CHAPTER IV

RESULTS

4.1 Total DNA and nuclear DNA extraction

In this study both of total DNA and nuclear DNA of *A. cerana* were extracted from individual worker pupae. The total DNA was pre-extracted immediatly at the collection-location and transported for future purification at the laboratory, whereas, all steps of nuclear DNA extraction were done at the laboratory. The initial preparation of DNA was previously tested for achievement of future analysis, so that the absorption spectrum of extracted DNA was measured from 200 to 400 nm (Appendix 5). The purity and concentration of DNA was estimated where the absorbance at 260 nm equal to 1 as equivalent to 50 μg/ml of double-standed DNA and the ratio of $OD_{260/280}$ between 1.65 and 1.85 showing the purity of DNA. Usually, about 3.9 and 2.9 μg were obtained single worker pupae total DNA extraction and nuclear DNA extractions, respectively. The extracted DNA was always dissolved in 40 μl of TE buffer. The $OD_{260/280}$ ratios of total and nuclear DNA ranged from 1.70 to 1.95. In addition, agarose gel electrophoresis of the undigested total and nuclear DNA migrated as the high molecular weight, larger than the 23.1 kb marker, and sheared fragments were minimal (Figure 7). Those results demonstrated that total DNA extraction and nuclear DNA extraction were suitable for subsequent experiments.

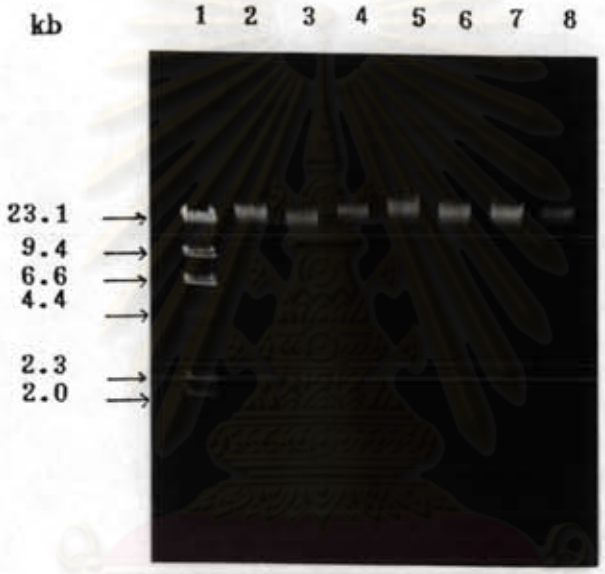


Figure 7 Agarose gel electrophoretic staining pattern of *A. cerana* total DNA and nucler DNA.

Total and nuclear DNA were extracted from individual worker pupae in the same colony and then subjected to 0.7% agarose gel electrophoresis at 100 V for 45 minutes;

lane 1 : λ/*Hind*III DNA standard

lane 2-4 : total DNA

lane 5-7 : nuclear DNA

4.2 Restriction digestion of honey bee DNA

The total DNA extracted from individual worker pupae from the same colony was then separately digested with and excess of ten restriction endonucleases; *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Hae*III, *Hind*III, *Nde*I, *Sau*3AI, *Sca*I and *Sma*I (800 ng/5 U) and incubated at 37 ^{o}C for 24 hours, then the reaction was stopped for electrophoresis. The result is shown in Figure 8, the distance of discrete bands of the restriction pattern are found when *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Hae*III, *Hind*III, *Nde*I, *Sau*3AI, *Sca*I and *Sma*I are used. Therefore the restriction endonucleases; *Bgl*II, *Cla*I, *Eco*RI, *Hae*III, and *Nde*I were selected for future study. This was because of their appropriate restriction pattern analysis which gave simple discrete bands.

Each of 5 U of five restriction endonucleases; *Bgl*II, *Cla*I, *Eco*RI, *Hae*III, and *Nde*I were used to digest 800 ng of individual *A. cerana* total DNA in 20 μl of reaction mixture. The incubation times were varied from 1, 2, 3, 4, 5, 6 and 24 hours respectively. Then the digested DNAs were separated by agarose gel electrophoresis on 1.0% agarose at 80 V for 3 hours. After staining with 2.5 μg/ml of ethidium bromide, the smear patterns lower than 23.1 kb in size appeared the same in every incubation time. For example, the digestions with *Eco*RI are shown in Figure 9. The results demonstrated that honey bee DNA could be completely digested with restriction endonucleases in 1 hour. Furthermore, this result clearly showed that the restriction pattern of individual honey bees is similar within this colony.

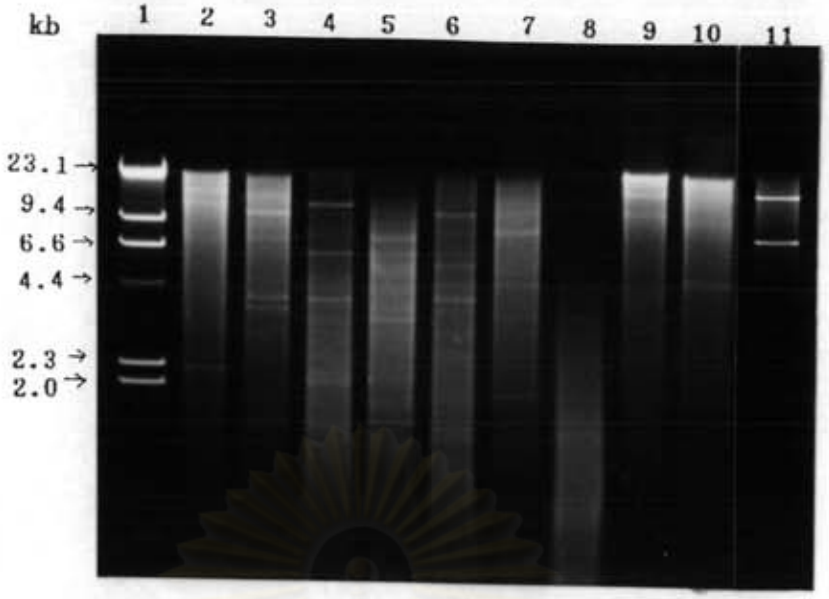


Figure 8 Agarose gel electrophoretic staining pattern of *A. cerana* total DNA from the same colony digested with various restriction endonucleases.

Total DNA extracted from single honey bee was used for 5 numbers of restriction endonuclease. Electrophoresis was performed on 0.8% agarose at 80 V for 3 hours and stained with 2.5 μg/ml of ethidium bromide;

lane 1 : λ/*Hind*III DNA standard

lane 2 : total DNA digested with *Bam*HI

lane 3 : total DNA digested with *Bgl*II

lane 4 : total DNA digested with *Cla*I

lane 5 : total DNA digested with *Eco*RI

lane 6 : total DNA digested with *Hae*III

lane 7 : total DNA digested with *Hind*III

lane 8 : total DNA digested with *Sau*3AI

lane 9 : total DNA digested with *Sca*I

lane 10 : total DNA digested with *Sma*I

lane 11 : total DNA digested with *Nde*I

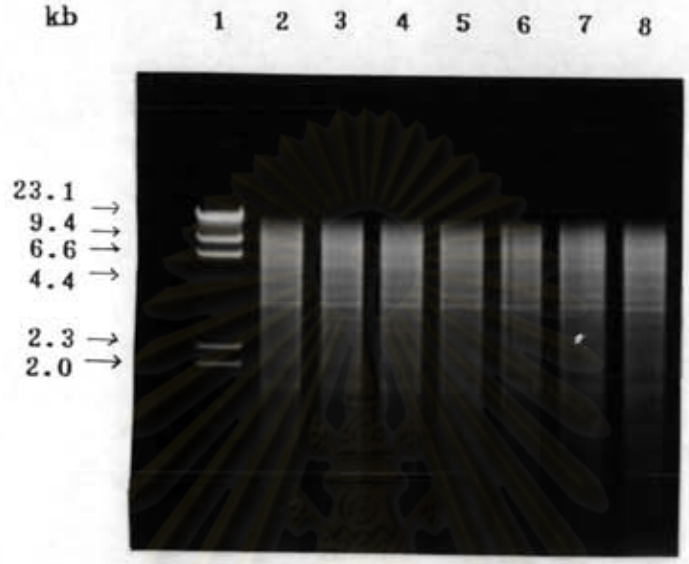


Figure 9 Agarose gel electrophoretic staining pattern of *A. cerana* total DNA digested with *Eco*RI and incubated on varying time.

Total DNA was extracted from individual worker pupae in the same colony. 800 ng DNA digested with 5 U of *Eco*RI. Electrophoresis was performed on 1.0% agarose at 80 V for 3 hours and stained with 2.5 μg/ml of ethidium bromide;

lane 1 : λ/*Hind*III DNA standard

lane 2 : total DNA digested with *Eco*RI for 1 hour

lane 3 : total DNA digested with *Eco*RI for 2 hours

lane 4 : total DNA digested with *Eco*RI for 3 hours

lane 5 : total DNA digested with *Eco*RI for 4 hours

lane 6 : total DNA digested with *Eco*RI for 5 hours

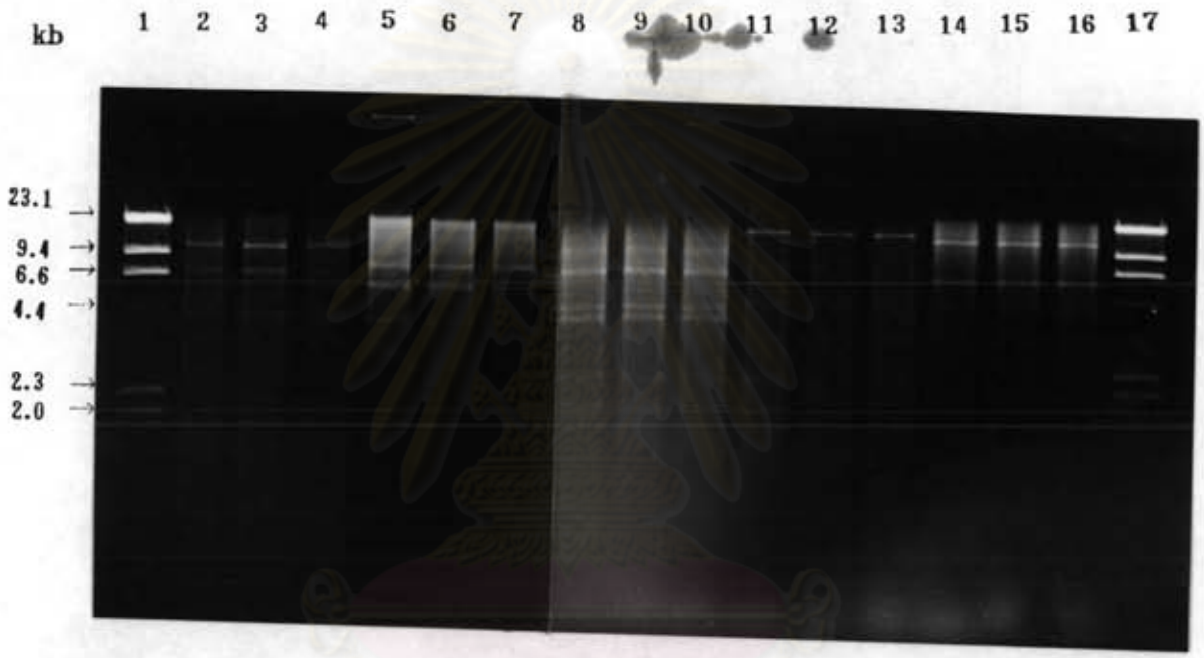
lane 7 : total DNA digested with *Eco*RI for 6 hours

lane 8 : total DNA digested with *Eco*RI for 24 hours

Because of the results from restriction endonucleases *Bgl*II,*Cla*I, *Eco*RI, *Hae*III and *Nde*I were selected to digest honey bee DNA for restriction pattern analysis, so each concentration of restriction endonuclease which a gave complete digestion was tested. Usually 800 ng of bee DNA was digested with 5, 10 and 15 U of each restriction endonucleases for 2 hours. The digested DNA fragments of each series for five restriction endonucleases gave the same patterns (Figure10). Therefore, 800 ng of each honey bee DNA could usually be digested with 5 U of each restriction endonuclease for subsequent restriction pattern analysis.

4.3 Comparison of restriction pattern between total DNA and nuclear DNA

The individual total DNA and nuclear DNA from the same colony was digested with each of five restriction endonucleases as previously presented. The resulting restriction patterns are shown in Figure 11 (some of 20 samples are shown), demonstrating that both total DNA and nuclear DNA extraction give the same patterns for each restriction endonuclease; *Bgl*II, *Cla*I, *Eco*RI, *Hae*III and *Nde*I. Therefore, the total DNA was used for restriction pattern. Since total DNA extraction method was convenient to pre-extracted DNA at the field and gave higher DNA than nuclear DNA extraction method, the total DNA extraction was selected for future study.



