CHARACTERIZATION AND DETECTION OF AND PHYLOGENETIC RELATIONSHIPS AMONG XANTHOMONAS ORYZAE PV. ORYZAE ISOLATES FROM SELECTED RICE VARIETIES IN PENINSULAR MALAYSIA

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By

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

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This work is dedicated to: *All I love Specially*

To the soul of my parents, mybeloved mother and father

In the heaven who regretfully did not live to see this work.

To my beloved wife Fariba for her help and her support, my sons, Ahmadreza and Omidreza

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

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Rice (*Oryza sativa*) is one of the most important food crops in the world and the major crop of Malaysia after oil palm. Bacterial leaf blight (BLB) disease caused by *Xanthomonas oryzae* pv *oryzae* (*Xoo*) is the most important disease in Malaysia. Paddy leaves or stems showing symptoms of disease were collected from rice fields in Penang, Kedah, Selangor, and Melaka in Peninsular Malaysia during the period from 2008 to 2010. The isolated bacteria were found to be oxidase negative, gram negative, anaerobic growth negative and did not produce fluorescent pigment. Thirty collected strains showed leaf blight symptoms on inoculated rice plants, hypersensitive reaction on tobacco, and hydrolysis on gelatin. Pathogenic tests on two rice varieties MR84 and IR8 showed that significant differences were observed between 30 strains. All strains of *Xoo* were classified into four groups, i.e. non-

virulent, slightly virulent, moderately virulent and highly virulent. Seven strains, including MXO1 and SXO8 were highly virulent, five strains (including SXO1 and MXO6) were moderately virulent, while 17 strains represented by PXO43 and KXO182 showed low virulence, and the only non-virulent strain was PXO36. It can be concluded that there is a relation between location and severity of disease that the most and the lowest severity was related to the strains of Melaka and Penang respectively and also the strains from the different rice-growing areas or states were diverse and differed in their degree of virulence. For detection of *Xoo*, infected tissues were crushed and the DNA was extracted using a modified CTAB method. A PCR product of about 470 bp was produced using the XOR-F/XOR-R2 primer pair that amplified the 16S-23S intergenic rDNA sequence region. Comparison indicated that the 30 strains have 98% to 99% nucleotide homology to the sequences of 16S-23S rDNA of *Xoo* accessions ABO26287 and AY251OO4 from Japan and China, respectively. Another PCR product of about 964 bp was generated by the primer pair TXT/TXT4R that amplified the insertion sequence element IS1113 and had 99% homology to the sequences of the *Xoo* accessions GU982970 and AF482989 from India and USA, respectively. According to these results, the causal agent of bacterial leaf blight of rice could be identified in less than three hours. Phylogeny analysis among Malaysian strains indicated genomic diversity of *Xoo* strains exist throughout Malaysia. Additionally, strains of *Xoo* could be divided into two main groups, namely Penang– Kedah and Selangor-Melaka that are neighboring states respectively an indication that geographical area is the predominant factor influencing strain variability. And also in the states the diversities of *Xoo* collected from rice cultivars generally were similar. The generated banding patterns resulting from the amplification of DNA templates of the 30 *Xoo* strains by REP, ERIC and BOX

primers were reproducible as was proven with at least three PCR replications, suggesting the suitability of using these primers as markers for genetic studies of *Xoo*. There was a slight difference between the phylogenetic tree patterns derived from respective REP, ERIC and BOX primer sets. The primers generated four common amplification bands in all strains tested, of the sizes 200, 400, 500 and 800 bp. Cluster analysis based on REP, ERIC and BOX primer sets and the pooled data resulting from all three primers (BER primer) indicated that the 30 *Xoo* strains were divided into two main groups with similarity between about 60% or into four sub-groups, representing strain from Penang, Kedah, Selangor and Melaka. Based on these results, there were significant similarities among 30 strains of *Xoo* that have been collected from the same origin in Malaysia, indicating that *Xoo* strains originating from different geographic regions in Peninsular Malaysia were phylogenetically different. Hence, this research indicated that strains of *Xoo* isolated from Malaysia are pathologically, genetically and geographically diverse.

