develop
531 disease symptoms reliably (O'Gara et al. 2004b). In contrast, Brooks (2008) found that there was no difference
532 in the infection of *P. colocasiae* on taro leaves.

533 It is hoped that more studies can be conducted to understand more on the pathogenicity and
534 aggressiveness of *P. palmivora* against oil palm. Artificial inoculation of oil palm seedlings using different *P.*
535 *palmivora* isolates should be repeated but with the same inoculum size for each isolate so that the
536 aggressiveness of the different isolates originating from different hosts and geographical regions can be
537 assessed without prejudice and probably can be correlated with the molecular characterization to see if the
538 isolates belonging to same clade have similar levels of aggressiveness against oil palm. It would also be good
539 if the assay can be conducted in a tropical environment; however, due to biosecurity constraints, it is difficult
540 to carry out such experiments in countries such as Malaysia and Colombia as it would involve introducing the
541 foreign isolates to the areas. However, the evaluation of the local isolates obtained from different hosts against
542 oil palm is possible.

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852 Table 2 Details of isolates collected in this study

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No.	Isolate	Species based on the source database	IDENTIFY IN THIS STUDY	Origin	Ex-host	Source*	GeneBank Accession Number				
							ITS	*B-Tubulin*	*CoxI*	*CoxII*	*EF-1α*
1	PPM1	*P. palmivora*	*P. palmivora*	Malaysia	*Theobroma cacao (cocoa)*	MPOB	KY197718	MH401213	MH760206	MH760244	MH760169
2	PPM2	unknown	*P. palmivora*	Malaysia	*Theobroma cacao (cocoa)*	MPOB	KY197719	MH401214	MH760207	MH760245	MH760170
3	PPM3	unknown	*P. palmivora*	Malaysia	*Theobroma cacao (cocoa)*	MPOB	KY197720	MH401215	MH760208	MH760246	MH760171
4	PPM4	unknown	*P. palmivora*	Malaysia	*Durio zibethinus (durian)*	MPOB	KY197721	MH401216	MH760209	MH760247	MH760172
5	PPM5	*P. palmivora*	*P. palmivora*	Malaysia	*Durio zibethinus (durian)*	MPOB	KY197722	MH401217	MH760210	MH760248	MH760173
6	PPM6	unknown	*P. palmivora*	Malaysia	*Theobroma cacao (cocoa)*	MPOB	KY197723	MH401218	na	na	na
7	P6948	*P. palmivora*	*P. palmivora*	Malaysia	*Hevea brasiliensis (rubber)*	WOGRC	KY475615	MH401219	MH760211	MH760249	MH760174
8	PPC28057 4	*P. palmivora*	*P. palmivora*	Colombia, Tumaco	*Elaeis guineensis (African oil palm)*	CENIPALMA	KY475616	MH401220	MH760212	MH760250	MH760175
9	P16828	*P. palmivora*	*P. palmivora*	Colombia, Central Zone	*Elaeis guineensis (African oil palm)*	WOGRC	KY475617	MH401221	MH760213	MH760251	MH760176
10	P16831	*P. palmivora*	*P. palmivora*	Colombia Tumaco	*Elaeis guineensis (African oil palm)*	WOGRC	KY475618	MH401222	MH760214	MH760252	MH760177
11	P8513	*P. palmivora*	*P. palmivora*	Colombia	*Theobroma cacao (cocoa)*	WOGRC	KY475619	MH401223	MH760215	MH760253	MH760178
12	P0497	*P. palmivora*	*P. palmivora*	Colombia	*Theobroma cacao (cocoa)*	WOGRC	KY475620	MH401224	MH760216	MH760254	MH760179
13	IMI382544	*P. palmivora*	*P. palmivora*	Indonesia	*Cocos nucifera (coconut)*	CABI Bioscience	KY475621	MH445343	MH760217	MH760255	MH760180

14	IMI382528	*P. palmivora*	*P. palmivora*	Indonesia	*Cocos nucifera (coconut)*	CABI Bioscience	KY475622	MH445344	MH760218	MH760256	MH760181
15	P3767	*P. palmivora*	*P. palmivora*	Indonesia	*Cocos nucifera (coconut)*	WOGRC	KY475623	MH445345	MH760219	MH760257	MH760182
16	CBS236.30	*P. palmivora*	*P. palmivora*	India	*Cocos nucifera (coconut)*	CBS-KNAW	KY475624	MH445346	MH760220	MH760258	MH760183
17	P16385	*P. palmivora*	*P. palmivora*	California, USA	*Howea forsteriana (kentia palm)*	WOGRC	KY475625	MH445347	MH760221	MH760259	MH760184
18	P11007	*P. palmivora*	*P. palmivora*	Guam	*Areca catechu (betel palm)*	WOGRC	KY475626	MH445348	MH760222	MH760260	MH760185
19	CBS179.26	*P. palmivora*	*P. palmivora*	Sri Lanka	*Theobroma cacao (cocoa)*	CBS-KNAW	KY475627	MH445349	MH760223	MH760261	MH760186
20	CBS298.29	*P. palmivora*	*P. palmivora*	Trinidad & Tobago	*Theobroma cacao (cocoa)*	CBS-KNAW	KY475628	MH445350	MH760224	MH760262	MH760187
21	PPG1	unknown	*P. palmivora*	Ghana	*Theobroma cacao (cocoa)*	OPRI	KY475629	MH445351	MH760225	MH760263	MH760188
22	PPG8	unknown	*P. palmivora*	Ghana	*Theobroma cacao (cocoa)*	OPRI	KY475630	MH445352	MH760226	MH760264	MH760189
23	PPG11	unknown	*P. palmivora*	Ghana	*Theobroma cacao (cocoa)*	OPRI	KY475631	MH445353	MH760227	MH760265	MH760190
24	PPG13	unknown	*P. palmivora*	Ghana	*Theobroma cacao (cocoa)*	OPRI	KY475632	MH445354	MH760228	MH760266	MH760191
25	CBS111346	*P. palmivora*	*P. palmivora*	South Korea	*Cymbidium* spp. (orchid)	CBS-KNAW	KY475633	MH445355	MH760229	MH760267	MH760192
26	PPC2614P	*P. palmivora*	*P. palmivora*	Colombia, Tumaco	*Elaeis guineensis (African oil palm)*	CENIPALMA	MH401198	na	MH760230	MH760268	MH760193
27	PPC3614L	*P. palmivora*	*P. palmivora*	Colombia, Tumaco	*Elaeis guineensis (African oil palm)*	CENIPALMA	MH401199	na	MH760231	MH760269	MH760194
28	CBS148.88	*P. arecae*	*P. arecae*	USA (Florida)	*Chamaedorea sefritzii (bamboo palm)*	CBS-KNAW	MH401200	na	MH760232	MH760270	MH760195
29	PPM7	unknown	*P. palmivora*	Malaysia	*Theobroma cacao (cocoa)*	MPOB	na	na	na	na	na
30	PPM8	unknown	*P. palmivora*	Malaysia	*Durio zibethinus (durian)*	MPOB	na	na	na	na	na
31	P19537	*P. palmivora*	*P. parasitica*	Colombia	*Elaeis guineensis (African oil palm)*	WOGRC	MH401208	MH760160	MH760233	MH760271	MH760196
32	P19538	*P. palmivora*	*P. parasitica*	Colombia	*Elaeis guineensis (African oil palm)*	WOGRC	MH401209	MH760161	MH760234	MH760272	MH760197