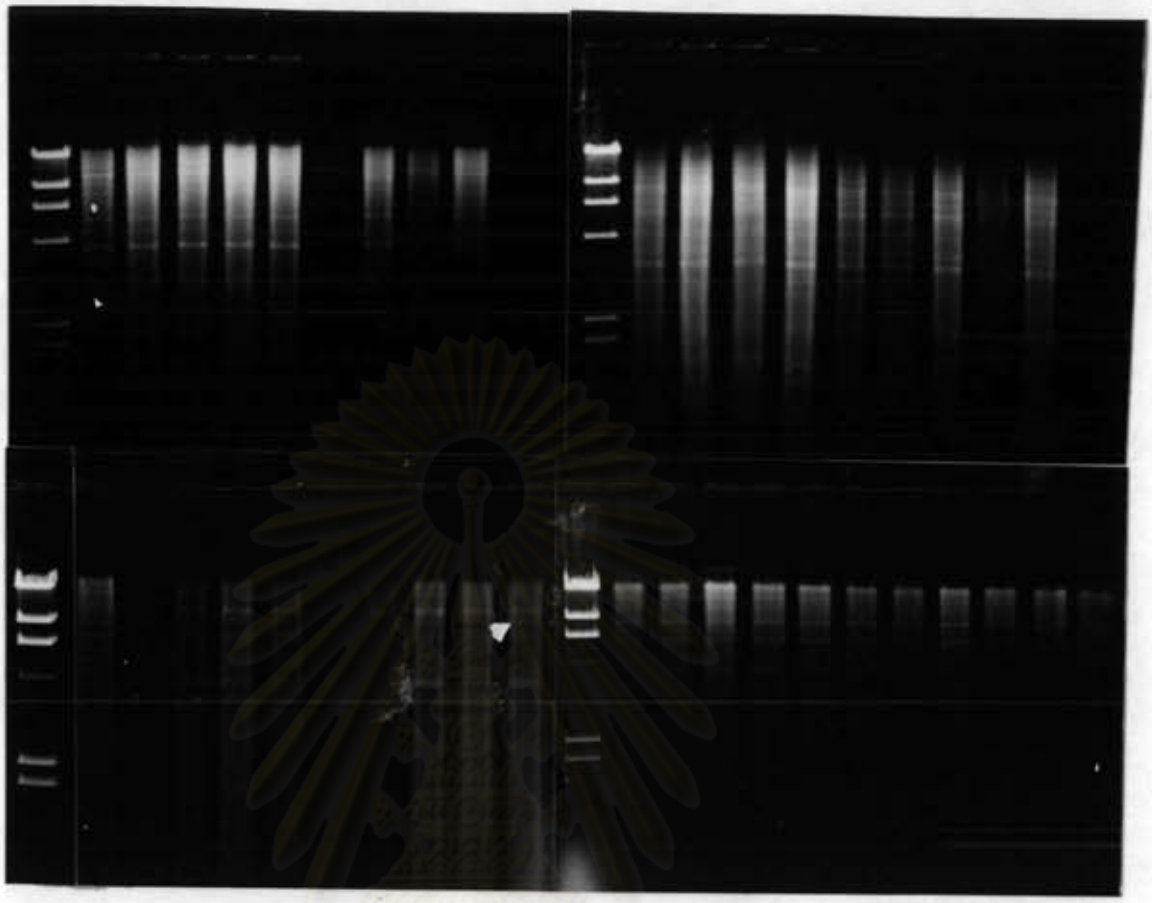


Figure 11 Agarose gel electrophoretic staining pattern of *A. cerana* total DNA and nuclear DNA digestedwith four restriction endonucleases.

Each of honey bee DNAs from the same colony was extracted from a single bee. 800 ng of DNA digested with 5 U of *Bgl*II (A), *Eco*RI (B), *Hae*III (C) and *Nde*I (D). Electrophoresis was performed on 0.8% agarose at 80 V for 3 hours and stained with 2.5 μg/ml of ethidium bromide.

lane 1 : λ/*Hind*III DNA standard

lane 2-5 : total DNA

lane 6-10 (A,B) : nuclear DNA

lane 6-11 (C,D) : nuclear DNA

A	B
C	D

4.4 Comparison of restriction patterns from total DNA of Asian honey bees

In order to test whether restriction pattern give the same result, the total DNA of various species of honey bees; *A. florea, A. mellifera* and *A. cerana* from individuals of the same colony were completely digested with restriction endonuclease *Eco*RI. The restriction pattern shows distinctly (Figure 12) that the *Eco*RI digested total DNA of *A. florea* having discrete bands presented at 21.0, 4.0 and 3.5 kb, *A. mellifera* has discrete bands at 14.5, 10.5, 4.0, 2.2 and 2.0 kb, whereas the *A. cerana* bands were at 6.0, 4.0, 3.7, 2.5 and 2.3 kb.

4.5 Comparison of restriction patterns from total DNA of *A. cerana* in the same colony

DNA from 20 individual bees from the same colony was isolated and purified. The total DNAs were then digested with each of five restriction endonucleases as previously described in section 4.2. The results from restriction patterns of individual honey bees within the same colony showed similar patterns (Figure 13, data is shown for only 9 samples) for each restriction endonuclease. Therefore, for subsequent experiments on restriction pattern and Southern hybridization analysis, less than 20 samples from each colony were used.

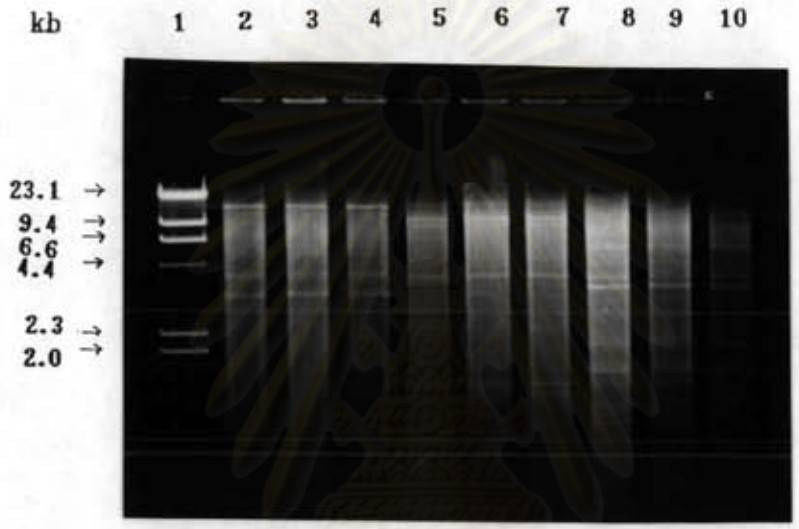


Figure 12 Agarose gel electrophoretic staining pattern of various species of *Apis* total DNA digested with *Eco*RI.

Total DNA was extracted from individual worker pupae in the same colony. 800 ng of total DNA digested with 5 U of *Eco*RI. Electrophoresis was performed on 1.0% agarose at 80 V for 3 hours and stained with 2.5 μg/ml of ethidium bromide;

lane 1 : λ/*Hind*III DNA standard

lane 2-4 : *A. florea*

lane 5-7 : *A. mellifera*

lane 8-10 : *A. cerana*

Figure 13 Agarose gel electrophoretic staining pattern of *A. cerana* of the same colony from different locations, total DNA digested with 5 restriction endonucleases.

Total DNA was extracted from individual worker pupae in the same colony. Electrophoresis was performed on 1.0% agarose at 80 V for 3 hours and stained with 2.5 μg/ml of ethidium bromide, 800 ng of total DNA digested with 5 U of restriction endonucleases;

lane 1 : λ/*Hind*III DNA standard

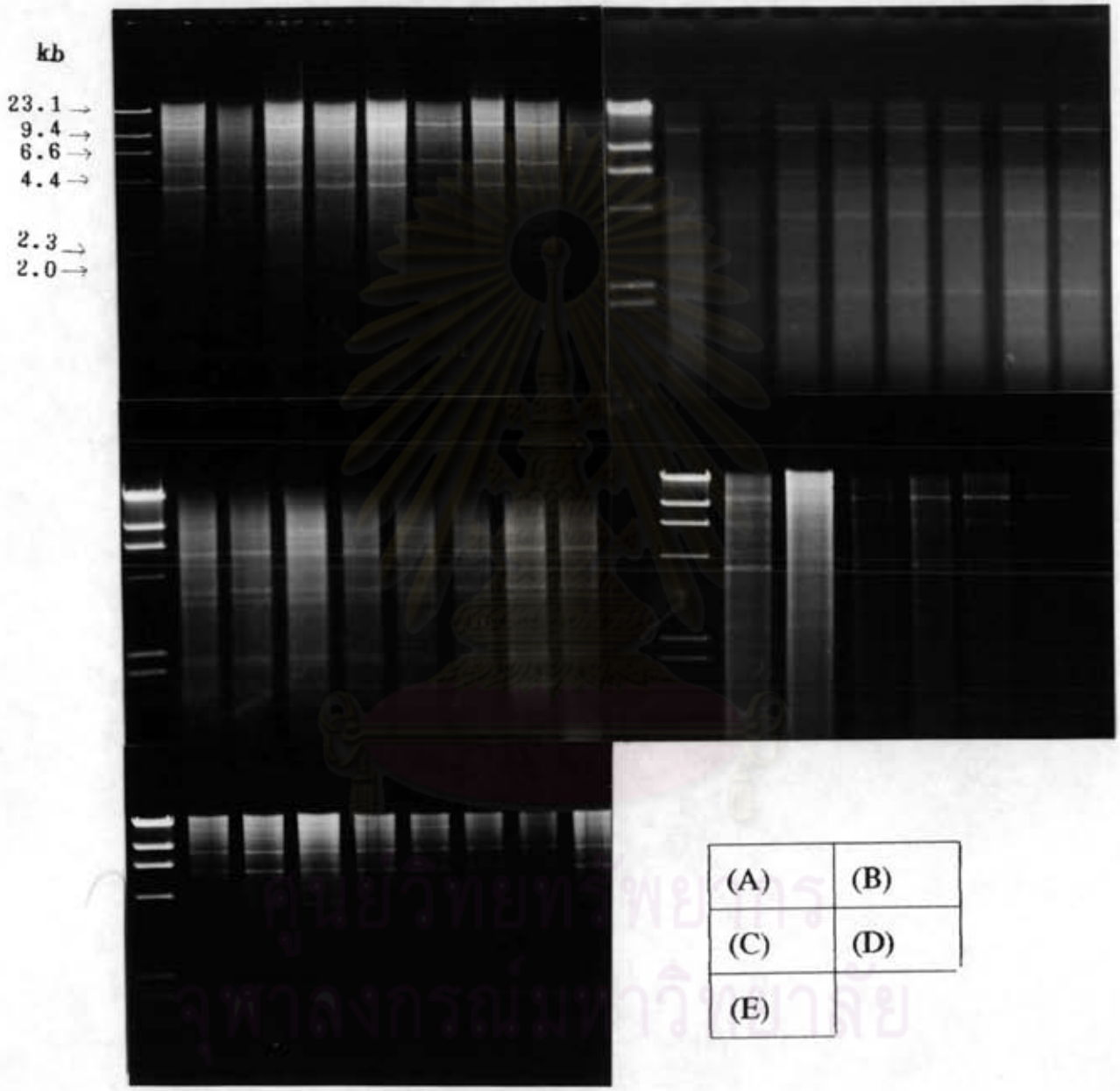
group A : total DNA digested with *Bgl*II

group B : total DNA digested with *Cal*I

group C : total DNA digested with *Eco*RI

group D : total DNA digested with *Hae*I

group E : total DNA digested with *Nde*I



(A)	(B)
(C)	(D)
(E)	

4.6 Comparison of restriction patterns from total DNA of A. cerana from different locations

Each DNA sample was collected from a worker pupae of *A. cerana*, colony and about 20 colonies of each location were then completely digested with each restriction endonuclease which was selected in section 4.2. The restriction pattern was compared within and among groups for the same restriction endonuclease.

The results of *Bgl*II which digested total DNA samples from all locations were divided into two groups (Figure 14) based on the positions of discrete bands presented at 10.3, 10.0, 8.7, 5.6 and 3.9 kb and at 10.3, 10.0, 8.7, 3.9 and 3.4 kb, namely groups B1 and B2 respectively. The Samui Island DNA patterns were presented as two groups; B1 and B2 (Figure 14 (E) with 45.5 and 54.5%, respectively, while the other locations had only group B1 (Table 3).

The *Cla*I which digested total DNA samples from all locations were divided into two groups; with discrete bands presented at 15.6, 9.1, 6.0, 4.1, 3.6 and 2.2 kb referred to group C1 and at 12.8, 9.1, 6.0, 4.3 and 3.6 kb refered to C2 (Figure 15). The DNA samples from all locations had group C1 but only the DNA samples derived from the Southern region had group C2. In the Southern region, the percentages of C1 and C2 were 47.4 and 52.6 respectively (Figure 15 (D)). The results are summarized in Table 4.

The restriction pattern obtained from *Eco*RI which digested total DNA is shown in Figure 16. The DNA smples from all location could be divided into five groups, E1, E2, E3, E4 and E5, based on the discrete

Figure 14 Agarose gel electrophoretic staining pattern of *A. cerana* from different locations, total DNA digested with *Bgl*II.

Total DNA was extracted from individual worker pupae in different colonies. 800 ng of DNA digested with 5 U of *Bgl*II Electrophoresis was performed on 1.0% agarose at 80 V for 3 hours and stained with 2.5 μg/ml of ethidium bromide;

lane 1 : λ/*Hind*III DNA standard

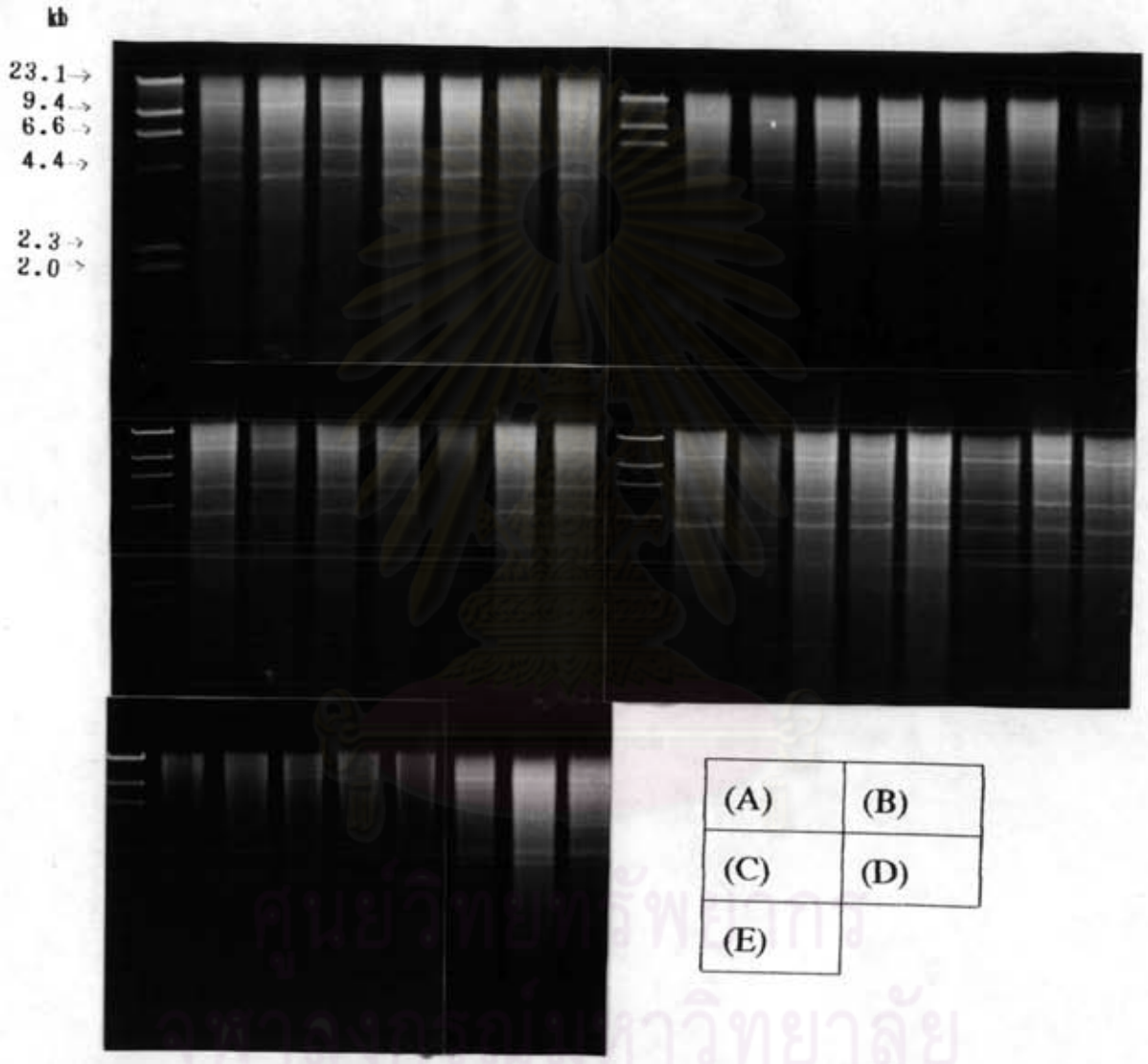
group A : total DNA of *A. cerana* from the Northern

