UNIVERSITI PUTRA MALAYSIA

DETECTION AND MOLECULAR CHARACTERIZATION OF PHYTOPLASMA ASSOCIATED WITH COCONUT YELLOW DECLINE

NAGHMEH NEJAT

FP 2009 23

DETECTION AND MOLECULAR CHARACTERIZATION OF PHYTOPLASMA ASSOCIATED WITH COCONUT YELLOW DECLINE

By

NAGHMEH NEJAT

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

October 2009

Dedicated to

All I love

Specially

The soul of my beloved mother in the heaven

who regretfully did not live to see this work.

My beloved father

Sister and brother

For their loving support

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

DETECTION AND MOLECULAR CHARACTERIZATION OF PHYTOPLASMA ASSOCIATED WITH COCONUT YELLOW DECLINE

By

NAGHMEH NEJAT

October 2009

Chairman: Kamaruzaman Sijam, PhD

Faculty: Agriculture

Phytoplasmas have been detected and characterized by molecular methods in coconut palm (*Cocos nucifera* L.) for the first time in Malaysia. Polymerase chain reaction (PCR) assays were used to determine whether a phytoplasma is associated with a yellow decline disease in different coconut ecotypes including Malayan Red Dwarf (MRD), Malayan Yellow Dwarf (MYD) and Malayan Tall (MT) palms. No amplification products were visible from symptomatic samples in first round PCR using phytoplasma universal primer pair P1/P7, but nested PCR with primer pairs R16F2n/R16R2 and fU5/rU3 resulted in amplification of products of approximately 1.2 kb and 890 bp respectively, from 8 out of 20 MRD, 9 out of 12 MYD and 12 out of 12 MT symptomatic palms tested. Sequence analysis of the 16S rDNA PCR products determined that the phytoplasma strain associated with coconut yellow decline (CYD) in MRD and MT ecotypes belongs to the '*Candidatus* Phytoplasma cynodontis' (16SrXIV) group of phytoplasmas. The phytoplasma derived from MYD presented high levels (97%) of homology with the sequences of the '*Candidatus* Phytoplasma trifolii' (16SrVI) group. The virtual RFLP analyses also confirmed that

MRD and MT CYD belongs to the '*Ca.* Phytoplasma cynodontis' group (16SrXIV), whilst MYD CYD does not belong to the identified groups based upon 16S rDNA virtual RFLP analysis.

Nested R16F2n/R16R2 PCR products from 6 spear leaves and 2 inflorescences from MRD palms showed high sequence similarity to the 16S rRNA gene from coconut chloroplasts, with a similar size (approximately 1.3 kb), and a further 5 R16F2n/R16R2 PCR products from MRD infloresences showed high sequence similarities to *Bacillus* spp. and *Bacillus megaterium* 16S rRNA gene sequences. These *Bacillus* PCR products also showed a similar RFLP profile to that obtained from the CYD phytoplasma when the restriction enzyme *Eco*RI was used. Trunk borings were the most reliable source of DNA for phytoplasma detection in coconuts using 16S rRNA gene primers, since there is less co-amplification of PCR products from other organisms when compared to spear leaves and inflorescences.

Real-time PCR using TaqMan probe was developed for sensitive, quantitative and rapid detection of coconut yellow decline (CYD) phytoplasma which is not related to the identified phytoplasma groups. Primers and probe were designed from the highly conserved 16S rRNA gene of CYD phytoplasma. The CYD primers were designed to amplify CYD phytoplasmas in genomic DNA extracts prepared from symptomatic MYD and MRD coconut ecotypes. The selected primers amplify specifically a target 112-bp fragment from the 16S rRNA gene region. The real-time PCR assay reliably detected the CYD phytoplasma in DNA from symptomatic MYD and MRD coconut palm ecotypes with the qCYD 16S probe. The result also shows that the concentration of the pathogen is typically low.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGESANAN DAN PENCIRIAN MOLEKUL FITOPLASMA YANG DIKAITKAN DENGAN PENYAKIT MATIROSOT KEKUNINGAN KELAPA

