CHAPTER 3

RESULTS

3.1 Honeybees total DNA extraction

A total of 177 *A. cerana* individuals from six geographically locations in Thailand was investigated. Total DNA was extracted from these specimens using a modification of Garnery's method (Garnery *et al.*, 1993) As can be seen from Figure 3.1, high molecular weight DNA with the molecular length greater than 23 kb was usually obtained. Since the specimens were preserved in absolute ethanol, slighly sheared DNA was observed. RNA was also found but it did not interfere the success of PCR reactions. The amount of total DNA extracted from each *A. cerana* individual was approximately 3-4 μg. This quantity was sufficient for at least 50 PCR reactions.

3.2 Screening of appropriate primers for PCR amplification

Various primers at the control region were designed from sequences of *A. mellifera* mitochondrial genome as list in Table 2.1. Using different primer combinations, the expected sizes of DNA fragments was specifically amplified from *A. mellifera* (1517, 1633, 1865, 1805 and 2413 bp from primers AM3-AM11, AM5-AM12, AM6-AM9, AM6-AM11 and AM8-AM11 , respectively) (Figure 3.2). Other pairs of primers generated both the expected amplification band and non-specific products. Some of these primers did not yield any amplifications products. The five primer pairs described above were then used to amplified the control region in *A. cerana*. As can be seen in Table 3.1, only a combination of AM8-AM11 provided consistent amplification

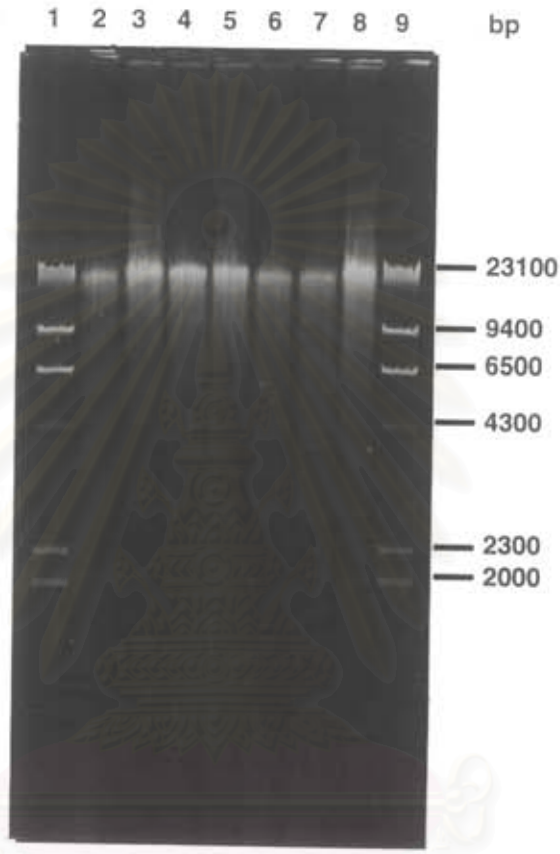


Figure 3.1 Total DNA extracted from *A. mellifera* and six *A. cerana* individuals

lanes 1,8 λ/*Hin*dIII DNA marker
lane 2 Total DNA isolated from *A. mellifera*
lanes 3-7 Total DNA isolated from six *A.cerana* individuals

Figure 3.2 The resulting PCR products amplified from *A. mellifera* total DNA using different combining primers

lanes 1,8 λ/*Hin*dIII DNA marker
lane 2 The product from primers AM6-AM9
lane 3 The product form primers AM6-AM11
land 4 The product form primers AM5-AM12
land 5 The product form primers AM3-AM11
land 6 The product form primers AM8-AM11
land 7 100 bp DNA marker

Table 3.1 Results for amplification of the control region using various combinations of primers listed in Table 2.1

H-strand primer	L-strand primer	Expected band size (bp)	PCR product	
			A. mellifera	*A. cerana*
AM1	AM9	1294	-	-
AM1	AM10	1277	-	-
AM1	AM11	1234	-	-
AM1	AM12	1223	-	-
AM2	AM9	1294	-	-
AM2	AM10	1277	-	-
AM2	AM11	1234	-	-
AM2	AM12	1223	-	-
AM3	AM9	1577	-	-
AM3	AM10	1560	-	-
AM3	AM11	1517	+	-
AM3	AM12	1506	-	-
AM4	AM9	1669	-	-
AM4	AM10	1652	-	-
AM4	AM11	1609	-	-
AM4	AM12	1598	-	-
AM5	AM9	1704	-	-
AM5	AM10	1687	-	-
AM5	AM11	1644	-	-
AM5	AM12	1633	+	-
AM6	AM9	1865	+	-
AM6	AM10	1848	-	-
AM6	AM11	1805	+	-
AM6	AM12	1794	-	-
AM7	AM9	2078	-	-
AM7	AM10	2061	-	-
AM7	AM11	2018	-	-
AM7	AM12	2007	-	-
AM8	AM9	2473	-	-
AM8	AM10	2456	-	-
AM8	AM11	2413	+	+
AM8	AM12	2402	-	-