group B : total DNA of *A. cerana* from the North-Eastern

group C : total DNA of *A. cerana* from the Central part

group D : total DNA of *A. cerana* from the Southern

group E : total DNA of *A. cerana* from the Samui Island



(A)	(B)
(C)	(D)
(E)	

Table 3 Summary of Restriction pattern of *Bgl*II digested total DNA of *A. cerana* from five locations of Thailand.

Sampling location	No. of colony of total DNA	% Classification based on size of the discreat bands (kb)	
		B_1 10.3, 10.0, 8.7, 5.6, 3.9	B_2 10.3, 10.0, 8.7, 3.9, 3.4
the Northern	20	100.0	-
the North-Eastern	20	100.0	-
the Central part	20	100.0	-
the Southern	20	100.0	-
the Samui Island	20	45.5[1a]	54.5[1b]

1 Number of colony from the Samui Island 1a; I_1, I_2, I_3, I_{10}, I_{14}, I_{16}, I_{17}, I_{22}, I_{23}, I_{24}, I_{26}, I_{27}

1b; I_4, I_8, I_9, I_{12}, I_{13}, I_{15}, I_{19}, I_{20}, I_{21}, I_{25}

Total DNA of *A. cerana* was extracted from individual worker pupa of each colony andabout 20 colonies for a location. The *Bgl*II 5 U digeted DNA about 800 ng. Electrophoresis was performed on 1.0% agarose at 80 V for 3 h. The λ phage DNA digested with *Hind*III was usedas standard molecular weight marker. The results were repeated about 2 to 4 times by using different pupae from the same colony.

Figure 15 Agarose gel electrophoretic staining pattern of *A. cerana* from different locations, total DNA digested with *Cla*I.

Total DNA was extracted from individual worker pupae in different colonies. 800 ng of DNA digested with 5 U of *Cla*I. Electrophoresis was performed on 1.0% agarose at 80 V for 3 hours and stained with 2.5 μg/ml of ethidium bromide;

lane 1 : λ/*Hind*III DNA standard

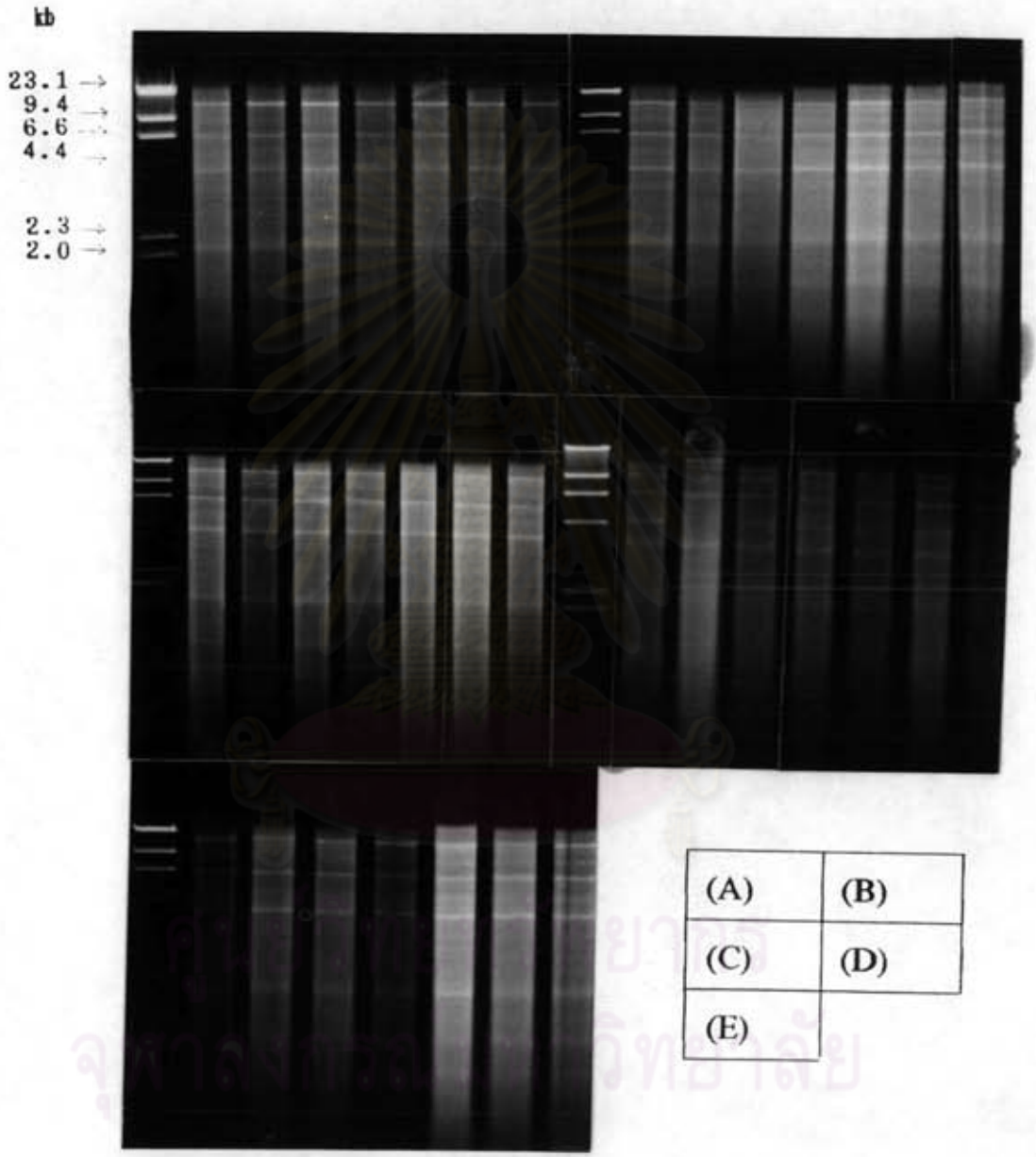
group A : total DNA of *A. cerana* from the Northern

group B : total DNA of *A. cerana* from the North-Eastern

group C : total DNA of *A. cerana* from the Central part

group D : total DNA of *A. cerana* from the Southern

group E : total DNA of *A. cerana* from the Samui Island



(A)	(B)
(C)	(D)
(E)	

Table 4 Summary of restriction pattern of *Cla*I digested total DNA of *A. cerana* from five locations of Thailand.

Sampling location	No. of colony of total DNA	% Classification based on size of the discreat bands (kb)	
		C_1	C2
		15.6, 9.1, 6.0, 4.1, 3.6, 2.2	12.8, 9.1, 6.0, 4.3, 3.6
the Northern	20	100.0	-
the North-Eastern	20	100.0	-
the Central part	20	100.0	-
the Southern	19	47.4[1a]	52.6[1b]
the Samui Island	20	100.0	-

1 Number of colony from the Southern 1a; S_1, S_4, S_9, S_{10}, S_{12}, S_{13}, S_{14}, S_{15}, S_{16}

1b; S_2, S_3, S_5, S_6, S_7, S_8, S_{11}, S_{17}, S_{19}, S_{20}

Total DNA of *A. cerana* was extracted from individual worker pupa of each colony and about 20 colonies for a location. The *Cla*I 5 U digeted DNA about 800 ng. Electrophoresis was performed on 1.0% agarose at 80 V for 3 h. The λ phage DNA digested with *Hind*III was used as standard molecular weight marker. The results were repeated about 2 to 4 times by using different pupae from the same colony.

bands at various positions.. Two locations in Thailand, the North-Eastern and the Central part, only had group E1 (Figure 16 (B) and (C)). In addition, two groups E1 and E4 were presented in DNA samples from the Southern at 80.0 and 20.0%, restpectively. DNA samples from the Northern had three groups, E1, E2 and E3, (Figure 16(A)) at 90.0, 5.0 and 5.0%, respectively. DNA samples from Samui Island were also divided into three groups, E1, E2 and E5, at 70.8, 12.5 and 16.7% respectively (Figure 16 (E)). The results are summarized in Table 5.

The restriction pattern of total DNA digested with *Hae*III were divided into three groups, H1, H2 and H3. DNA samples from the Northern, the North-Eastern and the Southern (Figure 14 (A), (B) and (C) respectively) were classified into two groups, H1 and H2, with percentages of 27.7 and 72.3, respectively, for the Northern; 26.3 and 73.7%, respectively, for the North-Eastern; and 68.4 and 31.6%, respectively, for the Southern. DNA samples from Samui island had two groups, H1 and H3 with each at 50% (Figure 17 (E)). The Central part had only group H3. The results are shown in Table 6.

The *Nde*I restriction patterns (Figure18) were divided into two groups; N1 and N2. The position of discrete bands were 8.1, 5.6, 4.1 and 3.3 kb refered to N1 and 13.3, 8.1 and 4.1 kb referred to group N2. The DNA samples derived from the Northern, the North-Eastern and the Central part had only group N1 (Figure 18; (A), (B) and (C)), whereas the Southern and Samui Island had both groups, with the percentages of 35.3 and 64.7, respectively, for the Southern; and 50% each for Samui Island (Figure 18; (D), (E)). More details are shown in Table 7.

Figure 16 Agarose gel electrophoretic staining pattern of *A. cerana* from different locations, total DNA digested with *Eco*RI.

Total DNA was extracted from individual worker pupae in different colonies. 800 ng of DNA digested with 5 U of *Eco*RI. Electrophoresis was performed on 1.0% agarose at 80 V for 3 hours and stained with 2.5 µg/ml of ethidium bromide;

lanes 1 : λ/*Hind*III DNA standard

group A : total DNA of *A. cerana* from the Northern

group B : total DNA of *A. cerana* from the North-Eastern

group C : total DNA of *A. cerana* from the Central part

group D : total DNA of *A. cerana* from the Southern

group E : total DNA of *A. cerana* from the Samui Island

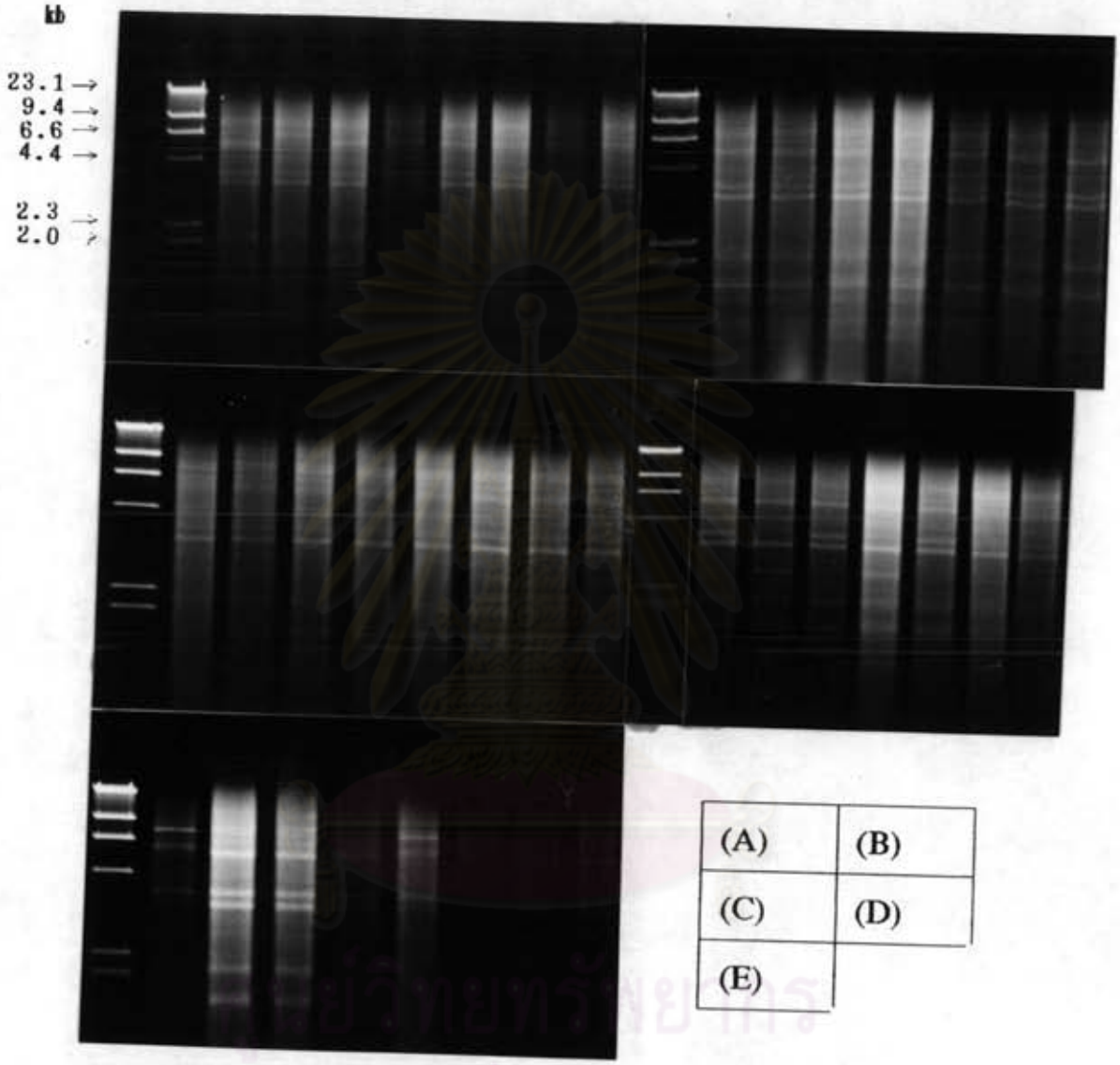


Table 5 Summary of restriction pattern of *Eco*RI digested total DNA of *A. cerana* from five locations of Thailand.