Oleh

Naghmeh Nejat

October 2009

Pengerusi: Kamaruzaman Sijam, PhD

Fakulti: Pertanian

Fitoplasma telah dikesan dan dicirikan dengan penggunaan teknik molekular bagi buah kelapa (*Cocos nucifera* L.) buat pertama kalinya di Malaysia. Ujian "Polymerase chain reaction" (PCR) telah digunakan untuk mengesan sama ada fitoplasma berhubung-kait dengan penyakit Yellow Decline pokok kelapa termasuk varieti Malayan Red Dwarf (MRD), Malayan Yellow Dwarf (MYD) dan Malayan Tall (MT) di Malaysia. Tiada produk amplifikasi dapat dilihat daripada sampel bersimptom dalam pusingan pertama PCR menggunakan pasangan primer universal phytoplasma P1/P7, tetapi "nested PCR" menggunakan pasangan primer R16F2n/R16R2 dan fU5/rU3 menunjukkan produk amplikasi sekitar 1.2 kb dan 890 bp, untuk 8 daripada 20 sampel MRD, 9 daripada 12 sampel MYD dan 12 daripada 12 sampel Malaysian Tall kelapa bersimptom yang telah diuji. Analisis jujukan 16S rDNA produk PCR menunjukkan strain fitoplasma yang berhubung-kait dengan Coconut Yellow Decline (CYD) di dalam MRD dan MT di Malaysia adalah daripada kumpulan *Candidatus Phytoplasma cynodontis*' (16SrXIV) dan fitoplasma daripada MYD menunjukkan aras homologi yang tinggi (97%) dengan kumpulan jujukan

16SrVI (kumpulan Clover proliferasi, '*Ca. P. trifolii'*). Analisis RFLP juga membuktikan MRD dan MT CYD dipunyai oleh kumpulan '*Ca. Phytoplasma cynodontis'* (16SrXIV), manakala MYD CYD tidak dipunyai oleh kumpulan yang ditemui berpandukan analisis maya RFLP 16S rDNA. Produk nested PCR R16F2n/R16R2 daripada 6 sampel pucuk belum terbuka dan 2 sampel bunga daripada kelapa MRD menunjukkan jujukan yang mempunyai persamaan yang tinggi dengan 16S rRNA gen daripada kloroplas kelapa, dengan saiz yang sama (sekitar 1.3kb), dan produk PCR 5 R16F2n/R16R2 daripada sampel bunga MRD menunjukkan persamaan jujukan yang tinggi dengan *Bacillus* spp. dan *Bacillus megaterium* jujukan gen 16S rRNA. Produk PCR *Bacillus* juga menunjukkan persamaan profil RFLP yang di dapati daripada fitoplasma CYD setelah enzim restriksi *Eco*RI digunakan. Data ini menunjukkan spesimen yang didapati dari batang kelapa dengan menggunakan 'trunk boring' adalah sumber DNA paling baik bagi pengesanan fitoplasma daripada buah kelapa menggunakan 16S rRNA gen primer, kerana kurangnya ko-amplifikasi produk PCR daripada organisma lain berbanding sampel pucuk dan sampel bunga.

Kaedah Real-time PCR menggunakan prob TaqMan telah dihasilkan untuk pengesanan cepat, sensitif dan kuantitatif fitoplasma CYD di mana ianya tidak berkait dengan kelompok fitoplasma yang dikenalpasti. Primer dan prob di rekabentuk daripada gen 16S rRNA CYD fitoplasma. Primer CYD di rekabentuk untuk mengesan fitoplasma CYD daripada penyediaan ekstrak genomik DNA daripada buah kelapa MYD dan MRD yang bersimptom. Primer yang dipilih dikesan secara spesifik pada fragmen 112 bp daripada kawasan gen 16S rRNA. Templat tiruan yang mengandungi klon plasmid fragmen DNA 1240 bp pada 16S rRNA gen

bagi pemencilan MYD CYD, digunakan untuk lengkungan kalibrasi bagi mendapatkan bilangan amplifikasi per sampel. Kaedah real-time PCR dapat mengesan fitoplasma CYD di dalam DNA daripada jenis kelapa MYD dan MRD yang bersimptom dengan prob qCYD 16S. Kaedah Real-time PCR menunjukkan tahap sensitiviti yang tinggi berbanding nested PCR yang biasa digunakan untuk mengesan fitoplasma. Keputusan kajian ini juga menunjukkan kepekatan patogen adalah rendah.