33	CBS358.59	*P. palmivora*	*P. colocasiae*	Sri Lanka	*Hevea brasiliensis (rubber)*	CBS-KNAW	MH401210	MH760162	MH760235	MH760273	MH760198
34	PPG3	unknown	*P. megakarya*	Ghana	*Theobroma cacao (cocoa)*	OPRI	MH401202	MH760163	MH760236	MH760274	MH760199
35	PPG4	unknown	*P. megakarya*	Ghana	*Theobroma cacao (cocoa)*	OPRI	MH401203	MH760164	MH760237	MH760275	MH760200
36	PPG12	unknown	*P. megakarya*	Ghana	*Theobroma cacao (cocoa)*	OPRI	MH401204	MH760165	MH760238	MH760276	MH760201
37	PC01	*P. cryptogea*	*P. cryptogea*	unknown	unknown	UoN	MH401205	MH760166	MH760239	MH760277	MH760202
38	13-A2	*P. infestans*	*P. infestans*	United Kingdom	*Solanum tuberosum (potato)*	UoN	MH401206	na	MH760240	MH760278	MH760203
39	2009-7654A	*P. infestans*	*P. infestans*	United Kingdom	*Solanum tuberosum (potato)*	UoN	MH401207	na	MH760241	MH760279	MH760204
40	CBS581.69	*P. botryosa*	*P. botryosa*	Malaysia	*Hevea brasiliensis (rubber)*	CBS-KNAW	MH401211	MH760167	MH760242	MH760280	MH760205
41	PYT01	*Pythium aphanidermatum*	*Pythium aphanidermatum*	Unknown	unknown	UoN	MH401212	MH760168	MH760243	MH760281	na
42	CCO2083	*P. palmivora*	*P. palmivora*	unknown	unknown	FERA	MH401201	na	na	na	na

*****Abbreviations of culture centres and source agencies: CBS-KNAW, Westerdijk Fungal Biodiversity Institute, Netherlands; CABI, The Centre for Agriculture and Bioscience International, UK; WOGRC, The World Oomycetes Genetic Resource Collection, University of California, Riverside, USA; MPOB, Malaysian Palm Oil Board, Malaysia; OPRI, The Oil Palm Research Institute (OPRI) of Ghana, Ghana; CENIPALMA, Colombian Oil Palm Research Center, Colombia; FERA, The Food and Environment Research Agency, UK; UoN, University of Nottingham, UK.

Table 3 First summer inoculation using African oil palm seedlings (Tenera)

Isolates	Host and origin	No. of inoculated palms	No. of palms with lesions	% of seedlings with lesions	% of palms recovered after 6 months
Ctrl (dH2O)	-	5	0	0	100
PPC280574	Oil palm - Colombia	5	3	60	100
P16828	Oil palm - Colombia	5	2	40	100
P16831	Oil palm - Colombia	5	1	20	100
P8513	Cocoa - Colombia	5	1	20	100
PPM1	Cocoa - Malaysia	5	4	80	100
PPM4	Durian - Malaysia	5	3	60	100
P6948	Rubber - Malaysia	5	4	80	100
IMI382544	Coconut - Indonesia	5	2	40	100
CBS1113.46	Cymbidium - South Korea	5	4	80	100
P11007	Betel palm - Guam	5	3	60	100
P16385	Kentia palm - California	5	3	60	100
PPG1	Cocoa-Ghana	5	2	40	100

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878 Table 4 Second round summer inoculation using African oil palm seedlings (Tenera)

Isolates	Host and origin	No. of inoculated palms	No. of palms with lesions	% of seedlings with lesions	% of palms recovered after 6 months
Ctrl (dH2O)	-	10	0	0	100
PPM1	Cocoa - Malaysia	10	3	30	100
P6948	Rubber - Malaysia	10	4	40	100
PPC280574	Oil palm - Colombia	10	5	50	100
P8513	Cocoa - Colombia	10	5	50	100