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

PENCIRIAN, PENGESANAN DAN HUBUNGAN FILOGENI STRAIN *XANTHOMONAS ORYZAE* PV. *ORYZAE* DARI TANAMAN PADI DI SEMENANJUNG MALAYSIA

Oleh

KAVOUS KESHAVARZI

Januari 2012

Pengerusi: Profesor Madaya Dr. Kamaruzaman Sijam, PhD

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Padi (*Oryza sativa*) merupakan salah satu tanaman makanan penting dunia dan merupakan juga tanaman makanan utama Malaysia selepas kelapa sawit. Tanaman ini sering dijangkiti beberapa penyakit kulat dan bakteria termasuk penyakit hawar daun bakteria (BLB) yang disebabkan oleh *Xanthomonas oryzae* pv *oryzae* (*Xoo*) atau sebelum ini dikenali sebagai *Xanthomonas campestris* pv *oryzae*. Daun atau batang padi yang menunjukkan simptom penyakit hawar telah dikumpul dari sawah padi di Semenanjung Malaysia dari negeri-negeri Pulau Pinang, Kedah, Selangor dan Melaka dari tahun 2008 hingga 2010. Pengkulturan koloni bakteria yang dipencil telah dilakukan. Bakteria yang dipencil didapati bersifat oxidase negatif, Gram negatif, mempunyai pertumbuhan anaerobik negatif dan tidak menghasilkan pigmen pendarfluor. Tiga puluh isolat *Xoo* yang dikumpul telah memberikan simptom penyakit hawar daun bakteria pada tanaman padi yang diinokulat serta memberikan tindak balas hipersensitif ke atas pokok tembakau dan hidrolisis pada gelatin. Ujian

patogenik pada varieti padi MR84 dan IR8 menunjukkan bahawa strain-strain bakteria tersebut boleh dibahagikan kepada empat kumpulan atau strain berdasarkan kapada tahap kevirulenan mereka. Tujuh strain termasuk MXO1 dan SXO8 menunjukkan tahap kevirulenan yang sangat tinggi. Sebaliknya lima strain (termasuk SXO1 dan MXO6) menunjukkan tahap kevirulenan sederhana, manakala 17 strain yang diwakili oleh PXO43 dan KXO182 menunjukkan tahap kevirulenan rendah, dan PXO36 pula merupakan satu-satunya strain yang tidak virulen. Keputusan juga menunjukkan bahawa strain yang dikumpul dari kawasan geografi yang berlainan adalah berbeza dari segi kevirulenan mereka ke atas tanaman padi. Untuk pengesanan serta-merta jangkitan *Xoo,* sedikit tisu yang dijangkiti telah dihancurkan dan DNA diekstrak dengan menggunakan kaedah CTAB yang diubahsuai. Produk PCR bersaiz 470 bp telah dihasilkan dengan menggunakan pasangan primer XOR-F/XOR-R2 yang mengamplifikasi jujukan intergenik 16S-23S rDNA. Perbandingan menunjukkan bahawa 30 strain berkenaan mempunyai tahap homologi nukleotida antara 98% hingga 99% kepada jujukan intergenik 16S-23S rDNA strain-strain *Xoo* ABO26287 dan AY251OO4 dari Jepun dan China, setiap satunya. Satu lagi produk PCR bersaiz 964 bp telah juga dijana oleh pasangan primer TXT/TXT4R yang mengamplifikasi unsur jujukan IS1113 yang mempunyai tahap homologi setinggi 99% dengan jujukan strain-strain GU982970 dan AF482989 dari India dan Amerika Syarikat, setiap satunya. Menurut keputusan ini, agen penyebab penyakit bakteria hawar daun padi telah dapat dikenalpasti sebagai *Xoo* dalam masa kurang daripada tiga jam penganalisaan. Analisa filogenetik menunjukkan keujudan kepelbagaian genomik diantara strain-strain *Xoo* di Malaysia. Strain-strain *Xoo* dapat dibahagikan kepada dua kumpulan utama negeri berjiran iaitu Penang-Kedah dan Selangor-Melaka, yang menunjukkan bahawa kawasan geografikal merupakan faktor penentu