- not amplified

\+ successfully amplified

results for both *A. cerana* and *A. mellifera* . Nevertheless, the PCR product (mtDNA fragment containing the control region) of *A. cerana* was 2750 bp in length which is slightly larger than that of *A. mellifera* (2560 bp). This PCR product will be called the control region.

3.3 Optimization of PCR conditions

Several important factors need to be optimized for reproducible amplification results. These are Mg^{2+} and primer concentrations as well as the amount of DNA template used.

3.3.1 $MgCl_2$ concentration

Different $MgCl_2$ concentration (1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mM) were examined for amplification effects at an annealing temperature of 55°C. The yields of PCR product were increased when $MgCl_2$ was elevated upto 4.0 mM $MgCl_2$ concentration. Increasing the concentration of $MgCl_2$ higher than this level did not yield better results (Figure 3.3). Therefore, the optimal concentration of $MgCl_2$ for this primer was chosen at 4.0 mM.

3.3.2 DNA template

A series of two fold increase in amount of DNA template (12.5, 25, 50, 100 ng) was used to optimize the PCR reaction using conditions described in 2.9.2. Amplification of the control region was successful for all template concentration used. Nevertheless, a concentration of template at either 50 or 100 ng yielded comparable amount of the product. As a result, the optimal concentration of DNA template for amplification of the control region in *A. cerana* was chosen at 50 ng (Figure 3.4).

Figure 3.3 Optimization of $MgCl_2$ concentration used for amplification of the control region of *A. cerana*

lanes 1,8 λ/*Hin*dIII DNA marker
lanes 2-7 The resulting PCR product when $MgCl_2$ of 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mM was included in the amplification reactions

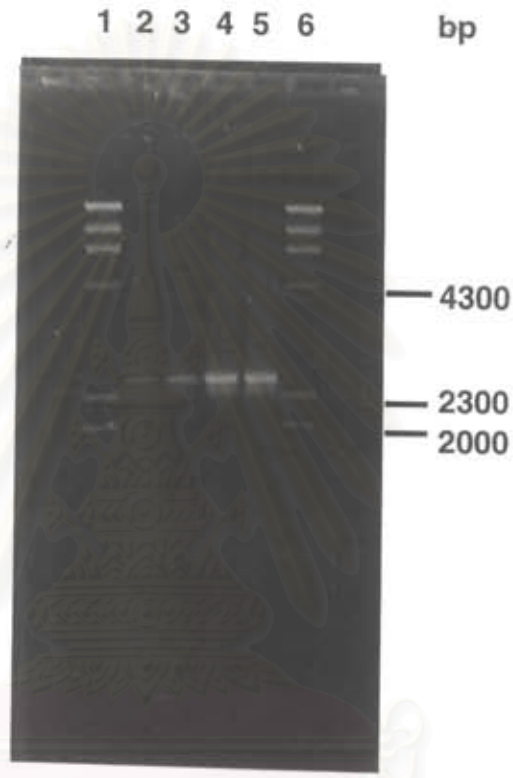


Figure 3.4 Optimization quantity of total DNA template used for amplification of the control region of *A. cerana*

lanes 1,6 λ/*Hin*dIII DNA marker
lanes 2-5 The resulting amplification product when different amounts of DNA templates (12.5, 25, 50 and 100 ng, respectively) were included in the PCR reactions

3.3.3 Concentration of primers

The optimal concentration of primers AM8 and AM11 was quantified using the standard conditions (2.9.2) with 50 ng of DNA template and 4.0 mM of $MgCl_2$. A faint control region product was observed at the concentrations of 0.1 μM of each primers. An increase of primer concentration to 0.2 μM yielded significant greater amount of the amplification product. Beyond this primer concentration, the PCR seemed to be amplified with equal efficiency (Figure 3.5).

