Restriction Endonuclease Pattern of Thai *Bombyx mori* L. Nucleopolyhedrovirus

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ABSTRACT

Bombyx mori nucleopolyhedrovirus (BmNPV) is a causative agent of grasserie which is the most destructive disease of silkworm (*Bombyx mori.* L.) Restriction profile of Thai BmNPV genomic DNA was studied by cleaving the DNA with *Bam*HI, *Bgl*II, *Hin*dIII, *Nco*I, *Pst*I, and the minimize of 6, 11, 19, 11 and 17 different DNA fragments were produced, respectively. Molecular size of the fragments was estimated by summation of the size of DNA fragments generated by restriction enzymes digestion as viewed on the electrophoresed gel. The Thai BmNPV genome was estimated to be in the range of 92.3-125.8 kb with the average of 110.6 kb. In comparison with genome of BmNPV T3 and D1 isolates from Japan, and N isolate from India, the Thai BmNPV genome is the smallest in size.

Key words: restriction pattern, nucleopolyhedrovirus, *Bombyx mori*, silkworm

Introduction

Bombyx mori nucleopolyhedrovirus (BmNPV) is a causative agent of grasserie disease of silkworm (*Bombyx mori* L.), the most destructive disease in Thai sericulture. The infected silkworm shows disease symptom during the final stage of larval development and dies without cocoon production resulting in the waste of expenditure, time and labour work. BmNPV belongs to genus Nucleopolyhedrovirus of the Baculoviridae family of insect viruses

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(van Regenmortel *et al.*, 2000). The family Baculoviridae is classified by the basic characteristic as an enveloped, rod-shaped virion containing a close circular double-stranded DNA genome (Bilimoria, 1986). Baculoviruses have been found in over 600 species of host arthropods, the majority are insects witHin the Order Lepidoptera (Hong *et al.*, 2000). They have also been isolated from the insects in Orders Diptera, Hymenoptera, Coleoptera, Neuroptera, Thysanura and Trichoptera and the crustacean in Order Decapoda (shrimp) (Hong *et al.*, 2000). 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). NPVs produce a large number of inclusion bodies called polyhedra during an epizootic stage which persist over seasons in the environment and therefore serve as a reservoir of inoculum to infect subsequent generation of host insects (Boucias and Pendland, 1998).

Polyhedral inclusion bodies and virions of BmNPV occured in different shapes and sizes among geographical isolates (Khosaka *et al.*, 1971). Extensive studies had revealed genotypic variation in field isolated of many types of baculovirus (Lee and Miller, 1978; Smith and Summers, 1981). The restriction endonuclease analysis of baculovirus DNA had been used to distinguish and characterize closely related genotypic variants and species of the virus (Smith and Summers, 1978). Isolates of BmNPV had been discovered in many countries and a number of research studies on restriction endonuclease pattern of BmNPV had been reported (Maeda and Majima, 1990; Singh *et al.*, 1996). However, the restriction endonuclease pattern of Thai BmNPV has not been investigated. This work aims to study the Thai BmNPV genomic DNA based on its restriction endonuclease pattern and compare with those of the other BmNPVs. Information on restriction endonuclease analysis or DNA fingerprint is useful for distinguisHing genotypic variants of BmNPVs and constructing the recombinant baculovirus for further beneficiary use.

Materials and Methods

Propagation of BmNPV

Fourth instar larvae of *B. mori* were inoculated with BmNPV. The virus was isolated from diseased larvae collected from

Udon Thani Sericultural Research Centre, Department of Agriculture, Thailand. The larvae were fed with BmNPV-contaminated mulberry leaves. Heavily infected larvae were collected in amber glass bottles containing distilled water. The infected larvae were allowed to putrefy and release the virus inclusion bodies or polyhedra which precipitated at the bottom of the container leaving most of the insect remains in the supernatant.

Purification of BmNPV

The polyhedra of BmNPV was purified following the method described by Attathom *et al.* (1988). In brief, the precipitate containing BmNPV polyhedra was filtered through four layers of cheesecloth, pelleted by centrifugation at 8,000 rpm for 15 min at 4 °C and washed with distilled water several times until the supernatant became clear. The polyhedra pellet was resuspended in distilled water and further purified using sucrose density gradient centrifugation (40-65% w/w) at 25,000 rpm for 1.5 hr at 4 °C. The polyhedral band was collected and washed afterward by centrifugation at 8,000 rpm for 15 min at 4 °C in distilled water to ensure removal of sucrose. The final pellet of purified polyhedra was resuspended in distilled water and kept at -20 °C for further uses.