Sampling location	No. of colony of total DNA	% Classification based on size of the discreat bands (kb)				
		E_1 8.0,5.6,3.7,3.5,2.2<2.2	E_2 8.0,5.0,3.7,3.5,2.2,<2.2	E_3 8.0,7.0,3.7,3.5,2.2,<2.2	E_4 8.0,7.0,5.6,3.5,2.8,2.2,<2.2	E_5 8.0,6.0,3.7,3.5
the Northern	22	90.0^{1a}	5.0^{1b}	5.0^{1c}	-	-
the North-Eastern	20	100.0	-	-	-	-
the Central part	20	100.0	-	-	-	-
the Southern	20	80.0^{2a}	-	-	20.0^{2b}	-
the Samui Island	24	70.8^{3a}	12.5^{3b}	-	-	16.7^{3c}

1 Number of colony from the Northern 1a; $N_1, N_2, N_3, N_4, N_5, N_7, N_8, N_9, N_{10}, N_{11}, N_{13}, N_{14}, N_{15}, N_{16}, N_{17}, N_{18}, N_{19}, N_{21}, N_{22}$ 1b; N_6 1c; N_7

2 Number of colony from the Southern 2a; $S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_8, S_9, S_{11}, S_{14}, S_{15}, S_{16}, S_{18}, S_{19}, S_{20}$ 2b; $S_{10}, S_{12}, S_{13}, S_{17}$

3 Number of colony from the Samui Island 3a; $I_1, I_2, I_3, I_6, I_7, I_{14}, I_{16}, I_{17}, I_{18}, I_{19}, I_{20}, I_{22}, I_{24}$ 3b ; $I_4, I_{11}, I_{12}, I_{13}, I_{15}$ 3c; $I_{21}, I_{23}, I_{25}, I_{26}, I_{27}$

Total DNA of *A. cerana* was extracted from individual worker pupa of each colony and about 20 colonies for a location. The *Eco*RI 5 U digeted DNA about 800 ng. Electrophoresis was performed on 1.0% agarose at 80 V for 3 h. The λ phage DNA digested with *Hind*III was used weight marker. The results were repeated about 2 to 4 times by using different pupae from the same colony..

Figure 17 Agarose gel electrophoretic staining pattern of *A. cerana* from different locations, total DNA digested with *Hae*III.

Total DNA was extracted from individual worker pupae in different colonies. 800 ng of DNA digested with 5 U of *Hae*III. Electrophoresis was performed on 1.0% agarose at 80 V for 3 hours and stained with 2.5 μg/ml of ethidium bromide;

lane 1 : λ/*Hind*III DNA standard

group A : total DNA of *A. cerana* from the Northern

group B : total DNA of *A. cerana* from the North-Eastern

group C : total DNA of *A. cerana* from the Central part

group D : total DNA of *A. cerana* from the Southern

group E : total DNA of *A. cerana* from the Samui Island

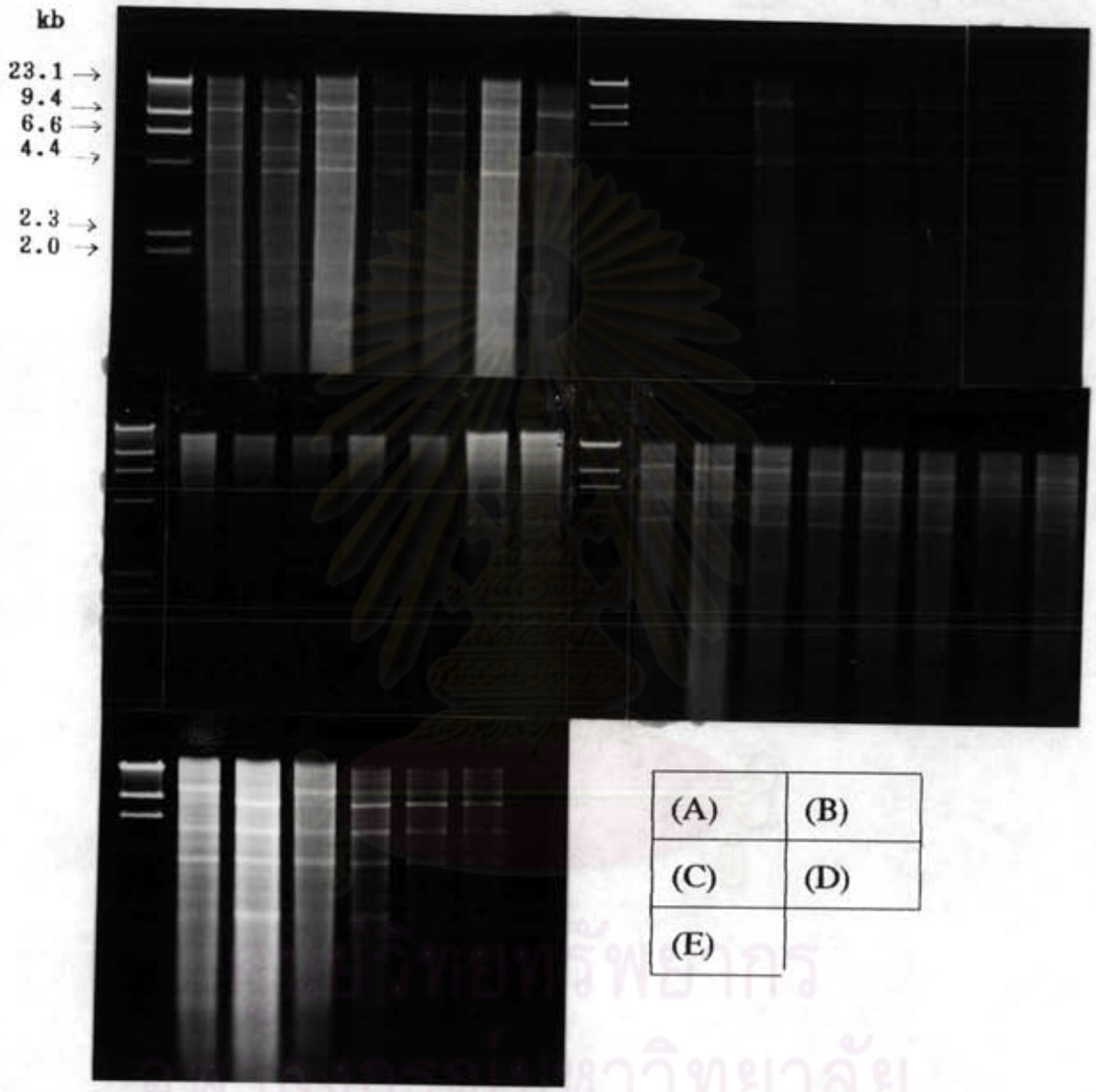

kb
23.1
9.4
6.6
4.4
2.3
2.0
(A)
(B)
(C)
(D)
(E)

Table 6 Summary of restriction pattern of *Hae*III digested total DNA of *A. cerana* from five locations of Thailand.

Sampling location	No. of colony of total DNA	% Classification based on size of the discreat bands (kb)		
		H1 11.4, 9.0, 5.6, 4.1	H2 11.4, 9.0, 6.5, 4.1	H_3 7.0, 5.6, 3.6, 3.0
the Northern	19	27.7^{1a}	72.3^{1b}	
the North-Eastern	19	26.3^{2a}	73.7^{2b}	-
the Central part	19			100.0
the Southern	19	68.4^{3a}	31.6^{3b}	-
the Samui Island	24	50.0^{4a}		50.0^{4b}

#1 Number of colony from the Northern 1a; N_1, N_2, N_5, N_{17}, N_{19}, N_{20} 1b; N_3, N_6, N_8, N_9, N_{10}, N_{11}, N_{12}, N_{13}, N_{14}, N_{16}, N_{18}, N_{21}, N_{22}

#2 Number of colony from the North-Eastern 2a; E_6, E_7, E_{12}, E_{15} 1b; E_1, E_2, E_3, E_4, E_5, E_8, E_9, E_{10}, E_{11}, E_{14}, E_{16}, E_{17}, E_{18}, E_{21}

#3 Number of colony from the Southern 3a; S_6, S_7, S_8, S_9, S_{10}, S_{11}, S_{12}, S_{13}, S_{14}, S_{15}, S_{17}, S_{19}, S_{20} 3b; S_1, S_2, S_3, S_4, S_5, S_{16}, S_{18}

#4 Number of colony from the Samui Island 4a; I_4, I_8, I_9, I_{11}, I_{12}, I_{13}, I_{15}, I_{19}, I_{20}, I_{21}, I_{25}, I_{26}

4b; I_1,I_3,I_6,I_7,I_{10},I_{14},I_{16},I_{17},I_{18},I_{22},I_{24},I_{27}

Total DNA of *A. cerana* was extracted from individual worker pupae of each colony and about 20 colonies for a location. The *Hae*III 5 U digeted DNA about 800 ng. Electrophoresis was performed on 1.0% agarose at 80 V for 3 h. The λ phage DNA digested with *Hind*III was used as standard molecular weight marker. The results were repeated about 2 to 4 times by using different pupae from the same colony.

Figure 18 Agarose gel electrophoretic staining pattern of *A. cerana* from different locations, total DNA digested with *Nde*I.

Total DNA was extracted from individual worker pupae in different colonies. 800 ng of DNA digested with 5 U of *Nde*I. Electrophoresis was performed on 1.0% agarose at 80 V for 3 hours and stained with 2.5 μg/ml of ethidium bromide;

lanes 1 : λ/*Hind*III DNA standard

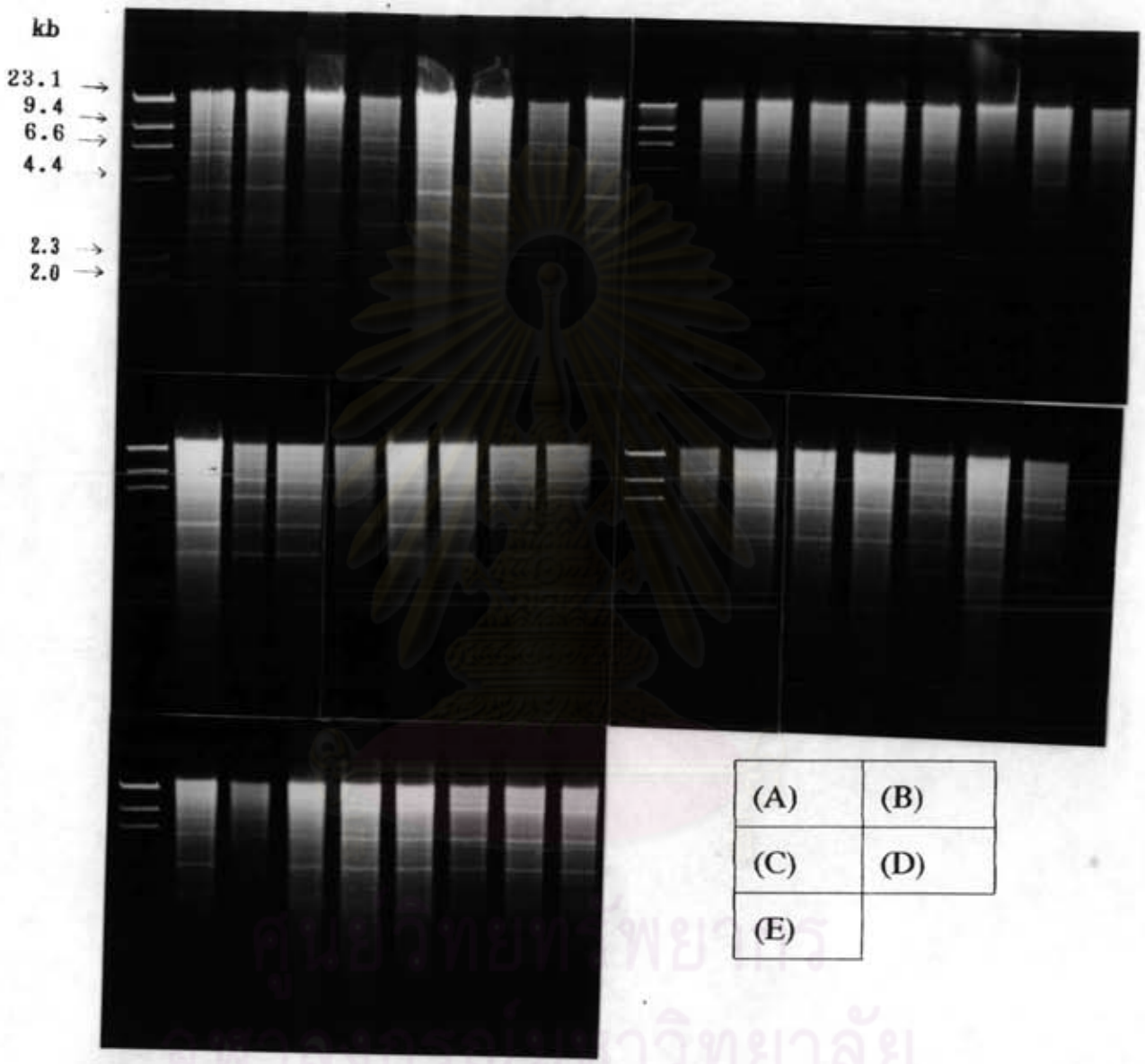
group A : total DNA of *A. cerana* from the Northern

group B : total DNA of *A. cerana* from the North-Eastern

group C : total DNA of *A. cerana* from the Central part

group D : total DNA of *A. cerana* from the Southern

group E : total DNA of *A. cerana* from the Samui Island



(A)	(B)
(C)	(D)
(E)	

Table 7 Summary of restriction pattern of *Nde*I digested total DNA of *A. cerana* from five locations of Thailand.