Therefore, the optimal condition for amplification of control region in *A. cerana* was the standard condition described in 2.9.2 with 4.0 mM $MgCl_2$, 0.2 μM of each primer and 50 ng of DNA template. The PCR reactions were carried out in a Gene Amp System 2400 (Perkin-Elmer) using the thermal profile constituting of 2 min at 94°C for predenaturation followed by 35 cycles of a 94°C denaturation for 30 sec, a 50°C annealing for 1 min and a 72°C extension for 2.5 min. The final extension was performed at the same temperature for 7 min. This condition was appropriate for amplification of control region of *A. mellifera* and *A. cerana* (Figure 3.6).

3.4 Characterization of PCR product

An inner primer (5′-AAA ATA AAT AAA TCA GTG GTA-3′) was designed from the ND_2 gene region of the complete mtDNA sequence of *A. mellifera*. This was used to partially sequence of the 5′ region of amplified PCR products from *A. mellifera* and *A. cerana* (Figure 3.7). Approximately one hundred bases was obtained. The sequence was then compared with those previously deposited in the GenBank using Blast

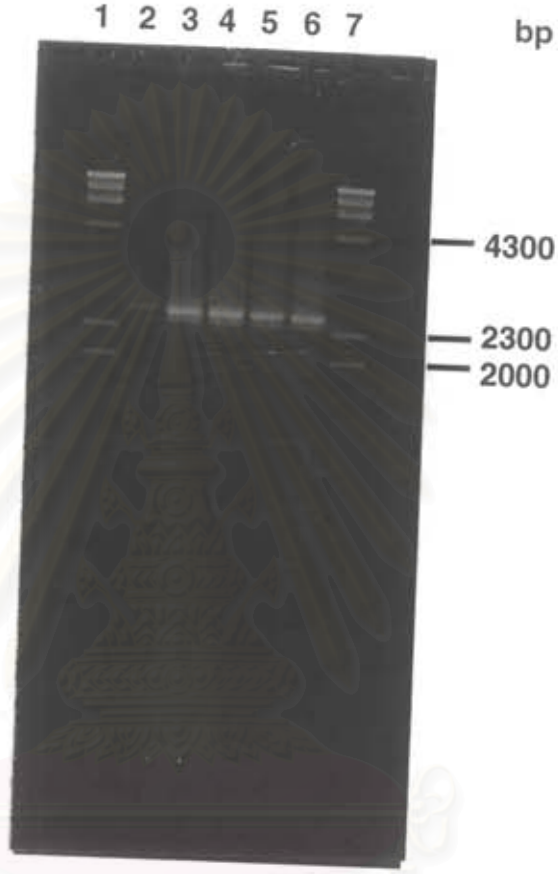


Figure 3.5 Optimization of primers concentration used for amplification of the control region of *A. cerana*

lanes 1,7 λ/*Hin*dIII DNA marker
lanes 2-6 The resulting PCR product when primers concentration at 0.1, 0.2, 0.3, 0.4 and 0.5 μM was included in the amplification reactions

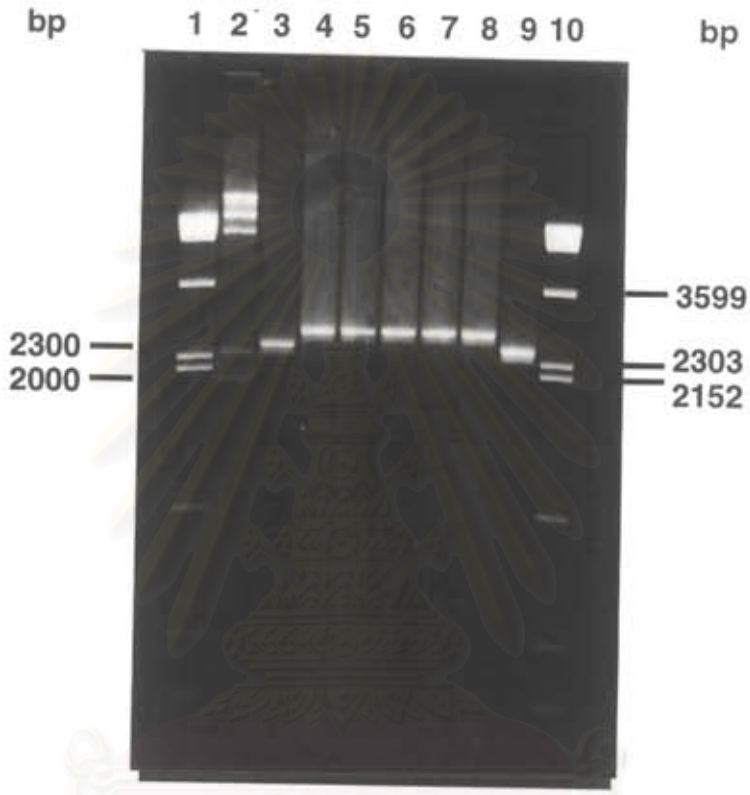
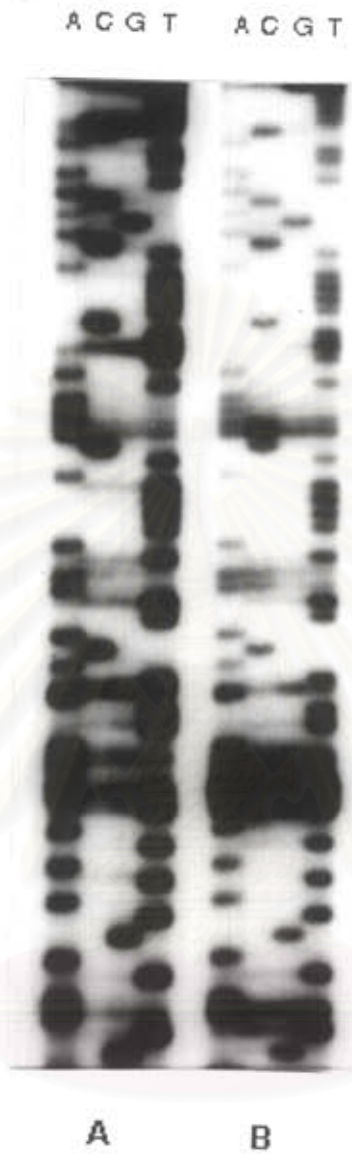


