

การโคลนและลักษณะการแสดงออกที่แตกต่างของยีนในเชื้อ *Pythium insidiosum*
สายพันธุ์ประเทศไทย



นางสาวพัชรี กัมมารเจษฎากุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาจุลชีววิทยาทางการแพทย์ (สหสาขาวิชา)
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ปีการศึกษา 2552
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**CLONING AND CHARACTERIZATION OF GENES DIFFERENTIALLY
EXPRESSED IN *PYTHIUM INSIDIOSUM* THAI STRAIN**

Miss Patcharee Kammarnjassadakul

**A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Medical Microbiology
(Interdisciplinary Program)**

Graduate School

Chulalongkorn University

Academic Year 2009

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522341

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THAI STRAIN

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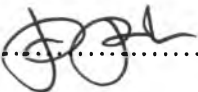
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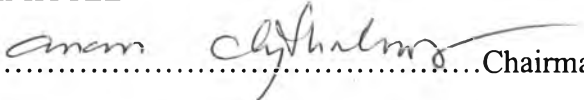
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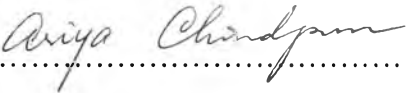
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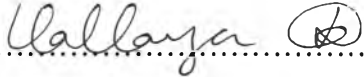
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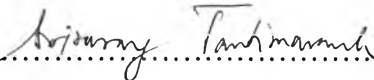
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พัชรี กัมมารเจษฎากุล : การโคลนและลักษณะการแสดงออกที่แตกต่างของยีนในเชื้อ *Pythium insidiosum* สายพันธุ์ประเทศไทย (CLONING AND CHARACTERIZATION OF GENES DIFFERENTIALLY EXPRESSED IN *PYTHIUM INSIDIOSUM* THAI STRAIN) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. อริยา จินคามพร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.ดร.ธนาภัทร ปาลกะ และ อ.ดร.กัลยาณ์ ศรีธัญญลักษณ์ - แดงดีป, 235 หน้า