879

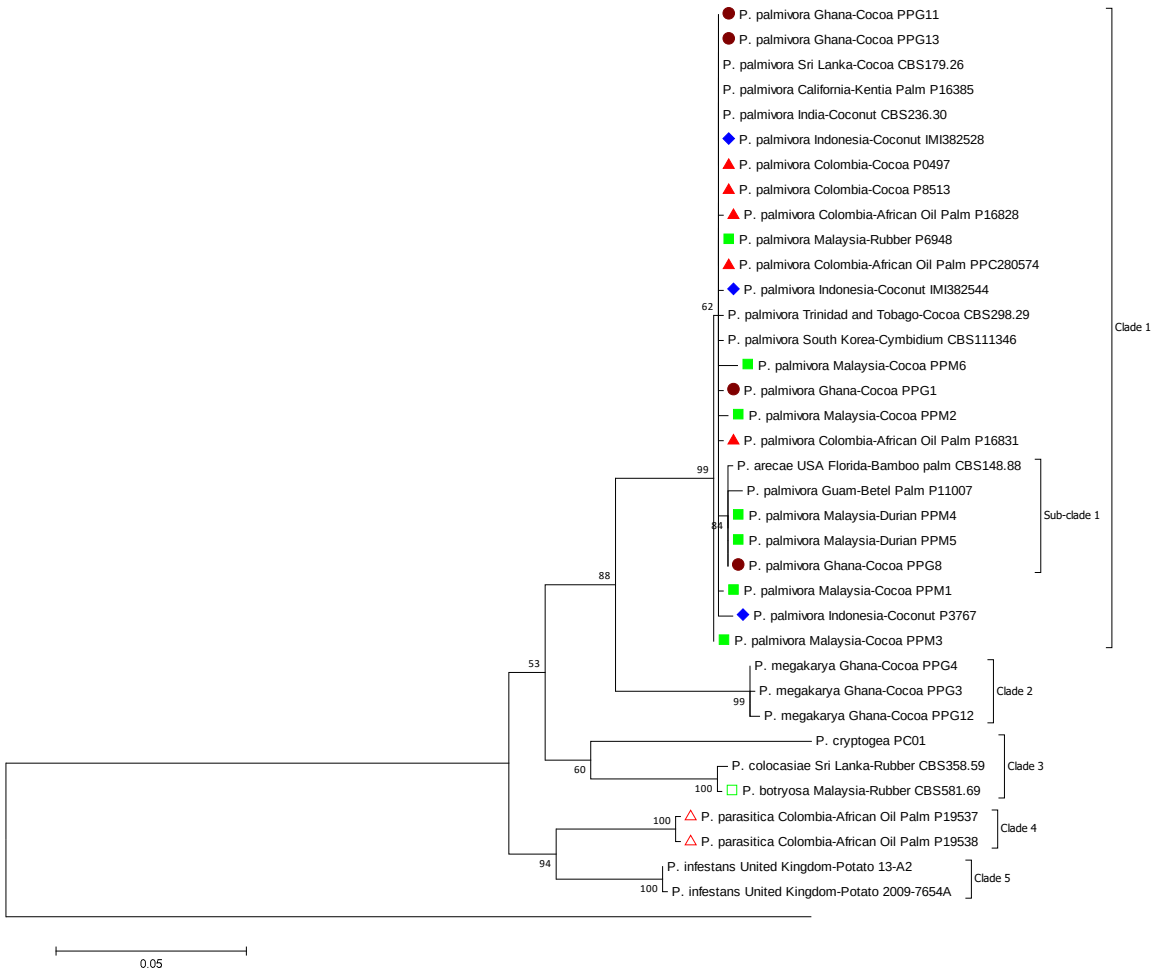


Figure 1 Molecular phylogenetic tree showing the relationship *P. palmivora* and other *Phytophthora* from different hosts and demographic origin constructed from ITS rDNA data using Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-3074.7150) is shown.