Sampling location	No. of colony of total DNA	% Classification based on size of the discreat bands (kb)	
		N1 8.1, 5.6, 4.1 3.3	N2 13.3, 8.1, 4.1
the Northern	20	100.0	-
the North-Eastern	20	100.0	-
the Central part	20	100.0	-
the Southern	17	35.0[1a]	64.7[1b]
the Samui Island	22	50.5[2a]	50.0[2b]

#1 Number of colony from the Southern 1a; S_1, S_2, S_3, S_4, S_5, S_6

1b; S_8, S_{10}, S_{11}, S_{13}, S_{14}, S_{15}, S_{16}, S_{17}, S_{18}, S_{19}, S_{20}

#2 Number of colony from the Samui Island 2a; I_3, I_8, I_9, I_{12}, I_{13}, I_{15}, I_{19}, I_{20}, I_{21}, I_{25}, I_{26}

2b; I_1, I_2, I_6, I_7, I_{16}, I_{17}, I_{18}, I_{22}, I_{23}, I_{24}, I_{27}

Total DNA of *A. cerana* was extracted from individual capped worker pupae of each colony and about 20 colonies for a location. The *Nde*I 5 U digeted DNA about 800 ng. Electrophoresis was performed on 1.0% agarose at 80 V for 3 h. The λ phage DNA digested with *Hae*III was used as standard molecular weight marker. The results were repeated about 2 to 4 times by using different pupae from the same colony.

4.7 DNA-DNA hybridization to total DNA of *A. cerana* by *A. mellifera* probes

In order to test wheather *A. mellifera* probes; # 24 and # 47 which had been used to differentiate between Africanized bees and European bees, The *A. mellifera* probes were dot hybridized with *A. cerana* total DNA. Dot hybridization was performed by using labled *A. mellifera* probes # 24 and # 47 with *A. cerana* total DNA from five locations There was evident hybridization of the # 24 probe shown in Figure 19 (A) that they were homology. This result was shown like probe # 47 (Figure 19 (B)). Therefore, Southern hybridizations were obtained by individual total DNA of *A. cerana* from each location completely digested with restriction endonuclease *Eco*RI and hybridized to probes # 24 and # 47. After colorimetric detection was performed, the results of hybridization with probe # 47 were shown unclear bands with high background (Figure 20 (B)). The results with probe # 24 were shown in Figure 20 (A) that any bands were not appeared. Therefore the future study of Southern hybridization, *A. cerana* brobes were prepared and used to replace the *A. mellifera* probes.

4.8 Preparation of *A. cerana* probes

*Bgl*II which digested *A. cerana* total DNA was separated by 0.8% low melting agarose gel electrophoresis. The DNA fragment range from 5 to 7 kb and 2 to 4 kb (Figure 21), labeled as the first and second groups, were individual cut, purified and then ligated into the *Bam*HI site of the

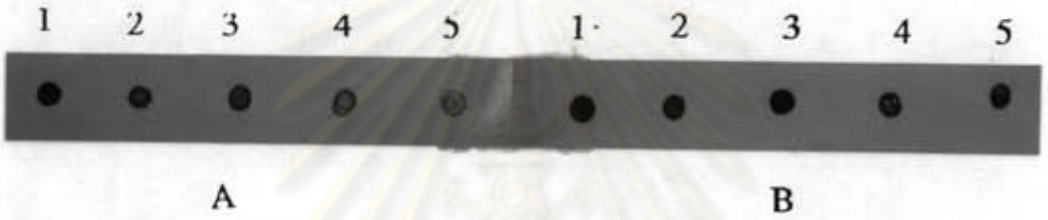


Figure 19 Dot hybridization between *A. mellifera* probes and total DNA of *A. cerana*.

Total DNA was extracted from individual worker pupae in the same colony. 500 ng DNA hybridized with # 24 probe (A) and # 47 probe (B) (about 10 ng/ml). The hybridization was performed at 65-68 °C for 12 hours and detected by colorimetric detection for 8 hours.

dot 1 : total DNA of *A. cerana* from the Northern

dot 2 : total DNA of *A. cerana* from the North-Eastern

dot 3 : total DNA of *A. cerana* from the Central part

dot 4 : total DNA of *A. cerana* from the Southern

dot 5 : total DNA of *A. cerana* from the Samui Island

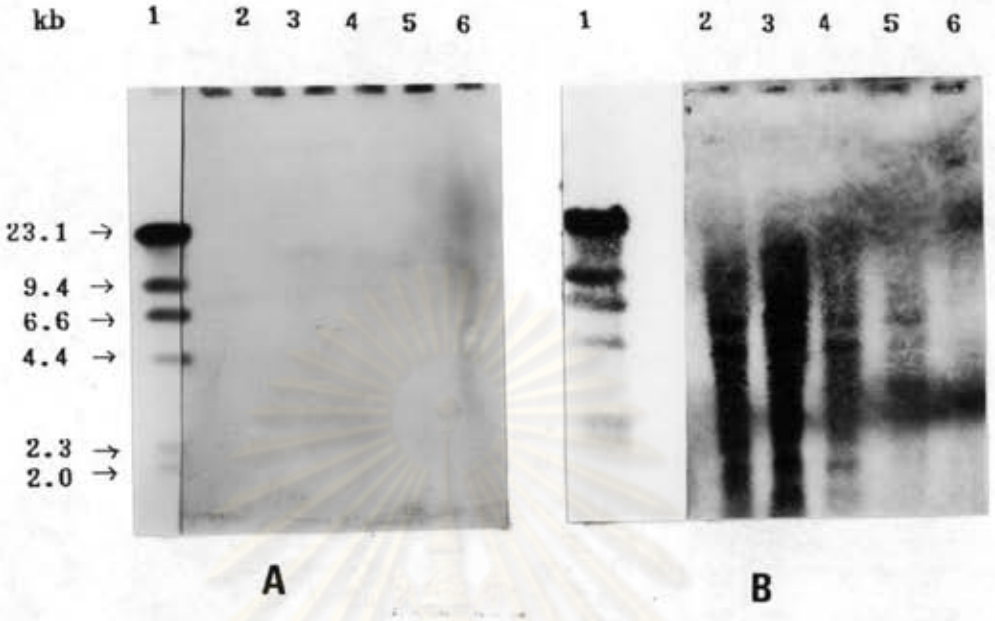


Figure 20 Southern hybridization between *A. mellifera* probes and total DNA of *A. cerana* digested with *Eco*RI.

Total DNA was extracted from individual worker pupae in the same colony. 2 μg DNA digested with 10 U of *Eco*RI and hybridized with # 24 probe (A) and # 47 probe (B) (about 10 ng/ml). The hybridization was performed at 65-68 °C for 12 hours and detected by colorimetric detection for 8 hours.

lane 1 : λ/*Hind*III DNA standard

lane 2 : total DNA of *A. cerana* from the Northern

lane 3 : total DNA of *A. cerana* from the North-Eastern

lane 4 : total DNA of *A. cerana* from the Central part

lane 5 : total DNA of *A. cerana* from the Southern

lane 6 : total DNA of *A. cerana* from the Samui Island

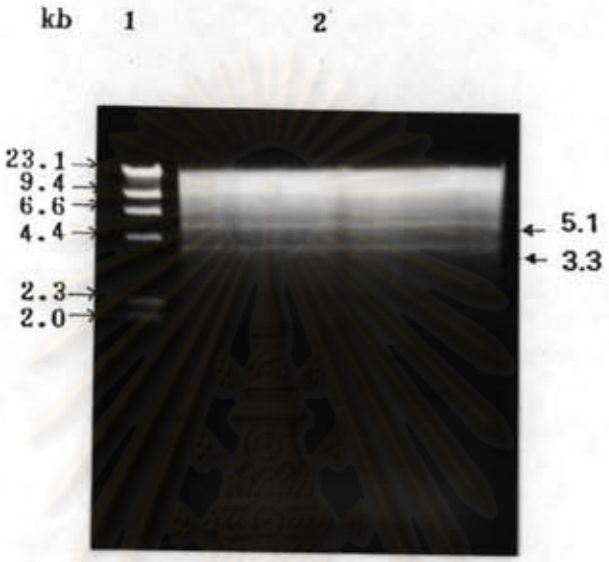


Figure 21 Agarose gel electrophoretic staining pattern of *Bgl*II completely digested total DNA of *A. cerana* for probe preparation.

Total DNA was extracted from individual worker pupae, 4 μg DNA digested with 20 U of *Bgl*II. Electrophoresis was performed on 1.0% low melting agarose at 50 V for hours and a half, and stained with 2.5 μg/ml of ethidium bromide;

lane 1 : λ/*Hind*III DNA standard

group 2 : total DNA of *A. cerana* digested with *Bgl*II

dephosphorylated plasmid pBR322. The recombinant plasmids were transformed into competent *E. coli* treated with $CaCl_2$ and grown on ampicillin-LB plate. According to the insertion inactivation (Ap^rTe^s), the numbers of recombinant plasmid clones from the first and second groups were 62 and 135 respectively. Both recombinant plasmid groups were then extracted by the minipreparation method and digested with *Pst*I and analysed by 0.7% agarose gel electrophoresis. The size of recombinant plasmids which is larger than 4.36 kb, directly confirmed recombination. Then, 7 and 6 clones of their groups, respectively, were randomly dot hybridized with the *A. cerana* chromosomal DNA labeled probe in order to selected the highly intense signal of the recombinant plasmids (Figure 22). The results of clones refered to # 5008, # 5043, # 3018, # 3035, # 3047 and # 3111 gave intense signals and their sizes were approximately 9.8, 9.8, 7.0, 7.0, 7.0 and 7.0 kb respectively (Figure 23). Therefore, these clones would be used as DNA probes for subsequent experiments. In order to be used as DNA probes, the recombinant plasmid DNAs carrying vector pBR322, the vector pBR322 had to previously proved not hybridized with *A. cerana* total DNA.

To confirm not having a previous hybridization with *A. cerana* total DNA of vector pBR322, the DNA of individual samples from different locations of Thailand, except from the Samui Island, were then digested with *Eco*RI and bounded on nylon membrane, and hybridized with pBR322 probe. The result as shown in Figure 24 (A) indicated that only positive controls which were pBR322 and recombinant plasmid # 3035. The intense bands were appeared as both DNA samples and

Figure 22 Dot hybridization between *A.cerana* genomic DNA probes and recombinant plasmid DNA.

Five hundred nanograms of recombinant DNA from randomly selected clones were spotted and hybridized with genomic DNA of *A. cerana* probe (about 10 ng/ml). The hybridization was performed at 65-68 °C for 12 hours and detected by chemiluminescence for 20 minutes;

row 1 no. 1-12 : I_{18}, # 5001, # 5004, # 5005 # 5007, # 5008, # 5024, # 5025, # 5037, # 5039, # 5040, # 5042, respectively

row 2 no. 1-12 : # 5043, # 5051, # 5058, # 5059, # 5060, # 5062, # 3001, # 3018, # 3027, # 3035, # 3037, # 3111 , respectively

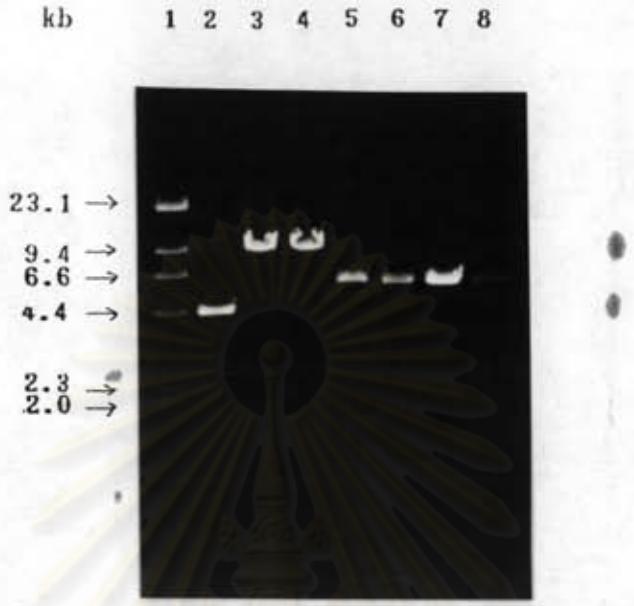


Figure 23 Agarose gel electrophoretic staining pattern of recombinant DNAs digested with *Pst*I.