Pythium insidiosum เป็นเชื้ออับติใหม่อาศัยอยู่ในน้ำมีลักษณะคล้ายเชื้อรา เป็นสาเหตุของโรค pythiosis พบในคนและสัตว์ บริเวณเขตร้อนจนถึงร้อนชื้น มีรายงานพบผู้ป่วย และเสียชีวิตจากโรคนี้อันตรายที่สุดจากประเทศไทย อย่างไรก็ตามข้อมูลเกี่ยวกับตัวเชื้อทางด้านพันธุกรรมและพยาธิสภาพการเกิดโรคนี้น้อยมาก งานวิจัยในครั้งนี้จึงมีวัตถุประสงค์ในการหาฮินจากเชื้อที่เจริญ ณ อุณหภูมิ 37°C น่าจะมีความสัมพันธ์กับการเกิดพยาธิสภาพของโรค การค้นหายีนดังกล่าวอาศัยวิธี PCR – select cDNA subtraction จากเชื้อที่เพาะ ณ อุณหภูมิ 27°C และ 37°C และจากข้อมูลด้านพันธุกรรมของเชื้อก่อโรคที่คล้ายคลึงจากฐานข้อมูลใน GenBank จากวิธี subtraction พบ 606 โคลนจาก subtracted cDNA libraries ของทั้งสองอุณหภูมิ เมื่อนำไปวิเคราะห์ลำดับเบสด้วยโปรแกรม BLASTN พบโคลนส่วนใหญ่มีลำดับเบสคล้ายกับ mitochondria ของกลุ่มราน้ำ และการวิเคราะห์การทำงานของโปรตีนด้วยโปรแกรม Swiss-Prot และ BLASTX พบ candidate genes ที่ควบคุมการทำงานของโปรตีนหลายชนิด ในที่นี้ผู้วิจัยได้ทำการศึกษาการแสดงออกของยีนที่ควบคุมการทำงานของ cytochrome c oxidase subunit II (*COX II*) β -tubulin (*TUB*) และ chitin synthase subunit II (*CHI II*) โดยอาศัยวิธี semi – quantitative PCR และยืนยันผลการศึกษาด้วยวิธี real time RT – PCR ในเชื้อจำนวน 16 สายพันธุ์ ที่เป็นตัวแทนของกลุ่มเชื้อที่มาจากแหล่งต่างกันได้จากการจัด phylogenetic distribution ผลการศึกษาพบยีน *COX II* เท่านั้นที่มีการแสดงออก ณ อุณหภูมิ 37°C สูงกว่า 27°C ถึง 2.5 เท่าโดยเฉลี่ย จัดว่ามีความแตกต่างอย่างมีนัยสำคัญ ($p = 0.0347$) และไม่พบการแสดงออกของยีน *TUB* และ *CHI II* ที่แตกต่างกันระหว่างสองอุณหภูมิ จากข้อมูลดังกล่าวนี้ชี้ให้เห็นว่าสามารถนำยีน *TUB* และ *CHI II* เป็น housekeeping gene ในการศึกษาการแสดงออกของยีนที่ไวต่ออุณหภูมิที่เปลี่ยนแปลงได้ สำหรับการแสดงออกของยีน *CHI II* ที่ 37°C พบว่าขึ้นกับแหล่งที่มาของเชื้อ นอกจากนี้ทางผู้วิจัยยังได้นำ *COX II* มาประยุกต์ใช้ในการศึกษา Phylogenetic relationship พบว่า *COX II* สามารถใช้ศึกษา Phylogenetic distribution ของเชื้อ *P. insidiosum* ได้นอกเหนือ ITS ที่มักนิยมใช้ โดยสรุปผลการศึกษาเป็นรายงานแรกที่แสดงว่า การแสดงออกของ *COX II* ที่ อุณหภูมิ 37°C สูงกว่า 27°C สำหรับความสัมพันธ์ระหว่างยีน *COX II* กับการก่อให้เกิดพยาธิสภาพ จำเป็นต้องศึกษาเพิ่มเติม

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิติ..... พชรี กัมมารเจษฎากุล.....
 ปีการศึกษา 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก..... อ.ริยา อริยา.....
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4889665020: MAJOR MEDICAL MICROBIOLOGY

KEYWORDS: *PYTHIUM INSIDIOSUM*/ SUPPRESSION SUBTRACTIVE HYBRIDIZATION/ REAL TIME RT – PCR/ SUBTRACTED CDNA LIBRARIES / PHYLOGENETIC TREE

PATCHAREE KAMMARNJASSADAKUL: CLONING AND CHARACTERIZATION OF GENES DIFFERENTIALLY EXPRESSED IN *PYTHIUM INSIDIOSUM* THAI STRAIN. THESIS ADVISOR: ASSOC. PROF. ARIYA CHINDAMPORN, Ph.D., THESIS CO-ADVISOR: ASST.PROF.TANAPAT PALAGA, Ph.D., KALLAYA SRITUNYALUCKSANA, Ph.D., 235 pp.

An emerging aquatic fungal like organism, *Pythium insidiosum*, causes the disease called 'pythiosis' in both human and animal from tropical, subtropical, and temperate zone. The highest mortality and morbidity in human pythiosis has been reported from Thailand. Very few genetic information and pathogenesis concerning this organism is not known. This study was to investigate genes expressed from culture at 37°C might involved in its pathogenesis. To isolate temperature-induced genes, PCR – select cDNA subtraction from cultures *P. insidiosum* at 27°C and 37°C and PCR-based searching the genetic information in GenBank Databases. Both subtracted cDNA libraries conditions, total six hundred and six clones with inserted cDNA fragments were detected. Nucleotide analysis by BLASTN showed mostly mitochondria DNA whereas the protein function predicted by Swiss-Prot and BLASTX programs demonstrated candidate genes encoding proteins. Here, gene encoding cytochrome c oxidase subunit II (*COX II*), β -tubulin (*TUB*), and chitin synthase subunit II (*CHI II*) were selected to analyze their expression using semi-quantitative PCR and confirmed by Real time RT-PCR. To demonstrate the expression level at 37°C and 27°C conditions, sixteen strains of *P. insidiosum*, represented three phylogeographic preference were recruited. The results showed that at 37°C growth condition, only *COX II* gene expressed 2.5 times over 27°C growth condition ($p = 0.0347$). Even though, *TUB* and *CHI II* show indistinguishable expression, these genes can be used as housekeeping genes for temperature susceptible gene expression studies. This is the new findings. However, it is of noted that the expression of *CHI II* at 37°C depended on the geographic distribution. Not only the expression was analyzed, the phylogenetic relationship was also performed using *COX II*. The result showed that *COX II* is the good candidate gene to reveal the genetic association among *P. insidiosum*. It also is an alternative gene of ITS which is commonly used for study the phylogenetic distribution. In conclusion, these data was the pioneer to demonstrate that *COX II* expressed at 37°C over 27°C. The insight of this gene and pathogenesis required to perform the further investigation.