Extraction of whole genomic BmNPV DNA

DNA extraction following the method of Chaeychomsri (2003) was performed. In brief, virions were released from the polyhedral inclusion bodies by alkaline dissolution (0.2 M Na_2CO_3, 0.5 M EDTA, 0.34 M NaCl). The released virus particles were treated with proteinase K and SDS overnight at 37 °C. 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. After centrifugation, the DNA pellet was washed with 70% ethanol, vacuum dried, resuspended in 50 µl of TE buffer (10 mM Tris-HCl, pH 8-1 mM EDTA), and stored at 4 °C.

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 hrs. DNA fragments were fractionated in 0.6% SeaKem GTG agarose in 1X TBE 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.

Results and Discussion

Extraction of whole genomic BmNPV DNA

Using the DNA restriction method described by Chaeychomsri (2003), provided a prominent DNA band that was a single clear band with no interference of RNA. The extracted BmNPV DNA was in high quantity and quality and can be used to demonstrate the differences of the restriction patterns when digestions with different enzymes were made.

Many methods had been described for harvesting viruses from the infected insect body. BmNPV could be collected from the insect haemolymph by cutting prolegs before filtering through layer of gauze (Singh *et al.*, 1996) or 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 simple and convenient for practicing as compared with other methods. Viral DNA extraction described in this study was quite similar to previously reported methods (Maeda and Majima, 1990; Hashimoto *et al.*, 1994; Singh *et al.*, 1996) in which the main chemicals used were alkaline solution for solubilizing the polyhedral inclusion bodies and proteinase K and SDS for releasing viral DNA. The methods of BmNPV DNA extraction used by various researchers were different, virtually in some minor steps. For example, there was a 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 the others.

Restriction pattern of Thai BmNPV

When the BmNPV DNA was cut with *Bam*HI, *Bgl*II, *Hin*dIII, *Nco*I and *Pst*I, about 6, 11, 19, 11 and 17 DNA bands were observed on the gel respectively (Figure 1). 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 (Table 1). The biggest bands were

