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DETECTION AND IDENTIFICATION OF CACAO BLACK POD CAUSAL AGENT BY POLYMERASE CHAIN REACTION (PCR)

Wael Alsultan, Ganesan Vadamalaı, Khairulmazmi Ahmad and Osamah Rashid

Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor Darul-Ehsan, Malaysia
ganesanv@upm.edu.my

Abstract: Among the *Phytophthora* species that cause black pod of cacao, *P. palmivora* is the most virulent, posing a serious threat to cacao production in Asia. Correct identification of the species causing the black pod and understanding the virulence factors involved are important for developing sustainable disease management strategies. The present study had revealed molecular characterization of thirty isolates of *P. palmivora* randomly obtained from infected cocoa pods in different states in Malaysia. All 30 isolates of *P. palmivora* were subjected to molecular identification methods using ITS rDNA, the mitochondrial cox1 and elongation factor 1α genes. Phylogenetic tree for *P. palmivora* was constructed using the sequencing of ITS rDNA, mitochondrial cox1 and elongation factor 1α genes. Different *Phytophthora* spp. sequencing had been chosen from NCBI and *Pythium undulatum* as outgroup. Molecular identification of rDNA confirmed that all isolates were *Phytophthora palmivora.* Phylogenetic analysis based on rDNA sequencing of different genes were separated different species of *Phytophthora* to various clades. *P. megakarya* is considered the closest species with *P. palmivora* based on ITS rDNA, translation elongation factor 1alpha and Cox subunit I. The significant of these findings are also viewed in terms of the increasing importance of *Phytophthora* diseases in this region.

Keywords: *Phytophthora,* black pod disease, *Theobroma cacao*, identification, phylogeny.

INTRODUCTION

Theobroma cacao L. is one of the most popular beverage crops next to coffee and tea in the world and its grown in about 57 countries. Ivory Coast, Ghana, Nigeria and Cameron are the major producer of cocoa beans in African continent while Indonesia and Malaysia are the top major producing countries in Asia. The demand of cocoa beans will be increased of 25% by 2020. In Malaysia, cocoa is the third important plantation crop after oil palm and rubber. It is stated as an industrial crop ear marked for imporoved productivitiy and expansion. Cocoa production in Malaysia had been reduced significantly from producing 9% of world production in the years of 1990-1991 to only below 2% of world production (Drenth and Guest, 2004). This decline was attributed to various factors including lower cocoa prices and the destruction of cocoa by the pests and diseases (Drenth and Guest, 2004). Cocoa is affected by various diseases caused by fungi and one of the most destructive diseases of cocoa is black pod. The main symptom of black pod disease of cocoa is a brown or black spot on the pod that quickly grows to cover the whole pod (Smith et al., 1992). Four species of *Phytophthora* are known to cause black pod rot: *P. megakarya, P. capsici, P. citrophthora and P. palmivora.* Pod rot disease causes 20– 30% annual losses in cocoa beans production globally, and much higher losses in specific locations during particularly wet and humid conditions (Erwin and Ribeiro, 1996). Unquestionably, characterization of *Phytophthora* spp. are

very essential to crop growers, environmentalists, legislators, policy makers and implementers to know the best way to managing and controlling diseases caused. Currently, DNA sequence-based molecular identification is considered more rapid, more sensitive and employed for specific identification of *Phytophthora* spp. (Drenth et al., 1994). Polymerase chain reaction (PCR)-based detection techniques have provided an alternative to microbiological identification of *Phytophthora* spp. (White et al. 1990; Cooke et al. 2000). PCR is a method for synthesising (amplifying) millions of copies of specific DNA sequences identified by two short oligonucleotides (primers) using a thermostable enzyme (Taq DNA polymerase) and repeated cycles of denaturation, polymerisation and elongation at different temperatures (Mullis and Faloona 1987; Ward et al., 2004).

METHODS

Survey, isolation, purification and maintenance of pathogen isolates

Phytophthora isolates were obtained from infected cocoa pods -2016- from different states, Sabah, Perak and Pahang in Malaysia. *Phytophthora* were isolated by the agar plate method. Diseased tissue samples were derived from the edge of lesions on cocoa pods and seeded on plates. 5 to 10 cm pieces of pod tissues were cleaned under running tap water, surface sterilized in 10% sodium hypochlorite for 5 min and rinsed twice in sterilized distilled water. The segments were then placed on sterilized filter paper to dry. A 0.5 cm section was cut from each end of the sterilized piece and on Corn Meal Agar medium. Four sections were placed on each petri dish. The plates were incubated at 25 ± 2°C for 5 days in the dark, subsequently Subcultures derived from pure colonies were maintained on CMA slants at 25 ± 2°C and used for future observations.

