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 APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Doctor of Philosophy (Agricultural Biotechnology)
DEGREE

Agricultural Biotechnology
FIELD

Interdisciplinary Graduate Program
PROGRAM

TITLE: Potential Application of PCR-Based Method for Early Detection of Grasserie Disease of Silkworm, *Bombyx mori*

NAME: Miss Mallika Kaewwises

THIS THESIS HAS BEEN ACCEPTED BY

Tipvadee Attathom **THESIS ADVISOR**
(Associate Professor Tipvadee Attathom, Ph.D.)

Sudawan Chaeychomsri **COMMITTEE MEMBER**
(Ms. Sudawan Chaeychomsri, Dr.Agr.)

Srimek Chowpongpang **COMMITTEE MEMBER**
(Mr. Srimek Chowpongpang, Ph.D.)

Pongthep A. **GRADUATE COMMITTEE CHAIRMAN**
(Associate Professor Pongthep Akratanakul, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON 3 / 11 / 2006

Vinai Artkongharn **DEAN**
(Associate Professor Vinai Artkongharn, M.A.)

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, *Hin*dIII, *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.

Mallika Kaewwises — Student's signature

Tipvadee Attathom — Thesis Advisor's signature

30 / 10 / 06

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 Chowpongpang 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

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (cont'd)

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

INTRODUCTION

The silkworm, *Bombyx mori* has been domesticated for silk production for over 4,000 years. Silk is "the queen of fibers" because it is a smooth, shining, fabulous and unique natural fiber produced by silkworm. Nowadays, silkworm is an insect of economic importance to Asian countries like China, India, Thailand, Vietnam and many other developing countries. In Thailand, sericulture is an important environmentally sustainable agro-industry activity practiced for centuries. Thailand is the most international reputation as a producer of high quality silk and silk products. Presently, the Thai silk has become world-famous for its quality and unique character. The popularity of Thai silk among customers in Thailand and in other countries all over the world stems from its distinctive beauty, consummate craftsmanship, soft and smooth texture, iridescent sheen, attractive colours and artistic designs which set it apart from silk elsewhere (Ministry of Agriculture and Cooperative, 2002). It provides substantial contributions to the national economy and serves as source of income for many farmers especially in the northeastern part of Thailand. In the past, sericulture was practiced only at family level as cottage industry. As a result in reign of King RamaV, he conserved the keep up and contributed the supportive to sericulture (Sikkhamondhol and Tengratanaprasert, 1993) and now it has become the nation-wide industry. The major obstacle in sericultural industry in Thailand is diseases of silkworm. Silkworm diseases that have been found in Thailand are aspergillus disease and muscadine disease caused by fungi, sotto disease caused by bacteria, pebrine disease caused by protozoa, flacerie disease caused by cytoplasmic polyhedrosis virus and grasserie disease caused by nucleopolyhedrovirus (United Nation, 1998). However, grasserie disease caused by *Bombyx mori* nucleopolyhedrovirus (BmNPV), is the most destructive disease of silkworm.

BmNPV belongs to genus Nucleopolyhedrovirus, family Baculoviridae (Van Regenmortel and Fauquet, 2000). The virus particle is composed of deoxyribonucleic acid (DNA) surrounded by capsid protein to form the nucleocapsid. The nucleocapsids are then

enveloped and are called virions (Tanada and Kaya, 1993). The two morphological subgroups within the NPVs are the single nucleocapsid NPV in which only one nucleocapsid is present per envelope, and the multinucleocapsid NPV in which several nucleocapsids are packed per envelope (Hunter-Fujita *et al*., 1998). The virions are occluded within polyhedral-shapes occlusion bodies called polyhedra that surround and protect the infectious virion (Blissard and Rohrmann, 1990). Polyhedra are mainly composed of a single polypeptide known as polyhedrin (Polh). Polh, which constitutes the crystalline matrix of baculovirus occlusion bodies, plays a significant role in the replication cycle of baculovirus. The Polh is encoded by an orthologous gene highly conserved among baculoviruses, and it is the gene from which most data from different isolates are available, so it is the most comprehensive option available for estimating the relationship among baculoviruses (Zanotto *et al.*, 1993).

Grasserie disease is difficult to cure or overcome as the life cycle of silkworm is short. The best way to control grasserie disease is to prevent disease infection. The infected silkworm expresses disease symptom during the final stage of larval development and die without cocoon production resulting in the waste of expense, time and labour work. The damage of this disease in Thailand is about 30-100% (Kaewwises and Niyomvit, 1995). In Thailand, silkworm can be reared throughout the year and grasserie disease is most severe in rainy season and least severe in the winter (Kaewwises and Sananvong, 1999). Recommendation for disease prevention from the Department of Agriculture is sanitation the rearing houses and equipments with formalin before rearing the next generation of silkworm. Farmers can use lime or chlorine in substitution to formalin (Sithisongkram and Ruksong, 1987). However, poor hygienic conditions and ineffective disease control as practiced by most of the farmers have often been followed by unstable sanitation results. Nowadays, sericulturists try to breed multivoltine and bivoltine silkworm varieties resistant to BmNPV. However, the presumable most effective solution for the control of grasserie disease is to detect viral infection as early as possible in order to stop spread of the disease in rearing house. Lack of rapid and accurate disease detection technique causes severe spread of grasserie disease annually. Many techniques have been developed to provide an early and accurate diagnosis method for this viral disease such as the enzyme-link immunosorbent assay (ELISA) (Vanapruk *et al.*, 1992; Shamim *et al.*, 1994), DNA hybridization (Attathom *et al.*,

1994), colloidal textile dye-based dipstick immunoassay (Nataraju *et al.*, 1994), monoclonal antibody (Nagamine and Kobayashi, 1991) and western blot analysis (Chaeychomsri *et al.*, 1995). Most of those detection methods were complicate and difficult to interpret the results.

The polymerase chain reaction (PCR) is a highly sensitive technique which amplifies target DNA sequences and PCR amplification of conserved fragment enabled the detection of low level of viral DNA. It has been employed for the detection of viral DNA such as human virus (Umlauft *et al.*, 1996), aminal virus (Peng *et al.*, 1998) and plant virus (Levesque, 2001). For NPV, the preferable gene employed for PCR detection was the polyhedrin gene (*polh*). This gene had been used to detect the NPVs of *Spodoptera literalis* (Chou *et al.*, 1996), *Autographa californica*, *Anticarsia gemmatalis*, *Bombyx mori*, *Orygia pseudotsugata*, *Spodoptera frugiperda*, *Spodoptera exigua*, *Anagrapha falcifera*, *Heliothis zea* (Moraes and Maruniak, 1997), *Perina nuda* (Wang *et al.*, 2000) and *Helicoverpa armigera* (Christian *et al.*, 2001). Moreover, PCR technique was employed to detect baculovirus DNA sequences from viral occlusion bodies contaminating the surface of gypsy moth (*Lymantria dispar*) eggs (Burand *et al.*, 1992). Many researchers brought advantage of PCR for baculovirus DNA detection in environment, for example detection of NPV DNA of *Anticarsia gemmatalis* and *Helicoverpa armigera* in soil (Moraes *et al.*, 1999; Christian *et al.*, 2001) and detection of baculovirus DNA in lake water (England *et al.*, 2001).

Detection of this viral disease at the early stage of infection will help to improve sanitary management, prevent the spread of the disease, and finally to eliminate this viral disease within silkworm population. Hence, farmers can obtain more silk productivity with high quality. In addition, the invention of the practical detection protocol will significantly eases the disease preventive strategy in sericultural industry. This study aims to explore the potential application of PCR-based methods for early detection of grasserie disease in silkworm and to develop PCR method for practical use in sericultural industry.

Objectives

1. To study BmNPV polyhedrin gene (*polh*) of the Thai isolate and determine its relatedness to other *polh*s of nucleopolyhedrovirus of some economic important insects.

2. To investigate the use of PCR-based method for early detection of grasserie disease of silkworm.

3. To evaluate the sensitivity and specificity of the developed PCR-based method.

4. To develop a simple protocol for practical application of PCR-based method for early detection of grasserie disease of silkworm.

LITERATURE REVIEW

1. Mulberry silkworm, *Bombyx mori*

1.1 Biology of *B. mori*

B. mori was originally in a wild state. As a result of being tamed over a long period by human beings and also due to artificial selection and rejection, it is now a domestic animal. This species of insect belongs to the phylum Arthropoda, class Insecta, order Lepidoptera, family Bombycidae. *B. mori* is monophagous insect that feed on only mulberry leaves. Its life cycle is completed by passing through four different stages; egg, larva, pupa and moth (Figure 1). During the period of pupation, the body is defenseless against external enemies and hence, the silkworm spins a protective shell around the pupa and this shell is known as the cocoon, the origin of silk tread.

Silkworm is an insect which undergoes complete metamorphosis. The duration of the whole life cycle is about 55-60 days at temperatures of 23-25°C (Tazima, 1962). The egg has an outer layer, the shell or chorion. Shape of the egg varies according to strain but in general it is unevenly elliptical. Colour of the egg after oviposition is usually pale yellow. After few days, the egg appears light brown in colour which is gradually darkens (Aruga, 1994). There are two types of egg; diapause and non-diapause. Silkworm races indigenous to temperate regions usually lay diapause eggs, while those of tropical regions lay mostly non-diapause eggs (Tazima, 1978). Once the embryonic growth inside the egg is complete, the young larva hatches. During hatching the young larvae eat up a part of the egg shell and emerge through the hole thus made. The newly hatched larvae are usually dark brown with fairly setae on all segments. Young larvae, soon after hatching so called the first instar, start feeding on mulberry leaves and grow. After feeding for a little over three days, they reach the end of their first instar, the larvae stop feeding completely and undergo ecdysis within 24 hours afterward. This period of ecdysis is called the first moult. After moulting they resume feeding, grow and repeat the moulting process. In the common strains of silkworm, this process repeated four times. There are four moults and

between two successive moults lies an instar. There are thus five instars in the life cycle of silkworm. About half of the whole life cycle is the larval stage, the only stage at which they take food. The range of food selection of this insect is very narrow, almost limited to mulberry leaves. Hence, the silkworm is classified as a monophagous insect (Aruga, 1994). In the fifth instar, the silkworms feed for six to eight days after which they stop feeding and are ready to spin cocoons. These, so called “ripe” or “mature” larvae, become very restless, raising their heads in search of a place to spin cocoon. In mature larvae, the silk glands are so large and weight about 40% of the body weight. The larvae are transferred to a spinning nest, which is usually made of straw or cardboard. The colour of the cocoon in commonly reared silkworm strains is mostly white. Besides white cocoons, there are cocoons of different colour such as golden yellow, flesh colour, crimson, bamboo leaf colour, and green (Tazima, 1978). It takes the larvae about 4 or 5 days after moulting to pupate and 9 to 14 days more until the emergence of the moths. Soon after that male and female moths mate. A male moth can mate with two or three females in one day, but the ability to mate deteriorate very quickly. The female moth begins to lay eggs shortly after the separation from the male and lays continuously for several hours until all eggs are deposited (Tazima, 1962).

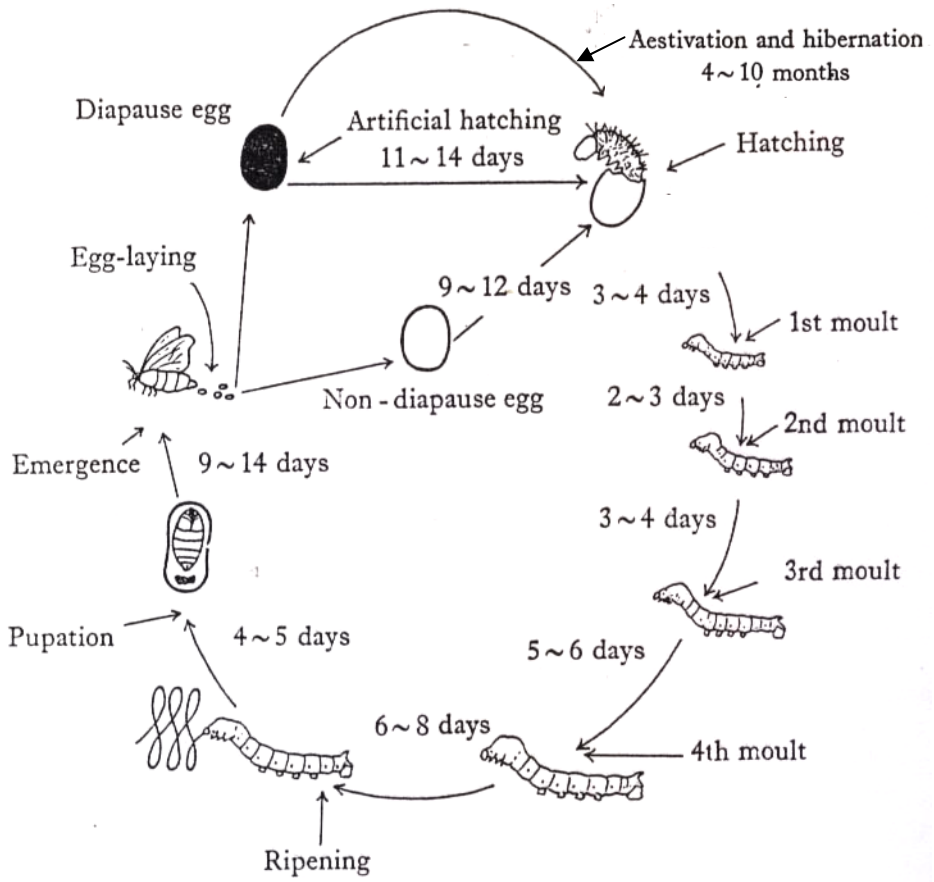


Figure 1 Life cycle of the mulberry silkworm, *Bombyx mori* which undergoes 4 developmental stages; egg, larva, pupa and moth.

Source: Tazima (1962)

1.2 Classification of *B. mori*

Mulberry silkworms are classified in different ways. Classification may be based on the native regions, the number of larval moult, the rearing period, body marking or pattern, body colour of mature larva, colour of cocoon and colour of egg (Department of Agriculture, 1980; Aruga, 1994).

1.2.1 Classification based on voltinism

Silkworms can be classified as univoltine, bivoltine and polyvoltine. Univoltine types have only one generation in a year and usually reared in spring. They have a long life cycle and their larvae and cocoons are large. Bivoltine types have two generations under natural climatic condition in any specific temperature. Compared to univoltine, the bivoltine silkworms are generally strong and healthy and are reared in spring and autumn. Their life cycle is shorter than that of univoltine silkworms. Polyvoltine or multivoltine types have many generations in a year. Larvae are generally strong and healthy with a short life cycle. Their cocoons are quite inferior to those of the univoltine and bivoltine silkworms. They are intermediate between univoltine and bivoltine rather than being pure bivoltine.

1.2.2 Classification based on native regions

On the basis of native regions, the silkworms are classified into Japanese, Chinese, European and Tropical races.

The Japanese race has univoltine and bivoltine silkworms. The larvae are usually strong and healthy, the cocoon mostly white and barrel-shaped. The cocoon fiber length is short and the reeling property is generally poor.

The Chinese race has univoltine, bivoltine and polyvoltine silkworms. They grow rapidly by feeding actively on mulberry leaves but are susceptible to high humidity. The cocoons of polyvoltine silkworm are spindle-shaped. The silk fibre is thin and its reeling property is good.

The European race has only univoltine silkworms. Generally, the eggs, larvae and cocoons are larger than those of the Japanese and Chinese races. The larval period is longer and the larvae grow fat by feeding actively on mulberry leaves. Cocoons are white and buff coloured and the fibre is longer with good reeling property.

The tropical race has polyvoltine silkworms. The larvae are strong and healthy and their laval period is short. Cocoons are yellow and the reeling property is poor.

1.2.3 Classification based on moulting

A few times during the larval stage, silkworms stop feeding, become inactive and shed their skin. This period is called moulting period and classification can be based on the number of larval moults which can be 3, 4, 5 moults or more. Silkworms with four larval moults are the ones which are widely reared. Those with two or six moults have a shorter life cycle and produce thin fibres whereas those with five moults have longer life cycle and their cocoons composed of thicker fibres.

1.2.4 Classification based on cocoon colour

The cocoon are broadly classified into white and coloured. The white ones are of two types; inferior white and superior white. Genetically some of these are inferior to yellow cocoons and others are superior. Among the white cocoons are pure white and somewhat dirty white cocoon. Coloured cocoons are of different colours; yellow, buff, straw and green.

1.2.5 Classification based on rearing period

According to the rearing period, silkworms are broadly classified into spring and summer-autumn silkworms. The summer-autumn silkworms are further categorized as summer, early autumn and very late autumn silkworms. Those reared even later, that is, after the very late autumn is referred to as early winter silkworms.

1.2.6 Classification based on larval marking

The commonly reared silkworms may appear to be plain or show pattern so called patterned or marked silkworms. Different types of larval marking such as stripes, dark colour, zebra bands, brown spots, quail marks, multistar, etc., have been known for a long time.

1. 3 *B. mori* in Thailand

In Thailand, there are three district silkworm strains, recommended by the sericulture research institute for sericulturists to rear in different parts of the country. The first is multivoltine strains or Thai strains, the second is bivoltine hybrid (Japanese x Chinese) and the third is multivoltine x bivoltine strains. As Thailand is in tropical region, temperature is quite high, over 30^{o}C in hot season, hence the bivoltine hybrids are usually difficult to rear but they produce high yield or cocoon whereas, the multivoltine strains are vice versa. However, the cross-breeds between multivoltine and bivoltine are conducted to combine the good characters from the two strains (Noppaseney, 2002). Multivoltine strain has the shorter life cycle with larval period of 18-23 days compare to 20-30 days of the bivoltine strain. A female multivoltine strain lays an average of 300 eggs while the bivoltine strain lays 400-500 eggs (Sungkompitak, 1989).

2. The causative agent of grasserie disease

2.1 Baculoviruses

2.1.1 Classification of baculoviruses

The causative agent of grasserie disease is *Bombyx mori* nucleopolyhedrovirus (BmNPV). This virus is classified in the genus Nucleopolyhedrovirus, family Baculoviridae. The family Baculoviridae is classified by the basic character as an enveloped, rod-shaped virion (approximately 50 x 250) containing a circular double-stranded DNA genome ranging from 50 to 100 million Daltons. They were previously divided into three subgenera based on their

morphological properties (figure 2). Subgenus A consists of the nuclear polyhedrosis viruses (NPVs). The characteristic feature of this group is that several virions are occluded in a polyhedral, proteinaceous inclusion body (IB). There are two morphological subgroups within the NPVs. The single-nucleocapsid NPV (SNPV) in which only one nucleocapsid is found per envelope and the multinucleocapsid NPVs (MNPVs) in which several nucleocapsids are packaged per envelope. Subgenus B consists of the granulosis viruses (GVs). In this group virions containing single nucleocapsid and only one virion is occluded in one inclusion body which is oval in shape. Subgenus C consists of virions which are not packaged into IBs at any stage of their life cycle, they are nonoccluded viruses (NOVs) (Bilimoria, 1986).

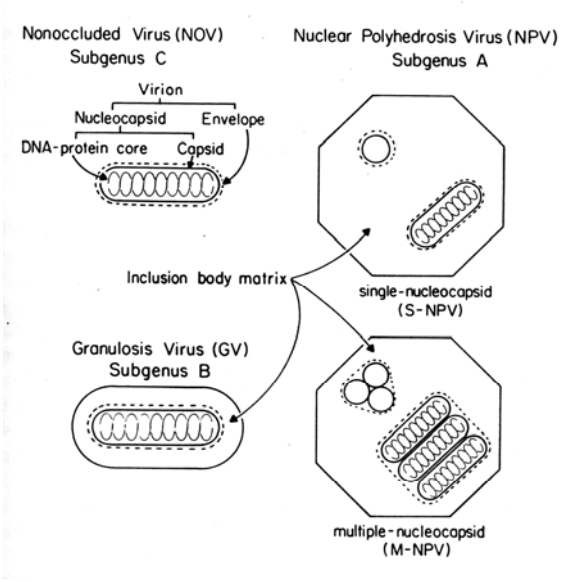


Figure 2 Structure diagram of insect viruses in the family Baculoviridae. It comprised three subgenera; subgenus A, B and C. The S- and M-NPV contain numerous virions per inclusion body

Source : Bilimoria (1986)

Currently, Baculoviridae comprises only two genera, nucleopolyhedrovirus (NPVs) and granulovirus (GVs). The terms nuclear polyhedrosis virus and granulosis virus have been replaced by nucleopolyhedrovirus and granulovirus, respectively (van Regenmortel *et al*., 2000). Baculoviruses have only been found in over 600 species of arthropod hosts. The majority

of baculovirus host are within the order Lepidoptera. They have also been isolated from the insects in orders Diptera, Hymenoptera, Coleoptera, Neuroptera, Thysanura and Tricoptera and the crustacean in order Decapoda (shrimp) (Hong *et al.*, 2000).

Morphology of NPVs, either multiply or singly enveloped does not appear to be crucial in taxonomy. For example, *Bombyx mori* SNPV (BmSNPV) and *Autographa californica* MNPV (AcMNPV) are far more closely related to each other than they are to most other NPVs whether SNPVs or MNPVs (Hunter-Fujita *et al*., 1998). The abbreviations commonly used for baculovirus are based on the first letter of the insect species and the viral subgroup, e.g. *Bombyx mori* SNPV as BmSNPV and *Pieris brassicae* GV as PbGV. Although baculoviruses are usually named after the host species from which they were isolated, this does not provide a satisfactory form of identification. In several instances, different baculoviruses have been isolated from the same insect species while a single virus, or a group of closely related strains, can occur in more than one insect species, e.g. *Autographa californica* MNPV (Hunter-Fujita *et al*., 1998).

2.1.2 Biology of baculoviruses

Baculoviruses are diverse group of viruses found mostly in insects. The baculo portion of the name refers to the rod-shaped capsids of the virus particles. The virus particle is composed of a protein shell (capsid) that surrounds the nucleic acid. Baculovirus capsids are usually 40-50 nm in diameter and 200-400 nm in length. Within the capsid, the DNA is condensed into a nucleoprotein structure known as the core. The capsid plus the core are collectively refered to as the nucleocapsid (O'Reilly *et al*., 1992). Nucleocapsids contain a single molecule of circular supercoil is double-stranded DNA. The length of baculoviral DNA is between 80-200 kb (van Regenmortel *et al*., 2000). Nucleocapsids are made in the nucleus of infected cells and are subsequently enveloped by one of two processes. Nuclocapsids can bud through the plasma membrane of the infected cell; such budded virus particles are releases into the extracellular fluid with a loosely fitting membrane envelope. Nucleocapsids may also acquire an envelope within the nucleus where they are produced. The envelope of virions embedded in

occlusion bodies fits closely around the nucleocapsids, and additional nuclear material may be compressed between the membrane and the nucleocapsids during the occlusion process. Viral occlusion bodies are formed in the nucleus and comprise enveloped nucleocapsids embedded in a crystalline protein matrix. Polyhedral occlusion bodies of NPVs are known as polyhedra, occlusion viruses, or polyhedral inclusion bodies. The protein making up the crystalline matrix of an NPV occlusion body is known as polyhedrin (O'Reilly *et al*., 1992).

A unique feature of the NPV life cycle is the production of two virion phenotypes. Those virions found within polyhedra are termed "polyhedra-derived virus" (PDV) and the other form, found in the hemocoel of the infected host insect, is termed "budded virus" (BV). Historically, a number of terms have been used for the two virion phenotypes. The PDV phenotype has also been called "occluded virus" (OV). Alternate names for the BV phenotype include "nonoccluded virus" (NOV) and "extracellular virus" (ECV or EV) (Blissard and Rohrmann, 1990). The composition of the envelopes of the two forms is different, PDV envelopes are synthesized within the nucleus during occlusion body morphogenesis, BV envelopes are acquired when newly synthesized nucleocapsids bud through the host cell cytoplasmic membrane and are released into the haemolymph. Spikes of a virus-coded glycoprotein (budded virus envelope fusion protein) protrude through the end of virus particle (Hunter-Fujita *et al.*, 1998).

The main features of the biology of NPVs are illustrated in Figure 3. Polyhedra are ingested by a susceptible insect and solubilized in the alkaline condition of the midgut. Virions of the polyhedra derived virus (PDV) phenotype are released and enter midgut epithelial cells by fusion with microvilli. Nucleocapsids are transported to the nucleus where uncoat of the viral DNA occurs. Progeny nucleocapsids bud through the nuclear membrane and are transported to plasma memrane but apparently lose the nuclear derived envelope in the cytoplasm. The nucleocapsids then bud through the cytoplasmic membrane into the hemocoel acquired the budded virus (BV) specific envelope that contains the virus-encoded envelope glycoprotein. These virions (of the BV phynotype) appear to be specialized for secondary infection of other host cells. A second group of progeny nucleocapsid becomes enveloped within

the nucleus by *de novo* assembled envelope. These virions are subsequently occluded within polyhedrin protein. Maturation of the polyhedra includes the addition of polyhedral envelope around the periphery of the forming occlusion bodies. Upon insect death and cell lysis, the polyhedra are released into the environment (Blissard and Rohrmann, 1990).

.

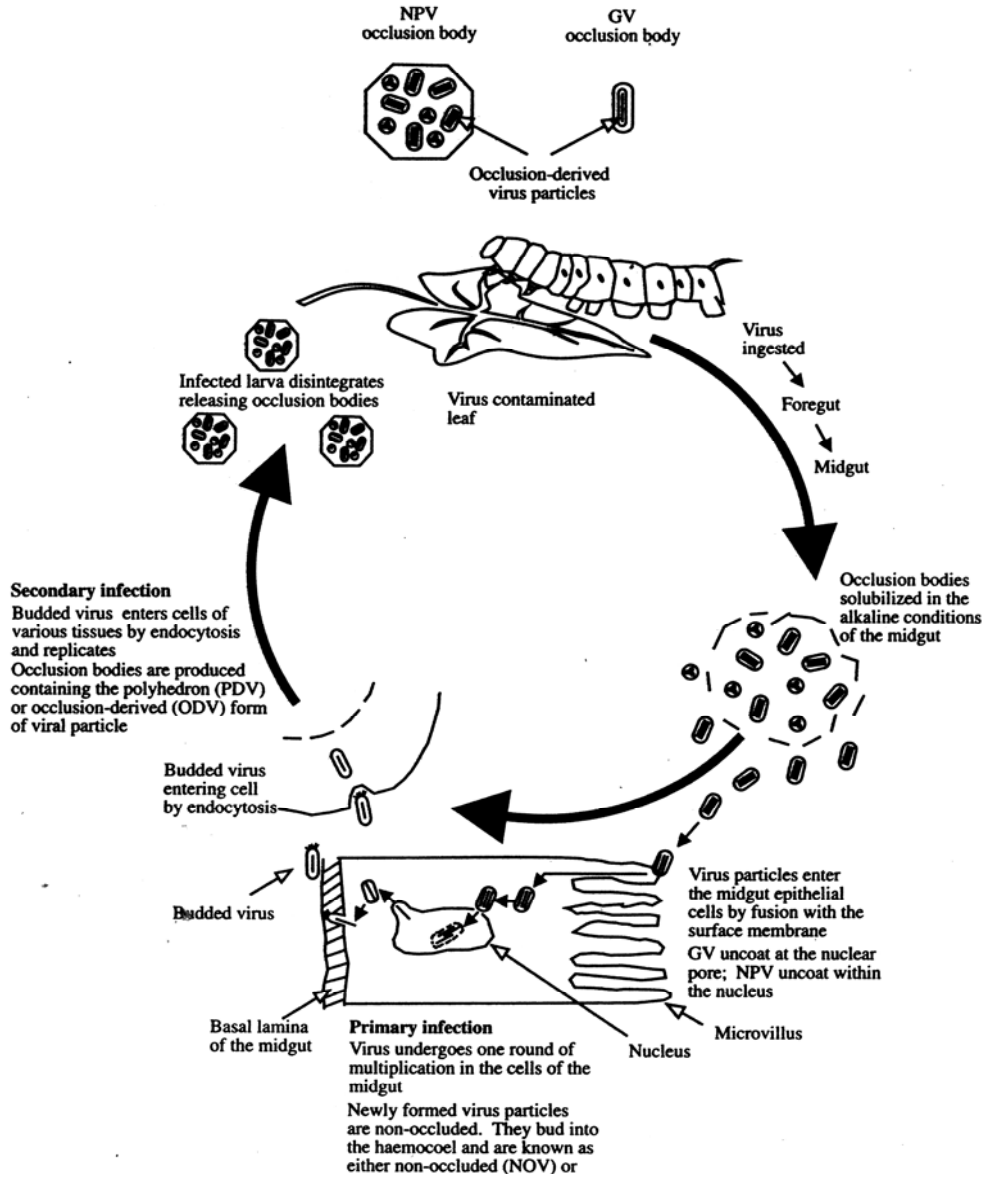


Figure 3 Typical cellular infection cycle of the nucleopolyhedrovirus. Polyhedra are ingested and are dissolved in the alkaline condition of the midgut, releasing infectious virions. Primary infection begins in which the virions infect midgut cell and budded virions (BV) are produced within the nucleus. The budded virions disseminate the virus within the insect host and start the secondary infection in which the pre-occludded virions (PDV) are produced. Subsequently, the PDV become embedded in the polyhedrin matrix and polyhedra are formed. The polyhedra are released into the environment when the insect dies and disintegrates.

