



THESIS

POTENTIAL APPLICATION OF PCR-BASED METHOD FOR EARLY DETECTION OF GRASSERIE DISEASE OF SILKWORM, *Bombyx mori*

MALLIKA KAEWWISES

GRADUATE SCHOOL, KASETSART UNIVERSITY

2006

THESIS

POTENTIAL APPLICATION OF PCR-BASED METHOD FOR EARLY DETECTION OF GRASSERIE DISEASE OF SILKWORM, *Bombyx mori*

MALLIKA KAEWWISES

**A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Agricultural Biotechnology)
Graduate School, Kasetsart University
2006**

ISBN 974-16-2382-8

Mallika Kaewwises 2006: Potential Application of PCR-Based Method for Early Detection of
Grasserie Disease of Silkworm, *Bombyx mori*. Doctor of Philosophy (Agricultural Biotechnology),
Major Field: Agricultural Biotechnology, Interdisciplinary Graduate Program.
Thesis Advisor: Associate Professor Tipvadee Attathom, Ph.D. 119 pages.
ISBN 974-16-2382-8

Grasserie disease caused by *Bombyx mori* nucleopolyhedrovirus (BmNPV) is the most destructive disease of silkworm, *Bombyx mori* in Thailand and other sericultural practising countries. Restriction profile of Thai BmNPV genomic DNA was studied by cleaving the DNA with *Bam*HI, *Bgl*II, *Hind*III, *Nco*I and *Pst*I. The Thai BmNPV genome was estimated to be in the range of 92.3-125.8 kb with the average of 109.8 kb. The full length of the polyhedrin gene (*polh*) of Thai BmNPV was cloned and sequenced. The *polh* sequence contained a 735 bp open reading frame (ORF) encoding a protein of 245 amino acids with a predicted molecular mass of 28.8 kDa. The nucleotide sequence of Thai BmNPV *polh* showed greater than 98% identity to the five different sequences of BmNPV *polh* previously characterized. The high degree of sequence identity with the *polh* sequences of other BmNPVs suggested that ORF sequence reported in this study is the Thai BmNPV *polh* gene. Comparison of Thai BmNPV *polh* sequence with other *polhs* of Lepidoteran NPVs (*Autographa californica*, *Helicoverpa armigera*, *Spodoptera litura* and *S. exigua*) indicated that the nucleotide and amino acid sequence identities were greater than 65% and 78%, respectively.

PCR-based method was developed for BmNPV detection in silkworm. Specific primers were designed from nucleotide sequence of Thai BmNPV *polh*. BmNPV DNA extraction was modified from the alkaline lysis method. Grasserie disease was detected in artificially inoculated and naturally infected silkworm samples. The developed PCR method could be used to detect BmNPV in every stage of silkworm's development. The method was sensitive enough to detect BmNPV using only one infected individual egg and larva. It could also be used to detect BmNPV in both sexes of silkworm pupa and moth. The whole process from DNA extraction to BmNPV detection could be done within 5 hours. It was considered suitable to be practiced as a routine measure for grasserie disease control by the government and private sectors in Thai sericulture. This study indicated that PCR method based on the *polh* sequence of BmNPV was efficient, specific and highly sensitive in detecting graserie disease in any stage of silkworm development.

Student's signature

Thesis Advisor's signature

ACKNOWLEDGMENTS

I would like to express my most appreciation and grateful thank to my advisor, Associate Professor Dr. Tipvadee Attathom for her guidance, dedicated efforts, valuable discussion and encouragement during my study and for her suggestion in completing the writing of papers and this thesis.

I would like to express my sincere thanks to my co-advisors, Dr. Sudawan Chaeychomsri and Dr. Srimak Chowpongpan for their advices and recommendations.

Sincere appreciation is expressed to Ms. Butsara Ravinoo (Nong Khai Sericultural Experiment Station), Mr. Wisit Fichun (Sericultural Extension Centre 9: Kanchanaburi Province), Ms. Jariya Mechuen (The Thai Silk Company), Ms. Busaya Cunvong (Chul Thai Silk Company) and Ms. Siripuk Suraporn (Maha Sara Kham University) who supported samples of silkworm.

My sincere thanks go to Mr. Rungrote Sochanthuk, Mr. Anon Thammasittirong, Ms Cheerapha Panyasiri and members of insect pathology laboratory for all advices, helpful and meaningful friendship.

This research work is partially supported by the Center for Agricultural Biotechnology through the fund from Subproject Graduate Study and Research in Agricultural Biotechnology under Higher Education Development Project, Commission on Higher Education, the Ministry of Education.

Finally, I would like to express all my love and gratitude to my beloved parents, my sisters and brothers for their support, understanding and encouragement throughout my life.