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ACKNOWLEDGEMENTS

First of all, I would like to express my feeling from the honesty to my great advisor: Assoc. Prof. Ariya Chindamporn, Ph.D. Throughout my candidate Ph.D lifetime, she gave wise advice, good care to support this thesis and other administrative challenges and the many constructive discussions. I am very to special thankful to my co- advisor: Assist. Prof. Tanapat Palaga, Ph.D. With his enthusiasm, his inspiration, and his great efforts to explain things clearly and simply, he provided a lot of suggestions from him improved my thesis and also my scientific thinking. Many thanks are address to my co-advisor to be loved: Kallaya Sritunyalucksana, Ph.D. for supervising this work and asking me to work in her research group at Centex Shrimp, Faculty of Science, Mahidol University, where I carried out a significant part of my thesis. With her talent and expertise for research, she helped me a lot with her great experience in Suppression Subtractive Hybridization System.

My special thanks go to the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund), Graduate School, Chulalongkorn University, the main financial support and I also thank Research Center, Faculty of Medicine, and Chulalongkorn University. I will forever be indebted to Prof. Dr. Boonsirm Withyachumnarnkul, MD., Ph.D. for the giving me the opportunity to use the excellence facilities at Centex Shrimp for more than three months. At Centex Shrimp, many people have supported me with word and deed. I am very special thankful to Prof. Nongnuch Vanittanakom, Ph.D., Faculty of Medicine, Chiang Mai University, Theerapong Krajaejun, MD., Ph.D. and Assoc. Prof. Srisurang Tantimavanich, Mahidol University for providing *P. insidiosum* references isolates and DNA samples. I am especially grateful to numerous people were involved and contributed in many ways in this thesis, especially Ms. Supranee Buranapraditkun, Ms. Sirada Koachareon, my sisters and brothers in Mycology research unit and all staffs of Department of Microbiology for providing a stimulating and fun environment in which to learn and grow. Last but not least and most importantly, I am extremely grateful to my parents, younger brother and my husband for all their love! and sharing their life with me. With their understanding, never lost patience with my impatience, they always raised and help me get through the difficult times; without them my work could not succeed.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	=	Percent
°C	=	Degree Celsius
×g	=	g - force
&	=	An ampersand is a logogram representing the conjunction word “and”.
β	=	Beta
Δ	=	Delta
μg	=	Microgram (s) = 10 ⁻⁶ gram
μl	=	Microliter (s) = 10 ⁻⁶ liter
μM	=	Micromolar
<i>A</i>	=	Absorbance Spectrum
Ag	=	Antigen
BLAST	=	Basic Local Alignment Search Tool
BLASTN	=	Nucleotide – nucleotide BLAST
BLASTX	=	DNA – protein BLAST
bp	=	Base pair
cm.	=	Centimeter
cDNA	=	Complementary DNA
Cat.	=	Catalog number
CA	=	Canada
<i>CHI II</i>	=	Chitin synthase subunit II gene
<i>COX II</i>	=	Cytochrome c oxidase subunit II gene
CMA	=	Cornmeal Agar