Figure 3.6 Control region from *A. mellifera* and *A. cerana* from six population in Thailand.

lanes 1,10 λ/*Dra*I DNA marker
lane 2 λ/*Hind*III DNA marker
lane 3 PCR amplified control region from *A. mellifera* (2560 bp)
lanes 4-8 PCR amplified control region from *A. cerana* (2750 bp)
lane 9 PCR amplified control region from the unidentified *Apis* species (S60)



A.

A. mellifera AAAATTTATN NNGAAGATAA GTAATAGTAT ATATAAAATT ATACATTAAT
ATTTTATCAA AATATTTCTT TTATCAGACA TATTACTTTT NNNAGATATA

B

A. cerana AAAATTTATN NNGAAGATAA GTAATAGTAT ATAAAAAATT ATACATTAAT
ATTTTATCAA AATATTTCTT TTATCAGACA TATTACTTTT TNNNGATATA

Figure 3.7 Nucleotide sequence in amplified control region of *A. mellifera* and *A. cerana*.

(Basic Local Alignment Search Tool). The results indicated that the 5′ sequence of PCR amplified products of both *A. mellifera* and *A. cerana* were homologous to ND_2 gene of *A. mellifera ligustica* (Crozier and Crozier, 1992). One hundred nucleotides of *A. mellifera* in this study was aligned with the complete mtDNA sequence of *A. mellifera ligustica*. The result from comparison of this 100 bases with a ND_2 sequence reported by Crozier and Crozier, 1992 revealed high simularity between then two sequences (>90%) (Figure 3.8). The PCR amplified product from *A. mellifera* was then subjected to restriction enzyme digestion using *Nde*I, *Swa*I, *Taq*I, *Hin*fI and *Hin*dIII. As can be seen from appendix F, the resulting digestion products were in consonant with those expected from the sequence of the same DNA portion in *A. mellifera ligustica* indicating that the PCR products obtained from both *Apis* species were orthologous fragments and can be further used for population genetic studies.

3.5 Analysis PCR products of control region by PCR-RFLP

The control region amplified from a total of 125 representative individuals (52 colonies did not yield PCR product) originating from six different geographic areas in Thailand (North, North/East, Central, South, Samui Island and Phuket Island) were digested with a panel of 15 restriction enzymes. Five restriction enzyme (*Eco*RI, *Kpn*I, *Sma*I, *Sau*3AI and *Bfr*I) did not digest the amplified product (Figure 3.9). Although, *Hin*dIII digested the control region of *A. mellifera* , no restriction site was available for that of *A. cerana* (Figure 3.10). Nine polymorphic restriction endonucleases were found. Four of which composing of *Dra*I, *Ase*I, *Ssp*I and *Hin*fI generated several digestion patterns. However,

