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IDENTIFICATION OF NATURAL HOST RANGE OF COCONUT CADANG-CADANG VIROID AND CHARACTERIZATION OF ITS SMALL RNAS FROM OIL PALM

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By

MOHAMMADREZA MOHAMMADI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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September 2014

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Coconut cadang-cadang viroid (CCCVd), a viroid from the genus *Cocadviroid* in the family *Pospiviroidae*, is the causal agent of orange spotting disorder in oil palm and variants of CCCVd have been detected and characterized from commercial oil palm plantations in Malaysia. It is considered as a potential threat to oil palm industry while epidemiological aspects of the viroid infection in oil palm are unknown. In order to investigate natural host range of the viroid, 64 leaf samples from 25 plant species from various locations in Selangor state and Kuala Lumpur were collected and tested by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) assay. Based on amplification of DNA molecules of ca. 250 bp representing basic monomeric form of the viroid ($CCCVd_{246}$), CCCVd was detected in 14 plant species including 7 palm, 3 monocotyledonous and 4 dicotyledonous plant species. From the palm species, CCCVd was detected from *Cocos nucifera*, *Corypha utan*, *Pritchardia pacifica*, *Ptychosperma macarthurii*, *Livistona chinensis*, *Saribus rotundifolius* and *Wodyetia bifurcata* but not from *Hyophorbe lagenicaulis, Cyrtostachys renda*, *Veitchia merrillii*, *Rhapis excels*, *Dypsis lutescens* and *Ravenala madagascariensis*. Fourteen out of 16 collected samples from coconut palms (*Cocos nucifera*) were found to contain CCCVd molecules. From other monocotyledonous and dicotyledonous plant species CCCVd was detected from *Heliconia* sp., *Maranta arundinacea, Etlingera elatior*, *Pseuderanthemum reticulatum*, *Codiaeum variegatum*, *Osmoxylon* sp. and *Carica papaya* but it was not detected from *Calathea luthea, Canna* sp., *Dracaena surculosa* and *Bauhinia* sp. Sequence analysis of 10 isolated CCCVd variants from 9 hosts revealed that there was no sequence variation among the variants. The consensus sequence of the variants was 246 nt in size with 100% homology to oil palm variant ($CCCVd\text{-}OP_{246}$) and exhibiting similar substitutions of C^{31} by U and G^{70} by C in P and CCR domains respectively compared to $CCCVd_{246}$ from coconut palms from the Philippines. Validation of results from RT-PCR was carried out through Two Dimensional-Polyacrylamide Gel Electrophoresis (2D-PAGE) analysis of nucleic acid extract of the samples and by hybridization assay. In 2D-PAGE analysis, bands representing

circular viroid RNAs were detected in the samples that were positive for CCCVd in RT-PCR and not in CCCVd negative samples. In hybridization assay these bands were hybridized with high stringency to full length digoxigenin (DIG) labeled cRNA probe and colorimetric signal was produced in their positions on membrane. In order to investigate the occurrence of Post-Transcriptional Gene Silencing (PTGS) and CCCVd small RNAs accumulation in oil palm, total small RNAs of 20-30 nt from two CCCVd infected asymptomatic and symptomatic oil palms were isolated and cloned. Small RNAs of full homology to CCCVd sequence were not detected among the sequenced clones. An attempt was made to establish Next Generation Sequencing (NGS) for detection of CCCVd variants from oil palm. Analysis of data obtained from deep sequencing of RNAs from a CCCVd infected oil palm showed that populations of CCCVd variants or other novel viroids were not detected but a 365 nt plant rRNA sequence was detected that was erroneously deposited in GenBank as Yucatan isolates of *Citrus exocortis viroid* (CEVd).

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia Sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENGENALPASTIAN PELBAGAI PERUMAH SEMULAJADI COCONUT CADANG-CADANG VIROID DAN PENCIRIAN RNA KECIL DARIPADA KELAPA SAWIT