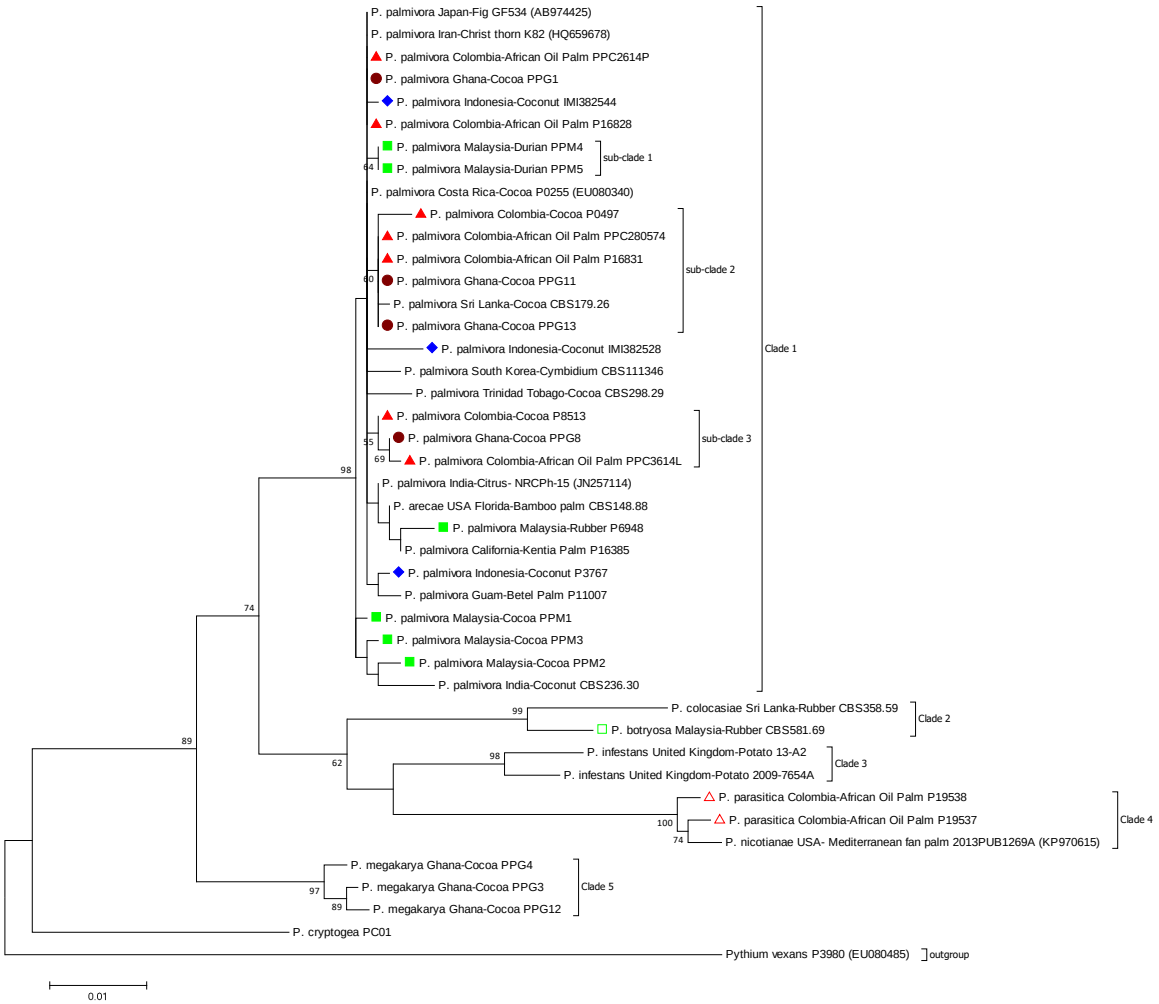


Figure 2 Molecular phylogenetic tree showing the relationship of *P. palmivora* and other *Phytophthora* from different hosts and demographic origin constructed from partial gene sequences of *translation elongation factor 1 alpha* (*EF-1α*) using maximum likelihood method based on the Tamura-Nei model. Note: The tree with the highest log likelihood likelihood (-2832.2482) is shown.

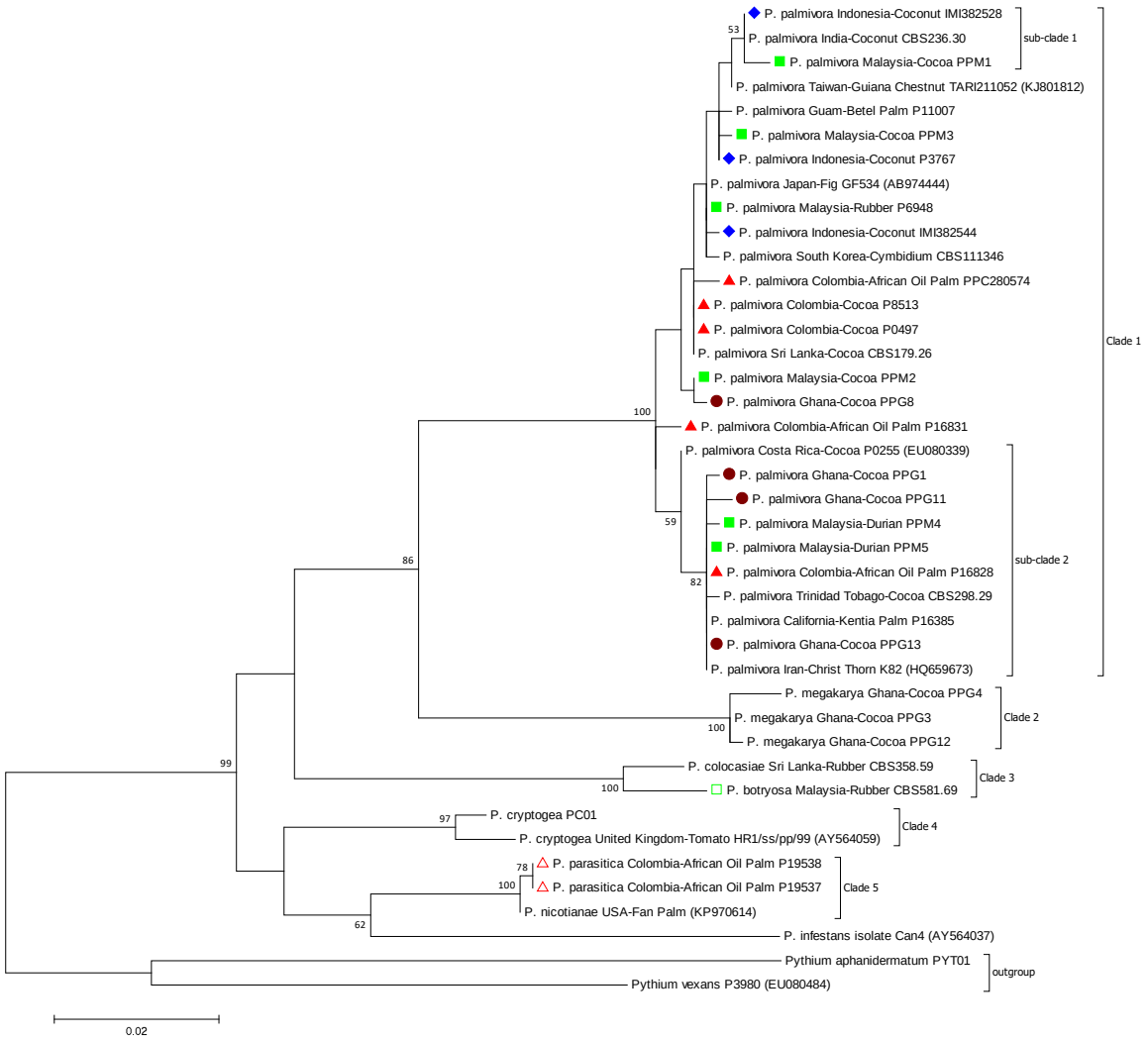


Figure 3 Molecular phylogenetic tree showing the relationship of *P. palmivora* and other *Phytophthora* from different hosts and demographic origin constructed from partial gene sequences of *β-tubulin* using maximum likelihood method based on the Tamura-Nei model. Note: The tree with the highest log likelihood likelihood (-2351.2154) shown.