A.

```
A.mellifera     GGATATTAGTTAATAAATAATAACATTTAAATTGCATTTAAAAATTAATATTTTATATAT
A.mell          ------------------------------------------------------------

A.mellifera     TATATCTAAAAAA-GTAATATGTCTGATAAAAGAAATATTTTGATAAAATATTAATGTAT
A.mell          TATATCTNNNAAAAGTAATATGTCTGATAAAAGAAATATTTTGATAAAATATTAATGTAT
                *******   *** *********************************************

A.mellifera     AATTTTATATATACTATTACTTATCTTCTTCATAAATTTTAAATACCACTGATTTATTTA
A.mell          AATTTTATATATACTATTACTTATCTTCNNNATAAATTTT--------------------
                ****************************   *********

A.mellifera     TTTTTTAATTACTATTTTTGTATTAATAATAAATTCCAATAATATTTTTATTCAATGAAT
A.mell          ------------------------------------------------------------
```

B.

```
A.mell          ---TATATCTNNNAAAAGTAATATGTCTGATAAAAGAAATATTTTGATAAAATATTAATG
A.cerana        ATATATATCNNNAAAAAGTAATATGTCTGATAAAAGAAATATTTTGATAAAATATTAATG
                   ******    ************************************************

A.mell          TATAATTTTATATATACTATTACTTATCTTCNNNATAAATTTT
A.cerana        TATAATTTTTTATATACTATTACTTATCTTCNNNATAAATTTT
                ********* ********************   *********
```

Figure 3.8 Comparisons of DNA sequence obtained from PCR amplified control region in this study with that from previously report using Clustal X

(A) Alignment of 5′ adjacent DNA sequence (located in ND_2) of *A. mellifera* published by Crozier and Crozier, 1992 and that from *A. mellifera* in Thailand (A. mell)

(B) Alignment of 5′ adjacent DNA sequence (in ND_2) between *A. mellifera* (A. mell) and *A. cerana* performed in the present study.

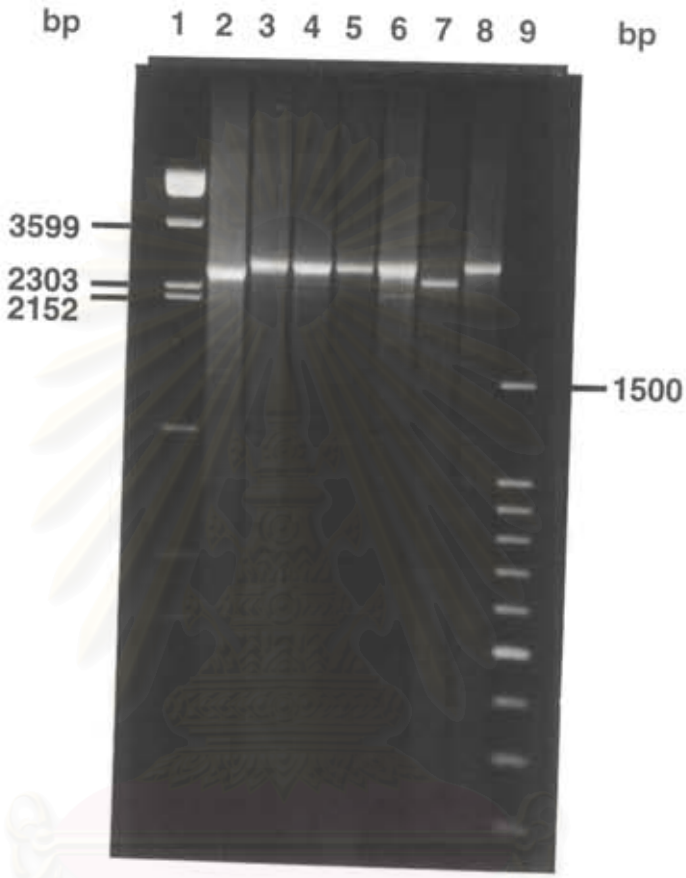


Figure 3.9 Agarose gel electrophoresis showing restriction fragment size of amplified control region of *A. cerana* digested with *Bfr*I. The results showed no restriction site for this restriction endonuclease

lanes 1,9	λ/*Dra*I and 100 bp DNA marker, respectively
lane 2	Undigested PCR product from *A. mellifera*
lanes 3-6,8	Undigested PCR products from *A. cerana*
lane 7	Undigested PCR product from unidentified *Apis* species (S60)