LIST OF ABBREVIATIONS AND SYMBOLS (continue)

C_T	=	Cycles threshold
CV	=	Coefficient of variation
dscDNA	=	Double strand cDNA
dNTPs	=	Deoxynucleoside 5'- triphosphates
DEPC	=	Diethyl pyrocarbonate
DNA	=	Deoxyribose nucleic acid
DNase	=	Deoxyribonuclease
DW	=	Distilled water
<i>et al.</i>	=	And others
<i>E</i> – value	=	Expected value
EDTA	=	Ethylenediaminetetraacetate
EtBr	=	Ethidium bromide
EtOH	=	Ethanol
FA gel	=	Formaldehyde agarose gel
g	=	Gram (s)
hr	=	Hour (s)
H ₂ O	=	Water
H ₂ O ₂	=	Hydrogen peroxide
Inc.	=	Incorporate
ITS region	=	Internal transcribed spacer region
IGS	=	Intergenic spacer
kb	=	Kilobases
KOH	=	Potassium hydroxide
KCl	=	Potassium chloride

LIST OF ABBREVIATIONS AND SYMBOLS (continue)

IPTG	=	Isopropyl- β -D-thiogalactopyranoside
L	=	Liter
<i>L.</i>	=	<i>Lagenidium</i>
LB	=	Luria-Bertani media
min	=	Minute (s)
mRNA	=	Messenger RNA
mg	=	Milligram(s)
ml	=	Milliliter (s)
mm	=	Millimeter
mM	=	Millimolar
mtDNA	=	Mitochondria DNA
M	=	Molar
MOP	=	3-[N-Morpholino] propanesulfonic acid]
MEGA	=	Molecular evolution genetics analysis
MIP	=	Munich Information Center for Protein Sequences
MgCl ₂	=	Magnesium chloride
ng	=	Nanogram (s) = 10 ⁹ gram
nm	=	Nanometer (s) = 10 ⁹ meter
no.	=	Number
NaOAC	=	Sodium acetate
NaOH	=	Sodium hydroxide
NCBI	=	National Center for Biological Information

LIST OF ABBREVIATIONS AND SYMBOLS (continue)

NJ	=	Neighbor-joining
nd	=	The second
OD	=	Optical density
pg	=	Pico gram (s)
pmol	=	Picomole
pH	=	Potential of hydrogen ion
PCR	=	Polymerase Chain Reaction
PDA	=	Potato dextrose agar
<i>P</i> – value	=	Probability value
<i>P.</i>	=	<i>Pythium</i>
<i>Ph.</i>	=	<i>Phytophthora</i>
rpm.	=	Revolution per minute
rRNA	=	Ribosomal RNA
rDNA	=	Ribosomal DNA
RNA	=	Ribonucleic acid
RT	=	Room temperature
RNase	=	Ribonuclease
RT – PCR	=	Reverse Transcriptase - PCR
s	=	Second (s)
spp	=	Specie (s)
st	=	The first
S	=	Sevedberg unit
SEM	=	Standard error
SD	=	Standard deviation

LIST OF ABBREVIATIONS AND SYMBOLS (continue)

SDA	=	Sabouraud dextrose agar
SDB	=	Sabouraud dextrose broth
SSH	=	Suppression Subtractive Hybridization
SSKI	=	Supersaturated potassium iodide
TBE	=	Tris – borate EDTA
TE	=	Tris – EDTA (buffer)
Tris	=	Tris (hydroxymethyl) amino – methane
<i>Taq</i>	=	<i>Thermus aquaticus</i>
<i>T_m</i>	=	Melting temperatures
<i>TUB</i>	=	β – tubulin gene
U	=	Unit (s) of enzyme
USA	=	United State of America
UK	=	United Kingdom
UV	=	Ultraviolet
V	=	Volts
V/cm	=	Volts per centimeter
wt/wt	=	Weight/weight
w/v	=	Weight/volume
X –gal	=	5-bromo-4-chloro-3-indolyl- β -D- galactopyranoside