Five hundred nanograms of recombinant plasmid DNA digested with 5 U of *Pst*I. Electrophoresis was performed on 0.7% agarose at 80 V for 3 hours and stained with 2.5 μg/ml of ethidium bromide;

lane 1 : λ/*Hind*III DNA standard

lane 2 : pBR322

lane 3 : # 5008

lane 4 : # 5043

lane 5 : # 3018

lane 6 : # 3035

lane 7 : # 3047

lane 8 : # 3111

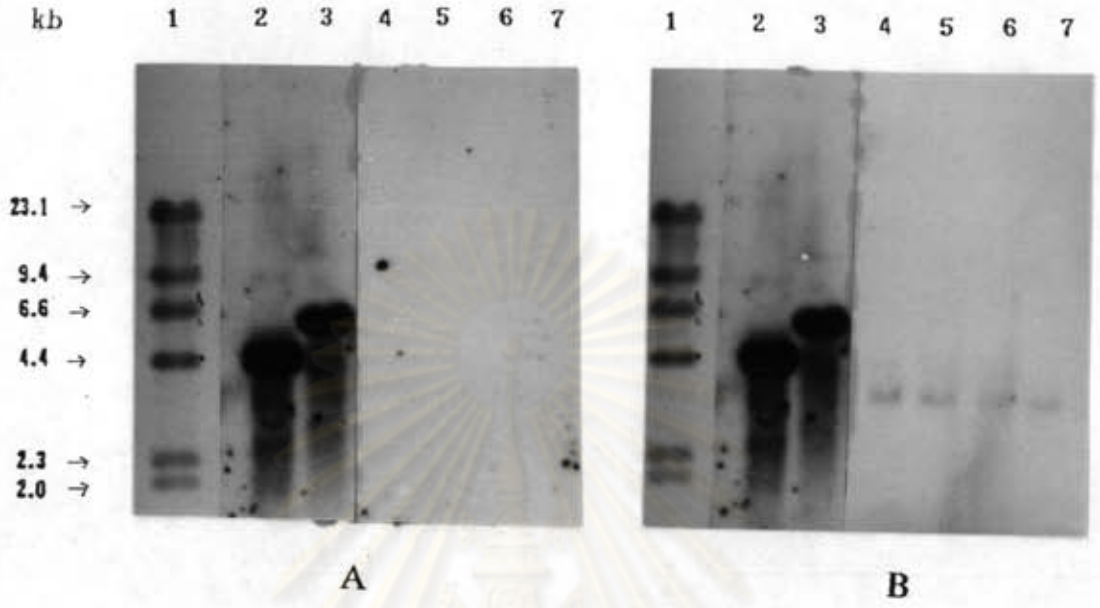


Figure 24 Southern hybridization between pBR322 probe (A) and # 3035 (B) and total DNA of *A. cerana* digested with *Eco*RI.

Two micrograms of total DNA of *A. cerana* were digested with 10 U of *Eco*RI, 500 ng of pBR322 and # 3035 digested with 5 U of *Pst*I and hybridized with pBR322 labeled probe (A) and # 3035 probe (B) (about 10 ng/ml). The hybridized was performed at 65-68 ^{o}C for 12 hours and detected by chemiluminescence for 20 minutes;

lane 1 : λ/*Hind*III DNA standard

lane 2 : pBR322 digested with *PSt*I

lane 3 : # 3035 digested with *Pst*I

lane 4 : total DNA of *A. cerana* from the Northern

lane 5 : total DNA of *A. cerana* from the North-Eastern

lane 6 : total DNA of *A. cerana* from the Central part

lane 7 : total DNA of *A. cerana* from the Southern

positive controls when vector pBR322 probe had beeen removed and rehybridized with probe # 3035 (Figure24 (B)).

Approximately one microgram of recombinant plasmid DNAs; # 5008, # 5043, # 3047, # 3035, # 3111 and # 3018 were then labeled with the Genius nonradioactive labeling system: random primed DNA labeling with DIG-dUTP. Finally, the labeled DNA concentrations were obtained at 1, 10, 100, 10, 10 and 1 ng/μl respectively (Figure 25).

Since the *A. cerana* DNA probes were prepared using 5 to 7 and 2 to 4 kb of total DNA of *A. cerana* from the Samui Island digested with *Bgl*II. Therefore probe # 3035 from the second group was randomly selected as DNA probe to hybridize with total DNA of *A. cerana* from Samui Island. The results was shown in figure 26 as the approximately 3 kb and 20 kb of intense bands were appeared, and confirmly indicated that this probe was the DNA fragment groups of 2 to 4 kb of *A. cerrana* total DNA.

4.9 Screening of suitable restriction endonucleases and DNA probes for Southern hybridization

The Southern hybridization analysis of *A. cerana* total DNA was previously determinded by using some of restiction endonucleases; *Bgl*II, *Hae*III and *Eco*RI being digested with the DNA. After being Southern transfered, *A. cerana* total DNA was hybridized with the recombinant plasmid probe in order to select the suitable of restriction endonucleases and DNA probes for future analysis.

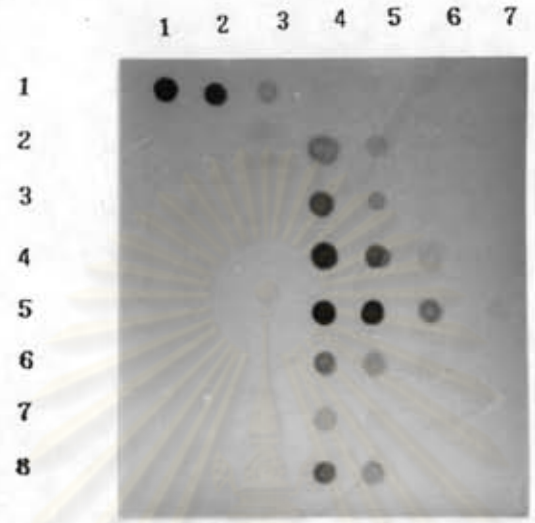


Figure 25 The yield estimated of tenfold dilution series of DIG-labled probes.

One microlitre of tenfold dilution of digoxigenated DNA probes spotted onto the nylon membrane and the signal intensities were visually compared with the labeled control DNA from the kit. The labeled control DNA was started from 4, 1, $1x10^{-1}$ dilution to the $1x10^{-6}$ μg/μl. Spotting was duplicated (A and B);

row 1 : control

row 2 : # 5008

row 3 : # 5043

row 4 : # 3047

row 5 : # 3035

row 6 : # 3111

row 7 : # 3018

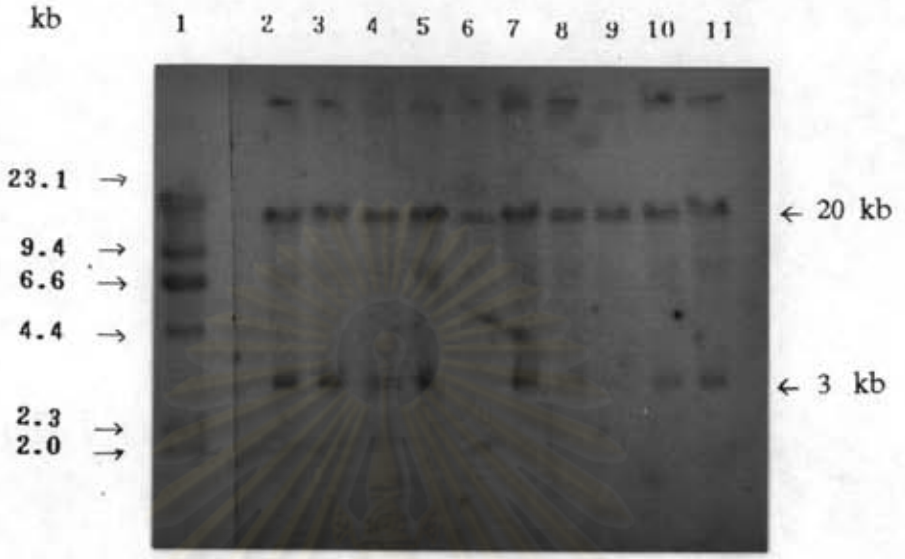


Figure 26 Southern hybridization between *A. cerana* probe # 3035 and total DNA of *A. cerana* from Samui Island digested with *Bgl*III.

Total DNA was extracted from individual worker pupae in the different colonies. 2 μg DNA digested with 10 U of *Bgl*II and hybridized with # 3035 (10 ng/ml). The hybridization was performed at 65-68 °C for 12 hours and detected by chemiluminescence for 20 minutes;

lane 1 : λ/*Hind*III DNA standard

lane 2-11 : total DNA of *A. cerana* from the Samui Island

From Figure 27, the results of hybridization with probe # 3018 showed that the total DNA of *A. cerana* digested with *Hae*III gave non simple intense bands. As a result, the analysis could not be determined. Whereas, the total DNA digested with *Eco*RI gave the clear and simple bands which could be useful for the Southern hybridization of the consequent experiments.

The Southern hybridization between total DNA of *A. cerana* digested with *Eco*RI and each probes (# 5008, # 5043, # 3018, # 3035, # 3047 and # 3111) were performed. The good experiment was considered from the characteristic of the bands. From experiments of various probes, # 3035 gave the clear and discrete bands (Figure 28). Therefore # 3035 probe was selected as the suitable probe for the future study.

4.10 Southern hybridization from total DNA of *A. cerana* from the same colony by *A. cerana* probe

Ten total DNAs sample extracted from ten individual pupae from the same colony of *A. cerana* from two locations of Thailand; the North-Eastern and the Southern were tested. Two micrograms of individual bee DNA were digested with 10 U of restriction endonuclease *Eco*RI. After having electrophoresed DNA was then bounded onto nylon membrane and hybridized with # 3035 probe (10 ng/ml). The results showed that they were all similar pattern if they are from the same colony (see Figure 29 A. and 30 A.). The results from # 3018 was also a specific pattern which was similar to the patterns of the same colony.

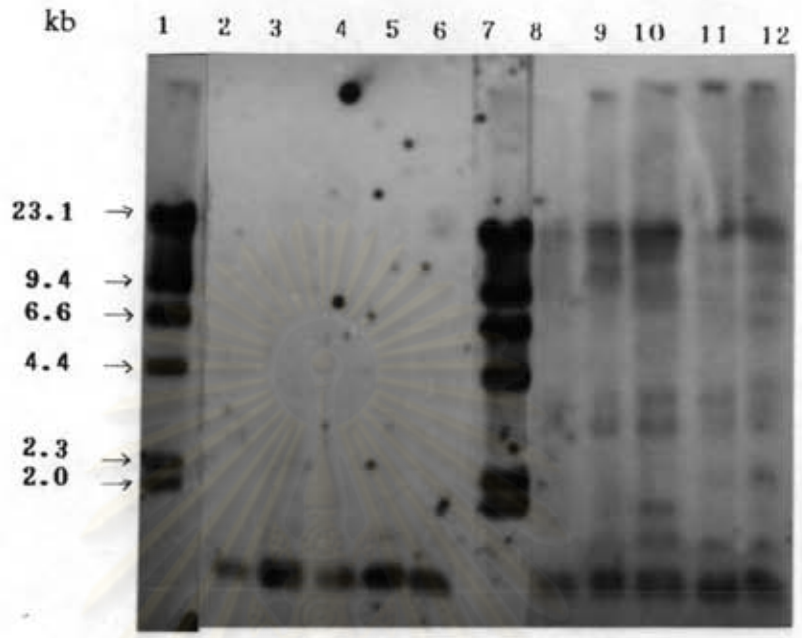


Figure 27 Southern hybridization between *A. cerana* probe # 3018 and total DNA of *A. cerana* digested with various restriction endonucleases.

Total DNA was extracted from individual worker pupae in different colonies. 2 μg DNA digested with 10 U of *Hae*III and *Eco*RI, hybridized with # 3018 probe (10 ng/ml). The hybridization was performed at 65-68 ^{o}C for 12 hours and detected by chemiluminescence for 20 minutes;

lane 1 : λ/*Hind*III DNA standard

lane 2-6 : total DNA of *A. cerana* digested with *Hae*III

lane 7 : total DNA of λ/*Hind*III DNA standard

lan 8-12 : total DNA of *A. cerana* digested with *Eco*RI

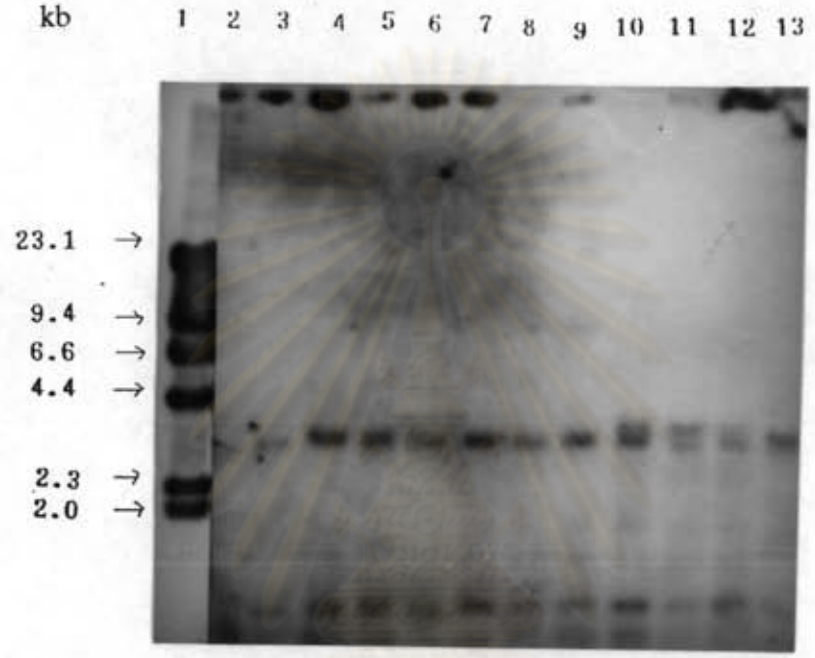


Figure 28 Southern hybridization between *A.cerana* probe # 3035 and total DNA of *A. cerana* digested with *Eco*RI.