Source: Hunter-Fujita *et al*. (1998)

2.2 *Bombyx mori* nucleopolyhedrovirus

Grasserie disease caused by *Bombyx mori* nucleopolyhedrovirus (BmNPV), is known under a variety of names. It is called “jaundice” in America, “grasserie” in France, “giallume” in Italy, “nobyo” in Japan and “gelbsucht” or “Fettsucht” in Germany and Austria (Tanada and Kaya, 1993). In Thailand, it is called “ rok tur”, “ rok taubeum”, “rok tauluang”, or “rok kati” (Kaewwises, 2000). There are two types of BmNPV particle, thick rod-shaped of 80 x 330 nm and slender rod-shaped with the size of 45 x 340 nm (Khosaka *et al*., 1971). In Thailand, the local strain BmNPV composed of rod-shaped nucleocapsids with the size of 45x340 nm, embedded within the polyhedral inclusion bodies (PIBs). The PIBs were 2-7 μm in diameter, and tetragonal shape with rather round angles. However, the globular and hexagonal PIBs were observed but unfrequently (Attathom and Sinchaisri, 1987).

Francki *et al.*(1991) had classified *Autographa californica* MNPV (AcMNPV) as the type species of the MNPV morphotype and *Bombyx mori* SNPV as the type species of the SNPV morphotype. The significance, however, of the SNPV classification of BmNPV is not clear since BmNPV can produce both MNPV and SNPV type virions depending upon the host or tissue in which it replicates. In general, NPVs produce two types of progeny, the budded virus (BV) and the occluded virus (OV), during its life cycle (Blissard and Rohrmann, 1990). BVs are predominantly produced during an early phase of infection and acquire their envelopes as they bud through the plasma membrane. BVs are highly infectious *in vitro* and are responsible for the systemic spread of the virus in the infected insect. OVs are thought to obtain their envelopes by *de novo* synthesis in the nucleus during a late phase of infection, and are subsequently occluded in polyhedra. In the field, polyhedra protect the occluded virions from the environment and are required for horizontal transmission (Rohrmann, 1992).

Infection mostly takes place through feeding of polyhedra-contaminated mulberry leaf, rarely through wounding. The factors influencing the outbreak of this disease are high temperature and humidity, or the sudden fluctuations of these two parameters, poor ventilation in the rearing room, and ineffective disinfection of rearing room and equipments and feeding of

tender mulberry leaves during late instars. Inadequate larval spacing, starvation and excessive moisture in the rearing bed also have been known to contribute towards the outbreak and spread of the disease (United Nation, 1990).

2.3 Symptomatology and pathology of *Bombyx mori* nucleopolyhedrovirus

Symptoms of grasserie disease caused by BmNPV were described as followed; during the early stage of infection, no symptoms are noticed except for the worms being slightly sluggish. Initially the skin shows oily and shining appearance. As the disease progresses, the skin becomes thin and fragile and the body becomes milky white with intersegmental swelling. The rupture of the fragile skin liberates the liquefied internal organs containing numerous numbers of polyhedra which become source of inoculum to the new host. The characteristic symptom of this disease is that the larvae become restless and crawl aimlessly along the ridges or rims of rearing trays, and subsequently falling on the ground and die. Death takes place after infection in about 4-5 days in the young larvae and 5-7 days in the grown up larvae (United Nation, 1990).

Johansen *et al.*(1986) reported that the infection of BmNPV was restricted to the nuclei of infected tissues. The evidence of NPV infection was the hypertrophied nuclei, almost filled the cells with numerous polyhedra. The fat body, tracheal matrix and hypodermis were heavily infected. Moderate infection was found in the nuclei of silk glands and the epithelial sheath of testes. There were no polyhedra in mid gut cells, muscle cells and malpighian tubules of silkworms. The follicular cells possess a relatively thick basement membrane at their basal surface which probably acts as a barrier to greatly retard the invasion of virus particle from the haemolymph. Attathom and Sinchaisri (1987) observed pathological changes occurred in NPV infected cells of silkworm. The hypertrophied nucleus undergoes chromatin and nucleolus disappearance. Nucleocapsids in group were observed in the diffuse mass of chromatin, the virogenic stroma, in the central area of the nucleus. As disease developed, the virions were membrane enveloped singly and occluded in the polyhedra. The polyhedra located randomly in the nucleus or in line along periphery of the nucleus. They also observed several ultrastructural

changes in associated with the BmNPV development, for example, the curved and vesicular membrane profiles appearing randomly in the infected nuclei.

2.4 Genes of *Bombyx mori* nucleopolyhedrovirus

BmNPV genome was 128,413 nucleotides long and contained 136 open reading frames (ORFs) encoding predicted proteins of over 60 amino acids (Figure 4) (Acharya *et al*., 2002). Gene expression pattern following baculovirus infection is regulated in a cascade fashion, where the activation of each set of genes relies on the synthesis of proteins from previous class. Based on this temporal regulation, the baculovirus genes are grouped into three classes; early, late and very late. Although most baculovirus genes can be placed into one of the above classes, some are transcribed in more than one phase. Early genes may be defined as those genes that are transcribed in the absence of any viral genes expression. The late and very late genes are dependent on early viral gene expression and on DNA replication. The transcription of very late genes, those expresses primarily during the occlusion phase, is activated approximately 10 to 12 hr after the initiation of late gene transcription (O'Reilly *et al*., 1992).

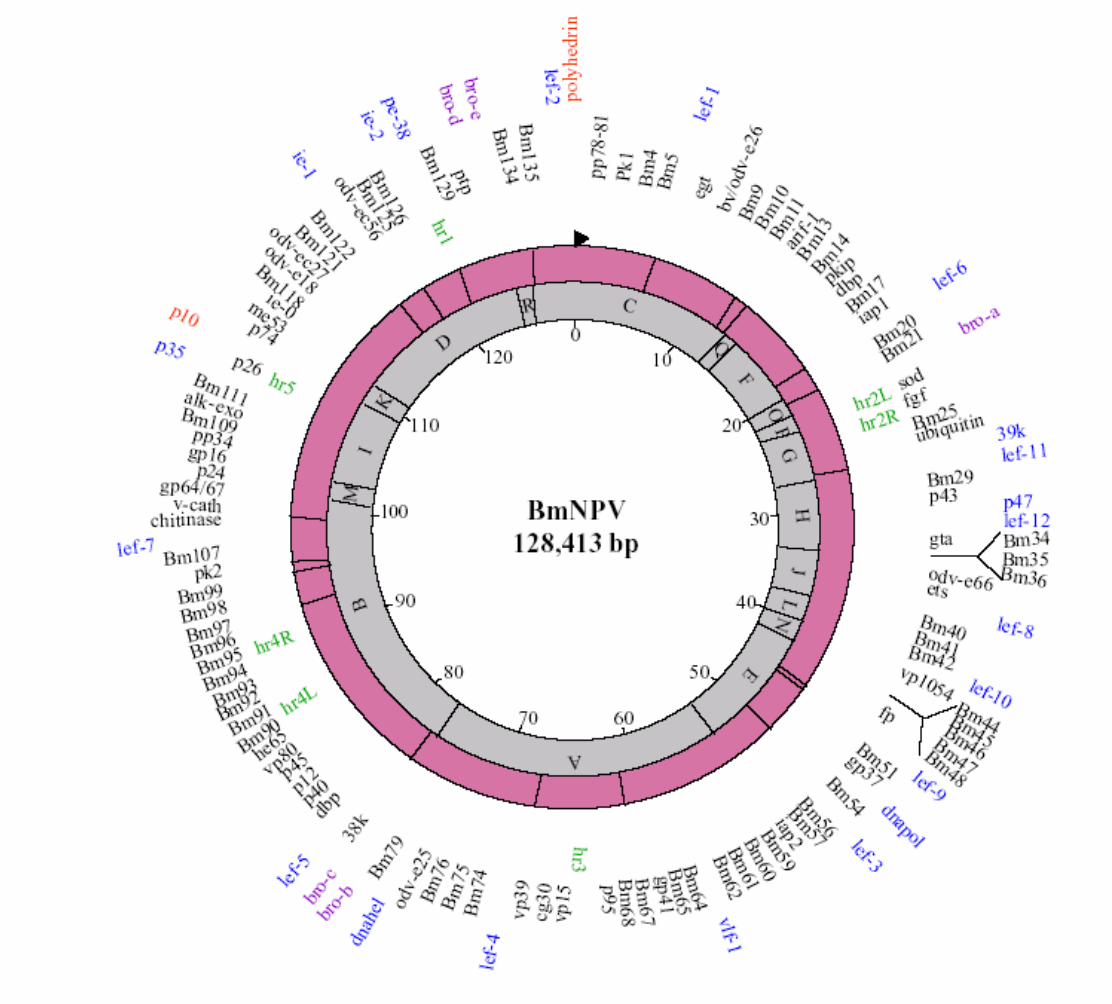
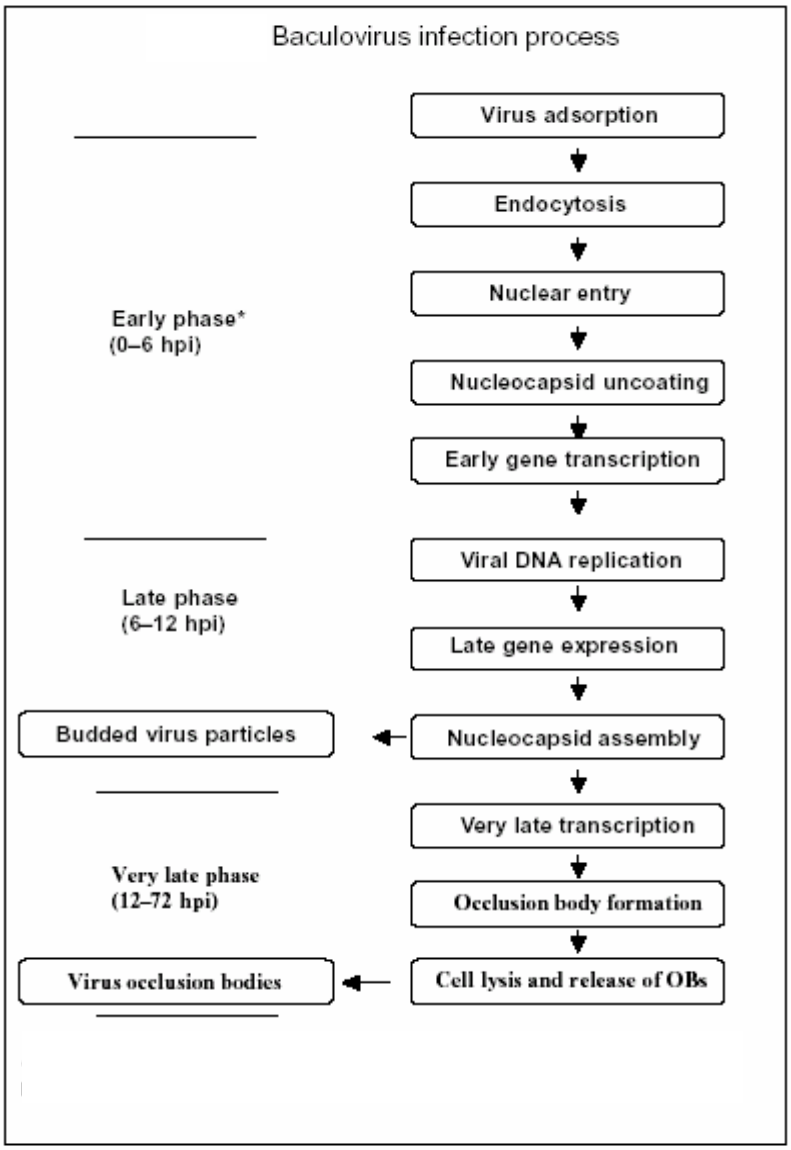


Figure 4 Genomic organization of BmNPV. BmNPV genome is a covalently closed circular double stranded DNA of 128,413 bp.

Source : Acharya *et al*. (2002)

There are three phases of gene transcription of baculovirus as illustrated in Figure 5 (O'Reilly *et al.*, 1992; Boucias and Penland; 1998, Acharya *et al.*, 2002).



Figture 5 Baculovirus infection process contains three phases; early, late and very late phase. The timing are based on AcNPV infection. The kinetics of BmNPV infection is a little slower; hpi = hours post infection. OBs = occlusion bodies.

Source : Acharya *et al*. (2002)

2.4.1 Early phase of gene transcription

In a permissive cell line, the baculovirus displays a temporally regulated biphasic life cycle. Phase I is marked by the production of the budded phenotype and requires the early and late gene transcription, whereas phase II results in the formation of the OV phenotype and the occlusion production.

The release and unfolding of viral DNA in the nucleus mark the initiation of gene transcription. Historically, the early transcription phase has been differentiated into the immediate-early and the delayed-early stages. Immediate early (IE) genes are those genes that can be transcribed by uninfected insect cells and require no viral gene products for their expression. Delayed early (DE) genes, on the other hand, require other viral gene products for their transcription. Early phase transcription, around 0-6 hrs post infection (h pi) results in the production of a series of structural and nonstructural proteins. These include the regulatory proteins (immediate-early, IE; late expression factors, Lefs), the cell and host modulator proteins (anti-apoptotic p35 and ecdysone UDP-glycosyl transferase), biosynthetic enzymes (viral RNA and viral DNA polymerase complexs), and various structural proteins. Early genes are transcribed by RNA polymerase II. Many early promoters contain a functional TATA box typical of most RNA polymerase II promoters of higher eukaryotes (Boucias and Pendland, 1998).

2.4.2 Late phase of gene transcription

The transition from early to late phase (>6 h pi) transcription is marked by two events; the replication of the viral DNA and the switch from early to late promoters. Late gene transcription is inhibited by aphidocolin, a DNA synthesis inhibitor, and cycloheximide, a protein synthesis inhibitor. All late genes possess the highly conserved late promoter (ATAAG) motif. which unlike the early promoters, is recognized by a novel viral RNA polymerase complex resistant to α-amanitin, an antibiotic known to bind to and inhibit RNA polymerase II. The viral RNA polymerse activity, extracted from infected cells by glycerol gradient ultracentrifugation , contains an array of peptides having sizes corresponding to various baculovirus-encoded late

expression factor (Lef) (Boucias and Penland, 1998). Many of late gene products are proteins that in concern with the early proteins produce the BV phenotype. Morphogenesis of the nucleocapsids takes place in the nucleus. Component capsid proteins, synthesizes on the ribosomes in the cytoplasm, are preferentially transported back into the nuclear region (Boucias and Penland, 1998).

The late phase extends from 6 h pi to approximately 20 to 24 h pi. During this phase, a distinct electron-dense structure known as the virogenic stroma forms in the nucleus. Capsid sheaths appear to assembly in pockets at the edge of the virogenic stroma and are filled, while associated end-on with the stroma, with a nucleoprotein core to form nucleocapsids. Progeny nucleocapsids leave the nucleus, possibly a variety of routes, and travel to the cytoplasm through the cytoplasmic membrane where they interact end-on with regions of this membrane having peplomerlike structures. Nucleocapsids usually bud individually from the cytoplasmic membrane although occasionally more than one nuclecapsid is observed in a budded virion (O'Reilly *et al*, 1992).

2.4.3 Very late phase of gene transcription

The very late phase or occlusion-specific phase begins around 20 h pi. Electron microscopy reveals the elaboration of membrane envelope segments within the nucleus during this occlusion process. Nucleocapsids interact end-on with membranes and eventually become enveloped, either individually (SNPVs) or in groups (MNPVs). Envelopment of the nucleocapsids appears to be an essential prior to embodiment within the polyhedrin matrix of an occlusion body. During the occlusion phase, the production of budded virions is greatly reduced, if not terminated (O'Reilly *et al*., 1992).

The first light microscopic indication of this occlusion phase is the formation of several polyhedral inclusion bodies (PIBs) in the nucleus. As they accumulate, the PIBs often form a ring around the inside of the nuclear membrane, eventually, the nucleus becomes virtually filled with occlusion bodies. The second phase of baculovirus replication involves the synthesis

and release of the viral occlusions. The polyhedrin and p10 are the two dominant very late genes. The very late promoters lack of the TATA and CATG motifs of the early gene but it possesses a group of 7 nucleotides upstream of the TAAG and AT-rich untranslated downstream region that mediates the hyper-expression of the very late genes (Boucias and Pendland, 1998).

The one important gene of the very late genes is polyhedrin gene. Polyhedrin is a phosphorylated molecule of 28.8 kDa (Gomi *et al*., 1999) which constitutes the matrix of baculovirus occlusion bodies so called polyhedra. Polyhedrin plays a significant role in the replicative cycle of baculovirus. It is a late protein which is synthesized in the last infection step in large amounts and this in turn reflects a corresponding abundance in the amount of accumulated polyhedrin mRNA, probably achieved through high rates of mRNA synthesis. This suggests that heterologous genes can be introduced into the genomes of baculoviruses and highly expressed effectively under the control of the polyhedrin promoter (Iatrou *et al.*, 1985). This high level of expression from polyhedrin gene promoter makes recombinant baculovirus expression vector system very useful in foreign protein production (Patil and Dandin, 1990). The occlusion body facilitates virus survival and dispersal in the environment and, in the context of biological control, is a convenient, safe, and simply manipulated product (Burand *et al*., 1992). Studies of occlusion body protein suggest that the numerous different lepidopteran NPVs have evolved from a lepidopteran NPV rather than cross-infecting from other orders of arthopods. Gene sequence data relating to occlusion body proteins (polyhedrin or granulin) are proving to be an important tool for discerning phylogenetic relationships, allowing lepidopteran NPV evolution to be divided into two distinct branches, namely group I and II (Zanotto *et al*., 1993).

3. Grasserie disease of mulberry silkworm

3.1 Factors affecting the grasserie disease

There were many factors that accelerate outbreak of grasserie disease in Thailand (Attathom and Sinchaisri, 1987). First, many of Thai farmers raise multivoltine type silkworm, the more frequency of the rearing times per year, the less thorough disinfection of the rearing

places will be practiced. Therefore, inadequate sanitation is of fundamental reason for outbreak of the disease in those poor-management farms. Second, in the remote area where it is inconvenient to obtain silkworm eggs from the Government Experiment Station, the farmers maintain their own seed stocks. Such seeds apparently have no guarantee on the quality and disease. Continuous cultivation of silkworms from these seed stocks creates the problems of inbreeding as well as the outbreak of the diseases. Third, unsuitable rearing condition, for example inappropriate rearing places and tools, poor ventilation, overcrowding, poor quality of mulberry leaves and improper rearing techniques will all weaken the silkworms to the infection of viral disease. The well-management sericultural farms are practically unaffected by the viral disease.

3.2 The prevention and control of silkworm disease

3.2.1 The use of anti-disease varieties

In view of the inadequate disinfection and prevalence of unhygienic conditions in the rearing areas, the use of disease resistant silkworm varieties can be the better option.

Eguchi *et al* (1998) had succeeded in evolving a silkworm hybrid which is completely resistant to *Bombyx mori* densonucleosis virus type 1 (BmDNV 1). Since the resistance to BmDNV 1 is controlled by a single major gene. It is possible to introduce the resistance into a susceptible breed easily by cross breeding.

On the contrary, resistance of the silkworm to nuclear and cytoplasmic polyhedrosis virus (NPV and CPV) and also infectious flacherie (IFV) is controlled by polygenes (Watanabe,1986). Therefore, introduce of complete resistance to these viruses is practically impossible in the silkworm breeds. However, breeding of strains with comparatively more resistance to these viruses is possible by selection of survivors after virus exposure (Uzgawa and Aruga, 1966).

In India, attempts had been made to induce resistance to BmNPV into a susceptible silkworm stock Ka by mating with a relatively more resistant stock, g133 followed by the exposure of progenies to BmNPV and selecting the survivors. The F1 population was found to have a survival rate of 41.66% in which the average of resistant stock was 68.84% and susceptible stock was 20.55%. F2 generation was comprised of segregating population so a wide range of survival was observed ranging from 50-86% (Sen *et al.*, 1999).

In Poland, Kremky and Michalska (1988) studied on resistance of silkworm line to BmNPV. The studies were carried out during four sericultural seasons on six silkworm lines inoculated with BmNPV and submitted to a low temperature treatment. Survival rates were estimate on the 9^{th} day after the treatment, and before pupation. Survival rate of the group inoculated with the virus in every generation was higher than the group exposed to a single inoculation. Low temperature treatment to the larvae activated latent form of the virus. Survival rate in different tested lines was similar, but reaction to inoculation and low temperature was different.

The utilization of disease-resistant varieties to prevent different kinds of disease has many advantages. No specific measures and chemical reagents are needed. The method saves labour power, material and expenses, reduces the environmental contamination, and obtains an obvious effect for disease prevention. With these advantages, it can easily be accepted, be used and be popularized. In recent years, the research on breeding of disease-resistant varieties has received much attention and shows more and more significant advantages in the field of agriculture. Up to now some disease-resistant varieties to different kinds of diseases have been bred by some silkworm breeding units in Japan. According to the epidemic situations in different areas and seasons, they use different varieties which are able to resist diseases correspondingly so that the epidemic disease are under control (Liu and Zhong, 1989).

3.2.2 Chemical method

In the past, it was considered as a non-realistic way to control the diseases of silkworm by chemical treatment, however, with the development of silkworm pathology in recent years, these methods have been used in silkworm production scale, and some of them have shown a gratifying prospect. Nevertheless, the chemical treatment needs to be improved. The major reason is that the time of these chemicals remaining in the silkworm's body is very short, so that the reagent needs to be applied day by day, and this affects silkworm health and brings a lot of troubles to the sericultural management.

The chemicals affect the metabolism of pathogens through oxidation of tissue or by denaturation and precipitation of protein of causal organisms. Disinfection of the rearing house is done 8-10 days before starting the rearing operation. The effectiveness of chemical disinfectant depends upon three factors i.e., the concentration, the duration and ambient temperature. Some of the chemicals commonly used as disinfectant are chlorine compounds, formalin, paraformadehyde and lime power (Pallavi and Kamble, 1997).

3.2.3 Physical methods

The physical methods include simple and effective physical treatment such as sunlight, streaming, burning, burying. Virus diseases are the most disastrous of silkworms' disease which could be brought under control, through integrated several control measures suitable to the existing conditions, such as destruction of pathogens through disinfection, breaking the transmission cycle, segregation of moulting worms, improvement of feeding and management to increase vigor and disease resistance, and selection of disease resistant varieties. To prevent the spread of virus, disinfection is rigorously implemented immediately after harvesting the cocoons as dead worms are the most concentrated source of infection. (Liu and Zhong, 1989).

3.2.4 The quarantine of silkworm disease

The grasserie disese predictive inspection have been developed to provide an early and accurate diagnosis method for this vial disease such as the enzyme-link immunosorbent assay (ELISA) (Vanapruk *et al.*, 1992; Shamim *et al.*, 1994), DNA hybridization (Attathom *et al.*, 1994), colloidal textile dye-based dipstick immunoassay (Nataraju *et al.*, 1994), monoclonal antibody (Nagamine and Kobayashi, 1991) and western blot analysis (Chaeychomsri *et al.*, 1995).

For pebrine disease of silkworm, the quarantine of silkworm diseases is an essential step to prevent the embryonic infection. Several predictive inspection and early diagnosis of pebrine disease have been developed, such as monoclonal antibody-sensitizes latex for pebrine spore diagnosis (Shi and Jin, 1997), multiprimer PCR for the early and simultaneous detection of several kinds of microsporidia that cause silkworm pebrine (Hatakeyama and Hayasaka, 2003). The predictive inspection of pebrine disease can help to adopt a corresponding measure for disease prevention.

During the current decade, with the permeation and enhancement of other outstanding techniques, the predictive diagnosis of viral disease and pebrine disease has made great progress. The methods mentioned above are only some examples, these methods can be widely used in correct diagnosis of pathogens and to know the possibility of cross infection among different kinds of insects or animals, it will help the research of silkworm infection disease. However, many of these methods are just research measures suitable for laboratory diagnosis. The ordinary method is still need for large scale inspection of the production line. For example, the ordinary microscopy still can not be discarded in prebrine spore's inspection (Liu and Zhong, 1989). A method which is more convenient and useful for quarantine in large-scale production still requires a greater effort in research endeavor.

3.3 Detection of nucleopolyhedrovirus

Viral disease especially that caused by the nucleopolyhedrovirus is of fundamental importance disease of silkworm. It occurred prevalently in rural areas where the Thai local varieties of silkworm were cultivated, therefore, imposed serious economic damage to the Thai

sericulture. In some farms, the viral disease outbreak can destroy the whole colony of silkworm. This phenonema often occurred in the fifth instar larvae which caused serious loss in both economy and labour intensity to the farmers. Therefore, several detection methods have been employed to early detect the present of grasserie disease in order to stop spread of the disease within the rearing colony and solve the disease problem.

3.3.1 Light microscope

Although virion of BmNPV cannot be seen by the light microscope, the occlusion bodies which covered the virions can be seen. This method is convenient and cheap. However, it is a long procedure and is not suitable for many samples. At present, light microscope is still used at Sericulture Research Centres and Sericulture Experiment Stations for grasserie disease detection.

3.3.2 Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is the most commonly applied immunoassay, in which antigen-antibody complexes are absorbed into wells in plastic microtitre plates. This method is suitable for large sample numbers and can detect low concentration of virus. Moreover, results can be easily seen from colour change. ELISA has been proven to be a sensitive and reliable method for the detection of grasserie disease. Vanapruk *et al.* (1992) showed that viral detection by ELISA was more efficient than the light microscope. Using ELISA, viruses can be detected within 1 day after inoculation, whereas, by light microscopes the virus can be detected after 3 days post-inoculation. However, this method is rather complicated and costly.

3.3.3 Colloidal textile dye-based dipstick immunoassay

Colloidal textile dye-based dipstick immunoassay was developed for BmNPV detection. The purified anti-BmNPV IgG was used to capture the antigen on nitrocellulose

membrane attached to a dipstick, and antigen was detected with colloidal textile dye labeled anti-BmNPV IgG. Dipstick textile blue dye sensitized with 500 µg/ml of affinity purified and anti-BmNPV IgG, could detect 10 ng/ml of antigen by forming a clear blue dot in 30 min. The sensitized dye was observed to be stable for a 3 months period at 4°C (Nataraju *et al*., 1994).

3.3.4 Monoclonal antibody-based sandwich ELISA

Shamim *et al.*, (1994) studied the development and characteristics of five hybrid cell clones secreting Murine monoclonal antibodies (MCAs) directed against BmNPV. Antibodies recognized to a variable extent four different strains of nuclear polyhedra, e.g., *Borrelina bombycis, Amsaeta olbistriga, Heliothis armigera and Spodoptera litura.*

Murine monoclonal antibodies (MCAs) were produced against *Borrelina bombycis* nuclear polyhedra. In ELISA all five MCAs reacted with nuclear polyhedra of *B. bombycis* and to a variable extent with other strains of nuclear polyhedra, namely *Amsaeta olbistriga, Heliothis armigera and Spodoptera litura*. In western blots, these recognized antigens of 31 (polyhedron protein) and 67 kDa of nuclear polyhedra (MA-321 showed reduced binding). In sandwich enzyme immunoassay employing purified rabbit polyclonal antibodies, MA-321 as low as 100 nuclear polyhedra could be detected. The results obtained by sandwich ELISA on haemolymph samples collected from rearing places revealed 100% correlation with results by microscopic examination. Haemolymph samples collected on various days from second instar larvae infected in the laboratory revealed that the sandwich ELISA could detect infection as early as 96 hr after infection.