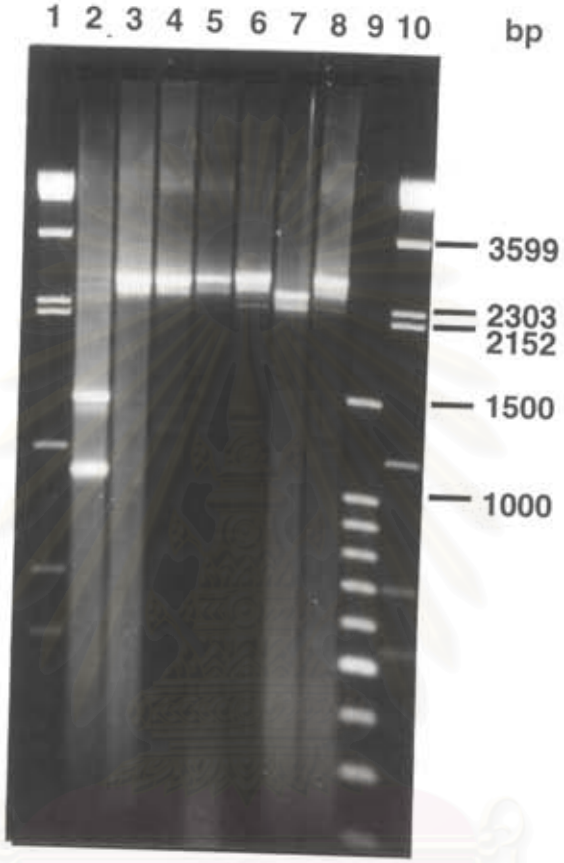


Figure 3.10 Agarose gel electrophoresis showing restriction patterns of the amplified control region of *A. mellifera* and *A. cerana* restricted with *Hin*dIII

lanes 1,10 λ/*Dra*I DNA maker
lane 2 Digested PCR product form *A. mellifera*
lanes 3-6,8 Uncut PCR products from *A. cerana*
lane 7 Uncut PCR product form unidentified *Apis* species (S60)
lane 9 100 bp ladder maker

the digestion fragments generated from *Ase*I, *Dra*I, and *Ssp*I (Figure 3.11-3.13) were too small to be unambiguously identified. In contrast, restriction patterns from *Hin*fI digestion were easy to score. The remaining restriction enzyme (*Swa*I, *Nde*I, *Alu*I, *Taq*I and *Rsa*I) produced lower number of digestion patterns (Figure 3.14-3.18). All except *Rsa*I digested PCR amplified control region from both *A. cerana* and *A. mellifera* as well as S60 whereas *Rsa*I did not digest that of *A. cerana* from the South and the unidentified *Apis* species (S60). Notably, the digestion pattern *A. mellifera* and S60 was not included for statistical due to length polymorphism of compared to that from *A. cerana*.

3.6 PCR-RFLP of amplified control region using *Taq*I, *Rsa*I and *Hin*fI

Three restriction endonuclease (*Taq*I, *Rsa*I and *Hin*fI) were selected to analysis the amplification control region of *A. cerana*.

3.6.1 PCR-RFLP analysis by *Taq*I

Two different haplotypes (A and B) were produced by *Taq*I digestion (Figure 3.17). Restriction profiles resulted from this enzyme digestion are shown by Table 3.2. The haplotype A and B were composed of three and two restriction fragments, respectively. A 2100 bp fragment was observed in both haplotype A and B. Considering restriction site loss, a single loss of restriction site generating 370 bp and 280 bp in haplotype A resulted in a 650 bp in haplotype B. All investigated specimens from North, North/East and Central possesed haplotype A. Whereas the Southern *A.cerana* had B haplotype.

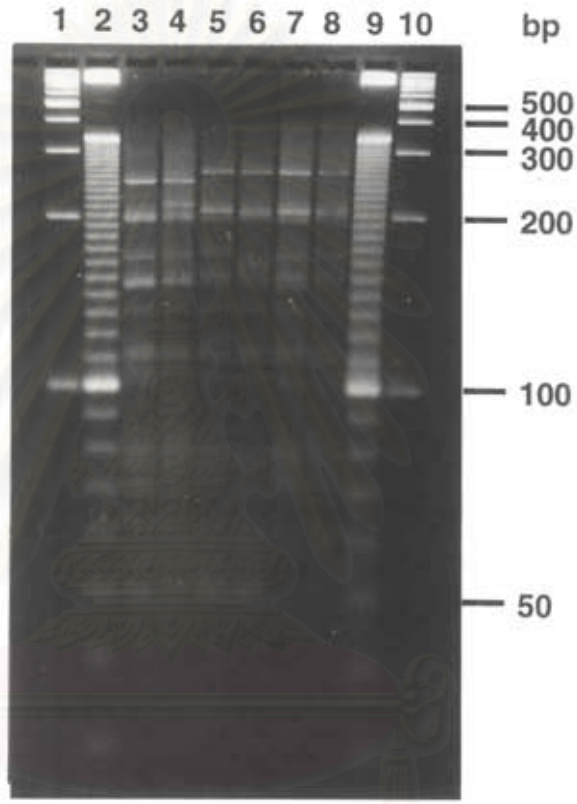


Figure 3.11 Agarose gel electrophoresis showing restriction patterns generated by digestion of control region of *A. cerana* with *Ase*I.