Total DNA was extracted from individual worker pupae in different colonies. 2 μg DNA digested with 10 U of *Eco*RI and hybridized with # 3035 probe (10 ng/ml). The hybridization was performed at 65-68 ^{o}C for 12 hours and detected by chemiluminescence for 20 minutes;

lane 1 : λ/*Hind*III DNA standard

lane 2-5 : total DNA of *A. cerana* from the North-Eastern

lane 6-8 : total DNA of *A. cerana* from the Central part

lane 9 : λ/*Hind*III DNA standard

lane 10-13 : total DNA of *A. cerana* from the Northern

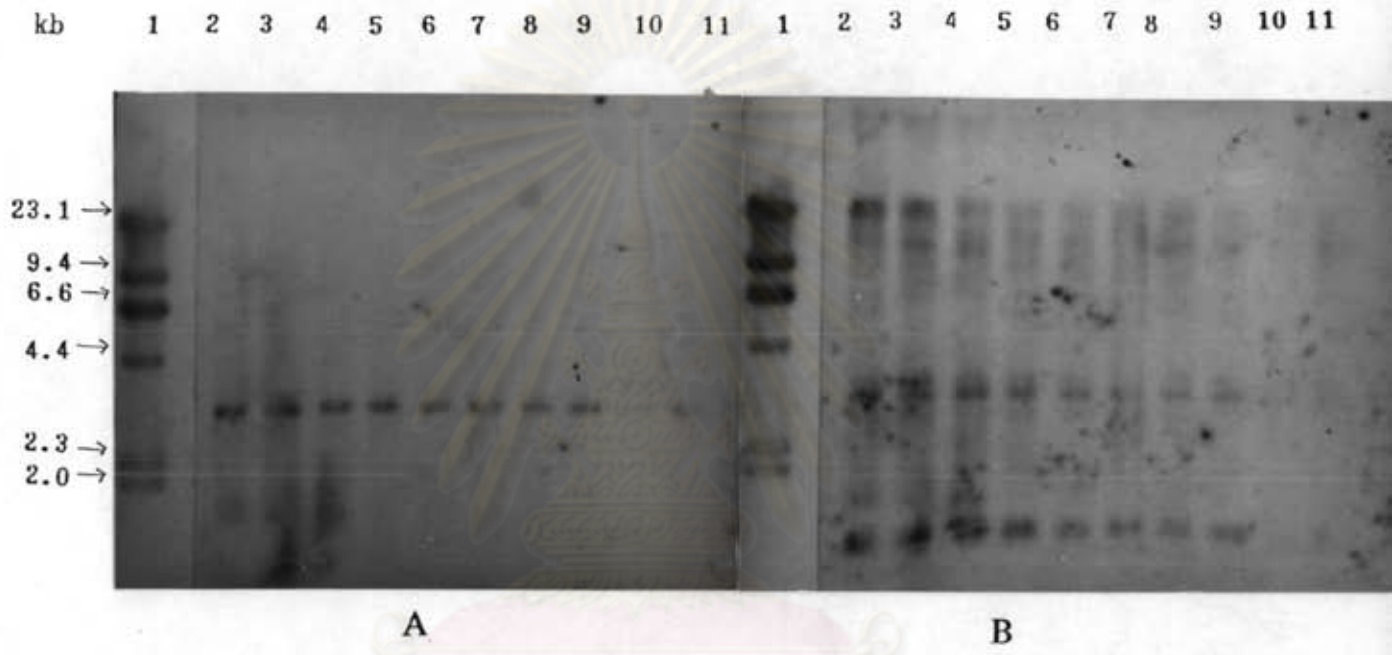


Figure 29 Southern hybridization between *A. cerana* probe # 3035 (A), probe # 3018 (B) with DNA of *A. cerana* within a colony from the North-Eastern digested with *EcoR*I.

Total DNA was extracted from individual worker pupa in the same colony, 2 μg DNA digested with 10 U of *EcoR*I, hybridized with # 3035 and # 3018 probe (10 ng/ml). The hybridization was performed at 65-68 ^{o}C for 12 hours and detected by chemiluminescence for 20 minutes;

lane 1 : λ/*Hind*III DNA standard

lane 2-11 : total DNA of *A. cerana* from the North-Eastern

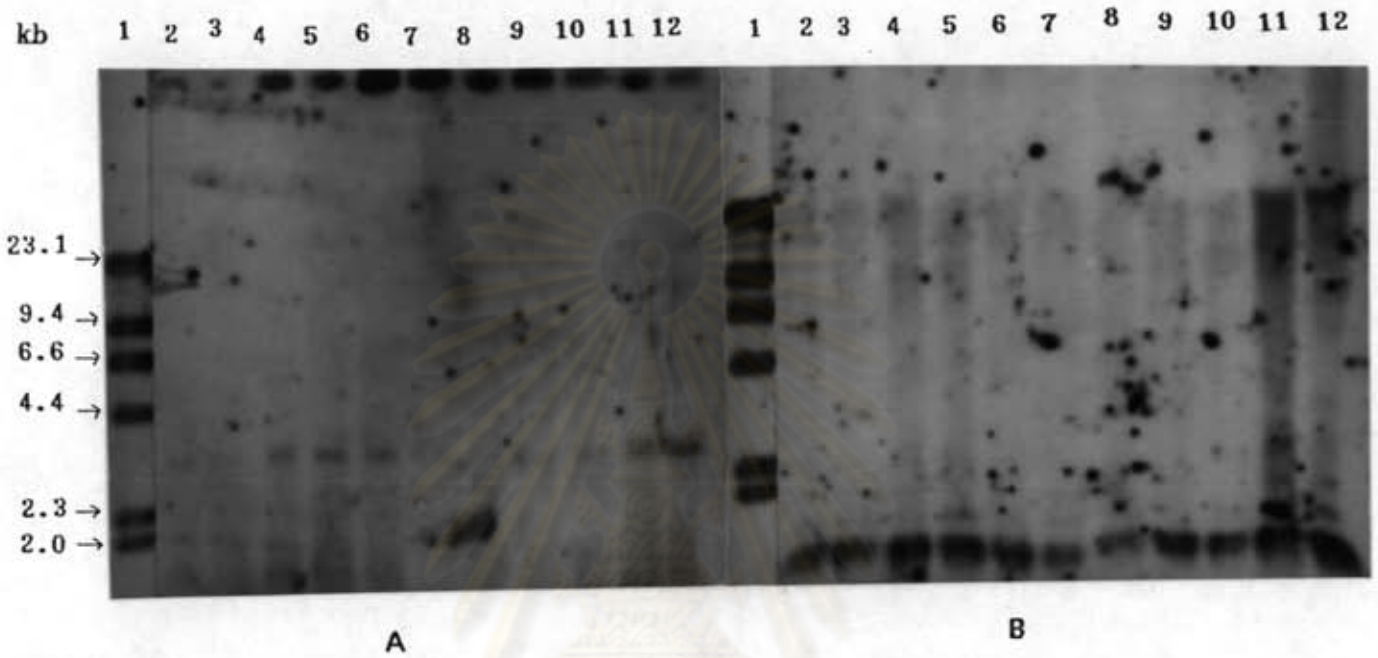


Figure 30 Southern hybridization between *A. cerana* probe # 3035 (A), probe # 3018 (B) with DNA of *A. cerana* within a colony from the Southern digested with *Eco*RI.

Total DNA was extracted from individual worker pupae in the same colony. 2 μg DNA digested with 10 U of *Eco*RI and hybridized with # 3035 and # 3018 probe (10 ng/ml). The hybridization was performed at 65-68 ^{o}C for 12 hours and detected by chemiluminescence for 20 minutes;

lane 1 : λ/*Hind*III DNA standard

lane 2-11 : total DNA of *A. cerana* from the Southern

4.11 Southern hybridization analysis of *A.cerana* total DNA from different locations

About twenty individual DNA samples from each of 20 colonies per location were digested with restiction endonuclease *Eco*RI (2 μg/10 U) following with Southern blot then hybridized with probe # 3035 as described in section 4.10. After chemiluminescent detection, each of DNA samples from 5 locations was compared base on the major of intense bands. The result was shown in Figure 31-35 that the results from the Samui Island were different from the other locations in that the major intense band of honey bees was presented at 2.1 kb whereas, the others were 3.1 kb. In addition, the minor intense bands presented at the different position could divide the DNA samples into nine groups. DNA samples from Samui Island appeared three groups in the following of intense bands; namely group VII) 2.1 kb; VIII) 2.1 and 2.2 kb; IX) 2.1, 2.2 and 5.5 kb (figure 35). The percentages of groups were 72.2, 22.2, and 5.6 respectively. In addition, the minor intense bands could divide DNA samples into six groups from four locations except the Samui Island. The results demonstrated that, The Northern were clasified into five groups (Figure 31) as following groups I) 3.1 kb; II) 3.1 and 3.3 kb; III) 3.1, 4.3 and 585 kb and IV) 3.1, 5.1, 6.2 and 12.8 kb at the percentages of 30.0, 20.0, 30.0, 15.0 and 5.0 respectively. The North-Eastern bees presented only group I (Figure 32). While the Central part bees were divided into three groups; I, II and VI; as the intense bands of group VI appeared at 3.1, 3.3 and 5.1 kb, with the percentages of 72.2, 22.2 and 5.6 respectively (Figure 33). Finally, Figure 34 showned the Southern which were classified only as group I. The results were summarized in Table 8.

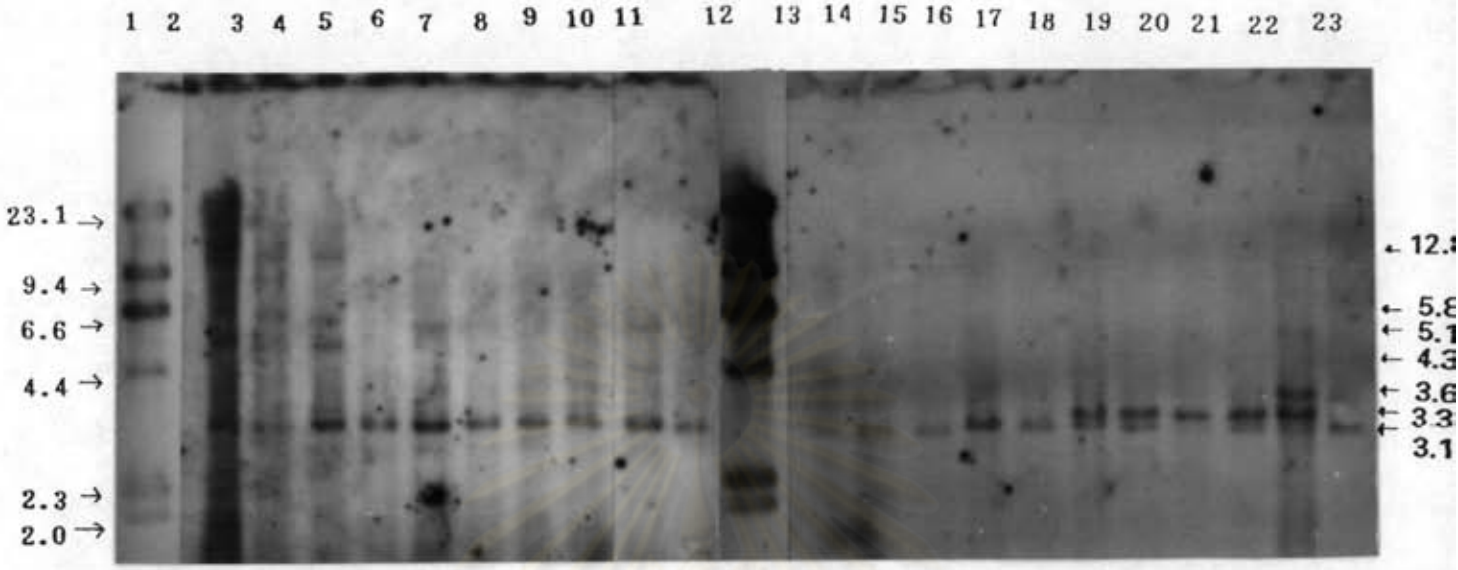


Figure 31 Southern hybridization between *A. cerana* probe # 3035 and total DNA of *A. cerana* from the Northern digested with *Eco*RI.

Total DNA was extracted from individual worker pupae in different colonies. 2 μg DNA digested with 10 U of EcoRI and hybridized with # 3035 probe (10 ng/ml). The hybridization was performed at 65-68 °C for 12 hours and detected by chemiluminescence for 20 minutes;

lane 1 : λ/*Hind*III DNA standard

lane 2-11 : total DNA of *A. cerana* from the Northern;
N_2, N_3, N_4, N_5, N_6, N_7, N_8, N_9, N_{10}, N_{11}, N_{12}

lane 12 : λ/*Hind*III DNA standard

lane 13-23 : total DNA of *A. cerana* from the Northern;
N_{13}, N_{14}, N_{15}, N_{17}, N_{18}, N_{19}, N_{20}, N_{21},
N_{22}, N_{23}, N_{24}

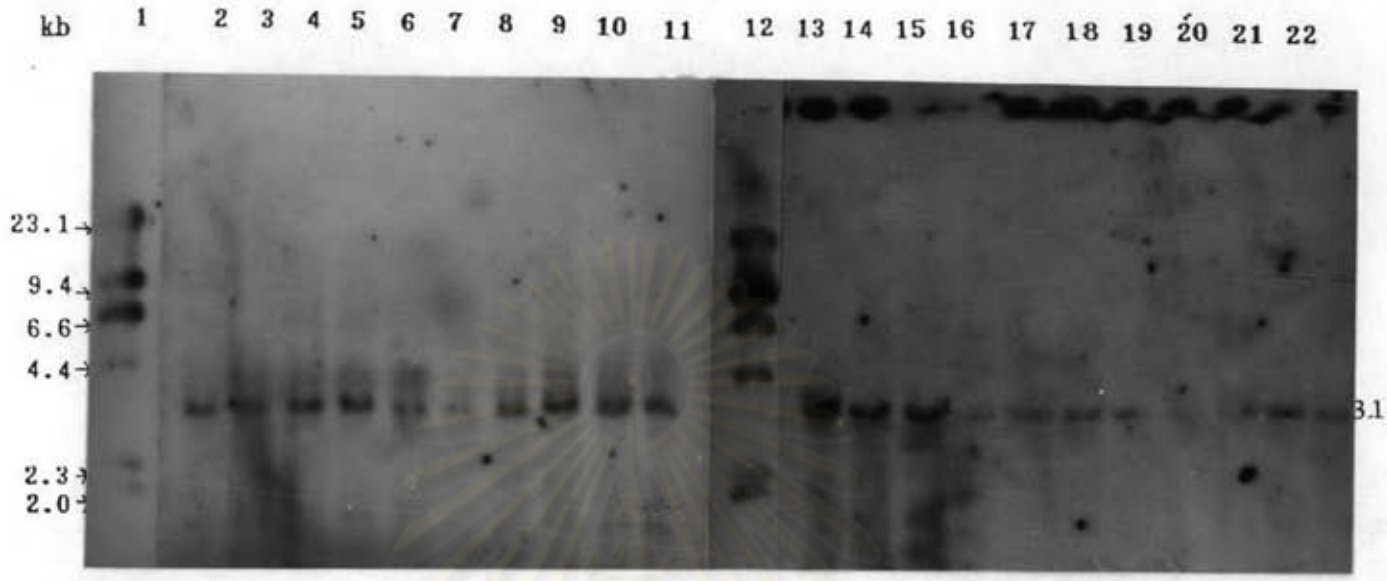


Figure 32 Southern hybridization between *A. cerana* probe # 3035 and total DNA of *A. cerana* from the North-Eastern digested with *Eco*RI.

