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)

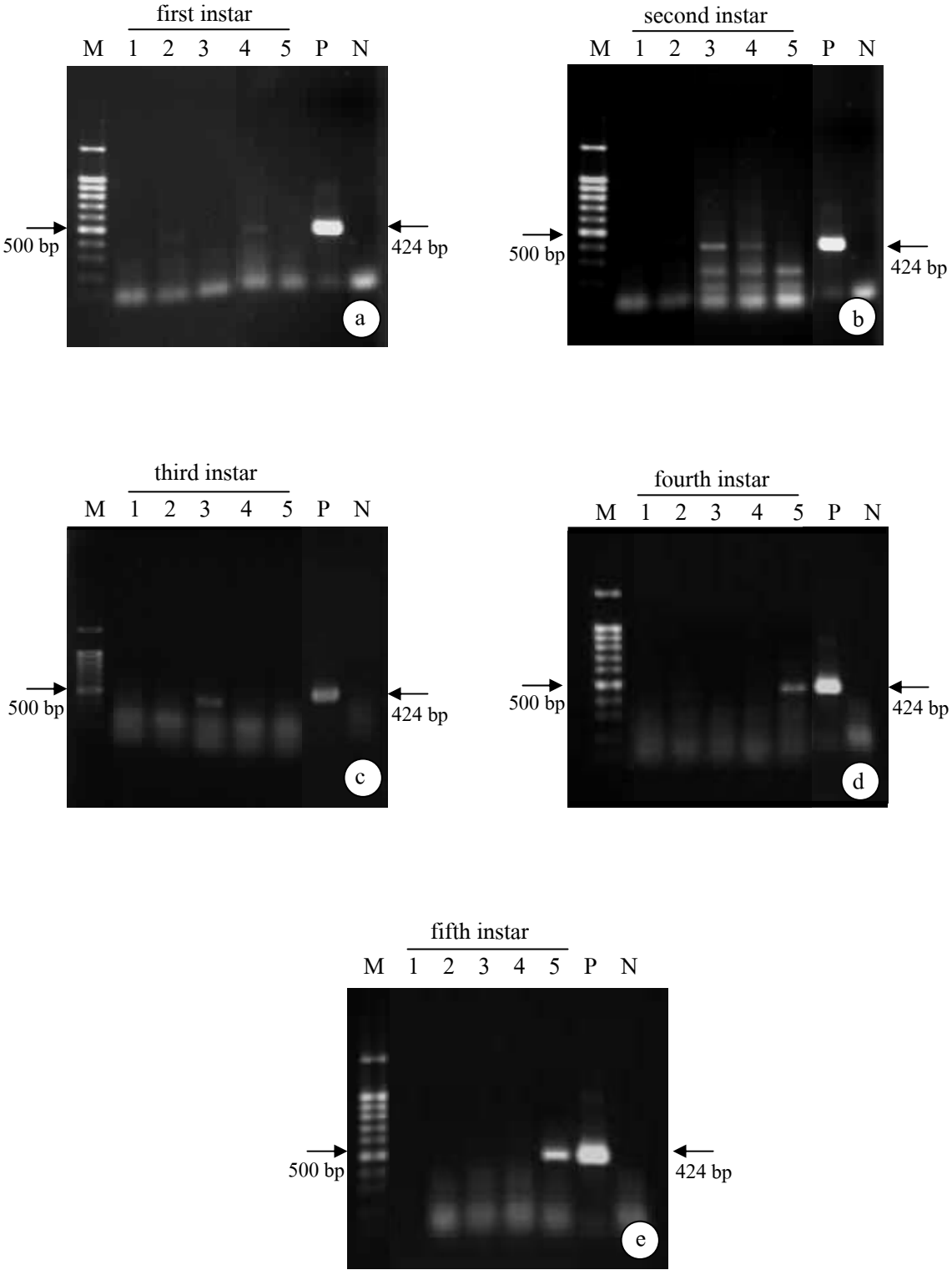

first instar
M 1 2 3 4 5 P N
500 bp
424 bp
a
second instar
M 1 2 3 4 5 P N
500 bp
424 bp
b
third instar
M 1 2 3 4 5 P N
500 bp
424 bp
c
fourth instar
M 1 2 3 4 5 P N
500 bp
424 bp
d
fifth instar
M 1 2 3 4 5 P N
500 bp
424 bp
e