utama dalam kepelbagaian strain. Strain-strain dari sesuatu negeri tidak menunjukkan perbezaan yang ketara dintara mereka. Corak penghasilan"band" dari amplifikasi templat DNA dari 30 strain *Xoo* dengan menggunakan primer-primer REP, ERIC dan BOX adalah konsisten dan boleh dihasil semula. Ini menyarankan kesesuaian penggunaan pasangan-pasangan primer berkenaan dalam kajian genetik *Xoo*. Terdapat sedikit perbezaan antara corak pokok filogenetik yang bersandarkan dari data REP, ERIC atau set primer BOX. Penggunaan primer-primer tersebut telah menghasilkan empat jujukan amplifikasi yang serupa dari semua strain yang dikaji iaitu yang bersaiz 200, 400, 500 dan 800 bp..Analisis kluster berdasarkan hasilan primer-primer REP, ERIC, BOX dan hasilan data dari ketiga-tiga pasangan primer yang disatukan (BER primer) menunjukkan bahawa 30 strain *Xoo* boleh dibahagikan kepada dua kumpulan utama dengan kesamaan diantara mereka pada tahap 60% atau kapada empat sub-kumpulan mewakili strain dari Pulau Pinang, Kedah, Selangor dan Melaka. Berdasarkan kepada keputusan ini bolehlah disimpulkan bahawa ujud ketaksamaan diantara 30 strain *Xoo* yang dikumpulkan dari lokasi berbeza di Semenanjung Malaysia. Strain dari setiap negeri dikelompokkan bersama. Strain-strain dari negeri jiran mempunyai tahap persamaan yang tinggi jika dibandingkan dengan strain dari negeri yang berjauhan, yang menunjukkan bahawa strain-strain *Xoo* yang berasal dari kawasan geografikal yang berlainan adalah berbeza secara filogenetik. Penyelidikan ini telah berjaya menunjukkan kepelbagaian strain *Xoo* yang dipencilkan dari Malaysia berdasarkan kapada aspek-aspek patologi, genetik dan kawasan geografikal.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
BLB	Bacterial leaf blight
BOX	Single primer sequence
bp	Base pair
CFU	Colony forming unit
CTAB	Cetyl trimethyl-ammonium bromide
DNA	Deoxyribonucleic acid
DNTP	Deoxyribonucleotides (dATP, dCTP, dGTP, dTTP)
EDTA	Ethylene diaminetetraacetic acid, disodium salt
ELISA	Enzyme linked immunosorbent Assay
ERIC	Enterobacterial repetitive intergenic consensus
NCBI	National Center for Biotechnological Information
PCR	Polymerase chain reaction
PVP	Poly vinyl pyrrolidone
RAPD	Randomly Amplified Polymorphic DNA
rDNA	Ribosomal DNA
REP	Repetitive extragenic palindromic elements sequence
RFLP	Restriction fragment length polymorphism
rpm	Revolution per minute
rRNA	Ribosomal RNA
TBE	Tris Boric acid EDTA
UV	Ultraviolet
W/V	Weight/volume

Xoo	*Xanthomonas oryzae* pv *oryzae*

CHAPTER 1

INTRODUCTION

Rice (*Oryza sativa* L) is one of the most important food crops in the world, being the staple diet of about half of the world population (Akhtar et al., 2008). This plant belongs to the grass family, and is related to other grass species such as wheat, oats and barley, which together produce food grains known as cereals. Grist, (1965) postulated that the cultivated species may have developed from species of wild rice. It was reported that at least twenty five wild rice species were well distributed in the tropical and subtropical regions in Asia, Africa, America and Northern Australia (Grist, 1965).

The world rice supply had more than doubled from 261 million tonnes in 1950 to 673 million tonnes in 1997. Rice production is expected to increase by 50 percent by the year 2025 (Khush and Virk, 2000). World rice production in 2004 was just less than 610 million tonnes. At least, 114 countries grow rice and more than 50 countries have an annual production of about or more than 100,000 tonnes. Asian farmers produce about 90% of the world's rice due to favorable hot and humid climate in their regions, with two countries, namely China and India, growing more than half of the total crop (International Rice Research Institute, 2005). Interestingly, less than 5% of the total rice production in the world is traded in the international market (Hossain, 1997) and because of that rice is considered as a security crop or commodity in many countries in Asia.

Rice cultivation has a long history in Malaysia. It is believed that rice had spread down from its original centre of origin during the period of 4000-5000BC (Abdullah et al., 1991: Othman et al., 1990) and became established as one of its center of diversity for

the species. Currently, rice has become the second major crop in Malaysia after oil palm. It is an important part of the diet of the people of Malaysia. The total planted area is about 667,310 ha, and the total rice production is about 2,030,000 tonnes (MOA, 2005).