DNA extraction

Total genomic DNA of the isolate was extracted from mycelium according to Schlenzig (2009) with minor modifications**.** For DNA extraction, 6 mm agar discs of actively growing mycelium from single hyphal tip cultures of *Phytophthora* isolates were transferred to 50 ml of V8 broth in a conical flask and incubated in dark for 3-4 days at 25°C. Mycelial mats were washed with sterile distilled water, dried briefly under vacuum and were ground in liquid nitrogen into fine powder with a sterile mortar and pestle. Total genomic DNA from 100 mg of ground mycelium was extracted using modified CTAB method. The quantity and purity of DNA were assessed using Nano Drop ND-1000 spectrophotometer UV absorbance at 260/280-nm ratio.

Primers and PCR amplification

Several primer pairs were employed for PCR amplification. The ITS regions of the isolates were amplified using the universal primers ITS-1 and ITS-4. Fragments of the EF-1α gene were amplified using the primers ELONG-F1/ ELONG-R1. The region containing the mitochondrial cytochrome c oxidase subunit 1 (cox1) gene fragment was amplified using COXF4N and COXR4N primers. All primers were purchased from 1st BASE, Serdang, Malaysia (supplier of IDT, USA oligos). Each PCR reaction contained 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. PCR amplifications were performed in Biometra Thermocycler. Thermal cycling consisted of initial denaturation for 3 min at 95°C, followed by 34 cycles of denaturation at 95°C for 30 sec, annealing at different conditions for various primers used in this study, an extension at 72°C for 1 min; and final extension of 72°C for 10 min. The PCR products were visualized in 1.5% agarose gels and documented.

Sequencing and phylogenetic analysis

PCR products (ITS, EF-1α and cox1) were sent for purification and sequencing at MyTACG company. The 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 to identify the closest related sequences. Phylogenetic tree for *Phytophthora* was constructed using the sequencing of (ITS, EF-1α and cox1). The maximum likelihood method had applied by Mega software version 7 (Tamura et al., 2013) Tamura 3-parameter model. Branch of the tree was obtained from phylogenetic analysis have been assessed through boot-strapping with 1000 replications. Different *Phytophthora* spp. sequencing had been chosen from NCBI. Nucleotide sequence of *Pythium* sp. (for all the 3 loci) was treated as an outgroup.

RESULTS AND DISCUSSION

Collection and maintenance of isolates

A total of 30 *Phytophthora* samples were isolated from three states of Malaysia during 2016. Agar plate method used yielded *Phytophthora* isolates from infected tissues of cocoa pods and after three days of incubation on CMA medium. Pure cultures were maintained on CMA agar slants. Examination of fungal cultures under light microscope showed sporangia identified to be *Phytophthora palmivora* with reference to Stamps et al (1990) (Figure 1).

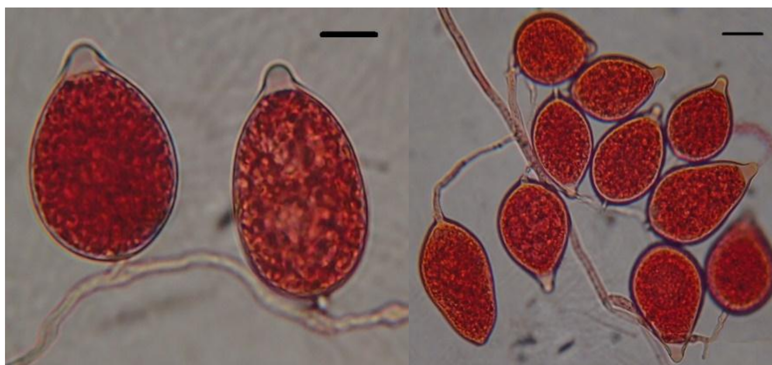

Figure 1: Sporangia of *Phytophthora palmivora* observed from 7 day old cultures.