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

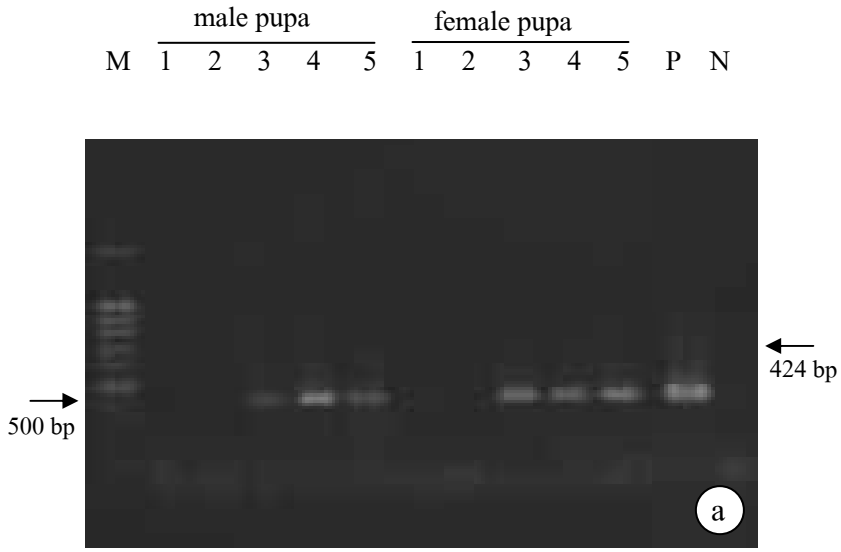
b) Moths of UB1 variety from Nong Khai province

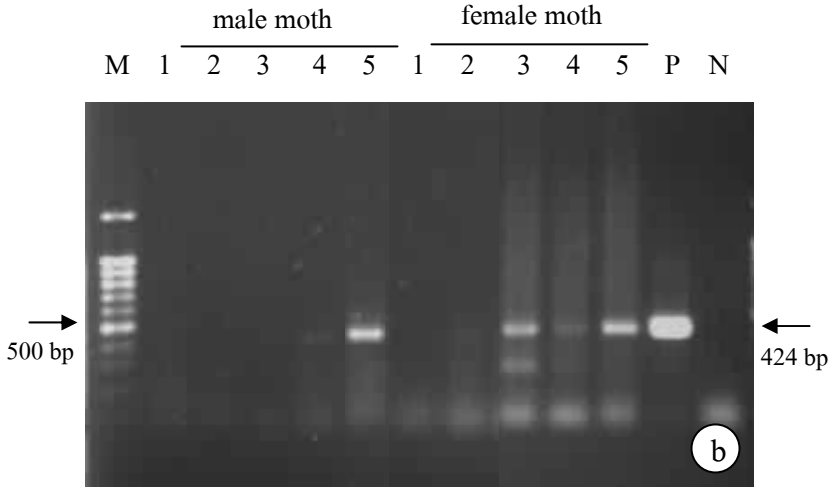
c) Male and female moths and their eggs of Nang Noi variety from Kalasin province

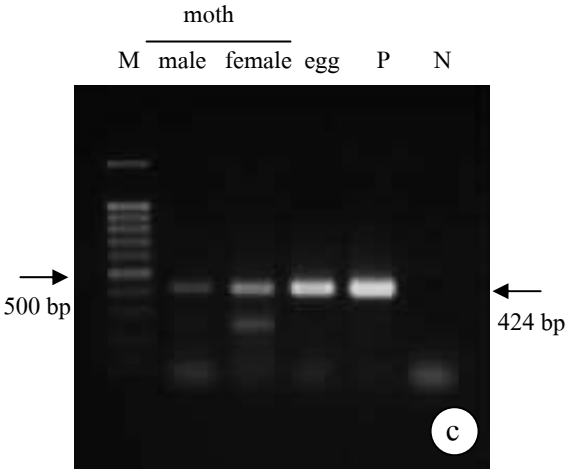
M=DNA marker (100 bp+1.5 kb)

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.