3.3.6 Agar diffusion technique

Krywienczyn and Bergold (1961) reported that upon comparing the serological findings with the taxonomic classification of the host insects, considerable differences existed among nine systems of Lepidoptera classification available to them. The comparison suggested that the polyhedron inclusion-body proteins isolated from Tortricidae and Pieridae were more

closely related to the inclusion-body protein from Bombycidae, Saturniidae and Geometridae than to those from Galleriidae, Noctuidae, or Lymantriidae.

3.3.7 Fluorescent antibody technique

The fluorescent antibody technique employed was developed the first by Coons and Kaplan in 1950, and has been successfully applied in clinical diagnostic work involving bacteria, fungi, protozoa, rickettsiae and viruses (Krywienczyk, 1963). Krywienczyk (1963) found which the fluorescence in the cytoplasm of infected cells before crystallization of polyhedra in the nuclei which suggested that the inclusion-body proteins are synthesized in the cytoplasm. In the later stage they concentrate in the nuclei and their crystallization starts after the virus particles have been form there.

3.3.8 PCR technique

Recent advances in molecular biology propose the more powerful techniques for baculovirus detection. Those methods basically based on the detection of the nucleic acid of the causative agent of the disease. One of DNA-based method is polymerase chain reaction (PCR). The PCR technique exploits the ability of the enzyme, DNA polymerase to synthesize many complementary strands of DNA from a very small amount of DNA template. The DNA sequence to be amplified is identified and two short oligonucleotide sequences (primers) are constructed, each being complementary to one or other of the 3' ends of the template sequence. By subjecting the reaction mix to cycles of heating and cooling at selected temperature, the two strands separate. Following this, the temperature is reduced allowing the two primers to anneal to complementary strands and synthesis of a new copy of the DNA takes place across the region flanked by the primers, beginning at the 3' end of each primer. Each strand and the amount of target DNA to which the primers can anneal have been doubled. By cycling through the separation, annealing and synthesis (or extension) temperature *n* times, it is theoretically possible to produce 2^n copies of the DNA region between the primers. At the end of the reaction the resulting PCR (or amplification) product can be electrophoreses and visualized on an agarose gel.

The successful amplification of a DNA fragment will be indicated by a discrete band of the same size as the target length, i.e. the sequence flanked by the primers (Hunter-Fujita *et al*., 1998). PCR-based method was developed for the detection of viral DNA in infected insect and shrimp (Faktor and Raviv, 1996; Otta *et al.*, 2003). Moreover, PCR technique was employed to detect baculovirus DNA sequences from viral occlusion bodies contaminating the surface of moth eggs (Burand *et al*., 1992). PCR technique was used in combination with other methods such as ELISA, so called PCR-ELISA (Sukkhumsirichart *et al.*, 2002). Nowadays real-time PCR was used to determine Baculovirus quantitatively (Rosinski *et al.*, 2002; Lo and Chao, 2004).

MATERIALS AND METHODS

Materials

1. Equipments

Autoclave	: HVE-110 (Hirayama, Japan)
Automatic micropipettes	: 10, P20, P100, P200 and P1000 (Gilson Medical Electronic S.A., France)
Electrophoresis apparatus	: i-Mupid (Cosmo Bio Co., Ltd., Japan)
	: Hoefer HE99X (Amershame Bioscience, USA)
Freezer	: -20 °C (Songserm Intercool, Thailand)
	: -86 °C Freezer (Sanyo, Japan)
Gel Documentation	: Gene Genius (SynGene, USA)
Incubator Shaker	: GFL 3032 (GFL, USA)
Laminar Flow Cabinet	: NU-440-300E (NuAire, USA)
Magnetic stirrer	: M21/1 (Franz-Morat KG GmbH, Germany)
Microcentrifuge	: A14 (Jouan, France)
Microwave oven	: EME 1960 (Electrolux, USA)
PCR Thermal Cycler	: PCT-100 (MJ Research, USA)
Power supply	: EPS-301 (Amershame Bioscience, USA)
Refrigerated microcentrifuge	: 3K18 (Jouan, France)
Shaking water bath	: WB/OB-45 (Memmert, Germany)
Spectrophotometer	: Spectronic GeneSys 5 (MiltonRoy,USA)
Ultracentrifuge	: L 8-60 M (Beckman, USA)

2. <u>Chemicals</u>

Absolute ethanol (BDH, England)

Agarose gel (FMC Bioproducts, USA)

Bacto-agar (Oxoid, England)

Bacto-tryptone (Oxoid, England)

Bacto-yeast extract (Oxoid, England)

Boric acid (Merck, Germany)

Bromophenol blue (Merck, Germany)

Chloroform (Merck, Germany)

100 mM dATP, dCTP, dGTP and dTTP (Fermentas, USA)

Ethidium bromide (Sigma Chemical Co., USA)

Etylene diamine tetraacetic acid, disodium salt dihydrate (Sigma Chemical Co., USA)

Hydrochloric acid (Merck, Germany)

Isoamyl alcohol (Merck, Germany)

2-Mercaptoethanol (Sigma Chemical Co., USA)

25 mM $MgCl_2$ (Perkin-Elmer Cetus, USA)

Marker : λ DNA- *Hin*dIII and ϕ X174 DNA-*Hae*III Mix. (Finnzymes, Finland)

Marker : λ DNA- *Hin*dIII (SibEnzyme, Russia)

Marker: 100 bp + 1.5 Kb DNA ladder (SibEnzyme, Russia)

10X PCR buffer (Finnzymes, Finland)

Phenol, redistilled (Aldrich Chemical Co., USA)

Potassium chloride (Merck, Germany)

Sodium acetate (Merck, Germany)

Sodium chloride (APS Chemical Limited, Canada)

Sodium dodecyl sulfate (Sigma Chemical Co., USA)

Sodium hydroxide pellet (Merck, Germany)

Tris (USB, Amershame Life Science, England)

QIAquick® PCR purification kit (QIAGEN, Germany)

3. Enzymes

DyNAzyme™ II DNA Polymerase (Finnzymes, Finland)

Proteinase K (QIAGEN, Germany)

Ribonuclease A (Sigma Chemical Co., USA)

Restriction endonucleases: *Bam*HI, *Bgl*II, *Hin*dIII, *Nco*I, *Pst*I, *Sal*I (New England Biolabs, USA)

4. Bacterial strain

Escherichia coli: strain DH5α

5. Cloning vector

pGEM®-T vector (Promega Corporation Medison, Wisconsin, USA)

Methods

1. Restriction pattern analysis of Thai BmNPV

1.1 Propagation of BmNPV

Fourth instar silkworm larvae of Nang Noi Sri Sa Ket variety obtained from Nong Khai Sericultural Station were inoculated with BmNPV isolated from diseased larvae collected from Udon Thani Sericultural Research Center. Silkworm were starved for 24 hr and then fed with BmNPV-contaminated mulberry leaves. Heavily infected larvae were collected and put in amber glass bottle contained distilled water and kept at room temperature. The silkworm cadavers were allowed to putrefy and release the virus inclusion bodies or polyhedra which precipitated at the bottom of the container leaving most of the remains in supernatant.

1.2 Purification of BmNPV polyhedra

BmNPV was purified following the method of Attathom (1978). After removal of insect remains in the supernatant, the crude suspension of BmNPV polyhedra was filtrated through 4 layers of cheesecloth. The polyhedra in the filtrate were pelleted by centrifugation at 8,000 rpm for 15 min at 4 °C. The precipitate contained polyhedra were washed by centrifugation with distilled water several times until the supernatant became clear. The precipitate was then resuspended in distilled water and 2 ml of crude polyhedra was overlaid on the top of 35 ml of a 40-65% (w/w) sucrose density gradient. The gradients were prepared by layering 5 ml aliquots of sucrose solutions in distilled water in decreasing density steps of 5%, one upon another, and kept at 4 °C overnight before use. Gradients were centrifuged at 25,000 rpm for 1.5 hr at 4 °C in a Beckman L 8-60 M centrifuge using SW 28 swinging rotor. The polyhedral band appeared as creamy-white band at 54% to 56% of sucrose gradient was collected and washed with distilled water by centrifugation at 8,000 rpm for 15 min at 4 °C. Washing process was repeated several times to ensure removal of sucrose. The pellet of purified polyhedra was resuspended in distilled water. At this stage, polyhedra were sufficiently pure for most purposes and kept at -20 °C for

further use. A drop of purified polyhedra suspension was placed on a slide, spead with a cover slip and the purity was examined under a compound microscope.

1.3 Extraction of whole genomic BmNPV DNA

DNA extraction following the method of Chaeychomsri (2003) was performed. Virions were released from the polyhedral inclusion bodies by dissolving polyhedral suspension (at concentration of approximately10^8 PIB/ml) with alkaline solution (0.2 M Na_2CO_3 , 0.5 M EDTA, 0.34 M NaCl) at the ratio of 1:1. The suspension was incubated on ice and stirred for 30 min or until the solution become clear. After centrifugation at 12,000 rpm for 1 min to precipitate contaminants in the solution, clear solution of 200 µl that contained the released virus particles was transferred to the new microtube. Then, 200 µl of 2% SDS (sodium lauryl sulphate) was added to disrupt the virus particles and release the DNA and 25 ul of Proteinase K (10 mg/ml) was added in order to digest any present nucleases. The mixture was incubated at 37 °C overnight. Then 8 µl of RNaseA (10mg/ml) was added and incubated at 37 °C for one hr. Genomic DNA was extracted with phenol, phenol/chloroform/isoamyl alcohol (25:24:1), and then with chloroform/isoamyl alcohol (24:1). DNA was precipitated with a mixture of 0.1 vol of 3 M sodium acetate, pH 5.2 and 2.5 vol of absolute ethanol and then stored at -20 °C overnight. After centrifugation at 12,000 rpm for 20 min, the DNA pellet was washed with 70% ethanol, vacuum dried for 15 min, resuspended in 50 µl of TE buffer (10 mM Tris-HCl, pH 8-1 mM EDTA), and stored at 4 °C. Purified BmNPV-DNA was analysed in 1% TAE agarose gel electrophoresis.

1.4 Restriction enzyme digestion

Viral DNA was digested with *Bam*HI, *Bgl*II, *Hin*dIII, *Nco*I and *Pst*I restriction endonucleases under conditions recommended by the supplier. The mixture was incubated at 37°C for 6 hr. DNA fragments were fractionated in 0.6% SeaKem GTG agarose in 1X TBE1 agarose gel electrophoresis. The gel was then stained for 15 min in 10 µg/ml solution of ethidium bromide. DNA fragments were visualized and photographed using a UV transilluminator. Data

were analyzed by using ID Image analysis software version 3.6.1, Eastman Kodak Company, New York.

2. Identification of polyhedrin gene of Thai BmNPV

2.1 PCR amplification of BmNPV *polh*

Primer set used in PCR for the amplification of the *polh* sequence was designed from the nucleotide sequence of BmNPV deposited in GenBank accession number L33180. This sequence contains whole genome of Japanese BmNPV (T3). The primers were designed from franking sequences of BmNPV(T3) *polh*. The oligonucleotide sequences of forward and reverse primers were:

Forward primer : 5'- CCCAAGATGTATAAACCA- 3'

Reverse primer : 5'- GCCTAACGCGCCCGATGTT- 3'

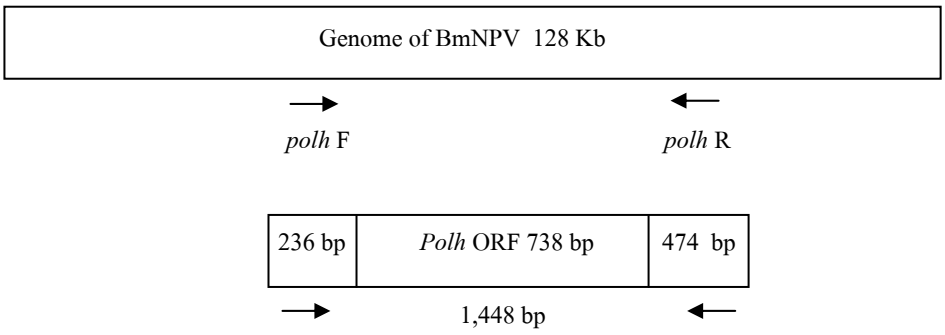


Figure 6 Diagram of the genome of BmNPV. Location of the primers used for PCR amplification is indicated. The predicted amplification product is 1,448 bp.

This primer pair amplified a 1,448 bp PCR product of *polh* ORF and flanking region. Each PCR reaction mixture consisted of 1 µl of DNA (~50 ng) sample, 1 unit of *Taq* DNA polymerase, 5 µl of 10x reaction buffer, 2 µl of 50 mM $MgCl_2$, 2 µl of 2.5 mM each of dNTPs and 1µl of 10 ρM of each forward and reverse primers in a total volume of 50 µl. The cycling

parameters for PCR were as follow; initial denaturation at 94 °C for 5 min, 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, for 35 cycles and followed by the final extension of 5 min at 72 °C in DNA thermal cycler PTC-100 (MJ Research, Inc). PCR products were fractionated in 1% TAE agarose gel electrophoresis.

2.2 Cloning and sequencing of BmNPV *polh*

PCR products of BmNPV *polh* were purified by QIAquick® PCR purification kit according to the method provided by the manufacturer. Purified products were ligated into pGEM®-T vector (Promega) at the ratio of 3:1. The reaction mix contained 3 µl PCR products, 1 µl of pGEM®-T vector and 5 µl of rapid ligation buffer. Reaction mix was made up to 10 µl with distilled water and incubated at 16 °C overnight.

The plasmid vector was transformed into competent *Escherichia coli* DH5α cells by heat shock. Two µl of ligation mix were added to 50 µl aliquots of *E. coli* strain DH5α competent cells and the mixture was incubated on ice for 30 min. Cells were then heat shocked at 42 °C for 90 sec, and placed on ice for 2 min. The cells were resuspended in 800 µl of LB (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl) and incubated in a shaking incubator at 37 °C for 60 min. The dilutions were plated out on LBA (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl and 1.5% bacto agar) containing 50 µg/ml ampicillin, 80 µg/ml X-gal and 60 µg/ml IPTG (isopropylthio-β-D-galactoside) and shaken at 250 rpm at 37 °C for16-18 hr for isolation of recombinant plasmids. Recombinant clones were identified by blue/white colony selection. The selected white colonies were then incubated overnight in LB medium containing 50 µg/ml ampicillin.

Plasmid DNA was extracted by alkaline lysis method of Sambrook and Russell (2001). Recombinant clone was grown overnight at 37 °C with vigorous shaking in 3 ml of LB containing 50 µg/ml of ampicillin. A 1.5 ml aliquot of cell suspension was centrifuged at 12,000 rpm for 1 min and the supernatant was discarded. The pellet was resuspended in 100 µl of chilled solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0) and incubated at

room temperature for 5 min. Two hundred microliters of freshly prepared solution II (0.2 N NaOH, 1% SDS) were added, and the content mixed by inverting the tube rapidly 2-3 times. The solution was then neutralized by adding of 150 µl of ice-colded solution III (3 M potassium acetate, 5 M glacial acetic acid) and placed on ice for 5 min. The content was centrifuged for 10 min at 12,000 rpm and the supernatant was extracted with equal volume of chloroform:isoamyl alcohol (24:1). The DNA was precipitated with 2 vol of ice-colded isopropanol, and centrifuged for 10 min at 12,000 rpm. The pellet was washed with 70% ethanol and centrifuged at 12,000 rpm for 5 min. The pellet was air dried, resuspended in 20 µl TE and stored at -20 °C. Before subjected to sequencing, clones of BmNPV *polh* were screened by PCR technique using the same primer set and *Hin*d III-*Sal*I restriction enzyme since the restriction sites of these two enzymes were present in BmNPV *polh* sequence.

Nucleotide sequences of the cloned PCR products were determined by cycle sequencing using a fluorescent dye dideoxy chain termination sequencing reaction kit and an applied ABI prism 377 sequencer. DNA sequencing service of DNA Technology Laboratory, BIOTEC, Thailand was employed through out this study.

2.3. Comparison and phylogenic analysis of the *polh* genes from different NPVs

A Lasergene®(DNASTAR, Inc) was used for analysis of the *polh* sequence. SeqMan program was used to assembly *polh* sequence from two strands DNA. Thai BmNPV *polh* sequence was submitted to the GenBank and the accession number AY779044 was designated. Two data sets, nucleotide sequence of the *polh* coding region and the predicted amino acid, were compared with data from GenBank. Thai BmNPV *polh* was compared with five BmNPV *polh*s; Japanese1 (T3, L33180) (Gomi *et al.*, 1999), Japanese 2 (M30925) (Maeda *et al*., 1985), Korean (K1, U75359) (unpublished), Chinese (X63614) (unpublished) and Canadian (M100430) (Iatrou *et al*., 1985). In addition, *polh* nucleotides and amino acid sequences of *Autographa californica* NPV(AcNPV; NC_001623) (Ayres *et al*., 1994), *Helicoverpa armigera* NPV (HaNPV; NC_002654) (Chen *et al*., 2001), *Spodoptera exigua* NPV (SeNPV; NC_002169) (IJkel *et al*., 1999) and *Spodoptera litura* NPV (SlNPV; NC_003102) (Pang *et al*., 2001) were

compared with Thai BmNPV *polh*. DNA and amino acid sequences were analyzed by Clustal Method of MegAlign. Phylogenetic tree and percent identity of nucleotide and amino acid sequence were generated by MegAlign .

3. Grasserie disease detection by PCR

3.1 Samples of BmNPV- infected silkworm

3.1.1 Artificially inoculated samples

In general, the older larvae are more resistant to virus infection than the younger larvae, therefore, silkworm of the 1^{st} , 2^{nd} and 3^{rd} instar (early instar) larvae were inoculated with BmNPV at the concentration of 10^6 PIB/ml and silkworm of the 4^{th} and 5^{th} instars (late instar) were inoculated with BmNPV at the concentration of 10^7 PIB/ml. Inoculation was accomplished by feeding the silkworm with BmNPV-contaminated mulberry leaves on day 1 of each instar. After the contaminated leaves were totally consumed, the larvae were reared as normal with fresh mulberry leaves. Infected larvae were collected in the last day of each instar period. Infected pupae, moths and eggs were prepared by inoculation of the fifth instar larvae on day 1 with 10^6 PIB/ml of BmNPV (expected to be sublethal dose for the fifth instar larvae). The larvae were reared with fresh mulberry leaves and allowed to pupate, emerge into adult, mate and lay eggs. Male and female pupae and emerging moths were collected on the last day of their developmental stages. Eggs laid by the silk moths were collected after laying for 5 days. Samples of larvae, pupae, moths and eggs were also collected from clean colony of silkworm reared in separate clean room.

3.1.2 Naturally infected samples

Naturally infected samples were randomly collected from silkworm rearing houses of

a. governmental institutes
   1) Nong Khai Sericultural Experiment Station, Department of Agriculture, Nong Khai province
   2) Sericultural Extension Centre 9, Kanchanaburi, Department of Agricultural Extension, Kanchanaburi province
b. Private company
   1) The Thai Silk Company (Jim Thompson Farm), Nakhon Ratchasima province
c. Contrated farm
   1) Jim Thompson Farm , Nakhon Ratchasima province
   2) Chul Thai Silk Company, Phetchabun province
d. local farmer
   1) Kalasin province.

The collections were made for all stages of silkworm from egg to silk moth. Pupae and moths were collected in both sexes. All samples were kept at -20 °C until use.

3.2 Extraction of BmNPV DNA from silkworm samples

BmNPV DNA was extracted from each sample of silkworm using the modified alkaline lysis method of Sambrook and Russell (2001). At least 50 µl of homogenated tissue was required for each sample or group of samples. Since egg and the first instar larva were very small, the optimum numbers of sample that would provide enough DNA for PCR amplification were determined. DNA was extracted from 1, 3, 5, 10, 15 and 20 eggs laid by infected silk moths and from 1, 2, 3 and 4 larvae of the first instar. For the second, third, fourth and fifth instar, only

one individual larva was used for DNA extraction. BmNPV DNA was also extracted from individual pupa and moth of both sexes.

In the extraction process, eggs were placed in a microtube and 50 µl of sterile distilled water was added, eggs were ground with a mini pestle. Individual sample of larva, pupa and moth was placed on mortar, steriled water was added at 1:1 ratio of body weight and ground. The 50 µl of homogenate was transferred to a new microtube, 200 µl of solution II (0.2 N NaOH, 1% SDS,) was added, the mixture was mixed thoroughly and 200 µl of cold Solution III (3 M potassium acetate, 5 M glacial acetic acid) was added. The solution was vortexed and added with 200 µl chloroform: isoamyl alcohol (24:1). A mixture was centrifuged at 12,000 rpm for 5 min and the upper phase was transferred to a new microtube. DNA was precipitated by adding 400 µl of isopropanol, mixed well and centrifuged at 12,000 rpm for 5 min at room temperature. Supernatant was discarded and DNA pellet was rinsed with 200 µl of 70% ethanol. DNA was allowed to vacuum dry up and were suspeneded in 50 µl TE (10 mM Tris-HCl pH 8).

3.3 Grasserie disease detection

For reliable and distinct PCR product for rapid detection, a set of specific primers was designed from the clone nucleotide sequence within Thai BmNPV polyhedrin gene (accession number AY779044). The oligonucleotide sequences of forward primer began from position 221-240 of *polh* ORF and reverse primers began from 616-644 of *polh* ORF. These primers amplified a 424 bp PCR product.

Forward primer: 5’ AATTCGCAGTGAAACCCG 3’

Reverse primer: 5’ AGAGTCTGTGCCGATGT 3’

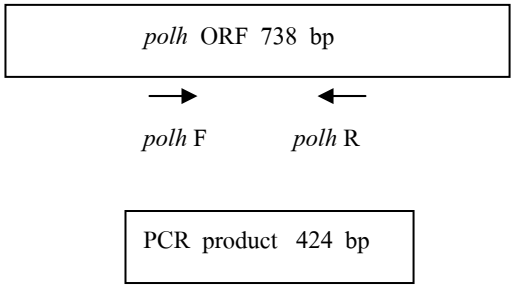


Figure 7 Diagram of the polyhedrin gene. Location of the primers used for PCR amplification is indicated. The predicted amplification product is 424 bp.

Each PCR reaction mixture was consisted of 1 µl of total DNA (~50 ng), 0.2 µl of *Taq* DNA polymerase, 2.5 µl of 10x reaction buffer, 1 µl of 50 mM $MgCl_2$, 1 µl of 2.5 mM dNTP and 1 µl of 5 ρM/µl of each forward and reverse primers in a final volume of 25 µl. The thermal profile for PCR was as follows; initiation at 94 °C for 5 min, and 35 repeated cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. PCR products were separated on 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV illuminator.

RESULTS AND DISCUSSION

1. Restriction pattern analysis of Thai BmNPV

1.1 Extraction of whole genomic BmNPV DNA

Determining the restriction enzyme pattern of the viral DNA is an essential tool in identifying and distinguishing isolates of virus. High quality and quantity of DNA are needed to demonstrate the restriction profile of each virus isolate. The BmNPV DNA extraction method used in this study following the method of Chaeychomsri (2003), provided a prominent DNA band on the gel (Figure 8). It is a single clear band with no interference of RNA. The obtained BmNPV DNA was in high quantity and quality and can be used to demonstrate the differences of the restriction patterns when digested with different enzymes.

There were many methods to harvest the virus, BmNPV from the insect body. BmNPV was collected from the insect haemolymph by cutting prolegs before filtering through layer of gauze (Singh *et al*., 1996). BmNPV was also collected by plaque purification (Hashimoto *et al*., 1994; Maeda and Majima, 1990). In this study, the virus was collected from infected larvae by allowing the insect body to putrefy and release polyhedra naturally. This method of viral harvesting was rather convenient to practice comparing with other methods. Viral DNA extraction described in this study was quite similar to previous methods (Maeda and Majima, 1990; Hashimoto *et al*., 1994; Singh *et al*., 1996) in which the main chemical used were alkaline solution for solubilizing the occlusion bodies and followed by proteinase K and SDS for releasing viral DNA. BmNPV DNA extraction used by various researchers were different in a few steps. For example, there was step of dialysation the DNA with SSC after extraction with phenol-chloroform-isoamyl alcohol in the study of Singh *et al*. (1996) while this step was omitted by others.

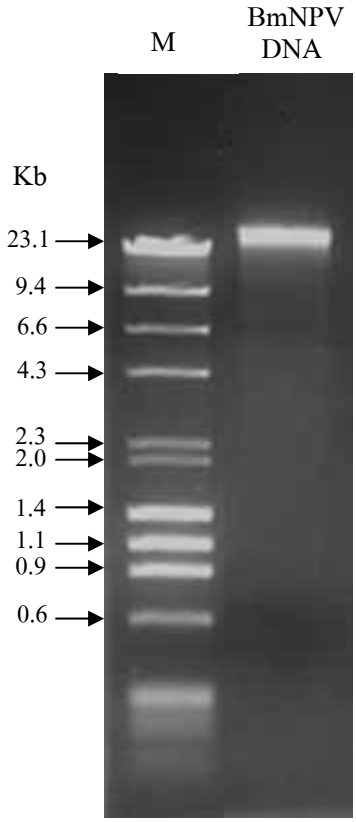


Figure 8 Gel electrophoresis of the DNA of Thai BmNPV extracted by the method of Chaeychomsri (2003).
M= marker : λ DNA- *Hin*dIII and ϕ X174 DNA-*Hae*III Mix

1.2 Restriction pattern of Thai BmNPV

The restriction pattern of BmNPV DNA was demonstrated in Figure 9. When the BmNPV DNA was cut with *Bam*HI, *Bgl*II, *Hin*dIII, *Nco*I and *Pst*I, 6, 11, 19, 11 and 17 DNA bands were observed on the gel, respectively. Molecular size of each DNA fragment was analyzed by comparison with the migration of the size marker and the fragments were assigned an alphabetical designation based on size as shown in Table 1. The biggest bands were 26.0 kb of fragment A and B digested with *Bam*HI. The smallest band that could observe was 0.3 kp of

fragment S digested with *Hin*dIII. The Thai BmNPV genome digested with *Bam*HI, *Bgl*II, *Hin*dIII, *Nco*I and *Pst*I was measured approximately 92.3, 102.6, 117.9, 114.3 and 125.8 kb, respectively. It was estimated to be in the range of 92.3-125.8 kb with the average of 109.8 kb. Genome estimation was based on the restriction fragments appeared on the gel. Some fragments may contain DNAs with little different in molecular size, therefore, they co-migrated in the same distance resulting in the overlapping DNA bands that cannot be differentiated. Therefore, bands which were more intense than the regular single bands were considered as two fragments of approximately same size.

Restriction patterns of genome of different strains of BmNPV had been previously reported. Maeda and Majima (1990) revealed the restriction pattern and genome size of BmNPV T3 isolate from Japan. The DNA was digested with *Eco*RI, *Hin*dIII, *Pst*I, *Bam*HI, *Kpn*I and *Sma*I. By summing the size of the fragments generated by these enzymes, the entire genome was estimated to be 130 kb. Hashimoto *et al.* (1994) showed restriction pattern of BmNPV D1 isolate from Japan digested with *Apa*I, *Bam*HI, *Bgl*I, *Hin*dIII, *Kpn*I and *Xho*I and the genome size was estimated to be 126.4 kb. Singh *et al.* (1996) reported the genome size of BmNPV N isolate from India as estimated from the restriction fragments of the genome digested with *Hin*dIII and *Eco*RI was measured approximately 118 kb. For complete genome of BmNPV, only T3 isolate was submitted in GenBank accession number L33180 and the genome was 128,413 nucleotides long (Gomi *et al*., 1999).