lanes 1,10 100 bp DNA marker
lanes 2,9 10 bp DNA marker
lanes 3-8 Digested PCR products from *A. cerana*

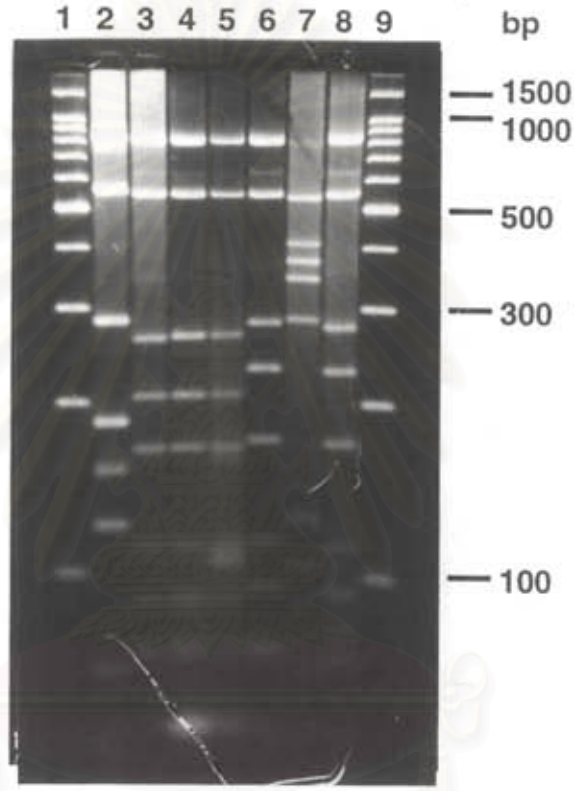


Figure 3.12 Agarose gel electrophoresis showing restriction patterns of amplified control region of *A. mellifera* and *A. cerana* digested with *Dra*I

lanes 1,9 100 bp DNA marker

lanes 2,7 Different of restriction profiles was observed in *A. mellifera* and the unidentified *Apis* species (S60), respectively.

lanes 3-6,8 Digested PCR product patterns from *A. cerana*

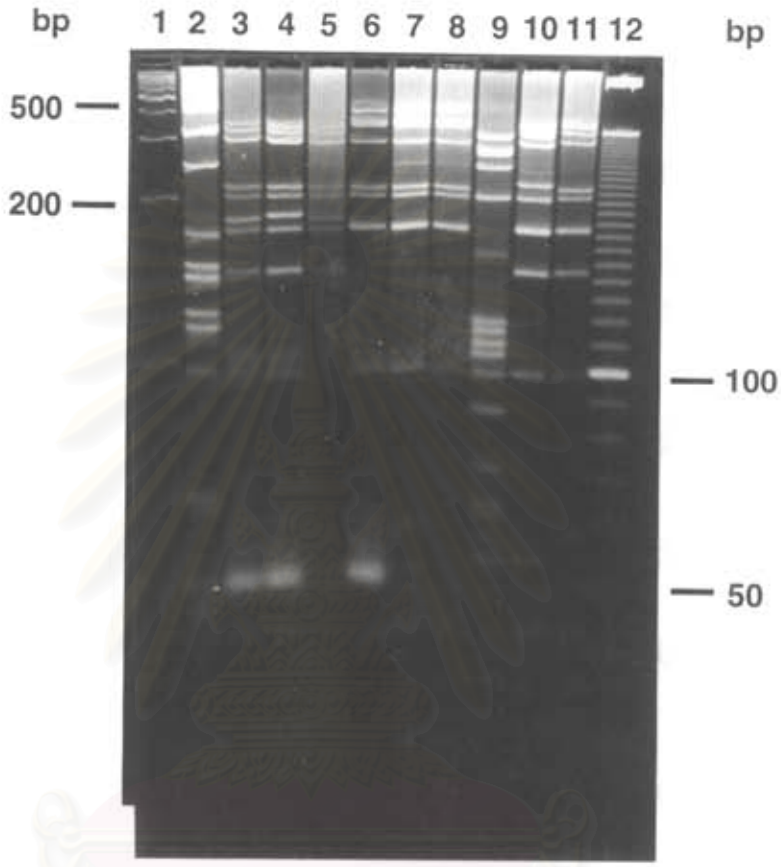


Figure 3.13 Agarose gel electrophoresis showing restriction patterns of amplified control region of *A. mellifera,* S60 and *A. cerana* digested with *Ssp*I

lane 1 100 bp DNA marker
lanes 2,9 Restriction PCR product patterns observed in *A. mellifera* and unidentified *Apis* species (S60), respectively.
lanes 3-8,10,11 Restriction PCR product patterns observed in *A. cerana*
lane 12 10 bp DNA marker