Oleh

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Coconut cadang-cadang viroid (CCCVd), sejenis viroid dari genus *Cocadviroid* dalam keluarga *Pospiviroidae*, agen penyebab penyakit bintik oren kelapa sawit dan varian CCCVd telah dikesan daripada ladang kelapa sawit komersial di Malaysia. la dianggap sebagai ancaman kepada industri kelapa sawit manakala aspek epidemiologi jangkitan viroid ini tidak diketahui. Dalam kajian perumah semula jadi viroid, sejumlah 64 sampel daun dari 25 spesies tumbuhan daripada pelbagai lokasi di negeri Selangor dan Kuala Lumpur telah dikumpul dan pencaman menggunakan Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Berdasarkan amplifikasi molekul DNA ca. 250 bp mewakili struktur asas monomeric CCCVd246, CCCVd dikesan di dalam 14 spesies tumbuhan termasuk 7 palma, 3 monokot dan 4 spesies tumbuhan dikot. Dari spesies palma, CCCVd dikesan dalam *Cocos nucifera*, *Corypha utan*, *Pritchardia pacifica*, *Ptychosperma macarthurii*, *Livistona chinensis*, *Saribus rotundifolius* and *Wodyetia bifurcate* tetapi bukan dari *Hyophorbe lagenicaulis, Cyrtostachys renda*, *Veitchia merrillii*, *Rhapis excels*, *Dypsis lutescens* dan *Ravenala madagascariensis*. Empat belas daripada 16 sampel yang diambil dari pokok kelapa (*Cocos nucifera*) didapati dijangkiti CCCVd. Dari lain spesies tumbuhan monokot dan dikot CCCVd dikesan dari *Heliconia* sp., *Maranta arundinacea, Etlingera elatior*, *Pseuderanthemum reticulatum*, *Codiaeum variegatum*, *Osmoxylon* sp. dan *Carica papaya.* tetapi ia tidak dikesan dari *Calathea luthea, Canna* sp., *Dracaena surculosa* dan *Bauhinia* sp. Analisis jujukan DNA 10 varian CCCVd dari 9 perumah menunjukan bahawa tidak terdapat sebarang perbezaan jujukan DNA antara varian-varian tersebut. Konsensi jujukan varian yang dipencil adalah bersaiz 246 nt dengan 100% homologi dengan CCCVd-OP_{246} dan mempamerkan penggantian yang sama di C^{31} oleh U dan G^{70} oleh C dalam domain P dan CCR masing-masing domain berbanding $CCCVd_{246}$ dari Filipina. Pengesahan keputusan daripada RT-PCR telah dijalankan melalui analisis ekstrak asid nukleik daripada sampel menggunakan "Two Dimensional-Polyacrylamide Gel Electrophoresis" (2D-PAGE) dan teknik penghibridan. Dari analisis 2D-PAGE, tompok dikesan di kedudukan di gel yang mewakili struktur bulat viroid dalam

sampel yang positif untuk CCCVd dalam RT-PCR dan tidak dalam sampel negatif CCCVd. Penghibridisasi menunjukan bahawa tompok tersebut telah hibridasi dalam keaadan stringency yang tinggi dengan probe cRNA yang dilabel dengan digoxigenin (DIG) dan isyarat kolorimetrik dihasilkan dalam kedudukan tompok pada membran. Dalam penyiasatan kejadian post-transcriptional gene silencing (PTGS) dan pengumpulan RNA kecil CCCVd dalam kelapa sawit, jumlah RNA kecil 20-30 nt dari dua kelapa sawit simptomatik dan asimptomatik yang dijangkiti oleh CCCVd telah diasingkan dan diklon. RNA kecil yang ber homologi penuh dengan jujukan CCCVd tidak dikesan di kalangan klon didapati. Penggunaan Next Generation Sequencing (NGS) untuk mengesan varian CCCVd dari kelapa sawit telah dicuba dalam kajian ini. Analisis data yang diperolehi daripada penjujukan dalam RNA dari kelapa sawit yang dijangkiti oleh CCCVd menunjukan bahawa, mana-mana populasi varian CCCVd atau viroid novel lain tidak dikesan tetapi urutan 365 nt rRNA telah dikesan yang sebelum ini telah dikemukakan pada Gen-bank sebagai *Citrus exocortis viroid* (CEVd) isolat Yucatan.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Acryl	Acrylamide
A	Ampere
AMV-RT	Avian myeloblastosis virus reverse transcriptase
APS	Ammonium persulphate
BCIP	5-Bromo-4-chloro-3'-indolyphosphate p-toluidine
BLAST	Basic local alignment tool
Bis	Bisacrylamide
bp	Base pair
CA	Chloroform-iso-amyl alcohol
CCCVd	*Coconut cadang-cadang viroid*
cDNA	Complementary deoxyribonucleic acid
CEVd	*Citrus exocortis viroid*
cRNA	Complementary ribonucleic acid
CY5	Cyanine 5
2D-PAGE	Two dimensional polyacrylamide gel electrophoresis
dATP	2'-Deoxy-adenosine-5'-triphosphate
dCTP	2'-Deoxy-cytosine-5'-triphosphate
dGTP	2'-Deoxy-guanosine-5'-triphosphate
dTTP	2'-Deoxy-thymidine-5'-triphosphate
dNTP	Deoxynucleoside-triphosphates
DDW	Double distilled water
DIG	Digoxigeinin
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DW	Distilled water
EB	Elution buffer
EDTA	Ethylenediamine tetra acetic acid
EtBr	Ethidium bromide
g	Gram
g	Centrifugal force
Gb	Giga base
HBO_3	Boric Acid
HCl	Hydrochloric acid
hr	Hour
IPTG	iso-Propyl- -D-thiogalactopyranoside
IPA	iso-Propyl alcohol
k	Kilo
L	Liter
LB	Lysogeny Broth (Luria Broth)
LBA	Luria Broth Agar
M	Molar
m	Meter
µ-	Micro (10^{-6})
m-	Milli (10^{-3})
min	Minutes
Mr	Relative molar mass