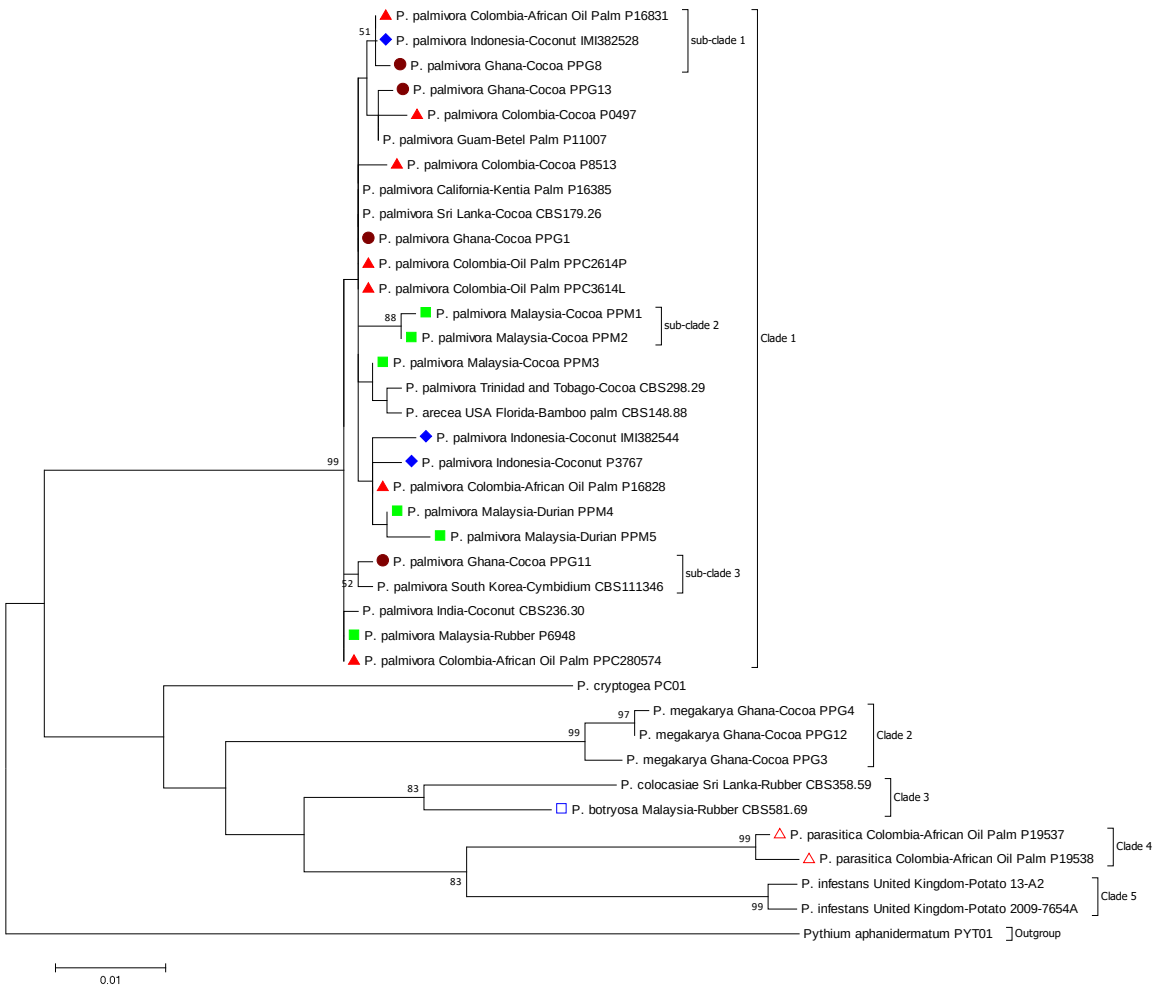


Figure 4 Molecular phylogenetic tree showing the relationship of *P. palmivora* and other *Phytophthora* from different hosts and demographic origin constructed from partial gene sequences of *cytochrome c oxidase subunit I (CoxI)* using maximum likelihood method based on the Tamura-Nei model. Note: The tree with the highest log likelihood likelihood (-2770.5889) is shown.