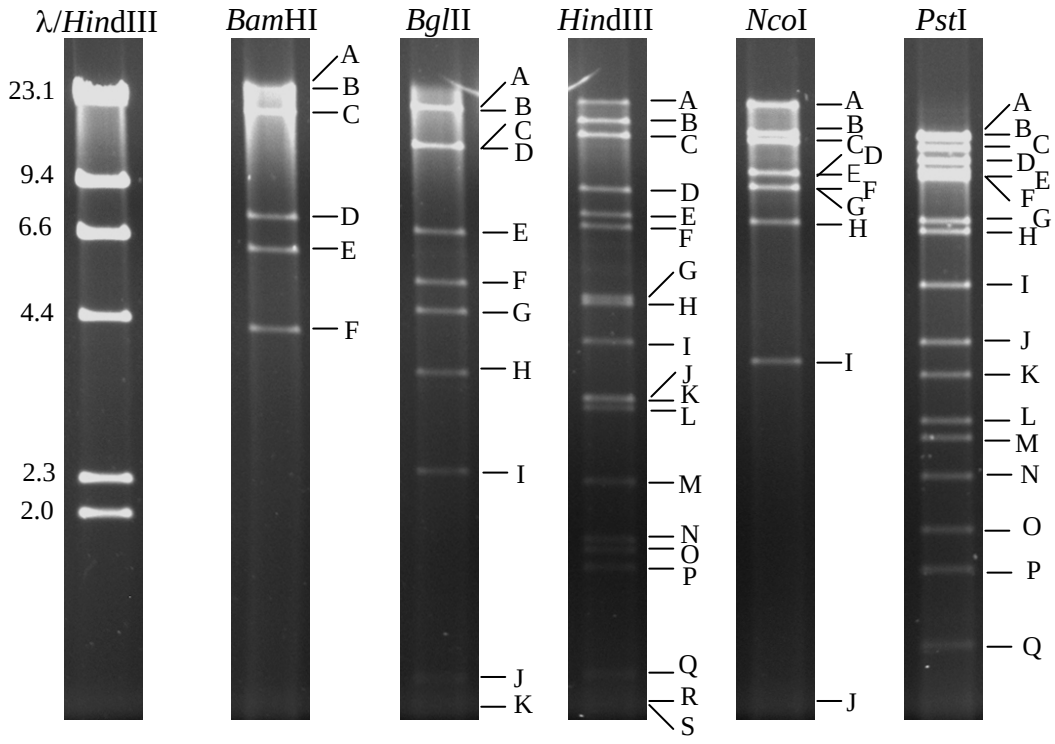


Figure 9 Cleavage patterns of Thai BmNPV DNA using the restriction endonuclease *Bam*HI, *Bgl*II, *Hin*dIII, *Nco*I and *Pst*I. Viral DNA was digested with these endonucleases and the cleaved fragments were separated on 0.6% SeaKem GTG agarose gel. Lane marked λ/*Hin*dIII showed molecular size marker pattern in kb. Each visible fragment was assigned a letter as shown.

Table 1 Restriction endonuclease cleavage fragments of the Thai *Bombyx mori* nucleopolyhedrovirus DNA

Fragment	Size (kb) of BmNPV restriction fragments				
	*Bam*HI	*Bgl*II	*Hin*dIII	*Nco*I	*Pst*I
A	26.0	22.9	23.1	22.3	17.4
B	26.0	22.9	20.1	17.9	17.4
C	22.5	16.2	17.8	16.5	15.4
D	7.5	16.2	9.2	11.6	13.1
E	6.1	7.1	7.9	11.6	11.2
F	4.2	5.5	7.3	9.3	10.4
G		4.7	4.9	9.3	7.4
H		3.7	4.8	7.5	6.8
I		2.4	4.1	7.5	5.3
J		0.6	3.4	3.8	4.1
K		0.4	3.4	0.4	3.7
L			3.2		3.1
M			2.3		2.9
N			1.8		2.4
O			1.7		2.0
P			1.5		1.8
Q			0.7		1.4
R			0.4		
S			0.3		
Total	92.3	102.6	117.9	114.3	125.8

Table 2 illustrated restriction endonuclease cleavaged fragments of Thai BmNPV DNA and DNA of other isolates of BmNPV such as T3 and D1 from Japan, and N from India. T3 and T3* are the same isolate but the fragment sizes of T3 were from the report of Maeda and Majima (1990) while fragment sizes of T3* of L33180 complete genome sequence were analyzed by computer software.

*Bam*HI restriction patterns showed that there were at least 6 DNA bands for BmNPV of Thai and T3 isolate while there were at least 5 DNA bands for the D1 isolate. Three small fragments digested with *Bam*HI of all BmNPVs were in similar size. The smallest fragments of Thai, T3, T3* and D1 were 4.2, 3.9, 4.2 and 4.2 kb, the next smaller fragments were 6.1, 6.0, 6.2 and 6.0 kb and the small fragments were 7.5, 7.3, 7.6 and 7.5 kb. When comparing fragment size of the Thai and T3 isolates, there were four fragments which gave the similar size, C, D, E and F. The largest fragment of Thai isolate was smaller than T3 and D1 isolate. The size marker used in this study was the low molecular weight size marker which perhaps too low and in narrow range until could not illustrate the DNA fragments that were bigger than 23.1 kb.

*Hin*dIII restriction patterns showed there were at least 19 bands for BmNPV of Thai and N isolate while there were at least 18 and 20 bands for the T3 and D1 isolate, respectively. DNA fragments of the Thai isolate were different in size from those of the T3, D1 and N isolate. In comparison between DNA fragments of the Thai and T3 isolate digested with *Hin*d III, fragment E, G, H, M, N, O, P and R of the Thai isolate were similar to fragment F, I, J, N, O, P and R of T3 isolate, respectively. Some DNA fragments of the Thai isolate were similar to fragments of D1 isolate. For example, fragment D, L, M, N and Q of the Thai isolate were similar to fragment D, M (N), O, P and T of the D1 isolate. In addition, there were some fragments of Thai isolate which were similar to fragment of N isolate. For example, fragment G, H, I, L, M, N and O of the Thai isolate were similar to fragment H, I, J, K, M, P and Q of N isolate.

*Pst*I restriction patterns showed there were at least 17 bands for BmNPV of Thai isolate while there were at least 19 bands for the T3 isolate. When digested BmNPV DNA with

*Pst*I, fragment A(B), G, M, N and O of Thai isolate were similar to fragment A(B), F(G), M, N(O) and P of the T3 isolate.

Even though T3 and T3* of BmNPV are the same isolate, some digested DNA fragments were different. For example, fragment A of T3 isolate digested with *Bam*HI was 54 while that of the T3* isolate was 51.1 kp and fragment A of T3 isolate digested with *Hin*dIII was 30 while that of T3* isolate was 27.3 kb. In addition, Fragment D of T3 isolate digested with *Pst*I was 12.5 kp while that of T3* isolate was 11.8 kb. This comparison study indicated that there were molecular size differences of the DNA fragments digested with restriction enzymes among or within viral isolates. The differences may result from the method of analysis.

In this study, size of the DNA fragments was estimated by comparison with the fragments of DNA marker that electrophoresed on the gel. This method was also used to estimate restriction fragments of the N isolate, while DNA fragments obtained from T3 and D1 isolate were estimated from the clone fragments which were probed by hybridization. Therefore, genome of Thai and N isolate were similar in size when digested with *Hin*dIII while the Thai BmNPV genome was smaller than that of the T3 and D1 isolate. Hence, DNA fragments with similar size of the T3, and D1 isolate can be distinguished while DNA fragments of the Thai isolate that have similar size cannot be clearly separated by gel electrophoresis. Even though bands which more intense than the regular single bands were considered as two fragments of same size, sometimes there were more than two of similar size fragments of fragment so it was difficult to determine correctly. However, this is only the preliminary investigation in order to compare restriction pattern of BmNPV with other isolates of previous reports. For intensive study of the whole genome, the co-migrated fragments should be elucidated by hybridization with different probes on the overlapping bands. However, the process comprises of several complicate work, is time consuming and expensive

The results showed that restriction enzyme that gave more fragments can be used for differentiation of isolate better than restriction enzyme that gave a few fragments. For example, *Hin*dIII can give more polymorphism of BmNPV isolates than *Bam*HI.

There were many reports on restriction pattern of other NPVs. Smith and Summers (1978) reported that restriction pattern of DNA of several NPVs (*Autographa californica* NPV, *Orgya pseudotsugata* NPV, *Rachiplusia au* NPV, *Portheria dispar* NPV, *Spodoptera exiqua* NPV, *Porthetria dispar* NPV, *Trichoplusia ni* NPV, *Heliothis zea* NPV, *H. armigera* NPV) can identify isolates of baculoviruses. Moreover, Lavina-Caoili *et al*. (2001) studied restriction pattern of 10 isolates of *Spodoptera litura* NPV from Japan, China and Philippines. The 10 isolates of SlNPV displayed similar overall restriction endonuclease pattern except for deletion or insertion of a few DNA fragments, indicated that there were minor differences among isolated genotypic variants in their genome organization. Restriction endonuclease analysis of a number of baculoviruses from different geographical regions has shown that each isolate has a unique set of DNA fragments which may indicate the variation in the genomic DNA sequence. Therefore, analysis of viral DNA by digesting with different restriction endonucleases and observing the restriction profiles is convenient, economy and one of useful tools in identification and classification of the viruses.

Table 2 Restriction endonuclease cleavaged fragments of the Thai BmNPV DNA and DNA of the other isolates of BmNPV*

Fragment	Size (kb) of BmNPV restiction fragment											
	*Bam*HI				*Hin*dIII					*Pst*I		
	Thai	T3	T3*	D1	Thai	T3	T3*	D1	N	Thai	T3	T3*
A	26.0	54	51.4	58.1	23.1	30	27.7	29.5	24.06	17.4	17.5	17.8
B	26.0	36	37.3	50.5	20.1	17	16.9	17.0	15.16	17.4	17.5	17.5
C	22.5	22	21.8	7.5	17.8	15.5	15.5	10.2	14.80	15.4	17.0	16.6
D	7.5	7.3	7.6	6.0	9.2	10.0	9.7	9.0	9.44	13.1	12.5	11.8
E	6.1	6.0	6.2	4.2	7.9	8.9	8.9	8.1	8.56	11.2	10.8	10.2
F	4.2	3.9	4.2		7.3	7.8	8.2	8.1	8.20	10.4	7.2	7.3
G					4.9	7.8	8.1	6.5	5.16	7.4	7.2	7.2
H					4.8	5.8	5.9	5.5	4.90	6.8	5.5	5.5
I					4.1	5.1	5.0	5.0	4.70	5.3	5.4	5.4
J					3.4	4.8	4.9	4.9	4.00	4.1	5.4	5.3
K					3.4	3.8	3.9	4.2	3.18	3.7	4.9	4.9
L					3.2	3.1	3.2	3.5	3.11	3.1	4.6	4.7
M					2.3	3.0	3.0	3.2	2.29	2.9	2.8	2.7
N					1.8	2.2	2.3	3.2	2.19	2.4	2.3	2.4
O					1.7	1.7	1.8	2.3	1.90	2.0	2.3	2.4
P					1.5	1.5	1.6	1.8	1.83	1.8	1.9	1.9
Q					0.7	1.0	1.0	1.7	1.74	1.4	1.5	1.6
R					0.4	0.7	0.8	1.0	1.23		1.5	1.6
S					0.3			1.0	1.05		1.3	1.3
T								0.7				
Total	92.3	129.2	128.5	126.3	117.9	129.7	128.4	126.4	117.5	125.8	129.1	128.1

* T3 = BmNPV isolate from Japan (Maeda and Majima, 1990)

T3*= BmNPV isolate from Japan (Gomi *et al*., 1999)

D1 = BmNPV isolate from Japan (Hashimoto *et al*., 1994)

N = BmNPV islate from India (Singh *et al*., 1996)

2. Identification of polyhedrin gene of Thai BmNPV

2.1 Cloning and sequencing of Thai BmNPV *polh*

To study the sequence of Thai BmNPV *polh*, specific primers for BmNPV *polh* were designed. When specific primers were used, PCR products of the expected size of 1,448 bp were observed on gel (Figure 10). Purified PCR product was cloned into pGEM®-T vector. Recombinant clones were identified by white colony selection. The selected clones were then screened for clone harboring the BmNPV *polh*.

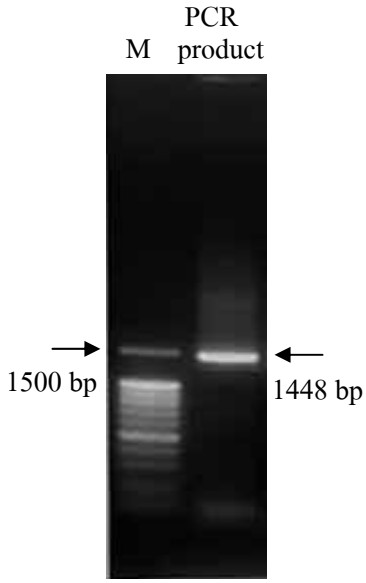


Figure 10 The expected PCR products of 1,448 bp amplified from the extracted DNA of Thai BmNPV.
M=DNA marker (100 bp+1.5kbp)

Two methods were used to screen clone harboring the BmNPV *polh* gene. The expected size of pGEM®-T vector with insert gene was shown in Figure 11 (a). Examples of clone selection for insert gene of BmNPV *polh* were illustrated in Figure 11 (b) and (c). At first, PCR was used to identify the recombinant clones. Figure 11 (b) showed three clones that were clarified by PCR and result indicated that only clone 1 revealed band of BmNPV *polh* of approximately 1,500 bp while clone 2 and clone 3 had no expected PCR products. Therefore, clone 1 may has an insert of *polh* gene. This result was rechecked by digestion recombinant clone with *Hin*dIII-*Sal*I. Figure 11 (c) showed *Hin*dIII-*Sal*I restriction pattern of the selected clone in which 3 bands with the size of 4.4 kb, 3 kb and 1.3 kb were observed. Fragment of 4.4 kb was the vector with insert gene, fragment of 3 kb was pGEM®-T vector and fragment of 1.3 kb was the insert gene.

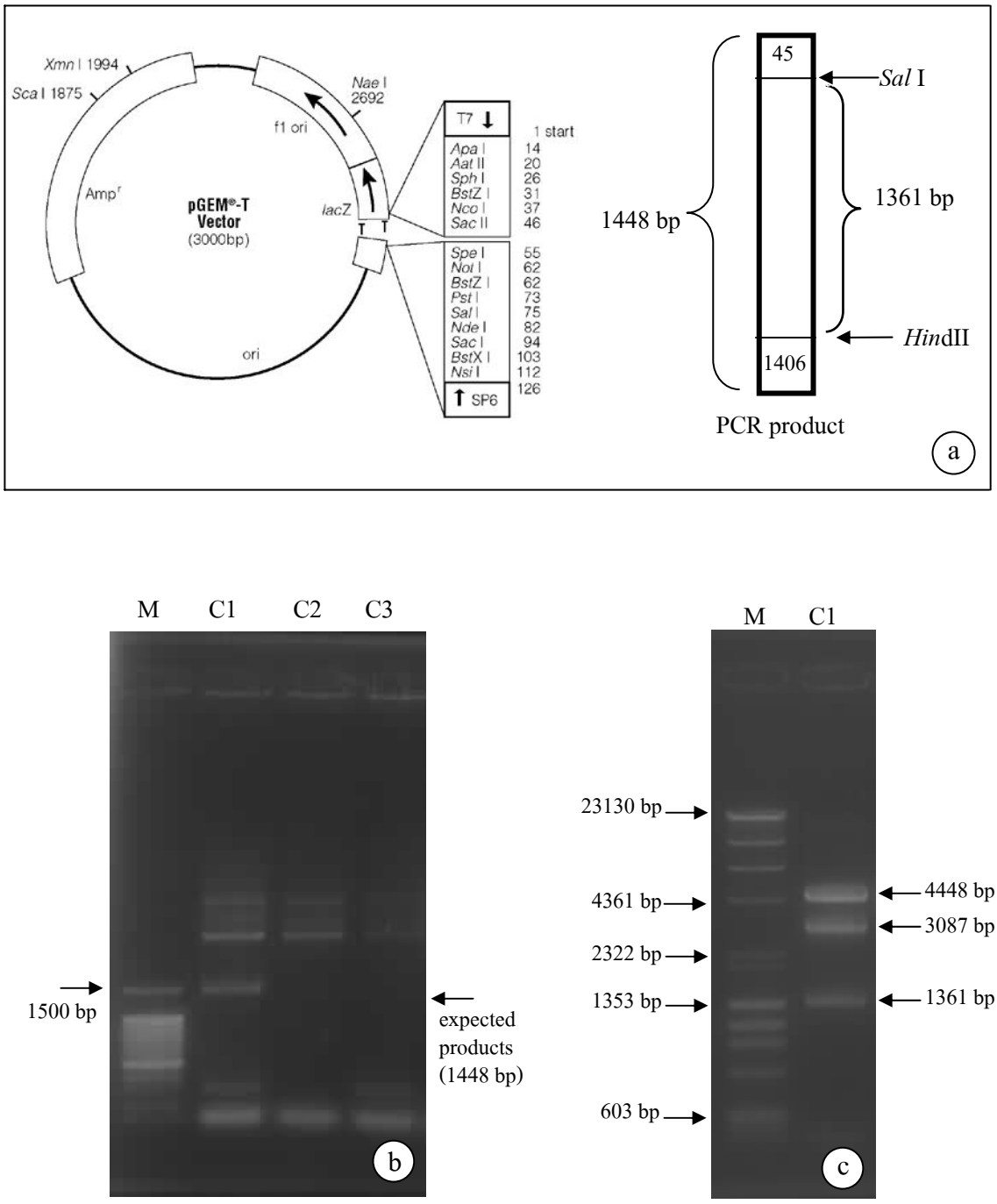


Figure 11 Screening of recombinant clones harboring the BmNPV *polh* gene.

a) Diagram of pGEM® -T vector and insert PCR product

b) Clone selection confirmed by PCR method, C=clones , M=marker 100 bp+1.5 Kb

c) Clone selection confirmed by *Hin*dIII-*Sal*I digestion, C=clones

M= marker :λ DNA- *Hin*dIII and ϕ X174 DNA-*Hae*III Mix

2.2 Nucleotide sequence of Thai BmNPV *polh*

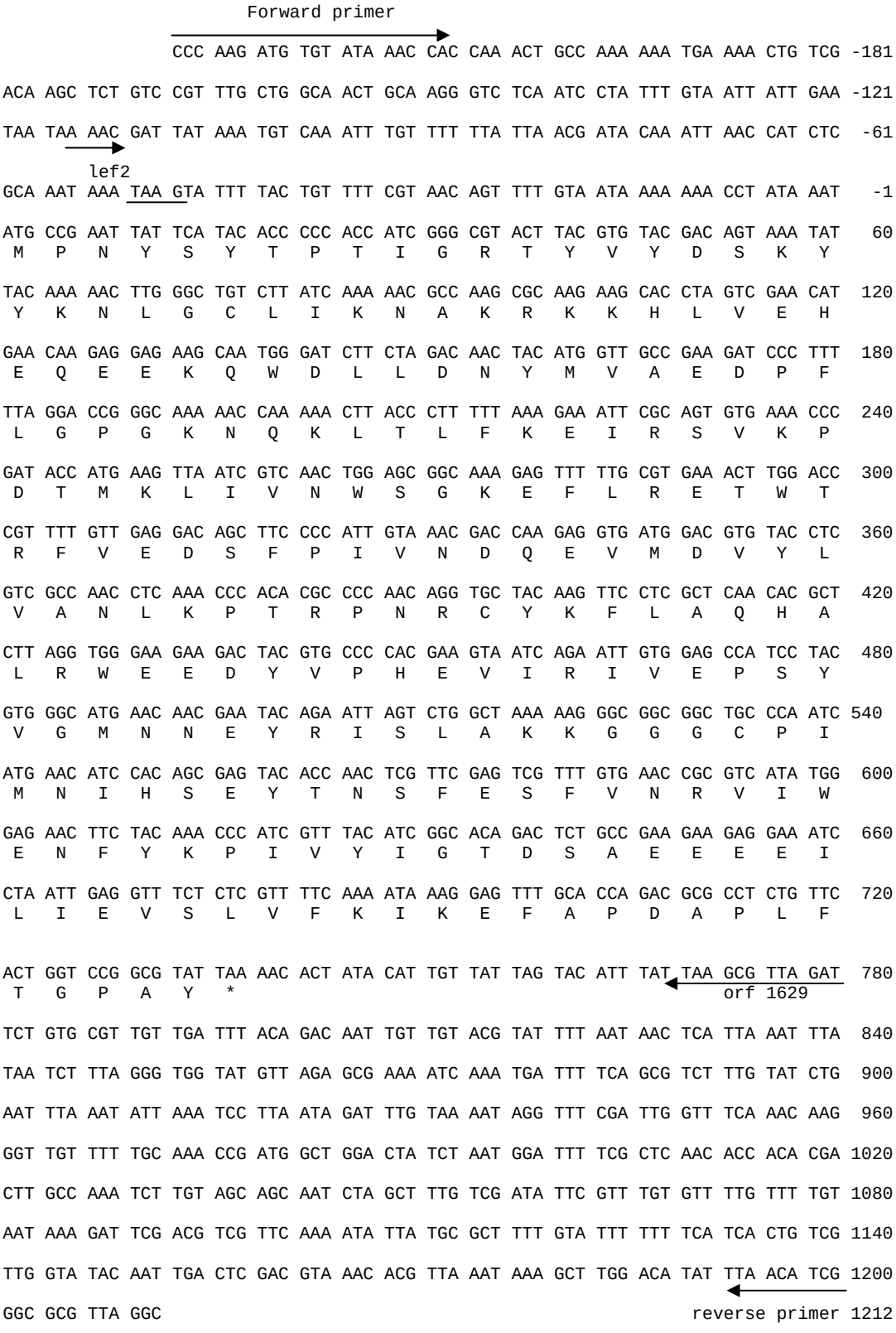
The PCR amplification products cloned into pGEM®-T vector showed the nucleotide sequence of 1,440-bp fragment of the Thai BmNPV DNA that contained the full-length coding region of the *polh*. The nucleotide sequence of Thai BmNPV *polh* and its flanking region was submitted to GenBank at electronic access (http://www.ncbi.nlm.nih.gov/) (Benson *et al*., 2000) and the accession number AY779044 was given as shown in Figure 12. The sequences and predicted amino acid were designed as shown in Figure 13. The *polh* ORF consisted of 735 nucleotides (not include stop codon) that encoded a polypeptide of 245 amino acids with the predicted molecular mass of 28.8 kDa. The submitted sequence contained 228 bp upstream of the translation initiation codon (ATG) and 474 bp downstream of the translation stop codon (TAA). Several characteristics of Thai BmNPV *polh* gene sequence were investigated. The immediate upstream sequence of the translation initiation site was AT rich and contained the unique conserved transcription start site TAAG motif which is similar to other baculovirus late gene promoters. There was no additional TAAG sequence in the sequence upstream of the initiation codon. As has been observed in all other hyperexpressed baculovirus late genes, there is a conserved sequence of 14 nucleotides upstream of the coding region with the consensus sequence of TAAATAAGTATTTT at position -42 to -56 (Leisy *et al*., 1986). DNA sequences similar to the consensus TATA and CAAT which represented important elements of eukaryotic gene promoters were observed at position -107 and -143. The canonical poly (A) signal AATAAA was present in the 3'end of the Thai BmNPV *polh* gene at position 1081. The Thai BmNPV *polh* ORF had the translation initiation codon, ATG, and the termination codon, TAA, as found in other baculoviruses (O'Reilly *et al*., 1992). The complete whole sequence of Thai BmNPV *polh* overlapped with *lef*-2 (late gene expression factor-2) in the 5' flanking region and *orf*1629 in the 3' flanking region. The *lef*-2 was located in the *polh* upstream region adjacent to the transcription start site in the same direction and the *orf*1629 was located in the *polh* downstream region in the reverse direction.

```
LOCUS       AY779044               1440 bp    DNA     linear   VRL 07-NOV-2004
DEFINITION  Bombyx mori nucleopolyhedrovirus from Thailand polyhedrin gene,
            complete cds.
ACCESSION   AY779044
VERSION     AY779044.1  GI:55247500
KEYWORDS    .
SOURCE      Bombyx mori NPV
  ORGANISM  Bombyx mori NPV
            Viruses; dsDNA viruses, no RNA stage; Baculoviridae;
            Nucleopolyhedrovirus.
REFERENCE   1  (bases 1 to 1440)
  AUTHORS   Kaewwises,M., Attathom,T., Chaeychomsri,S. and Chowpongpang,S.
  TITLE     The Polyhedrin Gene of Thai Bombyx mori nucleopolyhedrovirus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1440)
  AUTHORS   Kaewwises,M., Attathom,T., Chaeychomsri,S. and Chowpongpang,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (12-OCT-2004) Biotechnology Research and Development
            Office, Pathum Thani 12110, Thailand
FEATURES             Location/Qualifiers
     source          1..1440
                     /organism="Bombyx mori NPV"
                     /mol_type="genomic DNA"
                     /db_xref="taxon:271108"
                     /country="Thailand"
     CDS             229..966
                     /codon_start=1
                     /product="polyhedrin"
                     /protein_id="AAV48593.1"
                     /db_xref="GI:55247501"
                     /translation="MPNYSYTPTIGRTYVYDNKYYKNLGCLIKNAKRKKHLVEHEQEE
                     KQWDLLDNYMVAEDPFLGPGKNQKLTLFKEIRSVKPDTMKLIVNWSGKEFLRETWTRF
                     VEDSFPIVNDQEVMDVYLVANLKPTRPNRCYKFLAQHALRWEEDYVPHEVIRIVEPSY
                     VGMNNEYRISLAKKGGGCPIMNIHSEYTNSFESFVNRVIWENFYKPIVYIGTDSAEEE
                     EILIEVSLVFKIKEFAPDAPLFTGPAY"
ORIGIN
        1 cccaagatgt gtataaacca ccaaactgcc aaaaaatgaa aactgtcgac aagctctgtc
       61 cgtttgctgg caactgcaag ggtctcaatc ctatttgtaa ttattgaata ataaaacgat
      121 tataaatgtc aaatttgttt tttattaacg atacaaatta accatctcgc aaataaataa
      181 gtattttact gttttcgtaa cagttttgta ataaaaaaac ctataaatat gccgaattat
      241 tcatacaccc ccaccatcgg gcgtacttac gtgtacgaca ataaatatta caaaaacttg
      301 ggctgtctta tcaaaaacgc caagcgcaag aagcacctag tcgaacatga acaagaggag
      361 aagcaatggg atcttctaga caactacatg gttgccgaag atccctttt aggaccgggc
      421 aaaaaccaaa aacttaccct ttttaaagaa attcgcagtg tgaaacccga taccatgaag
      481 ttaatcgtca actggagcgg caaagagttt ttgcgtgaaa cttggacccg ttttgttgag
      541 gacagcttcc ccattgtaaa cgaccaagag gtgatggacg tgtacctcgt cgccaacctc
      601 aaacccacac gccccaacag gtgctacaag ttcctcgctc aacacgctct taggtgggaa
      661 gaagactacg tgccccacga agtaatcaga attgtggagc catcctacgt gggcatgaac
      721 aacgaataca gaattagtct ggctaaaaag ggcggcggct gcccaatcat gaacatccac
      781 agcgagtaca ccaactcgtt cgagtcgttt gtgaaccgcg tcatatggga gaacttctac
      841 aaacccatcg tttacatcgg cacagactct gccgaagaag aggaaatcct aattgaggtt
      901 tctctcgttt tcaaaataaa ggagtttgca ccagacgcgc ctctgttcac tggtccggcg
      961 tattaaaaca ctatacattg ttattagtac atttattaag cgttagattc tgtgcgttgt
     1021 tgatttacag acaattgttg tacgtatttt aataactcat taaatttata atctttaggg
     1081 tggtatgtta gagcgaaaat caaatgattt tcagcgtctt tgtatctgaa tttaaatatt
     1141 aaatccttaa tagatttgta aaataggttt cgattggttt caaacaaggg ttgtttttgc
     1201 aaaccgatgg ctggactatc taatggattt tcgctcaaca ccacacgact tgccaaatct
     1261 tgtagcagca atctagcttt gtcgatattc gtttgtgttt tgttttgtaa taaagattcg
     1321 acgtcgttca aaatattatg cgcttttgta ttttttttcat cactgtcgtt ggtatacaat
     1381 tgactcgacg taaacacgtt aaataaagct tggacatatt taacatcggg cgcgttaggc
```

Figure 12 Information of GenBank accession number AY779044, the submitted sequences of Thai BmNPV polyhedrin gene.