ACKNOWLEDGEMENTS

Most of all, all praises and my endless thanks be to God Almighty most beneficent and merciful for making it possible for me to complete this investigation.

It pleased me to take this opportunity to convey my deepest appreciation and gratitude to my supervisor Associate Professor Dr. Kamaruzaman Sijam for his generous help, invaluable guidance, patience and constant support throughout the completion of this thesis. My grateful appreciation is also due to Associate Professor Datin Dr. Siti Nor Akmar Abdullah for her kind permission to work at her laboratory, valuable advice and helpful suggestions. I especially want to extend my heartfelt thanks and deepest gratitude to Associate Professor Dr. Matthew Dickinson who was incredibly efficient, supportive and excellent in guiding me through this novel experience and his equally generous and scholarly guidance during its development. My deepest thankfulness goes to Professor Phil Jones for his kind cooperation. I wish to extend my sincere gratitude to Dr. Ganesan Vadamalai for his invaluable guidance, constant encouragement, constructive comments, understanding and critical discussion during the research work. I would like to extend my sincere appreciation to Mr. Zakaria Sidek for his support and kind cooperation. I am greatly thankful to the University Putra Malaysia, for granting me the fellowship to pursue this Ph.D programme in Malaysia. I am greatly thankful to Professor Izadpanah, Dr. Soufi and Dr. Salehi for their generous help, encouragement and a boost in my scientific endeavors. I would like to express my profound gratitude and honest thanks to the staff members of Microbiology and Agrobiotechnology laboratories and Plant Protection Department, particularly to Mrs. Junaina for always being so willing to render assistance throughout the course of the study, Mr. Mohd Yassin Yusof for his

help during sample collection, Mr. Shamsudin, Mrs. Sabariah Buang, Mrs. Norah Abdullah, Mr. Shahudin, Mr. Razali, Mrs. Azwana, Miss Rozila and Miss Siti Raziah for their assistance and convenience. I am certainly grateful to Sime Darby plantation in particular Mr. Hamdan Ibrahim and Mr. Mohaimi for valuable help in field and sampling. I wish to express my warmest, sincerest thanks and profound gratitude to my colleagues and friends Mr. Valdiani, Dr. Khakvar, Mr. Omidvar, Dr. Khairulmazmi Ahmad, Mr. Ebrahimi, Mr. Hashemi, Mr. Pishgahi, Mr. Kashiani, Mr. Kamaladiny, Mrs. Shamsi and Dr. Pourvakhshouri for their support and assistance in the research and good wishes.

My profound and heartiest thanks and love to my mother for her love, patience, encourangement and constant support until half of my study. My special and heartiest thanks and love towards my father for being an everlasting source of inspiration, his love, prayers, patience, continuous support and constant encouragement during the entire study period. I am forever indebted to my parents. My heartfelt thanks are extended to my sister and brother for their care, endless emotional and physical support, love and assistance throughout my course of study. My special and deepest thanks towards my grand mother for her love and prayers.

Last but certainly not least, I wish to express my sincere appreciation to all those who not mentioned here that helped me to ensure the completion of my research.

I certify that a Thesis Examination Committee has met on **9 October 2009** to conduct the final examination of Naghmeh Nejat on her thesis entitled "**Detection and molecular characterization of phytoplasma associated with coconut yellow decline**" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the degree of Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Zainal Abidin Mior Ahmad, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Halimi Mohd Saud, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Jugah Kadir, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal examiner)

Chung Jan Chang, PhD
Professor
College of Agricultural and Environmental Sciences
University of Georgia
United States of America
(External Examiner)

BUJANG BIN KIM HUAT, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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:

Kamaruzaman Sijam, PhD
Associate Professor
Deparment of Plant Protection
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Siti Nor Akmar Abdullah, PhD
Associate Professor
Department of Agriculture Technology
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Ganesan Vadamalai, PhD
Department of Plant Protection
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Matthew Dickinson, PhD
Associate Professor
School of Biosciences
University of Nottingham
(Member)

HASANAH MOHD GHAZALI, PhD
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia.