Mallika Kaewwises

October 2006

TABLES OF CONTENTS

	Page
TABLES OF CONTENTS.....	i
LIST OF TABLES.....	ii
LIST OF FIGURES.....	iii
INTRODUCTION.....	1
Objectives.....	4
LITERATURE REVIEW.....	5
Mulberry silkworm, <i>Bombyx mori</i>	5
The causative agent of grasserie disease.....	10
Grasserie disease of mulberry silkworm.....	23
MATERIALS AND METHODS.....	32
Materials.....	32
Methods.....	35
RESULTS AND DISCUSSION.....	44
Restriction pattern analysis of Thai BmNPV.....	44
Identification of polyhedrin gene of Thai BmNPV.....	53
Evaluation of PCR methodology for grasserie disease detection.....	68
Application of PCR for grasserie detection in collected samples.....	85
BmNPV DNA extraction.....	100
CONCLUSION.....	105
LITERATURE CITED.....	107

LIST OF TABLES

Tables	Page
1 Restriction endonuclease cleavage fragments of the Thai <i>Bombyx mori</i> nucleopolyhedrovirus DNA	48
2 Restriction endonuclease cleaved fragments of the Thai BmNPV DNA and DNA of the other isolates of BmNPV.....	52
3 Number of eggs laid by healthy and artificially BmNPV-infected silkworm moth, <i>Bombyx mori</i>	70
4 Percentage of several abnormal symptoms of pupae of silkworm, <i>Bombyx mori</i> artificially infected with nucleopolyhedrovirus.....	78
5 Number of <i>Bombyx mori</i> nucleopolyhedrovirus infected samples as detected by PCR.....	85
6 Characteristics of some silkworm varieties culturing in Thailand.....	87
7 Common sanitary procedures for silkworm diseases prevention by some silkworm rearing houses.....	89
8 Source and variety of collected silkworm and number of infected silkworm as detected by PCR.....	94
9 Comparison of the three methods of DNA extraction	102

LIST OF FIGURES

Figure	Page
1 Life cycle of the mulberry silkworm, <i>Bombyx mori</i> which undergoes 4 developmental stages.....	7
2 Structure diagram of insect virus in the family Baculoviridae.....	11
3 Typical cellular infection cycle of the nucleopolyhedrovirus.....	15
4 Genomic organization of BmNPV.....	19
5 Baculovirus infection process contains three phases; early, late and very late phase.....	20
6 Diagram of the genome of BmNPV.....	37
7 Diagram of the polyhedrin gene.....	43
8 Gel electrophoresis of the DNA of Thai BmNPV	45
9 Cleavage patterns of Thai BmNPV DNA using the restriction endonuclease <i>Bam</i> HI, <i>Bgl</i> II, <i>Hind</i> III, <i>Nco</i> I and <i>Pst</i> I.....	47
10 The expected PCR product of 1,448 bp amplified from the extracted DNA of Thai BmNPV.....	53
11 Screening of recombinant clones harboring the BmNPV <i>polh</i> gene.....	55
12 Information of GenBank accession number AY779044, the submitted sequences of Thai BmNPV polyhedrin gene.....	57
13 Nucleotide sequence of Thai BmNPV polyhedrin gene and its flanking regions	58
14 Comparison of flanking nucleotide of Thai BmNPV <i>polh</i> (AY779044) and Japanese 1 <i>polh</i> (T3)(L33180)	60
15 Nucleotide and amino acid sequence identity of BmNPV <i>polhs</i>	61
16 Alignment of the deduced amino acids of Thai BmNPV <i>polh</i> (AY779044) with other BmNPV <i>polhs</i>	62
17 A phylogenetic tree of 6 BmNPV <i>polhs</i>	63
18 Nucleotide and amino acid sequence identity of NPV <i>polhs</i>	64

LIST OF FIGURES (cont'd)

Figure	Page
19 Alignment of the deduced amino acids of Thai BmNPV <i>polh</i> (AY779044) with other NPV <i>polhs</i>	65
20 A phylogenetic tree of five NPV <i>polhs</i>	66
21 PCR products of DNA extracted from 1, 3, 5, 10, 15, 20 eggs laid by BmNPV-artificially infected silkworm moths.....	69
22 PCR products of DNA extracted from various numbers of BmNPV-artificially infected first instar larvae	72
23 PCR products of DNA extracted from various instars of BmNPV-artificially infected larvae	72
24 The healthy mulberry silkworm, <i>Bombyx mori</i> larvae	74
25 Symptoms of grasserie disease of silkworm, <i>Bombyx mori</i> caused by BmNPV	75
26 PCR products of DNA extracted from BmNPV-artificially infected male and female pupae	77
27 Symptoms of grasserie disease of silkworm, <i>Bombyx mori</i> caused by nucleopolyhedrovirus as appeared in the pupal stage.....	79
28 PCR products of DNA extracted from BmNPV-artificially inoculated male and female moths.....	82
29 Healthy and BmNPV-infected moths, <i>Bombyx mori</i>	83
30 PCR products of DNA extracted from naturally infected silkworm larvae	96
29 PCR products of DNA extracted from naturally infected pupae and moths of silkworm.....	98