Source: http://www.ncbi.nih.govlentrez/viewer.fcgi?db=nucleotide&val=55247500

Figure 13 Nucleotide sequence of Thai BmNPV polyhedrin gene and its flanking regions. A total of 1,440 base pairs was submitted to GenBank (AY779044). The first nucleotide of the polyhedrin gene translational start signal ATG is given number 1. The predicted amino acid sequence is indicated by one-letter code and displayed below the nucleotide sequence. The sequence at nt -51 initiates the 5' end of the mRNA. The putative transcription initiation motif (TAAG) is underlined. The potential polyadenylation signal (ATTAAA) is indicated by overlining. The primer used in the primer extension assay is also shown and the arrow indicates the direction of extension.




```
                    CCC AAG ATG TGT ATA AAC CAC CAA ACT GCC AAA AAA TGA AAA CTG TCG -181

ACA AGC TCT GTC CGT TTG CTG GCA ACT GCA AGG GTC TCA ATC CTA TTT GTA ATT ATT GAA -121

TAA TAA AAC GAT TAT AAA TGT CAA ATT TGT TTT TTA TTA ACG ATA CAA ATT AAC CAT CTC  -61

GCA AAT AAA TAA GTA TTT TAC TGT TTT CGT AAC AGT TTT GTA ATA AAA AAA CCT ATA AAT   -1

ATG CCG AAT TAT TCA TAC ACC CCC ACC ATC GGG CGT ACT TAC GTG TAC GAC AGT AAA TAT   60
 M   P   N   Y   S   Y   T   P   T   I   G   R   T   Y   V   Y   D   S   K   Y

TAC AAA AAC TTG GGC TGT CTT ATC AAA AAC GCC AAG CGC AAG AAG CAC CTA GTC GAA CAT  120
 Y   K   N   L   G   C   L   I   K   N   A   K   R   K   K   H   L   V   E   H

GAA CAA GAG GAG AAG CAA TGG GAT CTT CTA GAC AAC TAC ATG GTT GCC GAA GAT CCC TTT  180
 E   Q   E   E   K   Q   W   D   L   L   D   N   Y   M   V   A   E   D   P   F

TTA GGA CCG GGC AAA AAC CAA AAA CTT ACC CTT TTT AAA GAA ATT CGC AGT GTG AAA CCC  240
 L   G   P   G   K   N   Q   K   L   T   L   F   K   E   I   R   S   V   K   P

GAT ACC ATG AAG TTA ATC GTC AAC TGG AGC GGC AAA GAG TTT TTG CGT GAA ACT TGG ACC  300
 D   T   M   K   L   I   V   N   W   S   G   K   E   F   L   R   E   T   W   T

CGT TTT GTT GAG GAC AGC TTC CCC ATT GTA AAC GAC CAA GAG GTG ATG GAC GTG TAC CTC  360
 R   F   V   E   D   S   F   P   I   V   N   D   Q   E   V   M   D   V   Y   L

GTC GCC AAC CTC AAA CCC ACA CGC CCC AAC AGG TGC TAC AAG TTC CTC GCT CAA CAC GCT  420
 V   A   N   L   K   P   T   R   P   N   R   C   Y   K   F   L   A   Q   H   A

CTT AGG TGG GAA GAA GAC TAC GTG CCC CAC GAA GTA ATC AGA ATT GTG GAG CCA TCC TAC  480
 L   R   W   E   E   D   Y   V   P   H   E   V   I   R   I   V   E   P   S   Y

GTG GGC ATG AAC AAC GAA TAC AGA ATT AGT CTG GCT AAA AAG GGC GGC GGC TGC CCA ATC 540
 V   G   M   N   N   E   Y   R   I   S   L   A   K   K   G   G   G   C   P   I

ATG AAC ATC CAC AGC GAG TAC ACC AAC TCG TTC GAG TCG TTT GTG AAC CGC GTC ATA TGG  600
 M   N   I   H   S   E   Y   T   N   S   F   E   S   F   V   N   R   V   I   W

GAG AAC TTC TAC AAA CCC ATC GTT TAC ATC GGC ACA GAC TCT GCC GAA GAA GAG GAA ATC  660
 E   N   F   Y   K   P   I   V   Y   I   G   T   D   S   A   E   E   E   E   I

CTA ATT GAG GTT TCT CTC GTT TTC AAA ATA AAG GAG TTT GCA CCA GAC GCG CCT CTG TTC  720
 L   I   E   V   S   L   V   F   K   I   K   E   F   A   P   D   A   P   L   F


ACT GGT CCG GCG TAT TAA AAC ACT ATA CAT TGT TAT TAG TAC ATT TAT TAA GCG TTA GAT  780
 T   G   P   A   Y   *                                           orf 1629

TCT GTG CGT TGT TGA TTT ACA GAC AAT TGT TGT ACG TAT TTT AAT AAC TCA TTA AAT TTA  840

TAA TCT TTA GGG TGG TAT GTT AGA GCG AAA ATC AAA TGA TTT TCA GCG TCT TTG TAT CTG  900

AAT TTA AAT ATT AAA TCC TTA ATA GAT TTG TAA AAT AGG TTT CGA TTG GTT TCA AAC AAG  960

GGT TGT TTT TGC AAA CCG ATG GCT GGA CTA TCT AAT GGA TTT TCG CTC AAC ACC ACA CGA 1020

CTT GCC AAA TCT TGT AGC AGC AAT CTA GCT TTG TCG ATA TTC GTT TGT GTT TTG TTT TGT 1080

AAT AAA GAT TCG ACG TCG TTC AAA ATA TTA TGC GCT TTT GTA TTT TTT TCA TCA CTG TCG 1140

TTG GTA TAC AAT TGA CTC GAC GTA AAC ACG TTA AAT AAA GCT TGG ACA TAT TTA ACA TCG 1200

GGC GCG TTA GGC                                                 reverse primer 1212
```

The orientation of *polh*, *lef*-2 and *orf*1629 in BmNPV are the same as those found in AcNPV genome. The position of *orf1629* gene in HaNPV, SeNPV and SlNPV is next to *polh* similar to BmNPV but the position of *lef*-2 is different. In HaNPV, SeNPV and SlNPV, *lef*-2 is not located in *polh* upstream. In BmNPV *lef*-2 is essential for both viral DNA replication and late gene expression (Sriram and Gopinathan, 1998) and the *orf*1629 is essential for BmNPV viability (Je *et al*., 2001).

Flanking nucleotides of Thai BmNPV *polh* and BmNPV *polh* fragment which was used to design the primers, were compared. The major difference between the two isolates was the deletion of eight nucleotides in the upstream region of Thai BmNPV *polh* ORF while the rest of the nucleotide sequences were almost identical (Figure 14). Since the eight nucleotides are located upstream of the *polh* promoter, it might have no effect on the expression of *polh* promoter. A study on recombinant expression indicated that the suitable multiple cloning site (MCS) is located immediately downstream of the *polh* promoter (Acharya *et al*., 2002). In addition, there was a study on series of deletion in the upstream region of BmNPV *polh*s *(lef*-2, *orf*327, *orf*453 and *bro-e*) and the results revealed that the upstream region of *polh* has no effect on expression of *polh* promoter (Acharya and Gopinathan, 2001).

```
Thai                  CCCAAGATGTGTATAAACCACCAAACTGCCAAAAAATGAAAACTGTCGACAAGCTC -173
Japanese 1 (T3)       CCCAAGATGTGTATAAACCACCAAACTGCCAAAAAATGAAAACTGTCGACAAGCTC -181
                                                                  lef-2

Thai              TGTCCGTTTGCTGGCAACTGCAAGGGTCTCAATCCTATTTGTAATTATTGAATAATAAAA -103
Japanese 1(T3)    TGTCCGTTTGCTGGCAACTGCAAGGGCCTCAATCCTATTTGTAATTATTGAACAATAAAA -121

Thai              CGATTATAAATGTCAAATTTGTTTTTTATTAACGATACAAAT--------TAACCATCTC  -61
Japanese 1(T3)    CAATTATAAATGTCAAATTTGTTTTTTATTAACGATACAAATGGAAATAATAACCATCTC  -61

Thai              GCAAATAAATAAGTATTTTACTGTTTTCGTAACAGTTTTGTAATAAAAAAACCTATAAAT   -1
Japanese 1 (T3)   GCAAATAAATAAGTATTTTACTGTTTTCGTAACAGTTTTGTAATAAAAAAACCTATAAAT   -1
```

Figure 14 Comparison of flanking nucleotide of Thai BmNPV *polh* (AY779044) and Japanase 1 (T3) (L33180). The different nucleotide sequences are shaded. The deletion region in Thai BmNPV is indicated by dashes.

2. 3 Comparison of Thai BmNPV *polh* with other BmNPV *polh*s

The nucleotides of Thai BmNPV *polh* were compared with those of five other BmNPV; Japanese 1 (T3) (Gomi *et al*., 1999), Japanese 2 (Maeda *et al*., 1985), Korean (K1), Chinese and Canadian (Iatrou *et al*., 1985). All BmNPV *polh* ORFs contained 735 nucleotides that encoded a polypeptide of 245 amino acids. The coding portion of the *polh* of all BmNPVs was not interrupted by intervening sequences. The nucleotide and amino acid sequences of the BmNPV *polh* of Thai and Japanese 1 (T3) isolate were identical. Percentage identity of the BmNPV *polh* sequence of the Thai isolate compared with the isolates from Japan (Japanese 2), Korea, China, and Canada were 99.9%, 99.7%, 99.6% and 98.8%, respectively, and percent identity of Thai BmNPV Polh amino acid sequence compared with those of the above-mentioned BmNPV isolate were 100%, 99.6%, 99.6%, 99.6% and 96.7%, respectively (Figure 15).

Percentage nucleotide sequence identity								
	1	2	3	4	5	6	varieties	
1		100	99.9	99.7	99.6	98.4	1	Thai
2	100		99.9	99.7	99.6	98.4	2	Japanese 1(T3)
3	99.6	99.6		99.6	99.5	98.5	3	Japanese 2
4	99.6	99.6	99.2		99.3	98.1	4	Korean(K1)
5	99.6	99.6	99.2	99.2		98.0	5	Chinese
6	96.7	96.7	97.1	96.3	96.3		6	Canadian
	1	2	3	4	5	6		
Percentage amino acid sequence identity								

Figure 15 Nucleotide and amino acid sequence identity of BmNPV *polh*s. Percentage nucleotide identity is denoted above the diagonal and percentage amino acid identity is below. Sequence data of BmNPV *polh*s were retrieved from GenBank: 1) Thai (AY779044); 2) Japanese 1 (T3) (L33180); 3) Japanese 2 (M30925); 4) Korean (K1) (U75359); 5) Chinese (X63614) and 6) Canadian (M100430).

Five amino acid sequences of BmNPV Polhs were aligned with Thai BmNPV Polh and results showed variation of the amino acid sequence in the N-terminus (Figure 16). In comparison between the Polh amino acid sequence of Thai BmNPV with those of the Japanese 2, Korean (K1) and Chinese, there was only one amino acid difference. An amino acid substitution was observed at M^{56}/V in Japanese 2, at P^{5}/S in Korean, and at Q^{57}/E in Chinese Polh. Among the five isolates of *Bm*NPV, the Canadian isolate showed the most difference from the Thai *Bm*NPV Polh. There were 8 amino acid differences: substitution at position N^{7}/T, G^{26}/C, I^{38}/V, K^{42}/Q, V^{75}/I , N^{77}/S, D^{147}/E and M^{156}/V. The amino acid sequence beyond position 156 of BmNPV Polh was conserved in all BmNPVs studied. Comparison of the nucleotide and amino acid sequences among BmNPVs indicated that BmNPVs found in the Asian countries (Thailand, Japan, Korea and China) were more closely related than the isolate from the North American country (Canada).

```
           Thai MPNYSYTPTIGRTYVYDNKYYKNLGCLIKNAKRKKHLVEHEQEEKQWDLLDNYMVAEDPFLGPGK  65
Japanese 1 (T3) MPNYSYTPTIGRTYVYDNKYYKNLGCLIKNAKRKKHLVEHEQEEKQWDLLDNYMVAEDPFLGPGK  65
    Japanese 2  MPNYSYTPTIGRTYVYDNKYYKNLGCLIKNAKRKKHLVEHEQEEKQWDLLDNYMVAEDPFLGPGK  65
    Korean (K1) MPNYPYTPTIGRTYVYDNKYYKNLGCLIKNAKRKKHLVEHEQEEKQWDLLDNYMVAEDPFLGPGK  65
       Chinese  MPNYSYTPTIGRTYVYDNKYYKNLGCLIKNAKRKKHLVEHEQEEKQWDLLDNYMVAQDPFLGPGK  65
      Canadian  MPNYSYNPTIGRTYVYDNKYYKNLGGLIKNAKRKKHLIEHEKEEKQWDLLDNYMVAEDPFLGPGK  65
     Consensus  MPNYSYTPTIGRTYVYDNKYYKNLGCLIKNAKRKKHLVEHEQEEKQWDLLDNYMVAEDPFLGPGK  65

           Thai NQKLTLFKEIRSVKPDTMKLIVNWSGKEFLRETWTRFVEDSFPIVNDQEVMDVYLVANLKPTRPN 130
Japanese 1 (T3) NQKLTLFKEIRSVKPDTMKLIVNWSGKEFLRETWTRFVEDSFPIVNDQEVMDVYLVANLKPTRPN 130
     Japanese 2 NQKLTLFKEIRSVKPDTMKLIVNWSGKEFLRETWTRFVEDSFPIVNDQEVMDVYLVANLKPTRPN 130
    Korean (K1) NQKLTLFKEIRSVKPDTMKLIVNWSGKEFLRETWTRFVEDSFPIVNDQEVMDVYLVANLKPTRPN 130
        Chinese NQKLTLFKEIRSVKPDTMKLIVNWSGKEFLRETWTRFVEDSFPIVNDQEVMDVYLVANLKPTRPN 130
      Canadian NQKLTLFKEVRNVKPDTMKLIVNWSGKEFLRETWTRFVEDSFPIVNDQEVMDVYLVANLKPTRPN 130
     Consensus NQKLTLFKEIRSVKPDTMKLIVNWSGKEFLRETWTRFVEDSFPIVNDQEVMDVYLVANLKPTRPN 130

           Thai RCYKFLAQHALRWEEDYVPHEVIRIVEPSYVGMNNEYRISLAKKGGGCPIMNIHSEYTNSFESFV 195
Japanese 1 (T3) RCYKFLAQHALRWEEDYVPHEVIRIVEPSYVGMNNEYRISLAKKGGGCPIMNIHSEYTNSFESFV 195
     Japanese 2 RCYKFLAQHALRWEEDYVPHEVIRIMEPSYVGMNNEYRISLAKKGGGCPIMNIHSEYTNSFESFV 195
    Korean (K1) RCYKFLAQHALRWEEDYVPHEVIRIVEPSYVGMNNEYRISLAKKGGGCPIMNIHSEYTNSFESFV 195
        Chinese RCYKFLAQHALRWEEDYVPHEVIRIVEPSYVGMNNEYRISLAKKGGGCPIMNIHSEYTNSFESFV 195
      Canadian RCYKFLAQHALRWDEDYVPHEVIRIMEPSYVGMNNEYRISLAKKGGGCPIMNIHSEYTNSFESFV 195
     Consensus RCYKFLAQHALRWEEDYVPHEVIRIVEPSYVGMNNEYRISLAKKGGGCPIMNIHSEYTNSFESFV 195

           Thai NRVIWENFYKPIVYIGTDSAEEEEILIEVSLVFKIKEFAPDAPLFTGPAY               245
Japanese 1 (T3) NRVIWENFYKPIVYIGTDSAEEEEILIEVSLVFKIKEFAPDAPLFTGPAY               245
     Japanese 2 NRVIWENFYKPIVYIGTDSAEEEEILIEVSLVFKIKEFAPDAPLFTGPAY               245
    Korean (K1) NRVIWENFYKPIVYIGTDSAEEEEILIEVSLVFKIKEFAPDAPLFTGPAY               245
       Chinese  NRVIWENFYKPIVYIGTDSAEEEEILIEVSLVFKIKEFAPDAPLFTGPAY               245
      Canadian  NRVIWENFYKPIVYIGTDSAEEEEILIEVSLVFKIKEFAPDAPLFTGPAY               245
     Consensus  NRVIWENFYKPIVYIGTDSAEEEEILIEVSLVFKIKEFAPDAPLFTGPAY               245
```

Figure 16 Alignment of the deduced amino acids of Thai BmNPV *polh* (AY779044) with other BmNPV *polhs*; Japanese 1 (T3) (L33180), Japanese 2 (M30925), Korean (K1)(U75359), Chinese (X63614) and Canadian (M100430). The alignments of five BmNPV *polh*s were made using the Clustal method, MegAlign program of DNASTAR. The different amino acids are shaded.

Previous study on the Canadian and Russian BmNPV Polh indicated that there were 8 amino acid differences (Iatrou *et al*., 1985). The homology of other very late gene, *p10*, of BmNPV from different isolates has been reported. Among six BmNPV isolates, four from Korea and two from China, two of them had the identical P10 amino acid sequences while the rest had 8-14 amino acid differences from each other (Hong *et al.*, 2000). The percentage identity of five BmNPV *p10* from India, China, Japan (2 isolates) and Taiwan was higher than 95.7% (Palhan and Gopinathan, 2000). Functional studies on *p10* and *polh* of BmNPV have suggested *polh* is more conserved than *p10* since *polh* showed less differences and high percent identity than *p10*.

A phylogenetic tree was generated from the deduced amino acid sequence of BmNPV *polh* using DNASTAR (MegAlign) (Figure 17). The results showed that BmNPV could be divided into two groups. The Asian group composed of BmNPV isolates from Thailand, Japan 1 (T3), Japan 2, Korea (K1) and China. The North American group composed of BmNPV isolate from Canada.

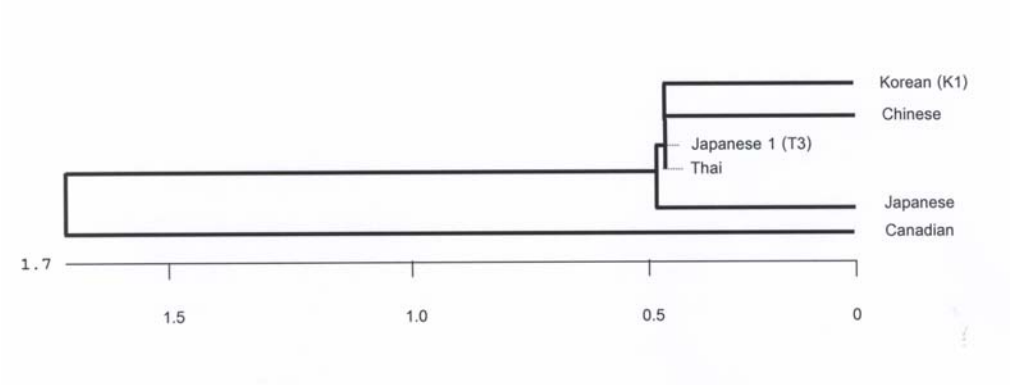


Figure 17 A phylogenetic tree of 6 BmNPV *polhs* (Thai: AY779044, Japanese 1 (T3): L33180, Japanese 2: M30925, Korean (K1): U75359, Chinese: X63614 and Canadian: M100430) constructed from amino acid sequences. The tree was generated by Clustal method, MegAlign program of DNASTAR.

Silkworm has been reared for centuries and has become a domestic animal species. Due to this life history, wings of silkworm have shortened and the silk moth can not longer fly. This immobility feature has restricted them to a confined region. Thus, it was believed that silkworms

and their NPVs may originally occur in the same place and during evolution they distributed and evolved individually. The limited vagility of the silkworm, biogeographic variation was observed between different isolates of NPVs in this species as demonstrated by phylogeographic differences between the Canadian and Asian isolates. However, the percentage identity of all BmNPV *polh* nucleotide sequences was greater than 98% which indicated that *polh* was a highly conserved gene.

2.4 Comparison of Thai BmNPV *polh* with other NPV *polh*s

Thai BmNPV *polh* nucleotides and amino acid sequences were compared with those of AcNPV (Ayres *et al*., 1994), HaNPV (Chen *et al*., 2001), SeNPV (IJkel *et al*., 1999) and SlNPV (Pang *et al*., 2001). AcNPV is a prototype of Baculovirus, and HaNPV, SeNPV and SlNPV are the viruses of economically important insect pests in Thailand. The percentage identities of Thai BmNPV *polh* gene sequence as compared with those of AcNPV, HaNPV, SeNPV and SlNPV were 75.2%, 65.3%, 70.1% and 65.9%, respectively, and percentage identities of the Polh sequence were 86.1%, 81.6%, 78.0%, and 79.6%, respectively (Figure 18).

Percentage nucleotide sequence identity							
	1	2	3	4	5		Virus
1		75.2	65.3	70.1	65.9	1	BmNPV
2	86.1		67.2	72.6	72.1	2	AcNPV
3	81.6	84.6		70.1	76.1	3	HaNPV
4	78.0	81.3	83.7		70.2	4	SeNPV
5	79.6	83.7	82.5	83.8		5	SlNPV
	1	2	3	4	5		
Percentage amino acid sequence identity							

Figure 1: Nucleotide and amino acid sequence identity of NPV *polhs*. Percentage nucleotide identity is denoted above the diagonal and percentage amino acid identity is below. Sequence data of NPV *polhs* were retrieved from GenBank: 1) BmNPV (AY779044); 2) AcNPV (NC_001623); 3) HaNPV (NC_002654); 4) SeNPV (NC_002169) and 5) SlNPV NC_003102).

ORF of BmNPV *polh* encoded a polyhedrin protein containing 245 amino acids similar to *polh* of AcNPV and HaNPV while the *polh* of SeNPV and SlNPV contained 246 and 249 amino acids, respectively. The amino acid sequence alignment of BmNPV Polh with other NPVs demonstrated that differences occurred more in the N-terminus than C-terminus (Figure 19). In HaNPV, the amino acid of Polh at position 40 is deleted. There are many substitution positions that make amino acids of BmNPV Polh differs from other NPV Polhs such as V^{30}/L, L^{50}/W, M^{128}/L, R^{129}/K, C^{147}/W, D^{148}/E, P^{149}/E, L^{186}/I, I^{199}/V and L^{226}/I. Amino acid contents of the putative BmNPV Polh indicated that it is rich in acidic amino acid residues, such as glutamic acid, especially at position 220-223 where four glutamic acids align consecutively. It is relative poor in cystein, tryptophan and histidine.

```
    BmNPV    ----MPNYSYTPTIGRTYVYDNKYYKNLGCLIKNAKRKKHLVEHEQEEKQWDLLDNYMVAEDPFL  65
    AcNPV    ----MPDYSYRPTIGRTYVYDNKYYKNLGAVIKNAKRKKHFAEHEIEEATLDPLDNYLVAEDPFL  65
    HaNPV    ---MYTRYSYSPTLGKTYVYDNKYFKNLGAVIKMPTQEH-LEEHEHEERNLDSLDKYLVAEDPFL  65
    SeNPV    ---MYTRYSYNPALGRTYVYDNKFYKNLGSVIKNAKRKEHLLQHEIEERTLDPLERYVVAEDPFL  65
    SlNPV    MYSRYSAYNYSPHLGKTYVYDNKYYKNLGHVIKNAKRKHDALEREADERELDHLDKYLVAEDPFM  65
Consensus        YT YSYSPTLGRTYVYDNKYYKNLGAVIKNAKRK HLLEHE EER LD LDKYLVAEDPFL  65

    BmNPV    GPGKNQKLTLFKEIRSVKPDTMKLIVNWSGKEFLRETWTRFVEDSFPIVNDQEVMDVYLVANLKP 130
    AcNPV    GPGKNQKLTLFKEIRNVKPDTMKLVVGWKGKEFYRETWTRFMEDSFPIVNDQEVMDVFLVVNMRP 130
    HaNPV    GPGKNQKLTLFKEIRSVKPDTMKLVVNWSGREFLRETWTRFMEDSFPIVNDQEIMDVFLSVNMRP 130
    SeNPV    GPGKNQKLTLFKEIRIVKPDTMKLVVNWSGKEFLRETWTRFMEDSFPIVNDQEIMDVFLVINMRP 130
    SlNPV    GPGKNQKLTLFKEIRNVKPDTMKLIVNWNGKEFLRETWTRFMEDSFPIVNDQEVMDVFLVVNMRP 130
Consensus    GPGKNQKLTLFKEIR VKPDTMKLVVNWSGKEFLRETWTRFMEDSFPIVNDQEVMDVFLVVNMRP 130

    BmNPV    TRPNRCYKFLAQHALRWEEDYVPHEVIRIVEPSYVGMNNEYRISLAKKGGGCPIMNIHSEYTNSF 195
    AcNPV    TRPNRCYKFLAQHALRCDPDYVPHDVIRIVEPSWVGSNNEYRISLAKKGGGCPIMNLHSEYTNSF 195
    HaNPV    TKPNRCYRFLAQHALRCDPDYIPHEVIRIVEPSYVGSNNEYRISLAKKYGGCPVMNLHAEYTNSF 195
    SeNPV    TRPNRCFRFLAQHALRCDPDYVPHEVIRIVEPVYVGTNNEYRISLAKKGGGCPVMNLHSEYTNSF 195
    SlNPV    TRPNRCFRFLAQHALRCDPEYVPHDVIRIVEPSYVGTNNEYRISLAKKGGGCPVMNLHAEYTTSF 195
Consensus    TRPNRCYRFLAQHALRCDPDYVPHEVIRIVEPSYVGSNNEYRISLAKKGGGCPVMNLHSEYTNSF 195

    BmNPV    ESFVNRVIWENFYKPIVYIGTDSAEEEEILIEVSLVFKIKEFAPDAPLFTGPAY            249
    AcNPV    EQFIDRVIWENFYKPIVYIGTDSAEEEEILLEVSLVFKVKEFAPDAPLFTGPAY            249
    HaNPV    EDFITNVIWENFYKPIVYVGTDSAEEEEILLEVSLIFKIKEFAPDAPLYTGPAY            249
    SeNPV    EEFINRVIWENFYKPIVYVGTDSGEEEEILLELSLVFKIKEFAPDAPLYNGPAY            249
    SlNPV    ESFIDKVIWYNFYKPIVYVGTDSAEEEEILLEVSLVFKIKEFAPDAPLYTGPAY            249
Consensus    E FI RVIWENFYKPIVYVGTDSAEEEEILLEVSLVFKIKEFAPDAPLYTGPAY            249
```

Figure 19 Alignment of the deduced amino acids of Thai *Bm*NPV *polh* (AY779044) with other NPV *polhs*; AcNPV (NC_001623), HaNPV (NC_002654), SeNPV(NC_002169) and SlNPV(NC_003102). The alignment of five NPV Polhs were made using the Clustal method, MegAlign program of DNASTAR. The different amino acids are shaded.

In general, percentage identity of NPV amino acid sequence was higher than nucleotide sequence which suggests that the genetic code is degenerated and the same amino acid is encoded by more than one codon. Previously, Chou *et al*. (1996) reported the percentage identities of amino acid sequence of the BmNPV Polh as compared to that of AcNPV, SeNPV, and SlNPV were 86%, 82% and 80%, respectively, which is similar to the results obtained from this study.

Phylogenetic tree of NPV Polhs showed that Thai BmNPV Polh was distinct from other NPVs. Based on multiple sequence alignment (MegAlign) analysis of the Polh, BmNPV separated from the group of AcNPV, HaNPV, SeNPV and SlNPV (Figure 20).

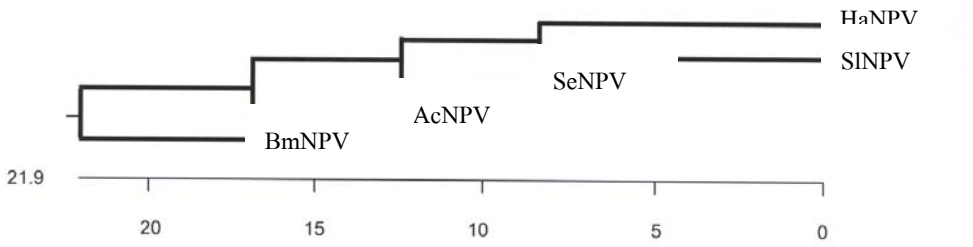


Figure 20 A phylogenetic tree of five NPV Polhs (BmNPV:AY779044, AcNPV:NC_001623, HaNPV: NC_002654, SeNPV:NC_002169 and SlNPV:NC_003102) constructed from amino acid sequences. The tree was generated by Clustal method, MegAlign program of DNASTAR.