Date: 14 January 2010

DECLARATION

I hereby declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

NAGHMEH NEJAT

Date: 1 February 2010

TABLE OF CONTENTS

		Page
DEDICATION		ii
ABSTRACT		iii
ABSTRAK		v
ACKNOWLEDGEMENTS		viii
APPROVAL		x
DECLARATION		xii
LIST OF TABLES		xvi
LIST OF FIGURES		xvii
LIST OF ABBREVIATIONS		xix
CHAPTER		
I	**INTRODUCTION**	1
II	**LITERATURE REVIEW**	5
	2.1 Coconut palm	5
	2.1.1 Usage of coconut	9
	2.1.2 Health benefits	10
	2.1.3 Nutritional benefits	11
	2.1.4 Coconut diseases	12
	2.2 Phytoplasmas	15
	2.2.1 Phytoplasma discovery	15
	2.2.2 Classification	17
	2.2.3 Taxonomy	19
	2.2.4 General properties of phytoplasma	19
	2.2.5 Host range	21
	2.2.6 Symptoms	21
	2.2.7 Transmissiom	22
	2.2.8 Management and control of phytoplasma associated diseases	23
	2.2.9 Detection methods	25
	2.3 Differentiation and classification of phytoplasmas	29
	2.3.1 Biological characteristics	29
	2.3.2 Serology (ELISA)	30
	2.3.3 DNA hybridization assay	31
	2.3.4 Southern hybridization assay	31
	2.3.5 RFLP analysis of ribosomal RNA	32
	2.3.6 Virtual RFLP analysis	35
	2.3.7 Sequencing and phylogenetic analysis of phytoplasmas	36
III	**DETECTION OF THE CAUSAL AGENT ASSOCIATED WITH YELLOWING OF COCONUT BY THE POLYMERASE CHAIN REACTION (PCR) AND NESTED PCR METHOD**	40

3.1 Introduction 40
3.2 Materials and Methods 42
3.2.1 Sample collection 42
3.2.2 Total nucleic acid extractions from coconut and oil palm tissues 43
3.2.3 PCR analysis 45
3.2.4 Agarose gel electrophoresis 48
3.3 Results 49
3.3.1 Disease symptoms 49
3.3.2 Detection of phytoplasma associated with CYD infected MRD ecotype 52
3.3.3 Nested-PCR Assays with two universal primer pairs 53
3.3.4 Detection of phytoplasma associated with CYD infected Malayan Tall ecotype 55
3.3.5 Detection of phytoplasma associated with CYD infected Malayan Yellow 56
3.4 Discussion 57
3.5 Conclusion 61

IV IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF THE CYD PHYTOPLASMA 62
4.1 Introduction 62
4.2 Materials and Methods 63
4.2.1 Cloning of PCR products 63
4.2.2 Sequence analysis 64
4.2.3 *In silico* restriction enzyme digestions and virtual gel plotting 65
4.3 Results 65
4.3.1 Sequence analysis 65
4.3.2 Amplification of nontarget sequences 68
4.3.3 Virtual RFLP Analysis 71
4.4 Discussion 75
4.5 Conclusion 78

V REAL-TIME PCR ASSAY FOR SENSITIVE AND ACCURATE DETECTION OF THE CYD PHYTOPLASMA 80
5.1 Introduction 80
5.2 Materials and Methods 80
5.2.1 Source of phytoplasma for TaqMan real-time PCR assay 80
5.2.2 Design of real-time PCR primers and probe 81
5.2.3 Preparation of plasmids 82
5.2.4 Real-time PCR amplification conditions 82
5.2.5 Specificity 83
5.3 Results 84

5.3.1 Real-time PCR 84
5.3.2 Real-time PCR sensitive detection and quantification of phytoplasmas in plant samples 84
5.3.3 Inhibitors 86
5.3.4 Sensitivity 86
5.3.5 Specificity 87
5.4 Discussion 87
5.5 Conclusion 89

VI SUMMARY, GENERAL CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH 90

REFERENCES 98
APPENDICES 123
BIODATA OF STUDENT 158
LIST OF PUBLICATIONS 159

LIST OF TABLES

Table		Page
5.1	Oligonucleotide primers and TaqMan probe sequences used for phytoplasma detection	82
5.2	Real-time PCR , nested PCR and conventional PCR results on healthy and infected CYD MRD and MYD and Malaysian periwinkle virescence infected sample	85