However, the rice crop is frequently infected by several fungal and bacterial diseases that affect its production. One of the important diseases of rice is the bacterial leaf blight (BLB) disease caused by *Xoo* (Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae). It is one of the most important bacterial diseases of rice, which usually infect the crop during the monsoon season (from June to September) in the south-east Asian and Indian subcontinent countries (Mew, 1987; Mew et al., 1993). Bacterial leaf blight was first detected in Japan in 1884, but was only identified as a disease in 1922 (Mew et al., 1993). The disease then spread to all continents of the world, except Europe (Ezuka and Kaku, 2000; Anonymous, 2007; Ghasemie et al., 2008). Rice is the main host of *Xoo*, which is also a seed-borne bacterium. The disease spreads through dispersion of plant straw, wind, rain, hail, wild rice, weeds (Nino-Liu et al.,2006), irrigation water and seeds (Nyvall, 1999). Bacterial inoculums enters the plant tissues either through wounds or water pores in the leaf and then travels systemically throughout the plant xylem (Nino-Liu et al., 2006). Infected plants show leaves wilting and rolls up, turning grayish-green to yellow, until the whole seedling dies. Surviving older plants have dried leaf blades. Lesions begin as water-soaked stripes on the leaf blades and eventually increase in length and width, turning yellow to grayish-white until the whole leaf dries up (Agrios, 1997). Infected plants also show either streaks or leaf blight symptoms (Akhtar et al., 2003). Infection by *Xoo* could cause yield loss of up to 50% in tropical Asia (Anonymous, 2007). In 1988 and 1994,

serious *Xoo* outbreaks in Malaysia were reported in the states of Penang, Kedah, Selangor and Perak, where more than 40% of planted areas were infected with the disease, causing an estimated yield loss of about 10-50% (Saad et al., 2003).

Biochemical tests (Ghasemie et al., 2008), pathogenic races of *Xoo* (Adhikari et al., 1999), fatty acids (Chase et al., 1992) and metabolic profiling (Jones et al., 1993) have been employed as tools for detection of the pathogen. The genome of a *Xanthomonas* strain, KAC10331 has been sequenced (Dharmapuri and Sonti, 1999). Several researchers have used polymerase chain reaction (PCR) technology as a tool to detect and study the variability of the pathogenic bacteria (Li and De Boer, 1995). The 16S ribosomal DNA sequence (16S rDNA) has been used for deducing phylogenic and evolutionary relationships among bacteria and other prokaryote species (Weisberg et al., 1991). Adachi and Oku (2000) used the XOR-F and XOR-R2 primer pair to amplify a fragment of the intergenic spacer region, 16S-23S rDNA, of *Xanthomonas* species. On the other hand, the TXT and TXT4R primer pair has been used to amplify the insertion sequence element IS1113 of *Xanthomonas oryzae* pathovars (Sakthivel et al., 2001).

Another PCR based technique, known as Rep-PCR has also been used in the identification and classification of bacteria (Versalovic et al., 1991). It is a repetitive sequence based genomic fingerprinting that uses primers that correspond to the endogenous interspersed repetitive sequences of the bacteria. These interspersed sequences are highly conserved elements in the prokaryotic genomes. Sequences of these elements have been extensively characterized in several prokaryotic microorganisms (Versalovic et al., 1991). Families of repetitive DNA sequences, like repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus

(ERIC), and single primer (BOX) elements, which are present in all prokaryotes, could be used for bacterial fingerprinting, and have been used effectively for analysis of several species of bacteria (Louws et al., 1994; Louws et al., 1999). As for the *Xanthomonas* species, rep-PCR was used to assess variations among several pathovars and has been shown to have low levels of intrapathovar diversity (Louws et al., 1995; Vauterin et al., 2000).

The occurrence of *Xoo* has been reported in Malaysia (Saad et al., 2003; Saad and Habibuddin, 2010), but information on the diversity of *Xoo* causing pathogen at the molecular level is still lacking. The present research was designed to focus on the detection and confirmation of *Xoo* infection using PCR method on samples of leaves and stems collected from rice fields. The study was also conducted to determine the diversity of Malaysian strains of *Xoo*, based on sequences of the intergenic region of 16S-23S rDNA and the insertion sequence element, IS1113 of the bacterial genome. In addition to that, this study was also designed to assess the phylogenetic relationships between local populations of Xoo strains from rice fields in Peninsular Malaysia using REP, ERIC and BOX PCR primers. The specific objectives of the study were to:

1. Characterize phenotypes of *Xanthomonas oryzae* pv *oryzae* from rice plants in Peninsular Malaysia.
2. Detect and identification *Xanthomonas oryzae* pv *oryzae* using universal and specific primers.
3. Investigate the diversity among the strains of *Xanthomonas oryzae pv oryzae* using molecular markers.

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