Nucleopolyhedrovirus clades were first identified by Zanotto *et al.* (1993). Based on the Polh, they constructed a phylogenetic tree of baculoviruses in which NPVs were divided into Group I and Group II. Subsequently, Bulach *et al*. (1999) supported these clades and revealed other subclades under Group II by analyzing the Polh and DNA polymerase. Both Zanotto *et al.* (1993) and Bulach *et al*. (1999) grouped BmNPV and AcNPV into Group I and HaNPV, SeNPV and SlNPV into Group II.

Harrison and Bonning (2003) constructed a phylogenetic tree of Polh sequence of many lepidopteran NPVs including BmNPV, AcNPV, HaNPV, SeNPV and SlNPV. They grouped HaNPV, SeNPV and SlNPV into Group II and BmNPV into Group I of the proposed tree of Zanotto *et al*. (1993). The AcNPV polyhedrin was put on a branch outside of the clade containing the other members of Group I. This could suggest that AcNPV may acquire its *polh* gene by recombination with another virus that is not closely related to other NPVs in Group I. In addition, Jehel (2004) used the Hidden Markov Model to explain that AcNPV *polh* is a chimeric gene which consists of a mosaic of the genome of Group I and II NPVs. From these results AcNPV can be grouped in both Group I and Group II depending on the method used for the analysis. Since several reports revealed that adding the AcNPV *polh* resulted in distortion and instability to the *polh* gene tree, therefore, many other genes were recently employed for phylogenetic analysis of baculovirus (Herniou *et al*., 2004). However, for simple molecular analysis, *polh* is still useful because a great number of *polh* gene sequences are available in GenBank (Lange *et al*., 2004).

3. Evaluation of PCR methodology for grasserie disease detection

To determine whether PCR is applicable for grasserie disease detection, preliminary tests were performed using BmNPV artificially inoculated samples.

3.1 Detection of BmNPV in silkworm eggs

When the specific primers were used to amplify fragment of BmNPV *polh*, PCR amplified products of the expected size (424 bp) were observed in the gel. As illustrated on the gel, PCR products of the Thai BmNPV *polh* had the same size of the clone of polyhedrin gene in pGEM®-T vector (positive control) while there was no amplified product when using PCR reaction mixture without DNA template (negative control). Eggs laid by artificially infected silkworm moths were used to illustrate the grasserie disease detection by PCR. The results revealed that genomic BmNPV DNA could be detected when using the sample as low as one egg. Increasing the number of eggs for DNA extraction provided higher concentration of DNA template resulting in high intensity of DNA band visualized on the gel (Figure 21). PCR products were specific to the virus used as the DNA template and no nonspecific sequences were observed. Strong intensity of PCR product bands were clearly visualized on the gel when using DNA extracted from 5 to 20 eggs. Thus, for accurate interpretation, 20 silkworm eggs were used to represent one sample of egg for all experiments in this study.

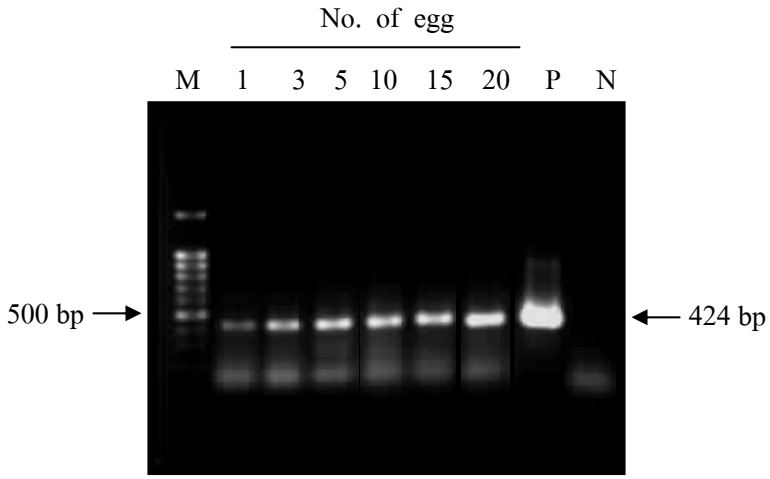


Figure 21 PCR products of DNA extracted from 1, 3, 5, 10, 15, 20 eggs laid by BmNPV-artificially infected silkworm moths

M=DNA marker (100 bp+1.5 kb)

P= positive control (pGEM®-T vector harboring polyhedrin gene)

N=negative control (no DNA template)

The colour and the characteristic of eggs laid by BmNPV- infected silkworm moth were not different from those laid by the healthy one. However, number of eggs laid by BmNPV-infected moth was about 40% less than those from healthy moth. Healthy female moth laid an average of 453 eggs while infected moth laid an average of 269 eggs (Table 3). This result was similar to the study of Khurad *et al*. (2004) who indicated that the number of eggs from healthy moth (580 eggs) was 40% more than the number of eggs from infected moth (360 eggs).

Table 3 Number of eggs laid by healthy and artificially BmNPV-infected silkworm moths, *Bombyx mori*

Couple of moths	No. of eggs laid by healthy moth	No. of eggs laid by BmNPV-infected moth
1	494	298
2	523	308
3	473	313
4	553	241
5	385	217
6	481	338
7	400	306
8	423	250
9	407	174
10	395	248
total	**4534**	**2693**
%	**100**	**59.38**

This study provided evidence that PCR is an efficient tool for detection of grasserie disease in silkworm. The detection could be made as early as in egg stage and only one egg can be used for the detection. In the past, scientists believed that only pebrine disease of silkworm is transmitted through eggs laid by infected silkmoth. Hence, most of the reports on disease detection in silkworm egg were dealing with pebrine disease and rarely with viral diseases. Attathom *et al.* (1994) reported that DNA probe was applicable for the detection of BmNPV in silkworm eggs of which the parents were artificially infected with BmNPV. PCR method was previously tested for *Lymantria dispar* NPV (LdNPV) detection on the surface of an egg. Samples of gypsy moth's egg were obtained by artificially contaminated surface of the eggs with LdNPV (Burand *et al*., 1992). In this study egg samples were those oviposited by mother moths infected with BmNPV during the larval stage. The existing of BmNPV in the eggs could suggest

that BmNPV may be transmitted vertically from parents to offsping. In recent study on BmNPV of silkworm, the result of PCR amplification of BmNPV DNA isolated from the viral-infected parents and the F1 offspring had confirmed that the viral infection was vertically transmitted to the progeny, however, the authors did not detect viral infection in egg (Khurad *et al.*, 2004). Ikuno *et al*. (2004) could detect BmNPV in infected eggs of silkworm using PCR with primers specific to polyhedrin gene. However, when compared with this study, their method comprised of many steps for DNA extraction. For example, eggs were washed with sodium hypochoride, crushed, and ground in STE-buffer, DNA was extracted from 40 mg of egg sample (approximately 11 eggs) by means of incubation with SDS and proteinaseK and followed by purification using DNA purification kit. However, in this study, DNA was extracted by grinding the eggs in solution II following the treatment with solution III, chloroform:isoamlyl alcohol, isopropanol and finally the DNA was washed by 70% alcohol.

3. 2 Detection of BmNPV in artificially inoculated silkworm larvae

The appropriate number of the first instar larvae for DNA extraction and subsequent PCR amplification was determined. The expected PCR products of 424 bp were obtained and there were no different of band clearness and intensity when using DNA template extracted either from 1, 2, 3 or 4 larvae (Figure 22). This result suggested that only one larva of the first instar provided adequate DNA template to be amplified by PCR method. DNAs extracted from individual artificially inoculated larva of the 1^{st}, 2^{nd}, 3^{rd}, 4^{th} and 5^{th} instar provided PCR products of expected size. No PCR products were observed when using DNA extracted from healthy larvae (Figure 23).

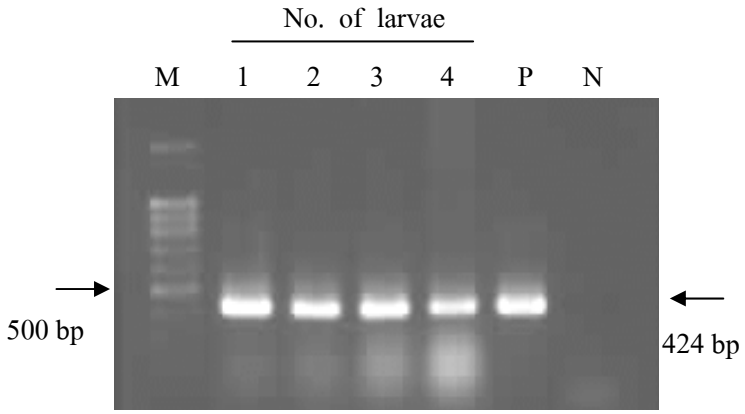


Figure 22 PCR products of DNA extracted from various numbers of BmNPV-artificially infected first instar larvae

M=DNA marker (100 bp+1.5 kb)

P=positive control (pGEM®-T vector harboring polyhedrin gene)

N=negative control (no DNA template)

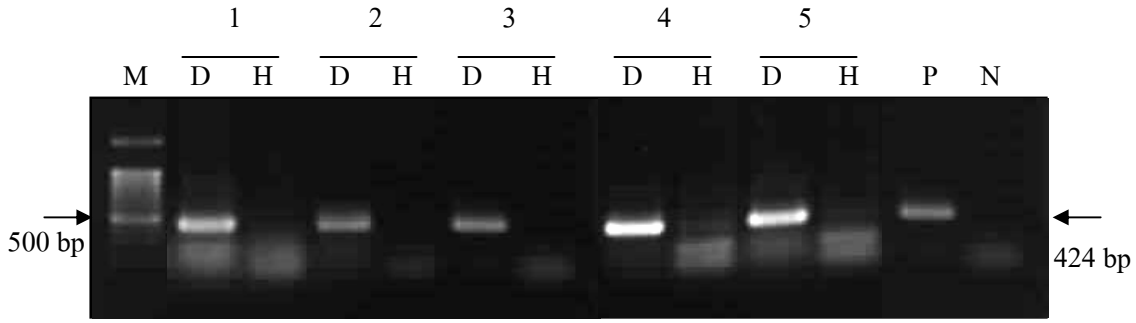


Figure 23 PCR products of DNA extracted from various instars of BmNPV-artificially infected larvae. Numbers on the top row indicated instar of the larvae.

M=DNA marker (100 bp+1.5 kb)

P=positive control (pGEM®-T vector harboring polyhedrin gene)

N=negative control (no DNA template)

H=healthy larva D=diseased larva

Silkworm, *Bombyx mori* undergoes four moults, thus there were five instars during the larval development (Figure 24). Symptoms of grasserie disease as normally shown in late instar of BmNPV infected larvae were illustrated in Figure 25. The symptoms could hardly be noticed during the early larval instar, except that the larvae were slightly sluggish. Disease symptoms started with the colour of larval body changed from white to yellow (Figure 25 a). As the disease progress, the body became swollen (Figure 25 b) and the colour of intersegmental region turned brown (Figure 25 c). In the later stage of infection, the skin ruptured, turbid haemolymph containing innumerable polyhedral bodies released and the larvae died (Figure 25 d). Frequently, characteristic symptom of NPV infection was observed. The larvae became restless and crawled aimlessly along the rims of rearing tray. Diseased larvae lost the clasping power of the abdominal legs except the caudal legs by which it hanged with the head downwards (Figure 25 e). Subsequently, they fell on the ground and died. Death took place after infection in about 3-5 days in the young larvae and 5-7 days in the late instar larvae.

Using PCR, BmNPV could be detected in the larval stage as early as the first instar larva. In all larval instar, only one individual infected larva provided adequate BmNPV DNA to use as PCR template. This result was similar to the study of Ikuno *et al*. (2004) who reported that PCR with complementary primers to the *polh* region could be used to detect BmNPV in haemolymph of all larval instars. Previous reports revealed methods for the detection of BmNPV in all larval instars including indirect ELISA (Vanapruk *et al.*, 1992) and DNA probe (Attathom *et al.*, 1994). However, these two methods were complicated and used rather expensive chemicals. In addition, some other techniques such as monoclonal antibody, 2-3 second instar larvae were needed for BmNPV detection (Kalsi *et al.*, 1998) and for sandwich ELISA, 5-6 second instar larvae were required for the detection (Shamim *et al.*, 1994).

Figure 24 The healthy mulberry silkworm, *Bombyx mori* larvae

a) first instar b) second instar

c) third instar d) fourth instar

e) fifth instar

Figure 25 Symptoms of grasserie disease of silkworm, *Bombyx mori* caused by BmNPV

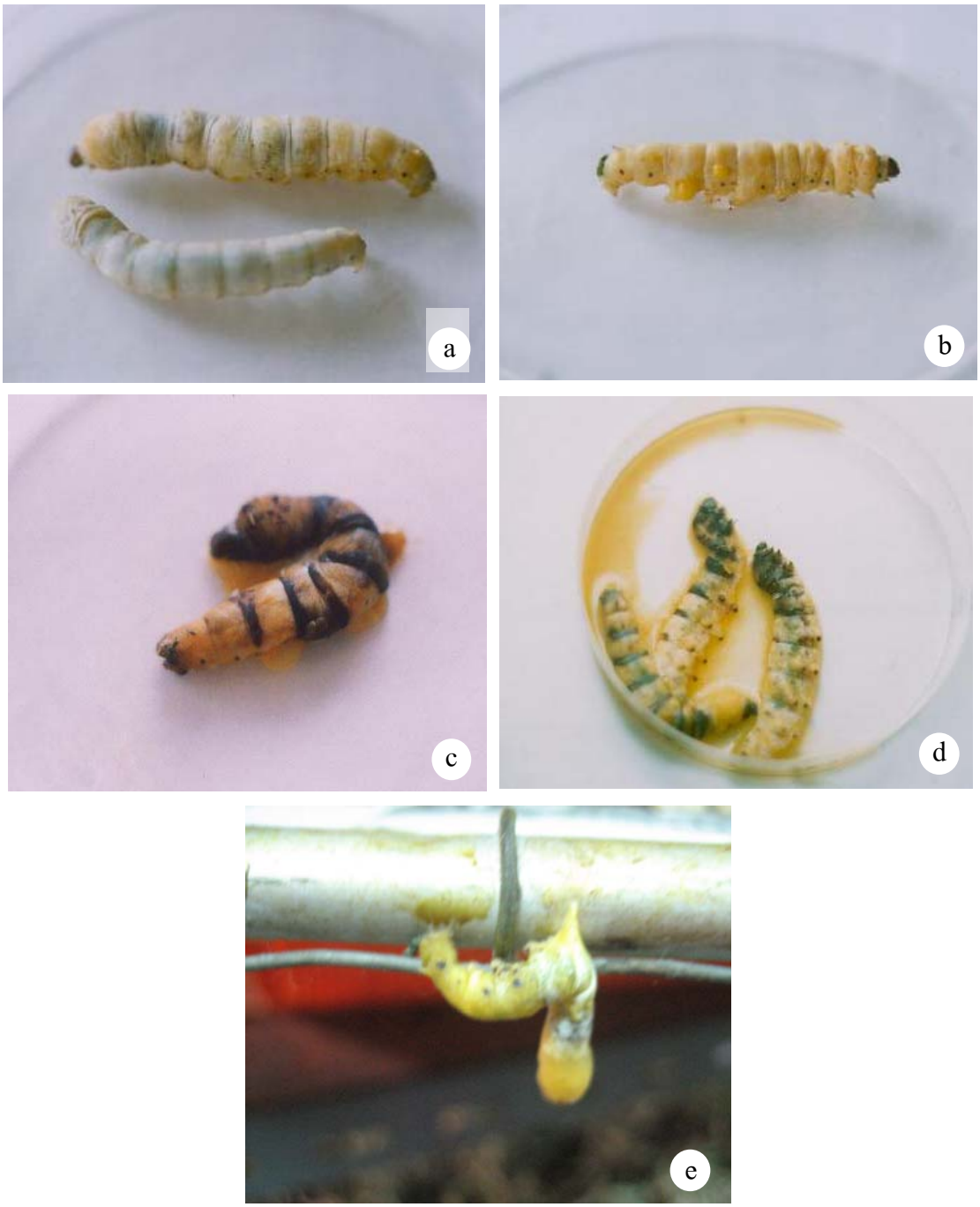
a) Healthy larva (top) compared with BmNPV infected larva (bottom) which appeared yellowish in body colour.

b) Larva showed robust body with swollen segments

c) Intersegmental area turned brown and the body became fluffy

d) Dead larvae marked by skin rupture and exudation of tubid haemolymph contained numerous polyhedra of the virus

e) Larva showed characteristic symptom of NPV infection. The larva hanged dead with head downwards.


a
b
c
d
e

3.3 Detection of BmNPV in silkworm pupae

With the developed PCR method, BmNPV could be detected in both infected male and female pupa while no BmNPV DNA was detected in healthy pupa (Figure 26). PCR products of expected size (424 bp) were observed in infected male and female pupae.

There were a few reports on BmNPV detection in pupal stage. In practical, grasseries detection at pupal stage is useless because if this disease occurred in pupal stage, it was too late to manage silkworm rearing. The only anticipated benefit is that one can eliminate infected pupae and keep, perhaps clean silkworm colony for next generation. Previously, Vanapruk*s* (1991) reported that the indirect ELISA could be used to detect BmNPV in the pupal stage after inoculation the fourth instar larvae with the virus at 10^6 PIB/ml. In addition, Attathom *et al*., (1994) reported the use of digoxiginin labeled probes to detect BmNPV DNA in infected pupae.

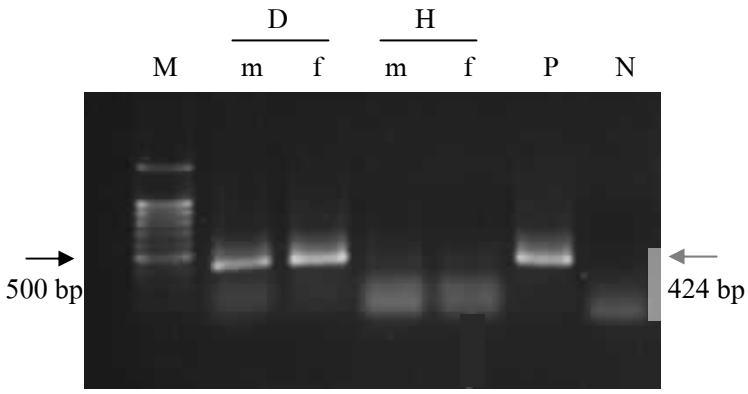


Figure 26 PCR products of DNA extracted from BmNPV-artificially infected male and female pupae

M=DNA marker (100 bp+1.5 kb)

P=positive control (pGEM® -T vector harboring polyhedrin gene)

N=negative control (no DNA template)

H=healthy pupa; D=diseased pupa

f= female pupa; m=male pupa

The yellow cocoons and healthy pupae of local silkworm race were shown in Figure 27 a and b. When the silkworms were artificially inoculated with BmNPV at their fifth larval instar, there were many symptoms appeared during the final moult and pupation. For example, some larvae could not change into pupal form and die within the old cuticle (4%) (Figure 27 c). However, some larvae changed into pupa but could not spin fibres (9%) (Figure 27 d), and some could not change into pupal form but spinned abnormal cocoon and finally died inside the cocoon (30%) (Figure 27 e). Several of the infected pupae showed abnormal pupal shape (22%) (Figure 27 g). Only 35% of the infected pupae appeared as normal pupae (Table 4).

Table 4 Percentage of several abnormal symptoms of pupae of silkworm, *Bombyx mori* artificially infected with nucleopolyhedrovirus.

Symptom	Percentage
1. normal pupa	35
2. larva cannot change into pupal form	4
3. larva can change into pupal form but cannot spin fibres	9
4. larva cannot change into pupal form but can spin fibres	30
5. abnormal pupa	22
total	**100**

The essential hormone involves in moulting process in insect is ecdysone. BmNPV was known to harbor *egt* gene encoded for EGT protein which was produced in insect cell during the viral infection. This protein transfers the sugar moiety from a UDP-sugar to ecdysone. The function of EGT protein is to block the moulting of insect host during infection (O'Reilly *et al*, 1992). Therefore, the deformed shape of BmNPV infected pupae, could occur from the action of the existing *egt* gene in BmNPV genome to inhibit normal development of the silkworm. In addition, NPV had some effect on pupal size as reported by Myers *et al.* (2000) who described that pupae of gypsy moth infected with *Lymantria dispar* nucleopolyhedrovirus (LdNPV) were smaller than the healthy pupae.

Figure 27 Symptoms of grasserie disease of silkworm, *Bomby mori* caused by nucleopolyhedrovirus as appeared in the pupal stage

a) Yellow cocoon of healthy pupae

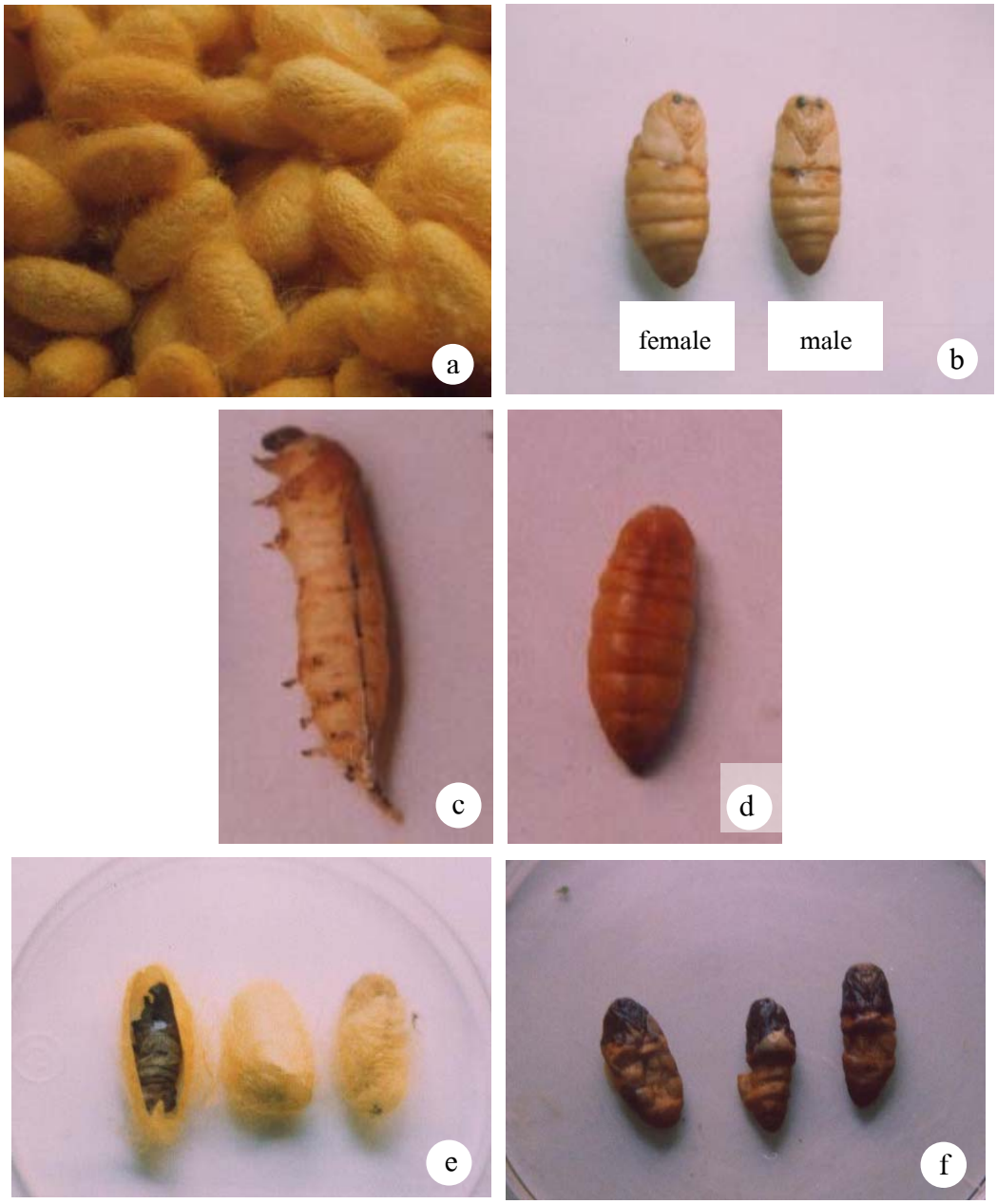
b) Healthy male and female pupae.

c) Larva cannot change into pupal form

d) Larva can change into pupal form but cannot spin fibres

e) Larva cannot change into pupal form but can spin fibres

f) Abnormal pupae


a
female
male
b
c
d
e
f

3.4 Detection of BmNPV in silkworm moths

Detection of grasserrie disease in silkworm moths by PCR gave similar result as found in pupae. DNA extracted from diseased male and female moths were amplified using primers specific to Thai *polh* gene, and PCR products of expected size were obtained. DNA from healthy samples gave no PCR products (Figure 28).

The healthy moths emerged by rupturing one end of the cocoon after which male and female moths started to mate. Normally, female is bigger than male (Figure 29 a). After mating, the female moth laid group of eggs that glued on paper (Figure 29 b). The BmNPV-infected moths were in irregular shape as compared to the healthy moths. Some moths could not emerge from the pupae (Figure 29 c) while the others had shortened or distorted wings (Figure 29 d). Some BmNPV infected moths were larger than the healthy moths. This is due to the fact that BmNPV infected nuclei of several insect cells and tissues. Numerous polyhedra were produced in the nuclei which caused the cells to undergo hypertrophy and hence the insect body swollen (Boucias and Rohrmann, 1998).

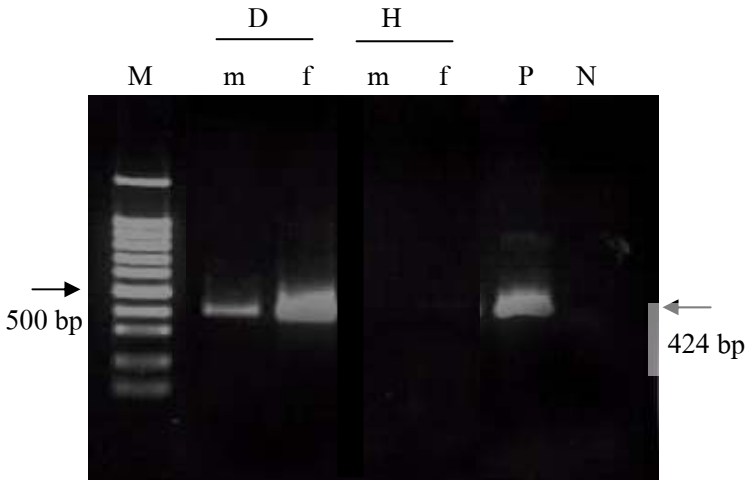


Figure 28 PCR products of DNA extracted from BmNPV-artificially inoculated male and female moths

M= DNA marker(100 bp+1.5kbp)

P= positive control (pGEM®-T vector harboring polyhedrin gene)

N=negative control (no DNA template)

H=healthy moth D=diseased moth

f= female moth m=male moth

Figure 29 Healthy and BmNPV-infected silkworm moths, *Bombyx mori*

a) Male and female healthy moths

b) Female moth lays group of eggs that glue on paper

c) Moth cannot emerge from pupal shell

d) Moth with abnormal body and wings

In this study, BmNPV-infected male and female silkworm moths were allowed to mate and laid their eggs. After that, BmNPV detection in the parent moths and their eggs by PCR was performed. From 30 samples, BmNPV was detected in 30% of the male moth, 10% of the female moth and 37% of the egg. BmNPV was detected less in male and female moths than in egg. In this study the whole body of moth was crushed and DNA was extracted and used as DNA template for PCR detection. Concentration of the viral DNA per volume of the solution may be lower than that obtained from eggs. For viral detection in moth, it may be necessary to detect the virus in each tissue or organ separately. Attention can be focused on tissues or organs that had records as sites of viral infection. The result of this study was similar to previous report by Vanapruk *et al.* (1992) who, using ELISA, detected BmNPV in all stages of silkworm except the adult moths which were inoculated with the virus at their 4^{th} larval instar. This may be due to the level of the virus in the sample was too low to be detected. However, Attathom *et al.* (1994) was able to detect BmNPV in adult silkworm moth using dot blot hybridization.