LIST OF FIGURES

Figure		Page
2.1	Phylogenetic relationships of several bacterial clades containing bacterial pathogens (A), and the 5 phylogenetic groups within the Class *Mollicutes* (B). Plant pathogenic / symbiotic bacteria are indicated in green. GL, gene loss; WL, loss of cell wall	18
3.1	A) The foliage turns light-yellow and eventually light-brown. B)Yellowing of the lower canopy starting with the oldest fronds and spreading rapidly to the younger ones and emerging spear leaf. C) Inflorescence necrosis. D) Premature nut fall.	50
3.2	Rot and collapse of the spear leaf in an immature palm	51
3.3	Amplification of coconut CYD infected samples with P1/P7 primers. Lanes (M) 1kb DNA ladder; (1) water control; (2,3,4) Coconut yellow decline infected samples; (5) Malaysian periwinkle virescence infected sample.	52
3.4	Amplification of CYD and other phytoplasma rRNA gene products with nested PCR primers (a) R16F2n/R16R2 and (b) fU5/rU3. (a and b) Lanes (M) 1kb DNA ladder; (1) Healthy coconut palm; (2,3) Coconut yellow decline infected sample; (4) Bermudagrass white leaf; (7) water control. (a) (5) Aster yellows; (6) Cape St Paul wilt; (b) (5) Cape St Paul wilt; (6) Aster yellows	54
3.5	Amplification of PCR products with nested PCR primers R16F2n/R16R2 from healthy MRD coconut samples. Lanes (M) 1kb DNA ladder; (1-3) Inflorescence samples; (4-6) Midribs of spear leaf samples.	55
3.6	Amplification of CYD from MT ecotype with nested PCR primers R16F2n/R16R2. Lanes (M) 1kb DNA ladder; (1) Malaysian periwinkle virescence infected sample; (2-6) Coconut yellow decline infected samples; (7) Healthy coconut palm.	56
3.7	Amplification of CYD from MRD ecotype with nested PCR primers R16F2n/R16R2. Lanes (M) 1kb DNA ladder; (1-3) Coconut yellow decline infected sample; (4) Malaysian periwinkle virescence; (5) Healthy coconut palm.	57
4.1	Dendrograms, constructed by the Neighbor-Joining method, showing the phylogenetic relationships between the Malaysian	

coconut yellow decline phytoplasmas with 40 phytoplasmas and *A. laidlawii* as out group constructed based on 16S rRNA gene sequences 67

4.2 Nucleotide sequence of fU5/rU3-primed rDNA product amplified from MRD from Serdang 68

4.3 Sequence of R16F2n/R16R2-primed rDNA product amplified from inflorescence of MRD 70

4.4 Sequence of R16F2n/R16R2-primed rDNA product amplified from inflorescence of MYD 70

4.5 RFLP analysis using *Eco*RI from cloned R16F2n/R16R2 PCR samples in the pCR2.1 vector. Lanes (M) 1 kb DNA ladder; (1-5) chloroplast rDNA from spear leaves; (6-8) *Bacillus* rDNA from inflorescences; (9) chloroplast rDNA from an inflorescence; (10-12) CYD phytoplasma rDNA from trunk borings. The large band of approx 4 kb is the pCR2.1 plasmid DNA and the smaller bands are from the rDNA PCR products 71

4.6 Virtual RFLP analysis with *Alu*I, *Hha*I, *Hinf*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, and *Taq*I restriction enzymes. Lanes 1: CYD from MRD ecotype (EU328159); 2: CYD from MT ecotype (EU636906); 3: CYD from MYD ecotype (EU498727); 4: oil palm phytoplsma (EU498728); 5: *Ca.* P. asteris (16SrI) [M30790] ; 6: Coconut lethal yellowing (16SrIV) [AF498307]; 7: *Ca.* P. Oryzae (16SRXI) [AB052873]; 8: *Ca.* P. cynodontis (16SrXIV) [AJ550984]; 9: Lethal decline Nigeria (16SrXXII) [Y14175]. M: phX174DNA-*Hae*III Digest DNA MW-marker 75

5.1 Standard curve used to quantify CYD phytoplasma based on the Ct-values obtained by the Taqman probe PCR assays, using 10-fold serial dilutions of plasmid as template by real-time PCR with primer/probe set qCYD-16S and triplicates, and quantification analysis of DNA extracted from MRD and MYD coconut ecotypes showing symptoms of CYD 85