mRNA	Messenger RNA
n-	Nano (10^{-9})
NaCl	Sodium chloride
Na_2EDTA	di-Sodium ethylenediamine tetra acetic acid
NaOAc	Sodium acetate
NBT	Nitro-blue tetrazolium chloride
NETME	Natrium Chloride-EDTA-Tris-HCl-Mercaptoethanol
nt	Nucleotide
NTC	Non template control
OS	Orange spotting
p-	Pico (10^{-12})
PAGE	Polyacrylamide gel electrophoresis
PCA	Phenol-chloroform-iso-amyl alcohol
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
ppm	Parts per million
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrrolidone
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RPA	Ribonuclease protection assay
rpm	Revolutions per minute
RT	Reverse transcription
RT-PCR	Reverse transcriptase polymerase chain reaction
SDDW	Sterile double distilled water
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
sec	Second
SSC	Saline-sodium citrate
SSPE	Saline sodium phosphate EDTA
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TEMED	N,N,N'-N'-Tetramethylethylenediamine
U	Unit
UV	Ultra violet
V	Volt
vol	Volume
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
X-gal	5-Bromo-4-chloro-3-indolyl-D-galactosidase

CHAPTER I

GENERAL INTRODUCTION

Viroids are the smallest known pathogens infecting and causing highly contagious diseases in higher plants. Viroids are defined as single stranded, circular and naked small RNA molecules composed of 246-401 nucleotides (Owens, 2008). *Coconut cadang-cadang viroid* (CCCVd), a viroid from the genus *Cocadviroid* in the family *Pospiviroidae* and one of the earliest discovered viroids, is the smallest known viroid with 246 nt length (Flores *et al*., 2005). It is the causal agent of cadang-cadang disease of coconut palm (*Cocos nucifera* L.) in the Philippines (Hanold and Randles, 1991). Cadang-cadang is an uncontrollable and lethal disease that has appeared as the most serious of all known viroid diseases in terms of lethality and it is estimated to have killed over 40 million coconut palms in the Philippines since it was first described in 1914 (Randles *et al*., 2009). The disease which is seemingly confined to the Philippines and has caused widely spread epidemic in this country is under strict quarantine surveillances by neighboring countries and also European authorities.

Experimental and field studies have shown that beside coconut, some other palm and monocotyledonous herbaceous plant species are susceptible and host to the viroid (Imperial *et al*., 1985; Hanold and Randles, 1991; Rodriguez, 1993). African oil palm (*Elaeis guineensis* Jacq.) is susceptible to CCCVd and natural and artificial infection of oil palm with CCCVd occurs (Randles *et al*., 2009). CCCVd has been frequently detected in oil palm plantations in Oceania and South East Asia and in these regions it has been observed to be associated with orange spotting (OS) disorder, also known as genetic orange spotting (GOS) (Hanold and Randles, 1991). Orange spotting disorder has been described since the early 19th century and its effects on growth and yield of oil palm have been well documented. The disorder which was initially described as nutrient deficiency has been recently confirmed to be caused by CCCVd (Joseph, 2012).

In Malaysia, CCCVd has been detected from OS diseased as well as symptomless oil palms and several variants of CCCVd have been characterized from commercial plantations (Vadamalai *et al*., 2006; Joseph, 2012; Wu *et al*., 2013). While it is considered to be a potential threat to oil palm industry, most of the epidemiological aspects of CCCVd infection in oil palm such as mode of transmission and spread in the field, involved vectors, natural host range as well as its potential economic impact are unknown and demand precise investigations. Therefore, considering the lack of information in these fields, the present study is conducted for the below objectives:

- to identify the natural host range of CCCVd
- to isolate and characterize CCCVd small RNAs from oil palm
- to detect CCCVd variants from oil palm by RNA deep sequencing

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