This evaluation study showed that grasserie disease of silkworm could be detected by PCR. Detection could be made in all developmental stages and in both sexes of silkworm. Therefore, PCR method was further used to demonstrate its efficacy in detecting grasserie disease in collected samples of silkworm from the rearing houses.

4. Application of PCR for grasserie detection in collected samples

4.1 Artificially inoculated samples

For comparison, BmNPV detections by PCR were made in both naturally collected samples and laboratory inoculated samples. For laboratory inoculated samples, the samples of each stage of silkworm development were collected and detected for grasserie disease by PCR. For egg stage, BmNPV was detected in four out of five egg samples (20 eggs per samples)(80%). For larval stage, BmNPV was detected in all artificially inoculated larval samples (100%). BmNPV was detected in 60% of the observed male and female pupae. The virus was also detected in 60% and 40% of the observed male and female moth respectively (Table 5).

Table 5 Number of *Bombyx mori* nucleopolyhedrovirus infected samples as detected by PCR

Source	Silkworm variety	Number of BmNPV detected samples/ Number of observed samples								
		Egg[1/]	Larva[2/]					Pupa[2/]		Moth[2/]	
			1st	2nd	3rd	4th	5th	Male	Female	Male	Female
Laboratory inoculated samples	Nang Noi	4/5	5/5	5/5	5/5	5/5	5/5	3/5	3/5	3/5	2/5
total		4/5	5/5	5/5	5/5	5/5	5/5	3/5	3/5	3/5	2/5
%		**80**	**100**	**100**	**100**	**100**	**100**	**60**	**60**	**60**	**40**

[1/] one sample = 20 eggs

[2/] one sample = individual larva, pupa or moth

BmNPV was detected in all samples of the larval stage of silkworm while in the other developmental stages, BmNPV was detected only in some samples. It was reported that silkworm has some mechanisms against virus infection, for example, viral inhibitory factor was found in silkworm haemolymph (Funakoshi and Aizawa, 1989) and a red fluorescent protein (RFP) in gut juice was found to possess antiviral activity against BmNPV (Nakazawa *et al*., 2004). Perhaps

with these antiviral mechanisms, viral infection was inhibited, therefore no viral DNA was detected in some of the samples.

4.2 Naturally infected samples

Samples of silkworm were collected from different rearing places. Each variety of collected silkworms exhibited different characteristics (Table 6). UB1 and Nang Noi Si Sa Ket varieties were bred and improved by Department of Agriculture. KSK was a breeding line of Department of Agricultural Extension. TSC and JT were Thai Silk Company (Jim Thompson Farm) breeding lines. Chul 1 and Chul 6 were bred by Chul Thai Silk Company. On the other hand, Nang Noi is the local Thai variety in which the local farmers prefer to culture. Each location prefers to raise its own silkworm variety. Normally bivoltine silkworm varieties produced white cocoon but cocoon of the TSC and the JT varieties of Thai Silk Company are yellow resulting from the breeding and line improving process of the company. The sericultural stations and companies in Thailand tried to breed bivoltine silkworms because their silk fibres are long and can be reeled by machine while those of the multivoltine varieties are shorter and can be reeled only by hand. In addition, the silk yarn from bivoltine varieties is more favorable for exporting to other countries.

Table 6 Characteristics of some silkworm varieties culturing in Thailand

Province	Silkworm variety	Voltinism	Cocoon colour
Nong Khai	UB1	bivoltine	white
	Nang Noi Si Sa Ket	multivoltine	yellow
Kanchanaburi	KSK	bivoltine	white
Nakhon	TSC	bivoltine	yellow
Ratchasima	JT	bivoltine	yellow
Phetchabun	Chul 1	bivoltine	white
	Chul 6	bivoltine	white
Kalasin	Nang Noi	multivoltine	yellow

To prevent silkworm from viral and other disease infections, precaution strategy is very important. Attention should be focused on sanitary practice before, during and after rearing courses. The rearing place and equipments should be thoroughly disinfected. Before rearing silkworm, it was recommended to spray 3% formalin in the rearing room. While rearing, the larvae should be sufficiently provided with healthy mulberry leaves. The temperature, humidity and other environmental conditions of the rearing place should be maintained at suitable level. If, in spite of these precautions, some silkworms were found to be infected, they should be immediately detected and discarded. The chemicals used for preventing disease outbreak were formaldehyde, paraformaldehyde, lime and chlorine. Paraformaldehyde is available in different trade names such as Pefsol and Chul F sol. The principle of silkworm disease prevention was similar in every place (Table 7) but the chemicals used were different from places to places. Formaldehyde was normally used by applying inside and outside rearing room for destroying pathogens before silkworm rearing. Lime and paraformaldehyde were used by scattering around the larvae before and after moulting (Table 7). Spraying with mild alkaline solution such as calcium oxide (CaO) may give substantial control of grasserie disease since viral polyhedra dissolved at high pH, subsequently released the viral particles exposing to the environment and finally lost their infectivity.

The early instar larvae are important stage of silkworm because they are susceptible to diseases. This stage needs special care and sanitation. If silkworm could survive early stage, the opportunity to successfully pass through all developmental stages and get high yield cocoon can be assured. Therefore, Chul Thai Silk Company takes responsibility in raising the early instar silkworm larvae and provides these healthy colony to farmers for cocoon production.

Table 7 Common sanitary procedures for silkworm diseases prevention by some silkworm rearing houses

Source	Method of silkworm disease prevention	
	Before silkworm rearing	**During silkworm rearing**
Nong Khai (Governmental institute)	Equipment : clean by detergent. Rearing room: spray with 3% formaldehyde.	Before moulting: treat with lime After moulting : treat with paraformaldehyde
Kanchanaburi (Governmental institute)	Equipment : clean by detergent and follow by chorine. Rearing room: spray with 3% formaldehyde.	Before moulting: treat with burned paddy husk After moulting : treat with paraformaldehyde
Nakhon Ratchasima (Private company)	Equipments : clean by detergent and follow by chorine Rearing room: spray with mixture of 3-4% formaldehyde and calcium oxide (CaO)] If BmNPV had occurred in the previous colony, spray with 3% CaO at first and follow by 3-4% formaldehyde.	Before moulting: treat with CaO After moulting : treat with paraformaldehyde During 5^{th} instar: treat with CaO and paraformaldehyde every the other day Rearing room : clean the floor with 0.3% chorine
Nakhon Ratchasima (Contracted farm)	Equipments : clean by detergent and follow by chorine Rearing room: spray with 3% formaldehyde.	Before moulting: treat with CaO After moulting : treat with paraformaldehyde
Phetchabun (Contracted farm)	Equipments : clean by detergent and follow by chorine. Rearing room: spray with chlorine 2 times at ratio 1 kg per 200 litres of water.	Before moulting: treat with chlorine After moulting : treat with paraformaldehyde
Kalasin (Local farmer)	Equipment : clean by detergent	No treatment

In this study, all stages of silkworm were collected from governmental institutes in Nong Khai and Kanchanaburi, Thai Silk Company in Nakhon Ratchasima, contracted farms in Nakhon Ratchasima and Phetchabun, and local farmer in Kalasin province. Numbers of the collected samples that gave positive result to BmNPV detection by PCR were shown in Table 8.

In egg stage, BmNPV was detected only in eggs collected from the local farmer rearing house in Kalasin province. It was not detected in eggs from other rearing houses. BmNPV infection was detected in eggs samples collected locally more than eggs samples collected from sericultural station and private company rearing houses. Normally eggs from sericultural stations and private companies were treated with formalin before rearing but no eggs treatment by the local farmers therefore the virus that contaminated on the surface of the eggs was not destroyed.

For larval stage, BmNPV was detected in all larval instars. It was detected in the following samples of laval stage which demonstrated the capacity of the PCR method for grasserie detection

First instar : Nang Noi Sri Sa Ket silkworm variety (Figure 30 a)
Second instar : Nang Noi Sri Sa Ket silkworm variety (Figure 30 b)
Third instar : KSK variety (Figure 30 c)
Fourth instar : Nang Noi variety (Figure 30 d)
Fifth instar : Nang Noi variety (Figure 30 e)

Some DNA samples provides more than one specific band, for example, PCR products of the DNA extracted from the second instar larvae of Nong Noi Sri Sa Ket variety from Nong Khai province (Figure 30 b). PCR products of BmNPV DNA extracted from the fifth instar were clearer and thicker than PCR products of BmNPV DNA extracted from the other larval instars. Furthermore, BmNPVs were detected at higher rate in late instar than in early instar. BmNPV detection in the fifth instar larvae was 12.8% while in the others were 2.6-5.1%. This supported the report of Shamim *et al*. (1994) which indicated that grasserie disease was mostly found in the final larval stage of silkworm.

By PCR, BmNPV was also found in both male and female pupae and moths. For pupal stage, BmNPV was detected in male pupae of KSK and Chul 6 varieties and in female pupae of JT and Chul 6 varieties. Examples of PCR products of DNA extracted from naturally infected male and female pupae were illustrated in Figure 31 a. For adult moth, BmNPV was also detected in both male and female moths of UB1 and Nang Noi varieties. However, BmNPV was detected only in female moth of JT and Chul 6 varieties. Examples of PCR products of DNA extracted from naturally infected male and female moths were illustrated in Figure 31 b. During the collection process, one couple of silkworm moths from Kalasin province was mating and laying eggs. BmNPV was detected in both male, female moths and their eggs (Figure 31 c). This result agreed with the study using artificially inoculated samples which indicated that BmNPV was detected in eggs laid by infected silkworm moth. This result could be the evidence to confirm that BmNPV can be transmitted via egg.

Samples from sericultural stations collected for PCR detection were the three varieties of silkworm; UB1 and Nang Noi Sri Sa Ket from Nong Khai Sericultural Experiment Station and KSK from Sericultural Extension Centre 9, Kanchanaburi province. The results revealed that BmNPV was detected in larva, pupa and adult stages. No BmNPV was detected in egg stage. In larval stage, the total of 6.7, 13.3, 6.7, 6.7 and 26.7% of the 1^{st}, 2^{nd}, 3^{rd}, 4^{th} and 5^{th} instar respectively were found to be infected by BmNPV. In pupal stage, 13.3 and 6.7% of male and female pupae were infected and in adult, 26.7 and 20% of male and female moth were infected.

Silkworm samples collected from private company rearing house, the Thai Silk Company in Nakhon Ratchasima province, was TSC variety. PCR detection indicated that no BmNPV was detected in all stages of those collected silkworm samples.

Three varieties of silkworm raised by contracted-farmers of the private companies were collected for grasserie disease detection. JT variety was collected from farmers under Thai Silk Company contraction in Kakhon Ratchasima province. Chul 1 and Chul 6 were collected from farmers under Chul Thai Silk Company contraction in Phetchabun province. The egg samples of JT and Chul 6 varieties used in this study were collected from eggs laid by silkmoths which were

reared by the farmers. They were not produced by the companies. Normally, colony of silkworm provided by the private company cannot be maintained by the farmers. There are two reasons, first, the eggs are diapause eggs, therefore, they cannot hatch naturally and need special treatment for hatching. Second, they are hybrids silkworm variety, the farmers cannot breed silkworm by themselves because they do not have the parental strain of silkworm. Grasserie disease detection by PCR showed that DNAs extracted from eggs and larvae collected from the contracted farmer's rearing house gave no PCR products. For pupal stage, BmNPV was detected in male pupae of Chul 6 variety and in female pupae of JT, Chul 1 and Chul 6 varieties. The total of 20 and 33% of male and female pupae were infected with BmNPV respectively. For adult moths, BmNPV was detected only in female moths of JT and Chul 6 varieties and the total of 20% of female moth were infected (Table 8).

Samples from the local farmer rearing house were collected only in Kalasin province. They were Nang Noi variety of silkworm. The results showed that BmNPV was detected in egg (50%), larval (20% of 4^{th} instar and 20% of 5^{th} instar) and adult stage (40% of male and 25% of female) but not in the pupal stage (Table 8). It could be explained that the pupal samples collected from the farmer decayed rapidly prior to DNA extraction, hence DNA's quality may be not good enough for PCR amplification. Moreover, the samples used in this study were randomly collected, therefore some samples may not be naturally infected with BmNPV.

BmNPV were not found in the samples collected from Thai Silk Company. The hygienic standard for the whole rearing system of the private company is very high. Only officers were permitted to get access to the working area. Therefore, opportunity that the silkworm will get contaminate with any pathogen is unlikely. It is interesting to note that BmNPV was not detected in larval samples collected from contracted farmers of the Thai Silk Company while the virus was detected in larval sample collected from the rearing houses of the governmental sericultural stations. Eventhough, silkworm rearing in the sericultural stations were normally taken care very well, the viral disease still occurred. The important factor caused the incidence of grasserie disease may be due to the variety of silkworm. Aruga (1994) reported that in silkworm, the F1 hybrids are more resistant to grasserie disease than parental strains. The governmental silkworm

varieties used in this study were parental strains while the varieties raising by private companies and contracted farms were hybrid strains which may be more resistant to grasserie disease.

This study indicated that PCR method worked efficiently well for grasserie disease detection. Viral DNA could be detected in the samples, eventhough, the samples which were collected randomly showed no symptoms of grasserie disease.

In Thailand, grasserie disease of mulberry silkworm in Udon Thani, Nong Khai and Nong Bou Lam Phu provinces was determined by Kaewwises and Niyomvit (1995) using light microscope. The silkworm samples were collected from 73 local farmer rearing houses. Gasserie disease was found in 64 larval samples or 88% of the total observed samples.

Table 8 Source and variety of collected silkworm and number of infected silkworm as detected by PCR

Source	Silkworm variety	Number of BmNPV detected samples/ Number of observed samples									
		Egg[1/]	Larva[2/]					Pupa[2/]		Moth[2/]	
			1st	2nd	3rd	4th	5th	Male	Female	Male	Female
1. Governmental institute											
Nong Khai	UB1	0/5	0/5	0/5	0/5	1/5	1/5	0/5	1/5	2/5	3/5
Nong Khai	Nang noi Sri Sa Ket	0/5	1/5	2/5	0/5	0/5	0/5	0/5	0/5	2/5	0/5
Kanchanaburi	KSK	0/5	0/5	0/5	1/5	0/5	3/5	2/5	0/5	0/5	0/5
Total		0/15	1/15	2/15	1/15	1/15	4/15	2/15	1/15	4/15	3/15
%		**0**	**6.7**	**13.3**	**6.7**	**6.7**	**26.7**	**13.3**	**6.7**	**26.7**	**20**
2. Private Company											
Nakhon Ratchasima	TSC	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Total		0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
%		**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
3. Contracted farm											
Nakhon Ratchasima	JT	0/5	0/4	0/4	0/4	0/4	0/4	0/5	1/5	0/5	2/5
Phetchabun	Chul 1	N	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5
Phetchabun	Chul 6	0/5	0/5	0/5	0/5	0/5	0/5	3/5	3/5	0/5	1/5
Total		0/10	0/14	0/14	0/14	0/14	0/14	3/15	5/15	0/15	3/15
%		**0**	**0**	**0**	**0**	**0**	**0**	**20**	**33**	**0**	**20**

[1/] one sample = 20 eggs

[2/] one sample = individual larva, pupa or moth

N = sample was not available

Table 8 (cont'd)

Source	Silkworm variety	Number of BmNPV detected samples / Number of observed samples								
		Egg[1/]	Larva[2/]				Pupa[2/]		Moth[2/]	
			1st	2nd	3rd	4th	5th	Male	Female	Male	Female
4. Local farmer											
Kalasin	Nang noi	1/2	N	0/5	0/5	1/5	1/5	0/4	0/3	2/5	1/4
Total		1/2		0/5	0/5	1/5	1/5	0/4	0/3	2/5	1/4
%		**50**		**0**	**0**	**20**	**20**	**0**	**0**	**40**	**25**
Total of all sources		**1/32**	**1/34**	**2/39**	**1/39**	**2/39**	**5/39**	**5/39**	**6/38**	**6/40**	**7/39**
%		**3.1**	**2.9**	**5.1**	**2.6**	**5.1**	**12.8**	**12.8**	**15.8**	**15**	**17.9**

[1/] one sample = 20 eggs

[2/] one sample = individual larva, pupa or moth

N = sample was not available

Figure 30 PCR products of DNA extracted from naturally infected silkworm larvae

a) First instar larvae of Nang Noi Sri Sa Ket variety from Nong Khai province

b) Second instar larvae of Nang Noi Sri Sa Ket variety from Nong Khai province

c) Third instar larvae of KSK variety from Kanchanaburi province

d) Fourth instar larvae of Nang Noi variety from Kalasin province

e) Fifth instar larvae of Nang Noi variety from Kalasin province

M=DNA marker (100 bp+1.5kb)

P=positive control (pGEM®-T vector harboring polyhedrin gene)

N=negative control (no template)

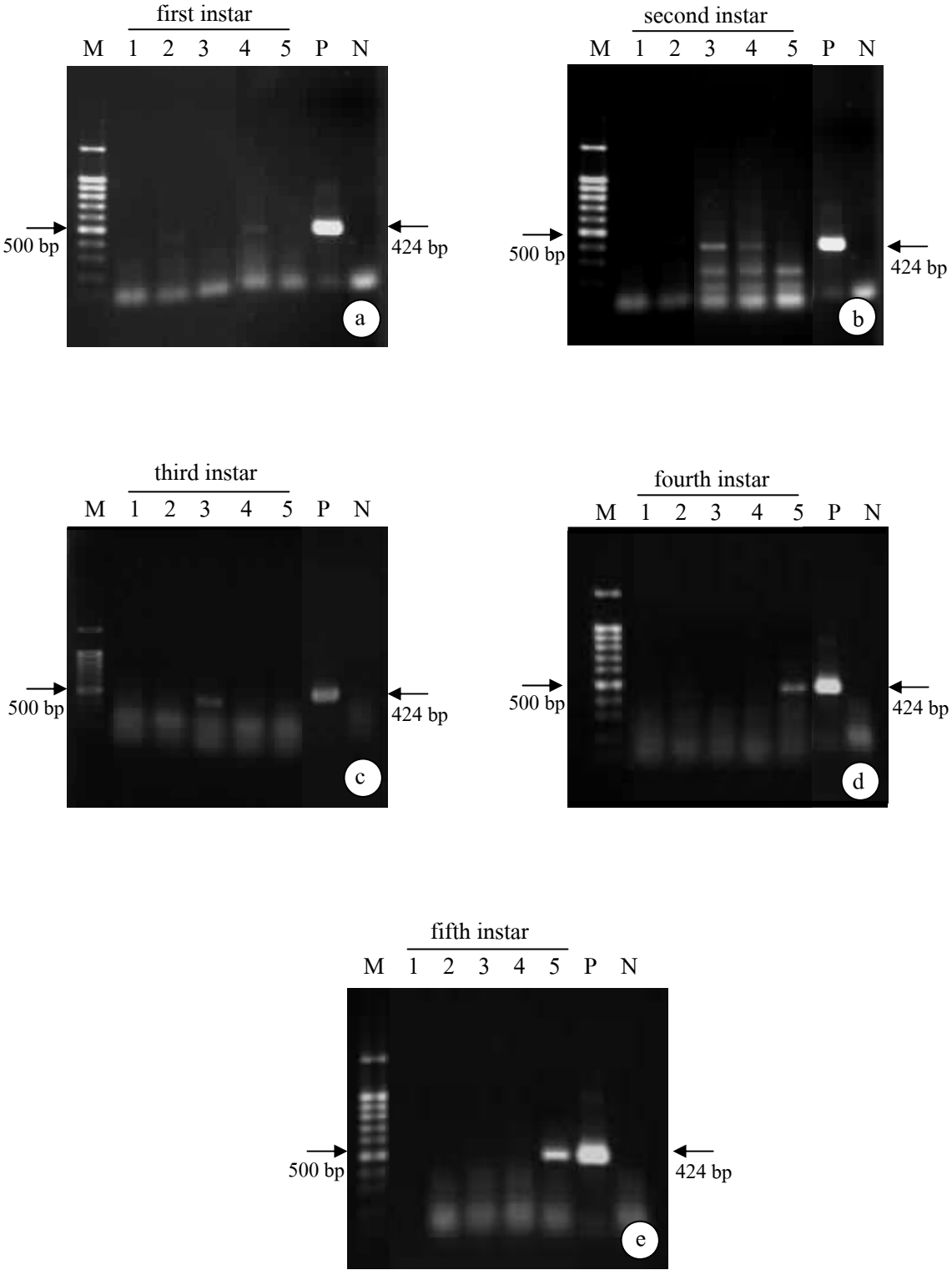

first instar
M 1 2 3 4 5 P N
500 bp
424 bp
a
second instar
M 1 2 3 4 5 P N
500 bp
424 bp
b
third instar
M 1 2 3 4 5 P N
500 bp
424 bp
c
fourth instar
M 1 2 3 4 5 P N
500 bp
424 bp
d
fifth instar
M 1 2 3 4 5 P N
500 bp
424 bp
e

Figure 31 PCR products of DNA extracted from naturally infected pupae and moths of Silkworm, *Bombyx mori*

a) Pupae of Chul 6 variety from Phetchabun province

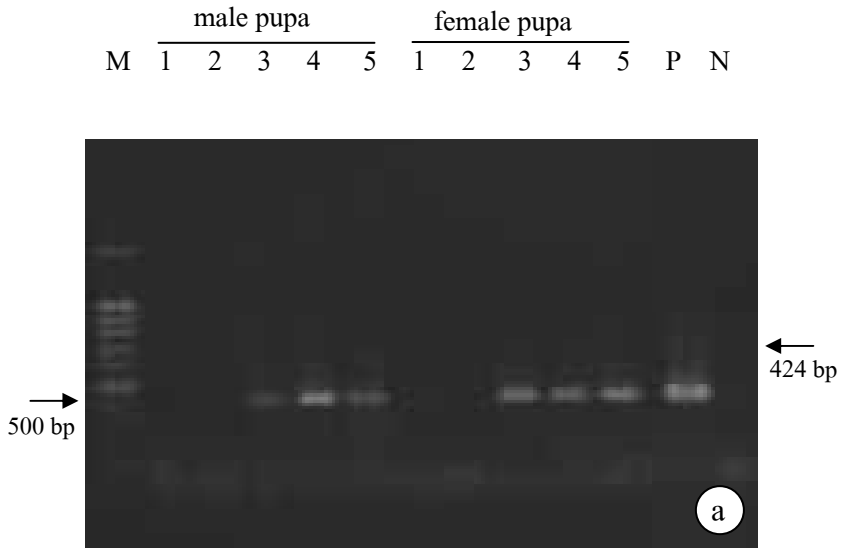
b) Moths of UB1 variety from Nong Khai province

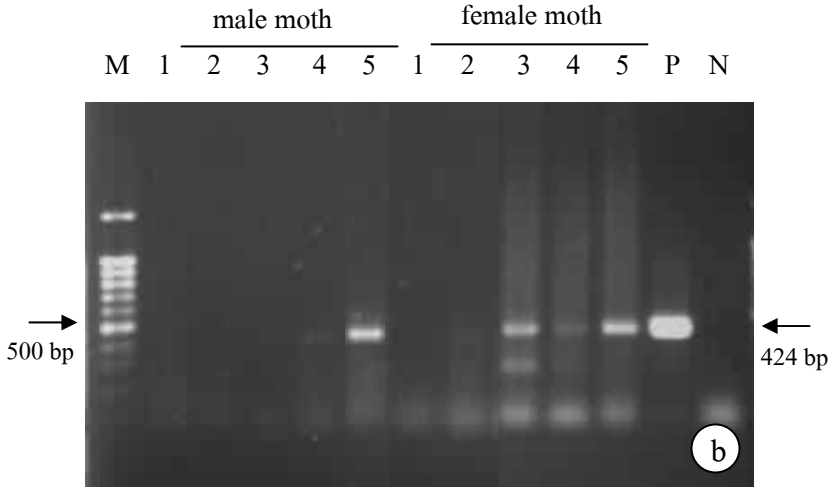
c) Male and female moths and their eggs of Nang Noi variety from Kalasin province

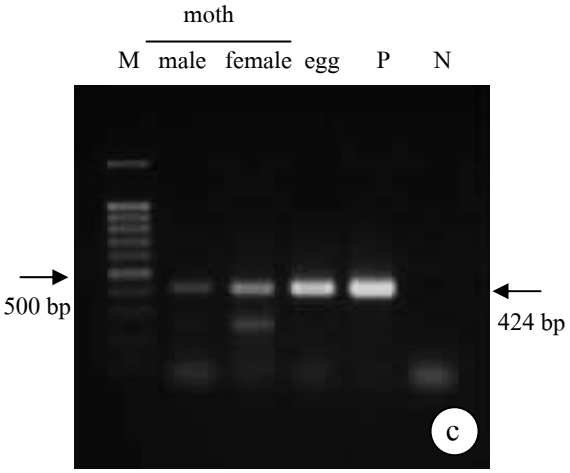
M=DNA marker (100 bp+1.5 kb)

P=positive control (pGEM® -T vector harboring polyhedrin gene)

N=negative control (no DNA template)


male pupa
female pupa
M 1 2 3 4 5 1 2 3 4 5 P N
500 bp
424 bp
a


male moth
female moth
M 1 2 3 4 5 1 2 3 4 5 P N
500 bp
424 bp
b


moth
M male female egg P N
500 bp
424 bp
c

5. BmNPV DNA extraction

There were many method for NPV DNA extraction (Iatrou *et al*., 1985; O'Reilly *et al*., 1992; Hunter-Fujita *et al*., 1998; Chaeychomsri, 2003). In this study, the BmNPV DNA extraction reported by Chaeychomsri (2003) (Method 1) was chosen and compared with alkaline lysis method described by Sambrook and Rusell (2001) (Method 2) and the modified alkaline lysis method (Method 3). The detail of steps and times of these three methods were presented in Table 9. Method 1 required 13 steps for DNA exraction which took more than 3 hrs 24 mins while Method 2 required 9 steps which took 47 mins. The modified alkaline lysis method, Method 3 composed of 7 steps and took 28 mins. This was clearly demonstrated that Method 3 used in this study required less steps and time for DNA extraction process.

Alkaline lysis method was used with little modification for NPV genomic DNA extraction for two reasons, 1) genome of NPV is closed-circular double stranded DNA that resemble plasmid which is usually extracted by alkaline lysis method. 2) In nature, the proteinaceous inclusion bodies or polyhedra of nucleopolyhedrovirus are dissolved readily by alkaline condition of mid gut juice of insect host, thus releasing the virus particles. In alkaline lysis method, the solution II contained SDS and NaOH which provided the solution of high pH. This resembles to the condition in insect gut which will facilitate the releasing of virus particle from the polyhedra. The important chemical used for BmNPV DNA extraction was SDS which is commonly used to extract polyhedra from infected cells and tissue. Lua *et al*. (2003) found that polyhedra treated with SDS had lost their polyhedron envelopes and virions were dislodged from the polyhedrin matrix, leaving empty spaces that were previously occupied by the occluded virions.

Modified alkaline lysis method is convenient and rapid as it takes only 28 mins to complete the whole process of DNA extraction. In previous procedure to obtain DNA as template for PCR amplification, the virus must be purified before DNA extraction which took at least 2-3 days. (Faktor and Raviv; 1996, Christian *et al.* 2001; Woo, 2001). However, with this modified method of alkaline lysis, step for polyhedra purification was omitted. Viral DNA could be

directly extracted from crude polyhedra suspension collected from diseased insects. In comparison, the modified alkaline lysis method presented in this study took less time (28 mins) than the Alkaline lysis method of Sambrook and Rusell (2001) (43 mins). Moreover, this method is very economical and safe as it utilized few common chemicals and did not take hazardous chemical such as phenol.