5.2 Real-time PCR products after electrophoresis. Lanes (M) DNA ladder mix; (1,2,3 and 4) Coconut yellow decline infected MRD; (5,6,7 and 8) Coconut yellow decline infected MYD; (9) Healthy coconut palm 86

LIST OF ABBREVIATIONS

bp	Basepair
BLAST	Basic Local Alignment Search Tool
CTAB	Cetyltrimethyl-ammonium bromide
CYD	Coconut yellow decline
dNTP	Deoxyribonucleotides (dATP, dCTP, dGTP, dTTP)
EDTA	Ethylene diaminetetraacetic acid, disodium salt
kb	Kilobase
LY	Lethal yellowing
MLO	Mycoplasmalike organism
MRD	Malayan red dwarf
MT	Malayan tall
MYD	Malayan yellow dwarf
NCBI	National Center for Biotechnological Information
PCR	Polymerase chain reaction
PVP	Polyvinylpyrrolidone
RFLP	Restriction fragment length polymorphism
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
UV	Ultraviolet
W/V	Weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

CHAPTER I

INTRODUCTION

The coconut palm (*Cocos nucifera* L.) is a member of the family Arecaceae (*Palmae)*, subfamily Cocoideae which includes 27 genera and 600 species. The sole species of the genus *Cocos* is coconut (Uhl and Dransfield, 1987). It includes more than 300 cultivars or varieties (Lebrun *et al.,* 1998). Coconut palm has different ecotypes or varieties such as Malayan yellow dwarf, Malayan green dwarf, Malayan red dwarf and Malayan tall in Malaysia. The precise coconut origin is not known for sure, but many historians believe Malaysia and Indonesia grew the world's first coconuts (Coconut, the Soul Food of the Tropics, 2003). Due to its popularity as a useful plant, it is known as 'King of Palms,' "tree of life" and *kalpa vriksha,* which translates as "tree that gives all that is necessary for living." Considered the most useful tree in the world, coconut has been used as a source of food, drink, clothing, shelter, heirloom history, and financial security for at least half a million years. It has been cultivated by man for 4000 years. The main products are coconut oil that is derived from dried kernel of the nut, coconut milk and copra. Coconut palm provides sustainable income to millions who are directly and indirectly dependant on coconut. The main source of food and income for about 10 million families relies on coconuts (IPGRI, 2004; Srinivisulu and Raghava Rao, 2007).

Coconut palm, despite its hardy nature and adaptability to different soil conditions, often succumbs to different diseases caused by fungi, bacteria, phytoplasmas, viruses and viroids (Harrison and Jones, 2003; Srinivisulu and Raghava Rao, 2007). Some of the most destructive diseases of coconuts such as cadang-cadang in the Philippines

and lethal yellowing in the Africas and Americas are amongst the most notorious plant diseases in the world (Dabek *et al.,* 1976; Del Rosario and Quiaoit, 1962; Harrison *et al.,* 1999; Ocfemia, 1937). Lethal yellowing (LY) is the most important coconut disease in terms of economic loss. This disease poses the most important threat to global palm and coconut production. Phytoplasmas are the causal agent of lethal yellowing disease (OEPP/EPPO).

The phytoplasmas (International committee on systematic bacteriology subcommittee on the taxonomy of mollicutes, 1993), which were originally described as mycoplasma-like organisms (MLO) (Doi *et al*., 1967), are a group of plant pathogenic bacteria that cause devastating damage to plants. Phytoplasmas are derived from Gram-positive bacteria which lack cell wall, have low G+C and are classified in the class *Mollicutes*, along with mycoplasmas, ureaplasmas, acheloplasmas and spiroplasmas (Agrios, 1997). Phytoplasmas constitute a unique, monophyletic clade of mollicutes more closely related to *Acholeplasma* than to the true *Mycoplasma* species based on sequence analysis of 16S ribosomal DNA (rDNA) and ribosomal protein genes (Gunderson *et al*., 1994; IRPCM, 2004).