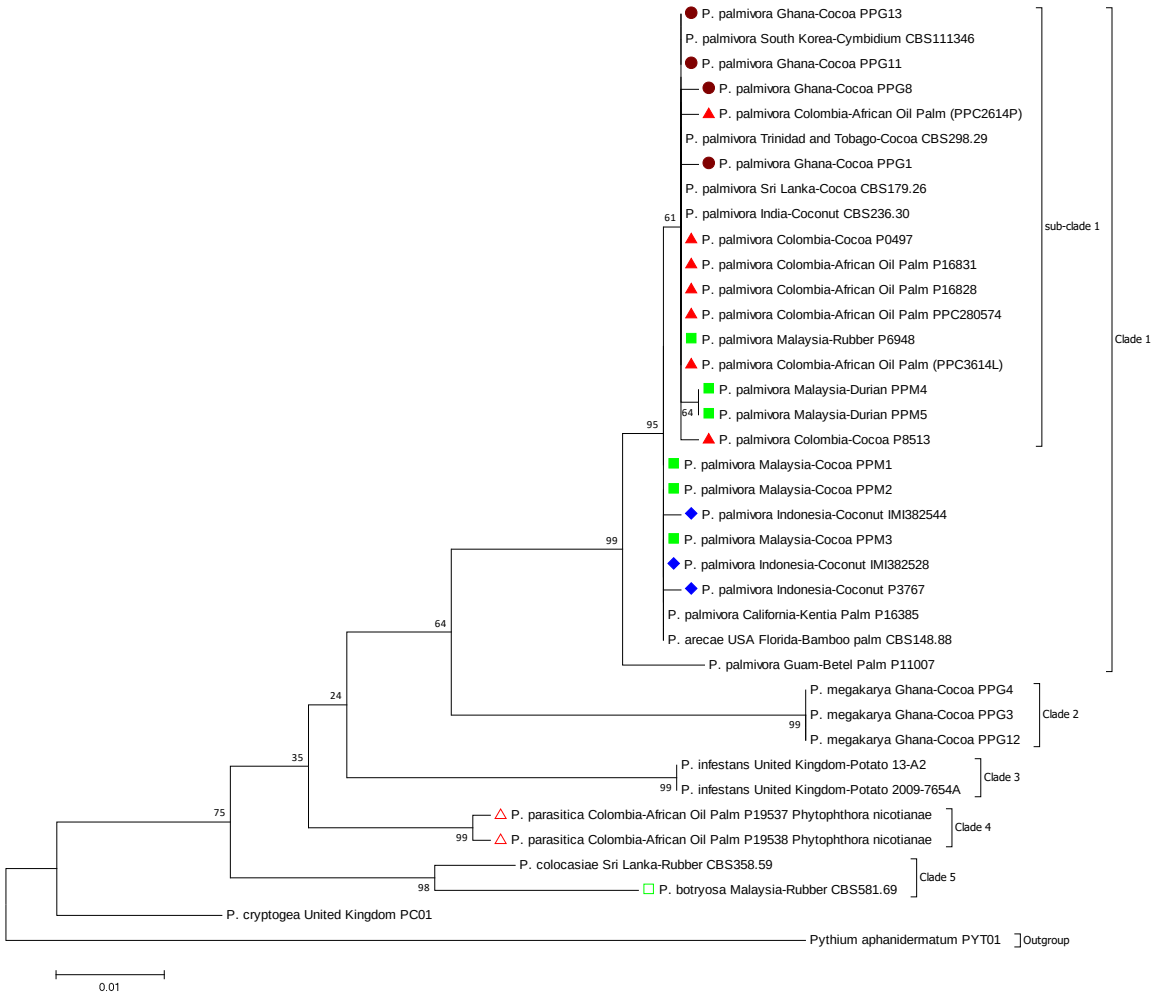


Figure 5 Molecular phylogenetic tree showing the relationship of *P. palmivora* and other *Phytophthora* from different hosts and demographic origin constructed from partial gene sequences of *cytochrome c oxidase subunit II (CoxII)* using maximum likelihood method based on the Tamura-Nei model.

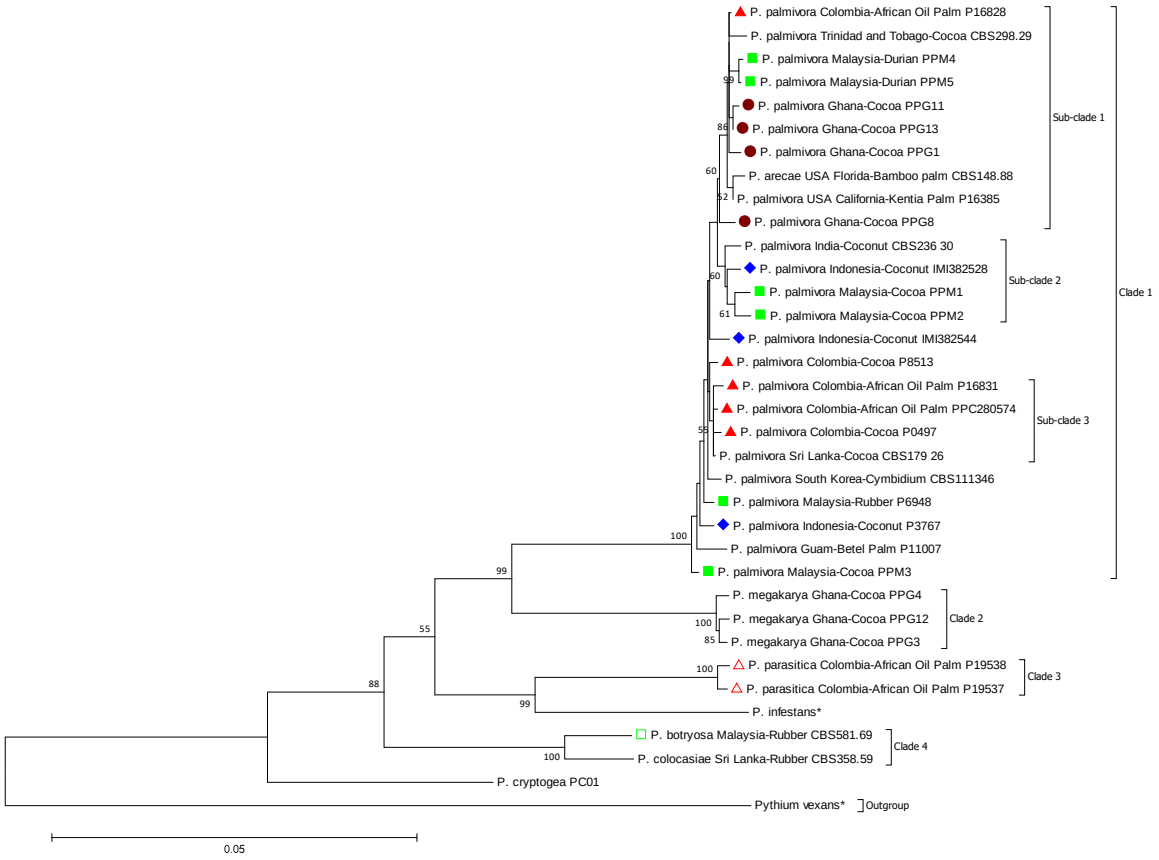


Figure 6 Molecular phylogenetic tree showing the relationship of *P. palmivora* and other *Phytophthora* from different hosts and demographic origin constructed from concatenated sequences of ITS, *EF-1α*, *β-tubulin*, *CoxI* and *CoxII* using maximum likelihood method based on the Tamura-Nei model.