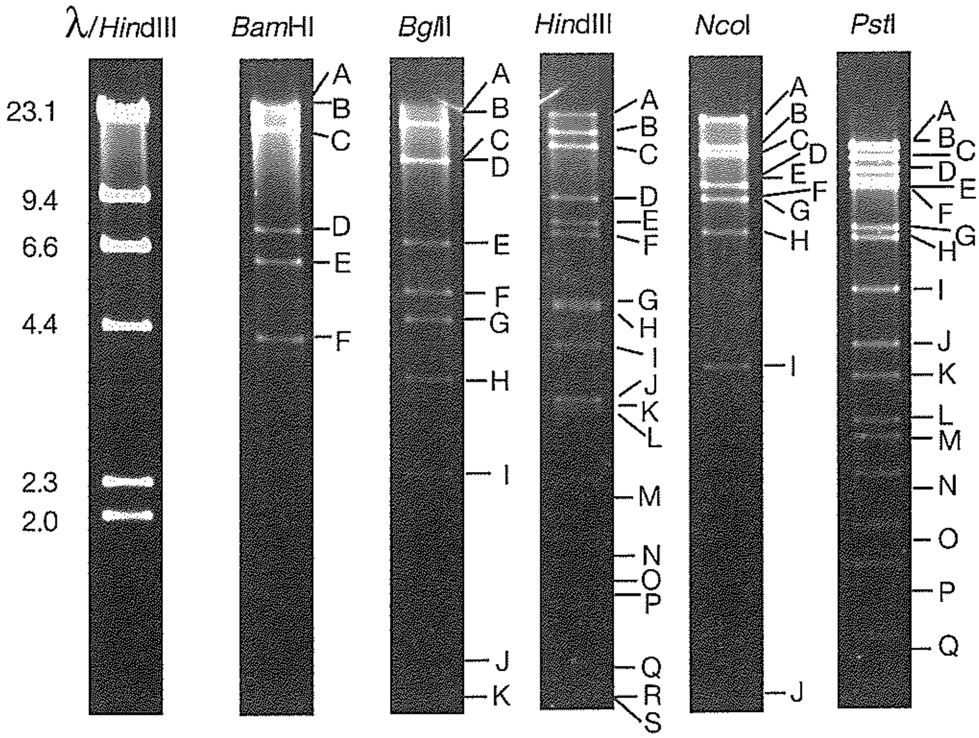

Figure 1. Restriction cleavage patterns of Thai *Bombyx mori* nucleopolyhedrovirus (BmNPV) DNA. The viral DNA was digested with *Bam*HI, *Bgl*II, *Hin*dIII, *Nco*I and *Pst*I and the cleavaged fragments were separated on 0.6% SeaKem GTG agarose gel. Lane marked λ/*Hin*dIII showed molecular size marker pattern in kb. Each visible fragment was assigned a letter as shown.

26.0 kb of fragment A and B when digested with *Bam*HI. The smallest band that could observe was 0.3 kb of fragment S when 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 (Table 1). Genome size estimation based on the restriction fragments appeared on the gel was in the range of 92.3-125.8 kb with the average of 110.6 kb. 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 band were considered as two fragments of approximately the same size.

Restriction patterns of genome of different strains of BmNPV had been previously analyzed and genome size was estimated by summing the size of the fragments generated by different restriction

Table 1. Restriction endonuclease cleavaged fragments of the Thai Bombyx mori nucleopolyhedrovirus (BmNPV) DNA

Fragment	Size (kb) of DNA fragments				
	*Bam*HI	*Bgl*I	*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**

enzymes. Genome of BmNPV T3 isolate from Japan digested with EcoRI, *Hin*dIII, *Pst*I, *Bam*HI, KpnI and SmaI was estimated to be 130 kb (Maeda and Majima,1990) while the genome of BmNPV D1 isolate from Japan digested with ApaI, *Bam*HI, *Bgl*I, *Hin*dIII, KpnI and XhoI was estimated to be 126.4 kb (Hashimoto *et al.*,1994). Singh *et al.* (1996) reported that genome of BmNPV N isolate from India as estimated from the restriction fragments of the genome digested with *Hin*dIII and EcoRI was measured approximately 118 kb. Among all reported BmNPVs, only genome of T3 isolate was submitted in GenBank and was assigned accession No. L33180 and the submitted genome was 128, 413 nucleotides long (Gomi *et al.*, 1999).

Restriction endonuclease cleavaged fragments of Thai BmNPV DNA and BmNPV DNA of T3 and D1 isolates from Japan, and N isolate from India were illustrated in Table 2. T3 and T3* were the same isolate but the fragment sizes of T3 were from the report of Maeda and Majima (1990) while fragment sizes of T3* (GenBank accession No.L33180), complete genome sequence were analyzed by computer software.

*Bam*HI restriction patterns showed that there were at least six DNA bands for BmNPV of Thai, T3 and T3* isolates while there were at least five DNA bands for D1 isolate. The last three small fragments viewed on the gel for the Thai, T3, T3* and D1 isolated were in similar size. They were 4.2, 3.9, 4.2 and 4.2 kb; 6.1, 6.0, 6.2 and 6.0 kb, and 7.5, 7.3, 7.6 and 7.5 kb respectively. In comparison, there were four DNA fragments of similar size, C, D, E and F for the Thai and T3 isolates. The largest fragment of the Thai isolate was smaller than that of all other isolates used in this study. The size marker used in this study was the low molecular weight size marker which may be too low and narrow in range until it could not illustrate the DNA fragments that were bigger than 23.1 kb which was the highest value of marker used in this study.

When digested with *Hin*dIII, there were at least 19 bands for BmNPV of Thai and N isolates while there were at least 18 and 20 bands for T3 and D1 isolates, respectively. DNA fragments of the Thai isolate were different in size from those of the T3, D1 and N isolates. However, fragments E, G, H, M, N, O, P and R of the Thai isolate were similar to fragments F, I, J, N, O, P and R of the T3 isolate, respectively and fragments D, L, M, N and Q of the Thai isolate were similar to fragments D, M(N), O, P and T of the D1 isolates, respectively. In addition, several fragments of the Thai isolate were similar to fragments of the N isolate, fragments G, H, I, L, M, N and O of the Thai isolate were similar to fragments H, I, J, K, M, P and Q of the N isolate, respectively.