Total DNA was extracted from individual worker pupae in different colonies. 2 μg DNA digested with 10 U of EcoRI and hybridized with # 3035 probe (10 ng/ml). The hybridization was performed at 65-68 °C for 12 hours and detected by chemiluminescence for 20 minutes;

lane 1 : λ/*Hind*III DNA standard

lane 2-11 : total DNA of *A. cerana* from the North-Eastern; E_1, E_2, E_3, E_4, E_5, E_6, E_7, E_8, E_9, E_{10}

lane 12 : λ/*Hind*III DNA standard

lane 13-22 : total DNA of *A. cerana* from the Northern-eastern; E_{11}, E_{12}, E_{13}, E_{14}, E_{15}, E_{17}, E_{18}, E_{19}, E_{20}, E_{21}, E_{22}

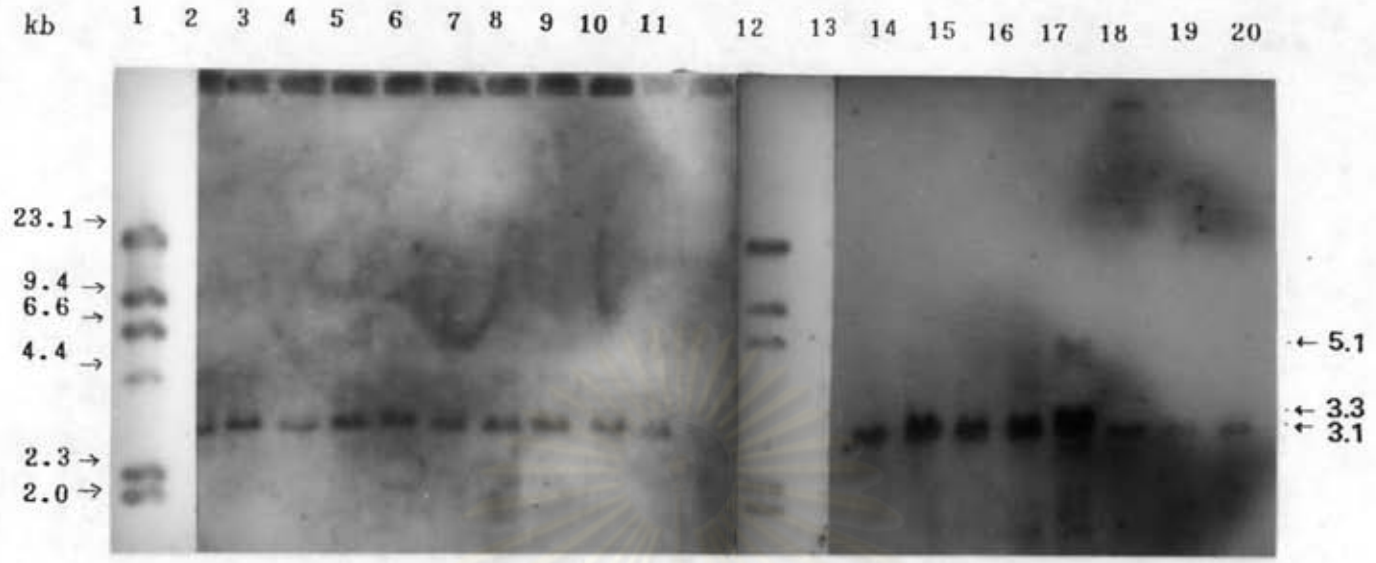


Figure 33 Southern hybridization between *A. cerana* probe # 3035 and total DNA of *A. cerana* from the Central part digested with *Eco*RI.

Total DNA was extracted from individual worker pupae in different colonies. 2 μg DNA digested with 10 U of EcoRI and hybridized with # 3035 probe (10 ng/ml). The hybridization was performed at 65-68 °C for 12 hours and detected by chemiluminescence for 20 minutes;

lane 1 : λ/*Hind*III DNA standard

lane 2-11 : total DNA of *A. cerana* from the Central part; C_{11}, C_{12}, C_{13}, C_{14}, C_{15}, C_{16}, C_{17}, C_{18}, C_{19}, C_{20}

lane 12 : λ/*Hind*III DNA standard

lane 13-20 : total DNA of *A. cerana* from the Central part; C_1 C_2, C_3, C_4, C_5, C_6, C_7, C_8

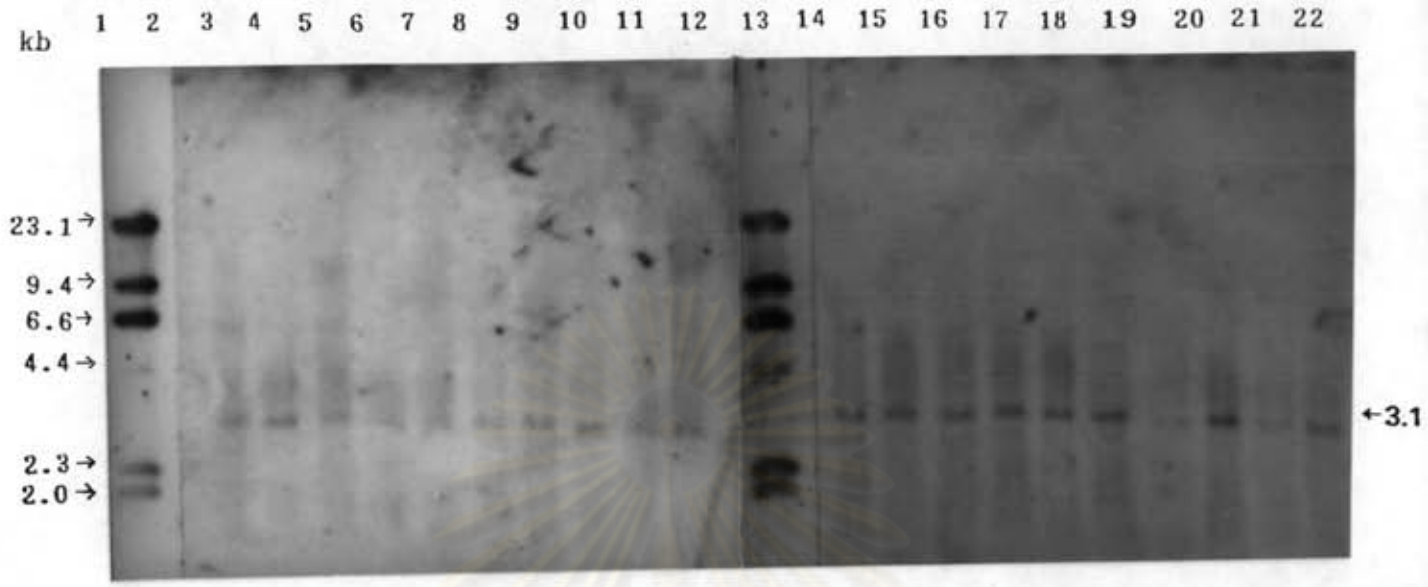


Figure 34 Southern hybridization between *A. cerana* probe # 3035 and total DNA of *A. cerana* from the Southern digested with *Eco*RI.

Total DNA was extracted from individual worker pupae in different colonies. 2 μg DNA digested with 10 U of EcoRI and hybridized with # 3035 probe (10 ng/ml). The hybridization was performed at 65-68 °C for 12 hours and detected by chemiluminescence for 20 minutes;

lane 1 : λ/*Hind*III DNA standard

lane 2-11 : total DNA of *A. cerana* from the Southern; S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_8, S_9, S_{10}

lane 12 : λ/*Hind*III DNA standard

lane 13-22 : total DNA of *A. cerana* from the Southern; S_{11}, S_{12}, S_{13}, S_{14}, S_{15}, S_{17}, S_{18}, S_{19}, S_{20}, S_{21}, S_{22}

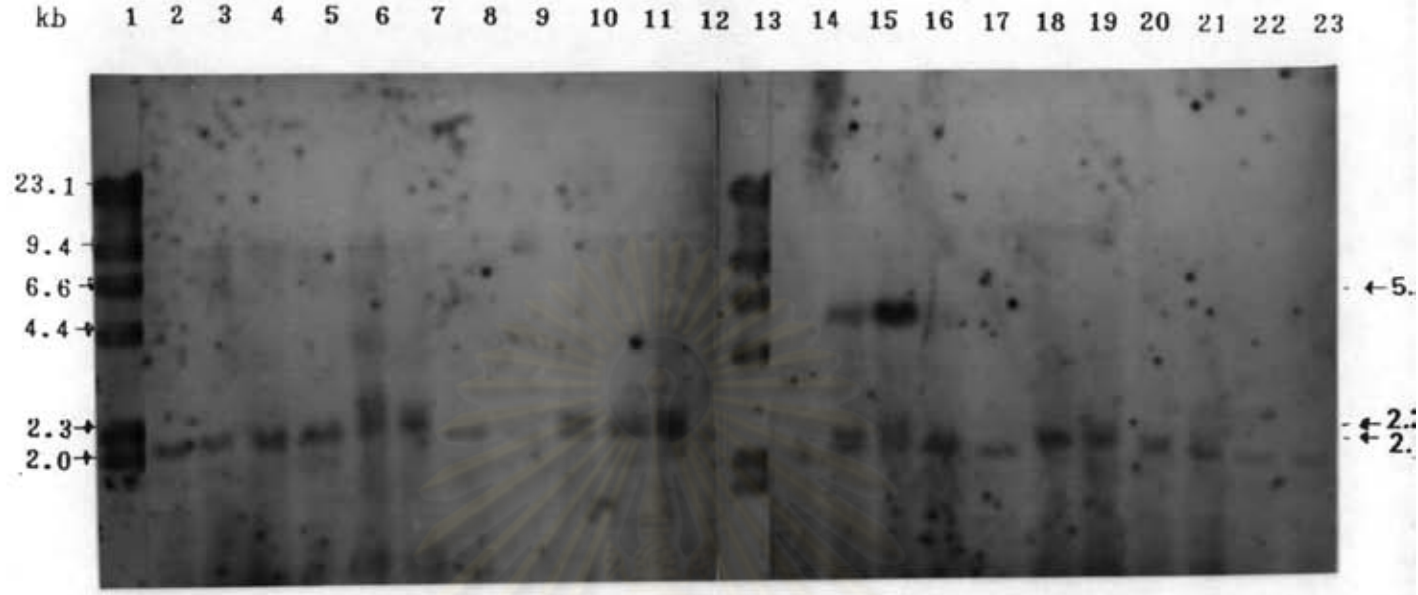


Figure 35 Southern hybridization between *A. cerana* probe # 3035 and total DNA of *A. cerana* from the Samui Island digested with *Eco*RI.

Total DNA was extracted from individual worker pupae in different colonies. 2 μg DNA digested with 10 U of EcoRI and hybridized with # 3035 probe (10 ng/ml). The hybridization was performed at 65-68 ^{o}C for 12 hours and detected by chemiluminescence for 20 minutes;

lane 1 : λ/*Hind*III DNA standard

lane 2-12 : total DNA of *A. cerana* from the Samui Island; I_1, I_2, I_4, I_{14}, I_{22}, I_{24}, I_{15}, I_{16}, I_{17}, I_{18}, I_{19}

lane 13 : λ/*Hind*III DNA standard

lane 14-24 : total DNA of *A. cerana* from the Samui Island; I_{20}, I_{13}, I_{13}, I_{21}, I_{23}, I_{25}, I_{26}, I_{27}, I_{16}, I_{17}

Table 8 Summary of Southern hybridization classification of *Eco*RI digested total DNA of *A. cerana* from five locations of Thailand.

Sampling location	No. of colony of total DNA	% Classification based on size of the intense bands (kb)								
		I	II	III	IV	V	VI	VII	VIII	IX
		3.1	3.1, 3.3	3.1, 4.3, 5.8	3.1, 5.1, 6.2, 12.8	3.1, 3.4, 3.6, 5.1	3.1, 3.3, 5.1	2.1	2.1, 2.2	2.1, 2.2, 5.5
the Northern	20	30.0[1a]	20.0[1b]	30.0[1c]	15.0[1d]	5.0[1e]	-	-	-	-
the North-Eastern	20	100.0	-	-	-	-	-	-	-	-
the Central part	18	72.2[2a]	22.2[2b]	-	-	-	5.6[2c]	-	-	-
the Southern	20	100.0	-	-	-	-	-	-	-	-
the Samui Island	18	-	-	-	-	-	-	72.2[3a]	22.2[3b]	5.6[3c]

1 Number of colony from the Northern 1a; N_5, N_{13}, N_{14}, N_{15}, N_{17}, N_{23} 1b; N_{18}, N_{19}, N_{20}, N_{21} 1c; N_6, N_7, N_8, N_9, N_{11}, N_{12} 1d; N_2, N_3, N_4 1e; N_{22}

2 Number of colony from the Central part 2a; C_6, C_7, C_8, C_9, C_{10}, C_{11}, C_{12}, C_{13}, C_{14}, C_{15}, C_{16}, C_{17}, C_{18} 2b ; C_1,C_2,C_3,C_4 2c; C_5

3 Number of colony from the Samui Island 3a; I_1, I_2, I_4, I_{14}, I_{15}, I_{16}, I_{18}, I_{20}, I_{21}, I_{23}, I_{25}, I_{26}, I_{27} 3b; I_{17},I_{19},I_{22},I_{24} 3c; I_{13}

Total DNA of *A. cerana* was extracted from individual worker of each colony and about 20 colonies for a location. The *Eco*RI 20 U digeted DNA about 2 μg. Electrophoresis was performed on 1.0% agarose at 80 V for 3 h. The λ phage DNA digested with *Hind*III was used as standard molecular weight marker. The results were repeated about 2 to 4 times by using different pupae from the same colony.