Identification of *Phytophthora palmivora* based on molecular characteristics

PCR amplification of the ITS region of the *Phytophthora* isolates gave products size approximately 750-850 base pairs, while EF-1α and cox1 regions gave products size 1000 base pairs. The result of sequencing analysis identified all isolates as *Phytophthora palmivora* with similarity ranging between 99-100%. All sequences of isolates were submitted to National Center for Biotechnology Information (NCBI) where accession numbers were obtained.

Phylogenetic analysis

Phylogenetic analyses were performed as described previously for the sequences of ITS, EF-1α, and cox1. According to phylogenetic analysis of *Phytophthora palmivora* based on rDNA sequencing of different genes, all isolates were clustered as one group without variation with high support of 100 bootstrap

(Figure 2). Phylogenetic analysis of ITS rDNA, Cox subunit I and translation elongation factor 1alpha were separated different species of *Phytophthora* to various clades. *P. megakarya* is considered the closest species with *P. palmivora* based on ITS rDNA, translation elongation factor 1alpha and Cox subunit I.

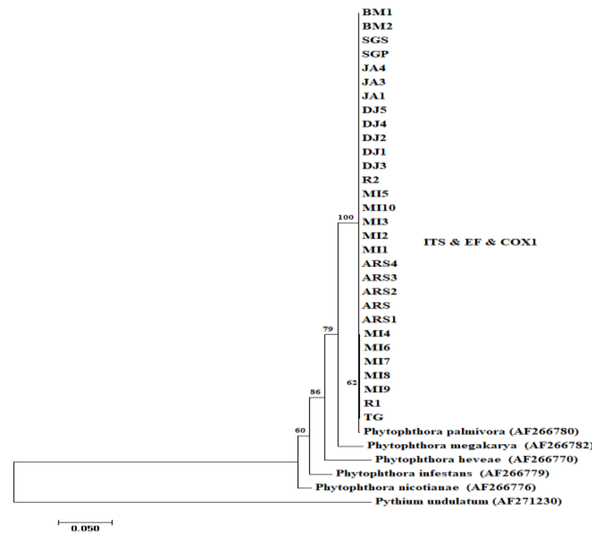

Figure 2: Phylogenetic analysis of *Phytophthora palmivora* of different type of genes using Maximum likelihood with substitutive model Kimura (1980).

The PCR-detection technique has several distinguishing features compared to the conventional diagnostic methods for plant pathogens. This technique has more sensitivity and versatility (Lee et al., 1993). The ITS region of rDNA have been reported to be beneficial for *Phytophthora* species identification. In other fungal groups ITS region was also useful in resolving taxonomic difficulties (Driver et al., 2000). The sequence information was used to study phylogenetic relationships between species level and below (White et al., 1990). In the present study, the rDNA analysis has been used successfully to differentiate *Phytophthora* species. Phylogeny of ITS, EF-1α, and cox1region or using another gene certainly provided an excellent opportunity to assess the traditional taxonomy of *Phytophthora* spp. and compare it with *Phytophthora* tree (Cocke et al., 2000). 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.

CONCLUSION

Molecular identification of rDNA confirmed that all the 30 isolates were *Phytophthora palmivora* and the study found that molecular method was an appropriate tool to accurately identify *Phytophthora* isolates from cocoa. The rRNA genes were highly conserved and hence these conserved sequences were used to support the identification of isolates as *Phytophthora palmivora*. ITS, EF-1α, and cox1 regions sequence analysis were found to be able to differentiate *Phytophthora* spp. at species level. This study confirmed that the nucleotide sequence analysis of rDNA is beneficial to provide information and knowledge on *Phytophthora* taxonomy. Therefore, molecular methods are needed to complement morphological identification as it is more rapid and can provide accurate identification for diverse fungal species. The recent fear that *Phytophthora* can cause a serious bud rot disease of palm oil in Colombia is considered

as a serious threat for Malaysia which is the second largest producer of palm oil in the world. It is of almost importance for the researchers in this country to update and upgrade the information and knowledge on the biology of *Phytophthora* spp. in order to understand aspects on pathogenicity and epidemiology of the disease caused. Such information will be valuable to assist more efficient management of disease caused by *Phytophthora* attacking a wide host range of crops including cocoa, durian, rubber, citrus, orchid, pepper and vegetable crops in Malaysia.

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