Phytoplasmas are systemic pathogens of important crops and cause diseases in more than 700 plant species belonging to 100 families. Phytoplasmas cause a wide variety of symptoms on infected plants that range from mild yellowing to death. Characteristic symptoms of phytoplasma infection include virescence and phyllody on herbal plants, poor taste and small sized fruits on infected trees and sterility of flowers, chlorosis of leaves, leaf curving, proliferation of axillary shoots, witches' broom, stunting and general decline on both herbal plants and trees (Heinrich *et al*.,

2001; McCoy *et al*., 1989). They are transmitted by phloem feeding insect vectors such as leafhoppers, some psyllids and planthoppers in a persistent propagative manner (Kirkpatrick, 1992; Lee *et al*., 2000; McCoy *et al*., 1989). Phytoplasmas are insect transmitted plant pathogens that are found exclusively in the phloem vessels of plants and they are not known to survive outside either the plant or insect hosts. They cannot be grown *in vitro* in cell-free media, unlike most of the other members of the *Mollicutes*. This has been the greatest barrier to characterizing these economically important plant pathogens. Despite their economic importance and unique biological features, phytoplasmas as plant pathogens remain the most poorly characterized (Namba *et al.,* 2005).

Heterogenous distribution and low concentrations of phytoplasmas in the plant and presence of putative inhibitors in phytoplasma-infected plant material also make their detection and identification difficult (Heinrich *et al*., 2001; Seemüller *et al*., 1998). The study of phytoplasmas by using molecular biological techniques has opened up new avenues for detection and diagnostics. The application of PCR for amplification of 16S rDNA from phytoplasmas provides a much more sensitive detection method than any other yet described. Oligonucleotide primers based on *Mollicutes* 16S rRNA genes have been used for specific detection of phytoplasmas in phytoplasma DNA mixtures with host DNA (Ahrens and Seemüller, 1992; Davis *et al*., 1992; Deng and Hiruki, 1990, 1991a and b; Lee *et al*., 1993b; Namba *et al*., 1993a). In some cases, including where there are low titres of the phytoplasma or PCR inhibitors present in the host, which are extracted along with the phytoplasma DNA, a single series of PCR with as many as 35-40 cycles may not be sufficient to detect the phytoplasma. In such cases, nested PCR in which a double round of amplifications is performed is

required. Recently, real-time PCR has been used to develop accurate, highly sensitive and specific assays to be employed in Apple proliferation destructive phytoplasmas detection and quantification (Baric and Dalla-Via, 2004).

No studies have been done on the causal agent associated with yellowing in coconut palm in Malaysia. In order to understand and manage the disease, ethiological studies and identification of the causal phytoplasmas and their characteristics is important. Therefore, inspection of commercial coconut production areas, collection of suspected samples with yellowing symptoms and surveying them by advanced molecular methods is necessary.

Therefore, the objectives of this study were:

1. To isolate and detect phytoplasma from coconut suspected yellowing by PCR and nested PCR with universal phytoplasma primers.
2. To classify phytoplasmas associated with disease of coconut palms in Malaysia, based on analysis of 16S rRNA gene operon sequences and virtual RFLP.
3. More specific and sensitive detection by real time PCR (qPCR) using TaqMan probe.

CHAPTER II

LITERATURE REVIEW

2.1 Coconut palm

The scientific name for coconut is *Cocos nucifera*. Based on the three little eyes at the base of the coconut's inner shell that reminded them of a goblin or grinning face, Spanish and Portuguese explorers named them *coco,* the word for goblin. The word coco has been translated to mean monkey face. Samuel Johnson's *Dictionary of the English Language* spelled the fruit cocoanut, Published in 1755. Then, the "a" was left out. Nucifera means "nut-bearing."

The coconut palm (*Cocos nucifera* L.) belongs to the family Arecaceae (*Palmae)*, subfamily Cocoideae which includes 27 genera and 600 species, and includes a large assemblage of monocotyledonous plants with a slender, unbranched stem and a crown of compound (pinnate or palmate) leaves. Coconut is the only species of the genus *Cocos*. It is diploid with 32 chromosomes (2n=32) (Uhl and Dransfield, 1987). As such, hybridization is mainly intraspecific. It consists of more than 300 cultivars or varieties (Lebrun *et al.,* 1998).

The exact coconut origin is not known for sure, but many historians believe Malaysia and Indonesia are the countries where coconut originated. Based on the long association of coconuts with agriculture and religions, and the presence of many varieties of coconut in the Asia-pacific region, some historians believe that the origin of coconut was in the Asia-Pacific region, more specifically the Malayan Archipelago (Menon and Pandalai, 1958). Today coconut cultivation encircles the