*Pst*I restriction patterns showed that there were at least 17 bands for BmNPV of

Thai isolate while there were at least 19 bands for T3 isolate. Fragments A(B), G, M, N and O of the Thai isolate were similar to fragments A(B), F(G), M, N(O) and P of the T3 isolate, respectively.

Some digested DNA fragments of T3 and T3* isolates were different in molecular size, even though they are the same BmNPV isolate. This was clearly elucidated by fragments A of T3 and T3* isolates which were measured 54 kb and 51.1 kb when digested with *Bam*HI and were measured 30 kb and 27.3 kb when digested with *Hin*dIII. In addition, fragment D of T 3 isolate digested with *Pst*I was estimated 12.5 kb 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 and wit*Hin* viral isolates. The differences may result from the methods of analysis which were different among researchers.

In this study, size of the DNA fragments was estimated by comparison with the fragments of DNA marker that electrophoresed on the gel. The same method was used to estimate restriction fragments of the N isolate (Singh *et al.*,1996) while those of T3 and D1 isolates were estimated from the clone fragments which were probed by hybridization (Maeda and Majima,1990; Hashimoto *et al.*,1994). Therefore, genome of the Thai BmNPV was similar in size with the N isolate and smaller than that of the T3 and D1 isolates when digested with *Hin*dIII. In comparison with genome of BmNPV T3 and D1 isolates from Japan, and N isolate from India, the Thai BmNPV genome is the smallest in size. DNA fragments with similar size of the T3 and D1 isolates could be distinguished while DNA fragments of the Thai isolate that had similar size could not be clearly separated by gel electrophoresis. It was rather difficult to correctly determine molecular size of BmNPV DNA solely by analyzing its restriction pattern. Bands which more intense than the regular single band were, even though considered as two fragments of relatively the same size, they might probably contain more than two fragments. This study is only the preliminary investigation of the Thai BmNPV restriction pattern in comparison with other previously reported isolates. 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

Table 2. Restriction endonuclease cleavaged fragments of the Thai *Bombyx mori* nucleopolyhedrovirus (BmNPV) DNA and DNA of the other isolates of BmNPV*

Fragment	Size (kb) of BmNPV DNA restiction fragments											
	*Bam*HI				*Hin*dIII					*Pst*I		
	Thai	T3	T3*	D1	Thai	T3	T3*	D1	N	Thai	T3	T3*
A	26.0	54.0	51.4	58.1	23.1	30	27.7	29.5	24.06	17.4	17.5	17.8
B	26.0	36.0	37.3	50.5	20.1	17	16.9	17.0	15.16	17.4	17.5	17.5
C	22.5	22.0	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 isolate from India (Singh *et al.*, 1996).

complicated work, is time consuming and expensive in sight of the equipments and chemical used.

The results suggested that restriction enzyme that gave more DNA fragments on the electrophoresed gel can be used for differentiation of isolates better than restriction enzyme that gave fewer fragments. This study showed that *Hin*dIII clarified polymorphism among BmNPV isolates better than *Bam*HI.

There were many reports on restriction patterns of other NPVs. Smith and Summers (1978) suggested that DNA restriction patterns of several NPVs (*Autographa californica* NPV, *Orgyia pseudotsugata* NPV, *Rachiplusia au* NPV, *Spodoptera exigua* NPV, *Porthetria dispar* NPV, *Trichoplusia ni* NPV, *Heliothis zea* NPVand *H. armigera* NPV) can be used to identify isolates of baculoviruses. Moreover, Lavina-Caoili *et al.* (2001) studied restriction patterns of 10 isolates of S. litura NPV (SlNPV) from Japan, C*Hin*a and the Philippines. The 10 isolates displayed similar overall restriction endonuclease patterns except for deletion or insertion of a few DNA fragments, indicated that there were minor differences in their genome organization among the isolated genotypic variants. 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 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 is one of the useful tools in identification and classification of the viruses.

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