Table 9 Comparison of the three methods of DNA extraction *

Method 1		**Method 2**		**Method 3**	
Step	Time	Step	Time	Step	Time
1.polyhedral dissolution with alkaline solution	30 min	1. cell suspension + solution I incubate at room temperature	5 min	1. homogenate tissue + solution II	1 min
2. centrifugation	1 min	2. add solution II	1 min	2. add solution III	1 min
3. clear solution + SDS+Proteinase K incubate at 37 °C	at least 1 hr	3. add solution III incubate on ice	5 min	3. chloroform/ isoamy alcohol extraction	5 min
4. add RNase A incubate at 37 °C	1 min	4. centrifugation	10 min	4. isopropanol extraction	5 min
5. phenol extraction	5 min	5. chloroform/ isoamyl alcohol extraction	5 min	5. rinse pellet with 70% ethanol	5 min
6. phenol/chloroform/isoamyl alcohol extraction	5 min	6. isopropanol extraction	5 min	6. dry pellet	10 min
7. chloroform/isoamyl alcohol extraction	5 min	7. rinse pellet with 70% ethanol	10 min	7. resuspend DNA with TE	1 min
8. DNA precipitation	1 min	8. dry pellet	1 min		
9. keep at -20°C	at least 1 hr	9. resuspend DNA with TE	1 min		
10. centrifugation	20 min				
11. rinse pellet with 70% ethanol	5 min				
12. dry pellet	10 min				
13. resuspend DNA with TE	1 min				
Total	**more than 3 hr 24 min**		**47 min**		**28 min**

* Method 1 : reported by Chaeychomsri (2003)

Method 2 : reported by Sambrook and Rusell (2001)

Method 3 : modified from that of Sambrook and Rusell (2001)

There were reports on BmNPV detection in all stages of silkworm by indirect ELISA (Vanapruk *et al*., 1992) and DNA probe (Attathom *et al*., 1994). The reports demonstrated that ELISA could be used to detect BmNPV in all larval stages and pupa but not in moths and eggs. However, the digoxigenin labeled probes using dot blot hybridization gave positive detection of viral DNA in an infected eggs, larvae, pupae and adults moths. Some technique, such as colloidal textile dye-based dipstrick immunoassay (Nataraju *et al*., 1994), monoclonal antibody-based sandwich ELISA (Shamim *et al*., 1994), monoclonal antibody (Nagamine and Kobayashi, 1991), and western blot analysis (Chaeychomsri *et al*., 1995) were also used to detect BmNPV in silkworm larvae. Those methods consisted of many sequential steps which were complicate, time consuming and required skillful scientists to perform those techniques. In addition, some methods were difficult to interpret the result while PCR method provided clear result and was rapid, sensitive and reliable.

Recently, Ikonu *et al*. (2004) reported BmNPV detection in silkworm by PCR using primers specific to polyhedrin gene, PCR products were obtained from DNA template extracted from the whole body of all larval instars, silkworm haemolymph and infected eggs. However, there was no detection on samples of pupae and moths demonstrated in their report. Ikonu *et al*. (2004) extracted DNA from silkworm using alkaline buffer, SDS and proteinaseK and then further purified DNA using putification kit. Their method was considered costly due to the purification kit they used.

PCR-technique has proved to be an effective and reliable method for early detection of grasserie disease in silkworm. The detection can be made as early as in the egg stage. Moreover, the developed PCR detection process as described here can be complete within five hours. The process composed of DNA extraction for 30 mins, PCR amplification for 3 hrs and result evaluation by gel electrophoresis for 1 hr. In previous reports, the whole process of viral detection by PCR took about 2-3 days (Moraes and Maruniak, 1997 Wang *et al*., 2000; Woo, 2001). Time reduction was obviously at the DNA extraction process (Table 9). BmNPV detection by PCR, therefore, proved to be suitable for promotion as common practice for governmental institutions and private sectors involving in sericulture. Rapid detection of the

grasserie disease will, especially help rearing management of the governmental institutions in which officers could make decision to eliminate the infected colony and avoid providing the farmers with the infected egg batches or infected young larvae.

CONCLUSION

Restriction pattern and the polyhedrin gene (*polh*) of the Thai *Bombyx mori* nucleopolyhedrovirus (BmNPV) were studied. PCR-based method was developed for early detection of BmNPV in all stages of silkworm development.

Restriction pattern of Thai BmNPV was analyzed by digestion with *Bam*HI, *Bgl*II, *Hin*dIII, *Nco*I and *Pst*I and compared with BmNPV isolates from Japan (T3 and D1 isolates) and from India (N isolate). This study showed that the restriction pattern of Thai BmNPV was different from other isolates of BmNPV isolates and size of Thai BmNPV genome was smaller than other isolates.

The full length of the polyhedrin gene (*polh*) of Thai *Bombyx mori* nucleopolyhedrovirus (BmNPV) was cloned and sequenced. The nucleotides sequence of Thai BmNPV *polh* and its flanking region was submitted to GenBank (accession number AY779044). 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 which were submitted to GenBank (Japanese 1-T3 (L33180); Japanese 2 (M30925); Korean-K1 (U75359); Chinease (X63614) and Canadian (M100430). 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 *Bm*NPV *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.

To develop PCR-based method for BmNPV detection in silkworm, specific primers were designed from nucleotide sequence of Thai BmNPV *polh.* *Bm*NPV DNA extraction was modified from the alkaline lysis method. PCR-based method was first evaluated for its potential for grasserie disease detection using samples of silkworm which were artifiacially inoculated with

BmNPV. It was found that by PCR method, BmNPV could be detected in all stages of silkworm. The method was sensitive enough to detect BmNPV in only one infected individual egg. In larval stage, BmNPV was detected in all instars and only one individual infected larva provided adequate BmNPV DNA to be used as PCR template. In pupal and adult stages, BmNPV was detected in both infected male and female.

Application of PCR-based method for grasserie disease detection in silkworm was demonstrated. Naturally infected samples were collected from silkworm rearing houses of the governmental sericultural stations, private company, contracted farms and local farmer. BmNPV could be detected in all stages of silkworm. The whole process of BmNPV genomic extraction and detection could be done within 5 hours. PCR technique has proved to be an effective and reliable method for early detection of grasserie disease of silkworm and can be routinely used to monitor and protect the spread of the disease in sericulture industry in Thailand.

LITERATURE CITED

Acharya, A. and K. P. Gopinathan. 2001. Identification of an enhancer-like element in the polyhedrin gene upstream region of *Bombyx mori* nucleopolyhedrovirus. **J. Gen. Virol.** 82: 2811-2819.

Acharya, A., S. Sriram, S. Sehrawat, M. Rahman, D. Sehgal and K. P. Gopinathan. 2002. *Bombyx mori* nucleopolyhedrovirus: Molecular biology and biotechnological applications for large-scale synthesis of recombinant proteins. **Curr. Sci.** 28: 455-465.

Aruga, H. 1994. **Principles of sericulture**. Balkema/Rotterdam, India.

Attathom, T. 1978. **A comparative Study of Six Baculovirus Isolates from Larvae of the Family Noctuidae (Insecta: Lepidoptera)**. Ph.D. Dissertation, University of California Riverside, California.

Attathom, T., S. Attathom, S. Kumpratueang and M. Audtho. 1994. Early detection of grassie disease of silkworm, *Bombyx mori* by DNA probe. pp. 257-271. *In* **Proceeding of 32nd Kasetsart University Anual Conference: Plant Science**, Kasetsart University, Bangkok, Thailand.

Attathom, T., S. Chaeychomsri, S. Chaichuchot, S. Attathom and P. Chiemsobat. 1988. Characterization of the nuclear polyhedrosis virus of the cotton bollworm, *Helothis armigera.* **Kasetsart J. (Nat. Sci. Suppl.)** 22: 14-23.

Attathom, T. and N. Sinchaisri. 1987. Nuclear polyhedrosis virus isolate from *Bombyx mori* in Thailand. **Sericologia** 37: 287-295.

Ayres, M. D., S. C. Howard, J. Kuzio, M. Lopez-Ferber and R. D. Possee. 1994. The compleate DNA sequence of *Autographa californica* nuclear polyhedrosis virus. **Virology** 202: 586-605.

Benson, D. A., I. Karsch-Mizrachi, D. L. Lipman, J. Ostell, B. A. Rapp and D. L. Wheeler. 2000. GenBank. **Nucleic Acids Res.** 20: 15-18.

Bilimoria, S. L. 1986. Taxonomy and identification of baculoviruses. Pages 37-60 in R. R. Granados and B. A. Ferderici, eds. **The Biology of Baculoviruses Volume I Biological Properties and Molecular Biology.** CRE Press, Inc., Florida.

Blissard, G. W. N., and G. F. Rohrmann. 1990. Baculovirus diversity and molecular biology. **Annu. Rev. Entomol.** 35: 127-155.

Boucias, D. G. and J. C. Pendland. 1998. **Principles of Insect Pathology**. Kluwer Academic, Massachusetts.

Bulach, D. M., C. A. Kuman, A. Zaia, B. Liang and D. E. Tribe. 1999. GroupII nucleopolyhedrovirus subgroups revealed by phylogenetic analysis of polyhedrin and DNA polymerase gene sequences. **J. Invertebr. Pathol.** 73: 59-73.

Burand, J. P., H. M. Horton, S. Retnasani and J. S. Elkinton. 1992. The use of polymerase chain reaction and shortwave UV irradiation to detect baculovirus DNA on the surface of gypsy moth eggs. **J. Virol. Methods** 36: 141-150.

Carstens, E. B., G. V. Williams, P. Faulkner and S. Partington. 1992. Analysis of polyhedra morphology mutants of *Autographa californica* nuclear polyhedrosis virus: molecular and ultrastructural features. **J. Gen. Virol.** 73: 1471-1479.

Chaeychomsri, S. 2003. Purification of virus and extraction of viral DNA. pp.1-8. *In* **A Laboratory Mannual of a Regional Workshop on Molecular Biology and Applications of baculovirus.** Central Laboratory and Greenhouse Complex Kasetsart University, Nakhon Pathom, Thailand.

Chaeychomsri, S., M. Ikeda and M. Kobayashi. 1995. Expression of viral DNA polymerase gene in larvae of silkworm, *Bombyx mori* (Lepidoptera: Bombycidae), during infection with nuclear polyhedrosis virus. **Appl. Entomol. Zool.** 30: 451-465.

Chang, P. S., C. F. Lo, G. H. Kou, C. C. Lu and S. N. Chen. 1993. Purification and amplification of DNA from *Penaeus monodon* type baculovirus (MBV). **J. Invertebr. Pathol.** 62: 116-120.

Chen, X., W. F. IJkel, R. Tarchini, X. Sun, H. Sandbrink, H. Wang, S. Peters, D. Zuidema, R. K. Lankhorst, J. M. Vlak and Z. Hu. 2001. The sequence of the *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus genome. **J. Gen. Virol.** 82: 241-257.

Cheng, X. W., G. R. Carner and H. W. Fescemyer. 1998. Polyhedrin sequence determines the tetrahedral shape of occlusion bodies in *Thysanoplusia orichalcea* single-nucleopolyhedrovirus. **J. Gen. Virol.** 79: 2549-2556.

Chou, C., C. Huang, C. Lo, G. Kou and C. Wang. 1996. Characterization of *Perida nuda* nucleopolyhedrovirus (*Penu*NPV) polyhedrin gene. **J. Invertebr. Pathol.** 67: 259-266.

Christian, P. D., N. Gibb, A. B. Kasprzak and A. Richards. 2001. A rapid method for the identification and differentiation of *Helicoverpa* nucleopolyhedrovirus (NPV *Baculoviridae*) isolated from the environment. **J. Virol. Methods** 96: 51-65.

Cowan, P., D. Bulach, K. Goodge, A. Robertson and D. E. Tribe. 1994. Nucleotide sequence of the polyhedrin gene region of *Helicoverpa zea* single nucleocapsid nuclear polyhedrosis virus: placement of the virus in lepidopteran nuclear polyhedrosis virus GroupII. **J. Gen. Virol.** 75: 3211-3218.

Department of Agriculture. 1980. **Mulbery - Silkworm**. Ministry of Agriculture and Coperatives, Bangkok, Thailand.

England, L. S., J. T. Trevors and S. B. Holmes. 2001. Extraction and detection of baculoviral DNA from lake water, detritus and forest litter. **J. Appl. Microbiol.** 90: 630-636.

Euchi, R.,W. Hara, A. Simazaki, K. Hirota, M. Ichiba, O. Ninagi and K. Nagayasu. 1998. Breeding of the silkworm race "Taisei" nonsusceptible to a densonucleosis virus type-1. **J. Seric. Sci. Jpn.** 67: 361-366.

Faktor, O. and D. Raviv. 1996. A polymerase chain reaction for the detection of nucleopolyhedroviruses in infected insects: the fate of the *Spodoptera litoralis* virus in *Locusta migratoria*. **J. Virol. Methods** 61: 95-101.

Franski, R., I. B. Fauquet and F. Brown. 1991. **Classification and nomenclature of viruses. Fifth report of the international committee on virus.** Fifth Report on the International Committee on Viruses. Arch. Virol. 2 (Suppl.).

Gomi, S., K. Majima and S. Maeda. 1999. Sequence analysis of genome of *Bombyx mori* nucleopolyhedrovirus. **J. Gen. Virol.** 80: 1323-1337.

Harrison, R. L., and B. C. Bonning. 2003. Comparative analysis of the genomes of *Rachiplusia ou* and *Autographa californica* multiple nucleopolyhedrovirus. **J. Gen. Virol.** 84: 1827-1842.

Hashimoto, Y., Y. Kanamori, T. HayaKawa, Y. Katayama, S. G. Kamita, S. Maeda and T. Matsumoto. 1994. Physical mapping of *Bombyx mori* nuclear polyhedrosis virus strain D1: identification novel interspersed homologous regions. **Appl. Entomol. Zool.** 29: 442-448.

Hatakeyama, Y. and S. Hayasaka. 2003. A new method of pebrine inspection of silkworm egg using multiprimer PCR. **J. Invertebr. Pathol.** 82: 148-151.

Herniou, E. A., J. A. Olszewski, J. S. Cory and D. R. O'Reilly. 2004. The genome sequence and evolution of baculovirus. **Annu. Rev. Entomol.** 48: 211-234.

Hong, H. K., S. D. Woo, J. Y. Choi, H. K. Lee, M. H. Kim, Y. H. Je and S. K. Kang. 2000. Characterization of four isolates of *Bombyx mori* nucleopolyhedrovirus. **Arch. Virol.** 145: 2351-2361.

Hunter-Fujita, F. R., P. E. Entwistle, H. F. Evans and N. E. Crook. 1998. **Insect Viruses and Pest Management**. Willey & Sons, Chichester.

Iatrou, K., K. Ito and H. Witkiewicz. 1985. Polyhedrin gene of *Bombyx mori* nuclear polyhedrosis virus. **J. Virol.** 54: 436-445.

IJkel, W. F., E. A. Strien, J. G. Heldens, R. Broer, D. Zuidema, R. W. Goldbach and J. M. Vlak. 1999. Sequence and organization of the *Spodoptera exigua* multicapsid nucleopolyhedrovirus genome. **J. Gen. Virol.** 80: 3289-3304.

Je, Y. H., J. H. Chang, M. H. Kim, J. Y. Roh, B. R. Jin and D. R. O'Reilly. 2001. The use of defective *Bombyx mori* nucleopolyhedrovirus genomes maintained in *Escherichia coli* for the rapid generation of occlusion-positive and occlusion-negative expression vectors. **Biotechnol. Lett.** 23: 1809-1817.

Jehle, J. A. 2004. The mosaic structure of the polyhedrin gene of the *Autographa californica* nucleopolyhedrovirus (*Ac*NPV). **Virus Genes** 29: 5-8.

Johansen, H. S., H. F. Evans, and N. E. Crook. 1986. Infection of silkworm follicular cell with nuclear polyhedrosis virus. **J. Invertebr. Pathol.** 48: 74-78.

Kaewwises, M. 2000. Silkworm disease and their control. pp.1-4. *In* K. Tsubouchi, V. Raksang and P. Chaosattakul (eds). **Proceedings of Workshop on Polyvoltine Sericulture and Postharvest Technique**. Nakhon Ratchasima Sericulture Research Center.

Kaewwises, M. and L. Niyomvit. 1995. Servey of grassery of silkworm in Udon Thani province. pp. 66-75. *In* **Annual Report of Silkworm Research**. Udon Thani Sericultural Research Center.

Kaewwises, M. and K. Sananvong. 1999. Comparison of NPV-resistance variety of multivoltine-bivoltine hybrid silkworm. pp. 56-65. *In* **Annual Report of Silkworm Research**. Udon Thani Sericultural Research Center.

Kalsi, G., M. Shamim, B. Nataraju, R. K. Datta and S. K. Gupta. 1998. Monoclonal antibodies based rapid immunoassay for the detection of nuclear polyhedrosis virus infection in *Bombyx mori* L. **Sericologia** 38: 401-405.

Kawakami, Y., H. Iwano, Y. Hatakeyama, T. Inoue, E. U. Canning and R. Ishihara. 2001. Use of PCR with the specific primers for discrimination of *Nosema bombycis*. **J. Seric. Sci. Jpn.** 70: 43-48.

Khosaka, T., M. Himno and K. Onerder. 1971. Separation and structure of component of nuclear polyhedrosis virus of the silkworm. **J. Virol.** 2: 267-273.

Khurad, A. M., A. Mahulikar, M. K. Rathod, M. M. Rai, S. Kanginakudra and J. Nagaraju. 2004. Vertical transmission of nucleopolyhedrovirus in the silkworm, *Bombyx mori* L. **J. Invertebr. Pathol.** 27: 8-15.

Kremky, T. and E. Michalska. 1988. Resistance of some mulbery silkworm lines to polyhedrosis. **Sericologia** 28: 61-70.

Krywienczyk, J. 1963. Demonstration of nuclear polyhedrosis in *Bombyx mori* (Linaeus) by fluorescent antibody technique. **J. Invertebr. Pathol.** 5: 309-317.

Lange, M., H. Wang, H. Zhihong, and J. A. Jehle. 2004. Toward a molecular identification and classification system of lepidopteran-specific baculovirus. **Virology** 325: 36-47.

Lavina-Caoili B.A., K. Kamiya, S. Kawamura, M. Ikeda and M. Kobayashi. 2001. Comparative *in vitro* analysis of geogrphic variants of nucleopolyhedrovirus of *Spodoptera litura* Isolated from China and the Philippines. **J. Insect Biotechnol. Sericol.** 70: 199-209.

Leisy, D., G. Rohrmann and G. Beaudreau. 1986. The nucleotide sequence of the polyhedrin gene region from the multicapsid baculovirus of *Orgyia pseudotsugata*. **Virology** 153: 280-288.

Levesque, C. A. 2001. Molecular methods for detection of plant pathogens-What is the future? **Can. J. Plant Pathol.** 24: 333-336.

Lin, G. Y., J. Zhong and X. Z. Wang. 2000. Abnormal formation of polyhedra resulting from a single mutation in the polyhedrin gene of *Autographa californica* multicapsid nuclepolyhedrovirus. **J. Invertebr. Pathol.** 76: 13-19.

Liu, S. X. and W. B. Zhong. 1989. The research channels in the prevention and control of silkworm disease. **Sericologia** 29: 287-295.

Lo, H. R. and Y. C. Chao. 2004. Rapid titer determination of baculovirus by quantitative real-time polymerase chain reaction. **Biotechnol. Prog.** 20: 354-360.

Lua, L. H. L, L. K. Nielsen and S. Reid. 2003. Sensitivity of *Helicoverpa armigera* nucleopolyhedrovirus polyhedra to sodium dodecyl sulfate. **Biological Control** 26: 57-67.

Maeda, S., T. Kawai, M. Obinata, H. Horiuchi, Y. Saeki, Y. Sato and M. Furusawa. 1985. Production of human alpha-interfteron in silkworm using a baculovirus vector. **Nature** 315: 592-594.

Maeda, S. and K. Majima. 1990. Molecular cloning and physical mapping of the genome of *Bombyx mori* nuclear polyhedrosis virus. **J. Gen. Virol.** 71: 1851-1855.

Ministry of Agriculture and Cooperatives. 2002. **Queen of Silk**. Ministry of Agriculture and Cooperatives.

Moraes, R. R. and J. E. Maruniak. 1997. Detection and identification of multiple baculovirus using the polymerase chain reaction (PCR) and restriction endonuclease analysis. **J. Virol. Methods** 63: 209-217.

Moraes, R. R., J. E. Maruniak and J. E. Funderburk. 1999. Methods for detection of *Anticasia gemmatalis* nucleopolyhedrovirus DNA in soil. **Appl. Environ. Microbiol.** 65: 2307-2311.

Myers, J. H., R. Malakar and J. Cory. 2000. Sublethal nucleopolyhedrovirus infection effects on female pupal weight, egg mass size, and vertical transmission in gypsy moth (Lepidoptera:Lymantriidae). **Environ. Entomol.** 29: 1268-1272.

Nagamine, T. and M. Kobayashi. 1991. Preparation and characterization of monoclonal antibodies against occluded virions of *Bombyx mori* nuclear polyhedrosis virus. **J. Invertebr. Pathol.** 57: 311-324.

Nataraju, B., V. Sivaprasad, R. K. Datta, S. K. Gupta and M. Shamim. 1994. Colloidal textile dye-based dipstick immunoassay for the detection of nuclear polyhedrosis virus (BmNPV) of silkworm, *Bombyx mori* L. **J. Invertebr. Pathol.** 63: 135-139.

Noppaseney, P. 2002. New-sex-linked multivoltine x bivoltine strains. pp. 116-121. *In* **XIXth Congress of The International Sericultural Commission Proceedings**, Queen Sirikit National Convention Center, Bangkok, Thailand.

O'Reilly, D. R., L. K. Miller and V. A. Luckow. 1992. **Baculovirus Expression Vectors: Laboratory Manual**. W. H. Freeman and Company, New York.

O'Reilly, D. R., R. S. Hails and T. J. Kelly. 1998. The impact of host developmental status on baculovirus replication. **J. Invertebr. Pathol.** 72: 269-275.

Otta, S. K., I. Karunasagar and I. Karunsagar. 2003. Detection of monodon baculovirus and whitespot syndrome virus in apparently healthy *Penaeus monodon postlarvae* from India by polymerase chain raction. **Aquacuture.** 220: 59-67.

Palhan, V. B. and K. P. Gopinathan. 2000. The *p*10 gene of *Bombyx mori* nucleopolyhedrosis virus encodes a 7.5-kDa protein and is hypertranscribed from a TAAG motif. **J. of Gent.** 79: 33-40.

Pallavi, S. N. and C. K. Kamble. 1997. Disinfection and hygiene in sericulture-a review. **Sericologia** 37: 401-415.

Peng, S. E., C. F. Lo, C. H. Ho, C. F. Chang and G. H. Kou. 1998. Detection of white spot baculovirus (WSBV) in giant freshwater prawn, *Macrobrachium rosenbergii*, using polymerase chain reaction. **Aquaculture** 164: 253-263.

Pang, Y., J. Yu, L. Wang, X. Hu, W. Bao, G. Li, C. Chen, H. Han, S. Hu, and H. Yang. 2001. Sequence analysis of the *Spodoptera litura* multicapsid nucleopolyhedrovirus genome. **Virology** 287: 391-404.

Patil, C. S. and S. B. Dandin. 1990. Review of literature on using nuclear polyhedrosis virus (baculovirus) of silkworm, *Bombyx mori*., as a vector for foreign gene expression. **Sericologia** 30: 269-275.

Rohrmann, G. F. 1986. Polyhedrin structure. **J. Gen. Virol.** 67: 1499-1513.

_____. 1992. Baculovirus structure proteins. **J. Gen. Virol.** 73: 749-761.

Rosinski, M., S. Reid and L. K. Nielsen. 2002. Kinetics of baculovirus replication and release using real-time quantitative polymerase chain reaction. **Biotechnol. Bioeng.** 77.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenic trees. **Mol. Bol. Evol.** 4: 406-425.

Sambrook, J. and D. W. Russell. 2001. **Molecular Cloning: a laboratory manual.** Cold Spring Habor, New York.

Sen, R., M. M. Ahsan and R. K. Datta. 1999. Induction of resistance to *Bombyx mori* nuclear polyhedrosis virus, into a susceptible bivoltine silkworm breed. **Indian J. Seric.** 38: 107-112.

Shamim, M., M. Baig, R. K. Datta and S. K. Gupta. 1994. Development of monoclonal antibody-based sandwich ELISA for the detection of nuclear polyhedrosis virus infection in *Bombyx mori* L. **J. Invertebr. Pathol.** 63: 151-156.

Shi, L. and W. Jin. 1997. Study on the differential diagnosis of *Nosema bombysis* of the silkworm, Bombyx mori, by monoclonal antibody-sensitized latex. **Sericologia** 37: 1-6.

Sikkhamondhol, B. and S. Tengratanaprasert. 1993. **The Situation on Sericulture in Thailand**. Department of Agriculture.

Singh, K. K., S. S. Ingalhalli, R. D. Sanakal, S. B. Hinchigeri, S. Basavarajappa and C. J. Savanurmath. 1996. Characterization of geographical isolate nuclear polyhedrosis virus of the silkworm, Bombyx mori, (L). **Sericologia** 36: 633-639.

Sithisongkram, T. and S. Ruksong. 1987. Study on method and period to denature grasserry virus rod. pp. 225-231. *In* **Annual Report of Silkworm Research**. Nakhon Ratchasima Sericultural Research Center.

Smith, G. E. and M. D. Summers. 1978. Analysis of baculovirus genomes with restriction endonucleases. **Virology** 89: 517-527.

Sriram, S. and K. P. Gopinathan. 1998. The potential role of a late gene expression factor, Lef2, from *Bombyx mori* nuclear polyhedrosis virus in very late gene transcription and DNA replication. **Virology** 251: 108-122.

Sukkhumsirichart, W., W. Kiatpathomchai, C. Wongteerasupaya, B. Withyachumnarnkul, T. W. Flegel, V. Boonseang and S. Panyim. 2002. Detection of hepatopancreatic parvovirus (HPV) infection in Penaeus monodon using PCR-ELISA. **Mol. Cell. Probes** 16: 409-413.

Sungkompitak, V. 1989. **New Technique of Sericultural Business**. Vacharin Printing, Bangkok.

Tanada, Y. and H. K. Kaya. 1993. **Insect Pathology**. Academic Press, Inc, California.

Tazima, Y. 1962. **The Genetics of the Silkworm**. Logos Press Academic Press.

_____. 1978. **The Silkworm, an Important Laboratory Tool**. Kodansha Ltd., Tokyo.

United Nation. 1990. **Handbook on Pest and Disease Control of Mulberry and Silkworm**. United Nation.

_____. 1998. **Silkworm Rearing, Disease and Their Control**. United Nation.

Umlauft, F., D. T. Wong, P. J. Oefner, P. A. Underhill, R. C. Cheung, T. L. Wright, A. A. Kolykhalov, K. Gruenewald and H. B. Greenberg. 1996. Hepatitis C virus detection by single-round PCR specific for the terminal 3' noncoding region. **J. Clin. Microbiol.** 34: 2552-2558.

Uzigawa, K. and H. Aruga. 1966. On the selection of resistance strains of the infectious virus in the silkworm, *Bombyx mori* L. **J. Seric. Sci. Jpn.** 35:23-26.

van Regenmortel, M. H. V. , C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle and R. B. Wickner. 2000. **Virus Taxonomy: Classification and Nomenclature of Viruses: Seventh Report of the International Committee on Taxonomy of Viruses**. Academic Press, San Diego, California.

Vanapruk, P., T. Attathom, K. Sanbatsiri and S. Attathom. 1992. Comparison of methods for the detection of nuclear polyhedrosis virus in silkworm, *Bombyx mori* Linn. pp 237-243. *In* **Proceeding of 30th Kasetsart University Anual Conference: Plant Science**, Kasetsart University, Bangkok, Thailand.

Veda, K., I. Nagai and M. Horikomi. 1997. **Silkworm Rearing**. Science publishers. Inc., New Hampshire.

Vlak, J. M. and G. F. Rohrmann. 1985. The nature of polyhedrin. pp. 489-542 *In* K. Maramorosch and K. E. Sherman, eds. **Viral Insecticides for Biological Control**. Academic Press, Florida.

Wang, C. H., H. N. Yang, H. C. Liu, G. H. Kou and C. F. Lo. 2000. Nested polymerase chain reaction and in situ hybridization for detection of nucleopolyhedrosis. **J. Virol. Methods** 84: 65-75.

Watanabe, H. 1986. Resistace of the silkworm, *Bombyx mori*, to viral infections. **Agric. Ecosyst. Environ.** 15: 131-139.

Woo, S. D. 2001. Rapid detection of multiple nucleopolyhedroviruses using polymerase chain reaction. **Mol. Cells.** 11: 334-340.

Zanotto, P. M. A., B. D. Kessing and J. E. Maruniak. 1993. Phylogenetic interrelationships among baculoviruses: Evulutionary rates and host associations. **J. Invertebr. Pathol.** 62: 147-164.