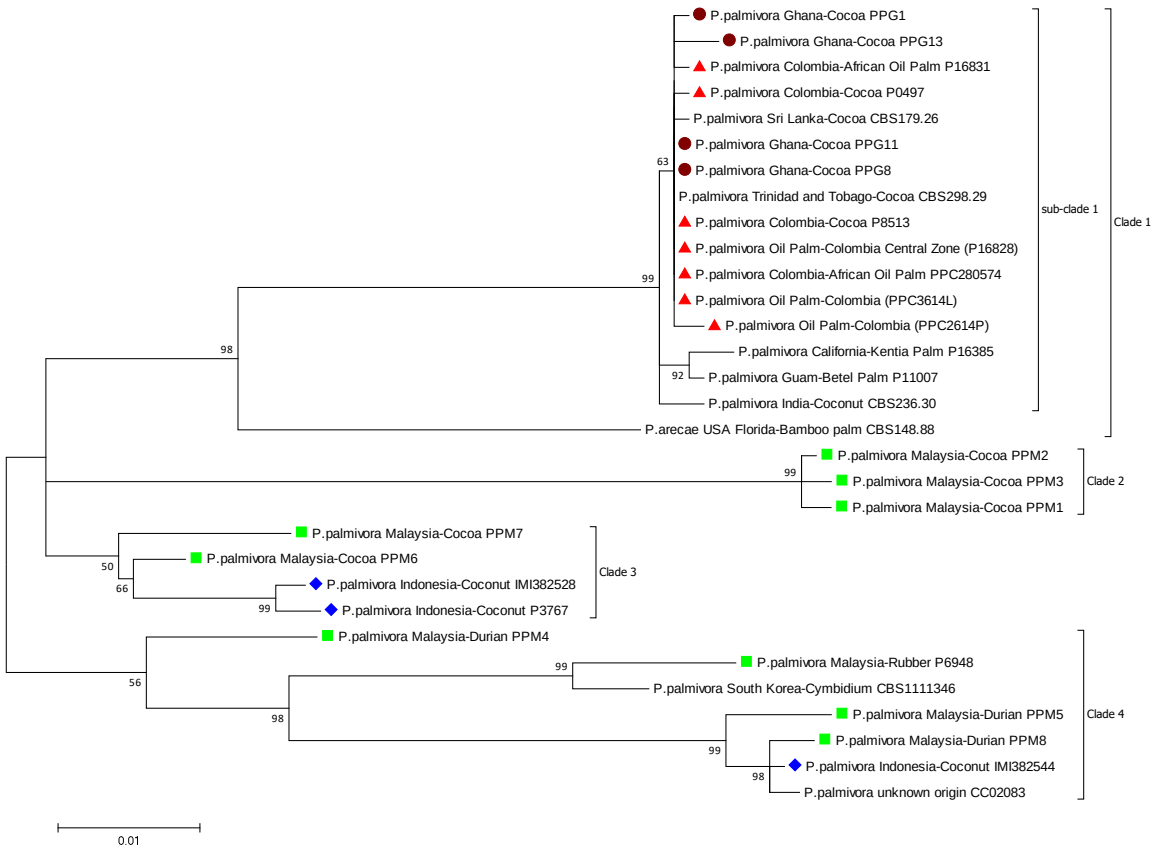
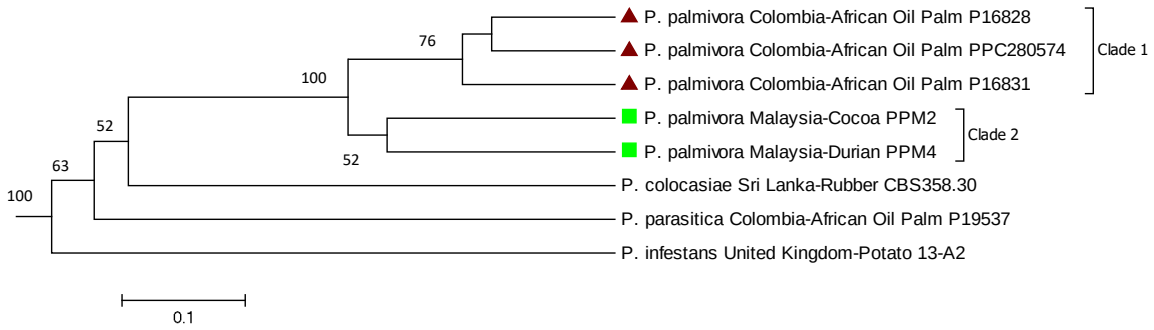
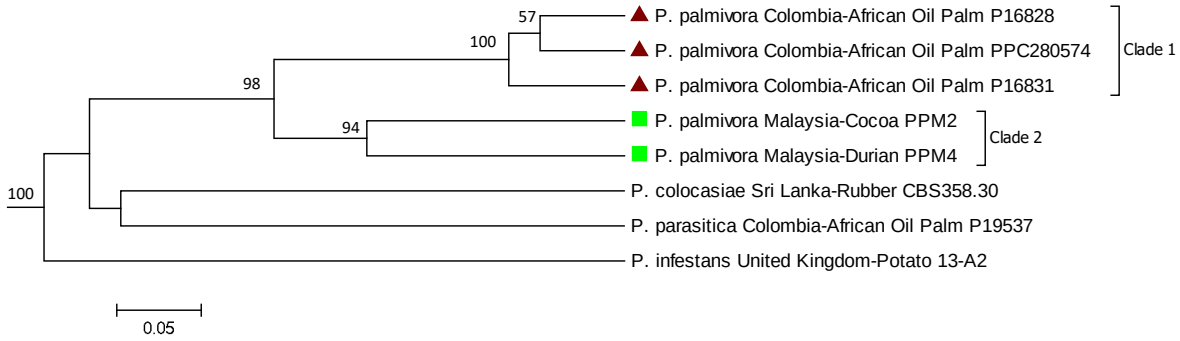


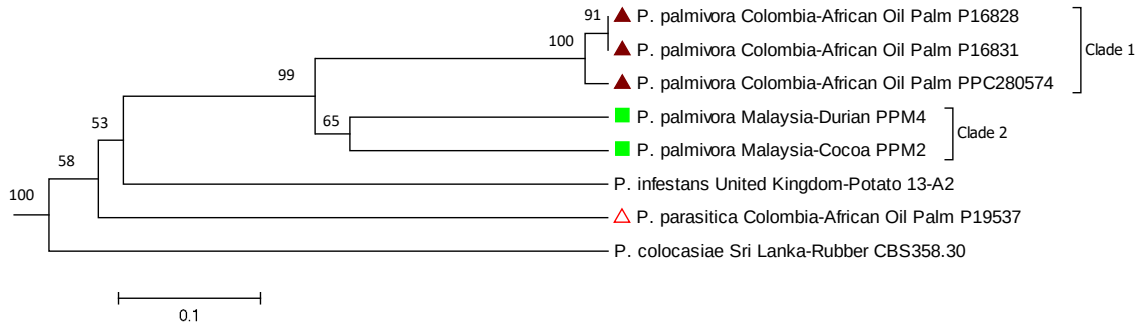
Figure 7 Molecular phylogenetic tree showing the relationship of *P. palmivora* isolated from oil palm originated from bud rot disease hotspot zone in Colombia with other isolates of *P. palmivora* from different hosts and demographic origin constructed from our new PpHPAV marker using maximum likelihood method based on the Tamura-Nei model. Note: The tree with the highest log likelihood likelihood is shown.



(a)



(b)



(c)

Figure 8 Phylogenetic tree constructed from AFLP data using primer (a) *Eco*RI-A/*MSe*I-AG, b). *Eco*RI-AC/*Mse*I-AG (c) *Eco*RI-TA/*Mse*I-AG.

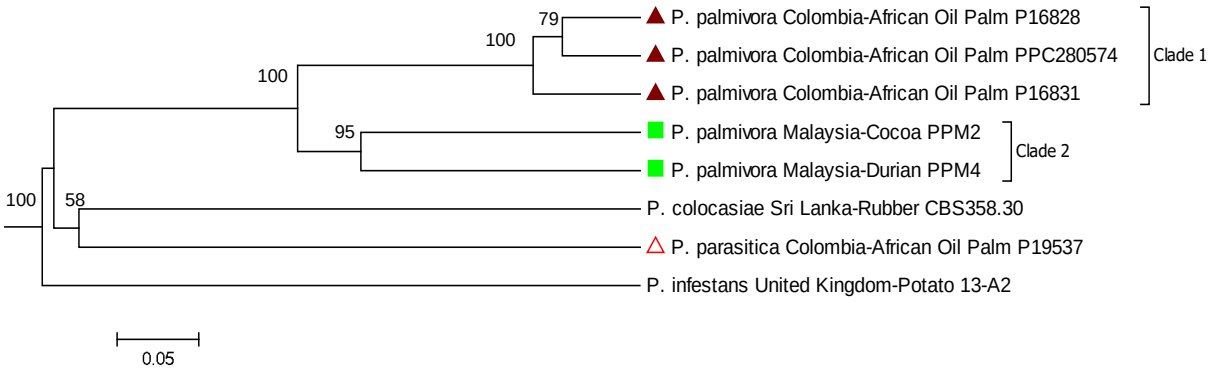
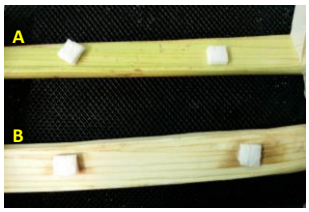
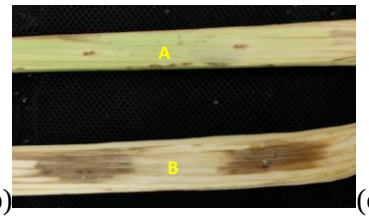


Figure 9 Phylogenetic tree constructed from concatenated AFLP data using three primer pairs of *Eco*RI-A/*MSe*I-AG, *Eco*RI-AC/*Mse*I-AG and *Eco*RI-TA/*Mse*I-AG.

(a) 

(b) 

(c) 

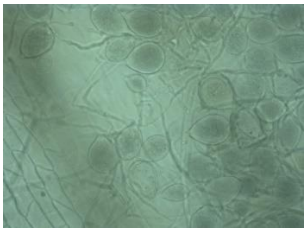
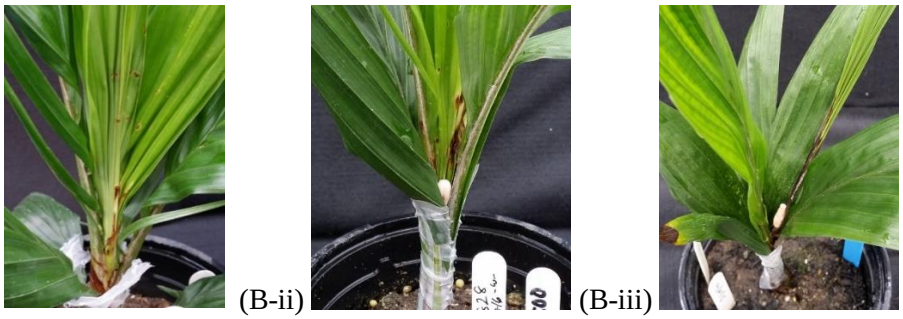
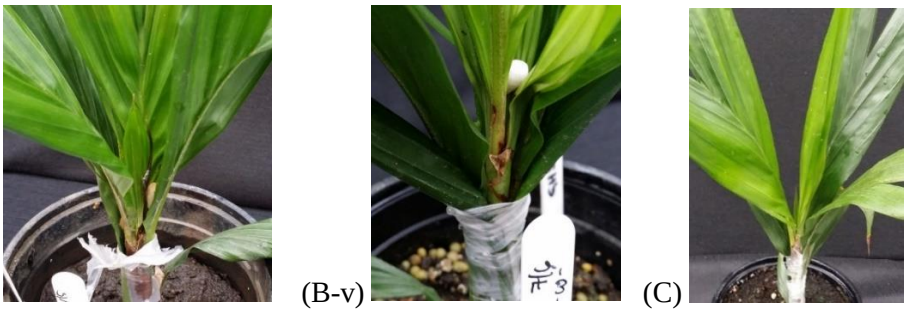
(d)  (e) 

Figure 10 (a) Brown lesions on the inoculation site of young oil palm spear leaves observed at the 4th day of inoculation, (b) lesion at 5th day and (c) control assay with water and (d) Mycelial growth from the re-isolation of the diseased tissue (brown lesion) and (e) sporangia observed on diseased tissue at the inoculation site of *P. palmivora* (Microscopic magnification: 40x10).

(A-i)  (A-ii)

(B-i)  (B-ii) (B-iii)

(B-iv)  (B-v) (C)

(D-i)  (D-ii)

Figure 11 Lesions observed on infected oil palm seedlings inoculated with *P. palmivora* isolate P16835 (A-i) and CBS111346 (A-ii) carried out in the middle of May. Similar disease symptoms were also observed on the subsequent inoculation repeated with the same isolates as in the previous inoculation. Shown are some examples of the symptoms that appeared on the seedlings

inoculated with; B-i) PPC280574, B-ii) P16828, B-iii) PPM4, B-iv) PPM1, B-v) P6896 and C) Control (distilled water spiked with carrot juice). Infected seedlings at 6 months after inoculation with (D-i) *P. palmivora* zoospores (D-ii) distilled water + carrot juice (control).