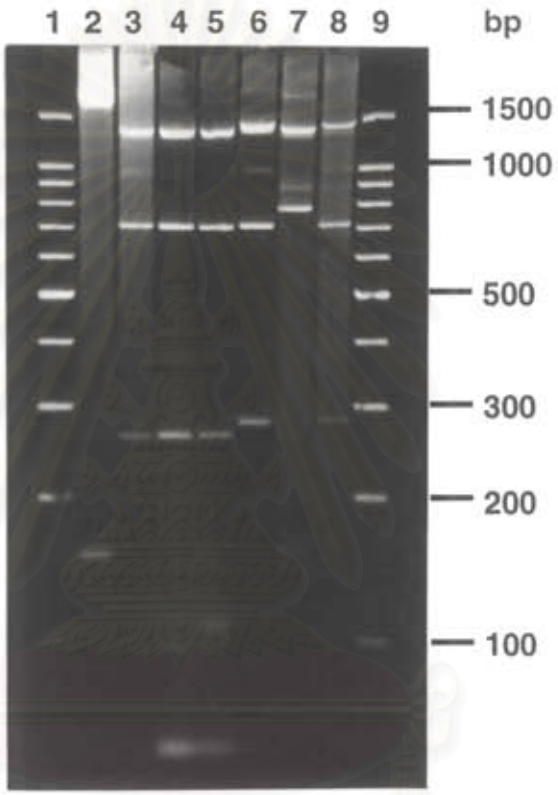


Figure 3.14 Agarose gel electrophoresis showing restriction patterns of amplified control region of *A. mellifera* and *A. cerana* digested with *Swa*I

lanes 1,9 100 bp DNA marker
lanes 2,7 Different restriction patterns of *A. mellifera* and S60, respectively.
lanes 3-6,8 Digested PCR products paterns of *A.cerana*

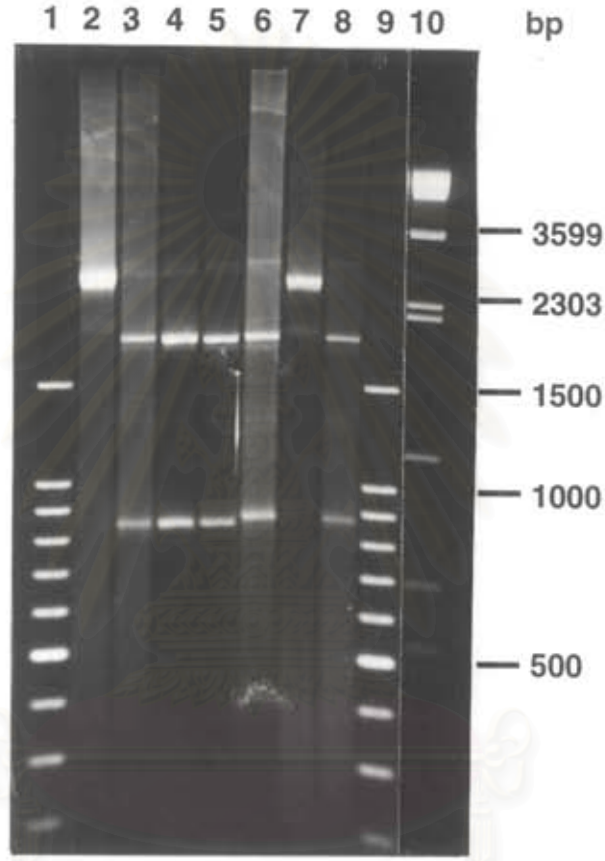


Figure 3.15 Agarose gel electrophoresis showing restriction patterns of amplified control region of *A. mellifera* and *A. cerana* digested with *Nde*I

lanes 1,9 100 bp DNA marker
lanes 2,7 Undigested PCR product from *A. mellifera* and unidentified *Apis* species (S60), respectively.
lanes 3-6,8 Digested PCR product patterns from *A. cerana*
lane 10 λ/*Dra*I DNA marker

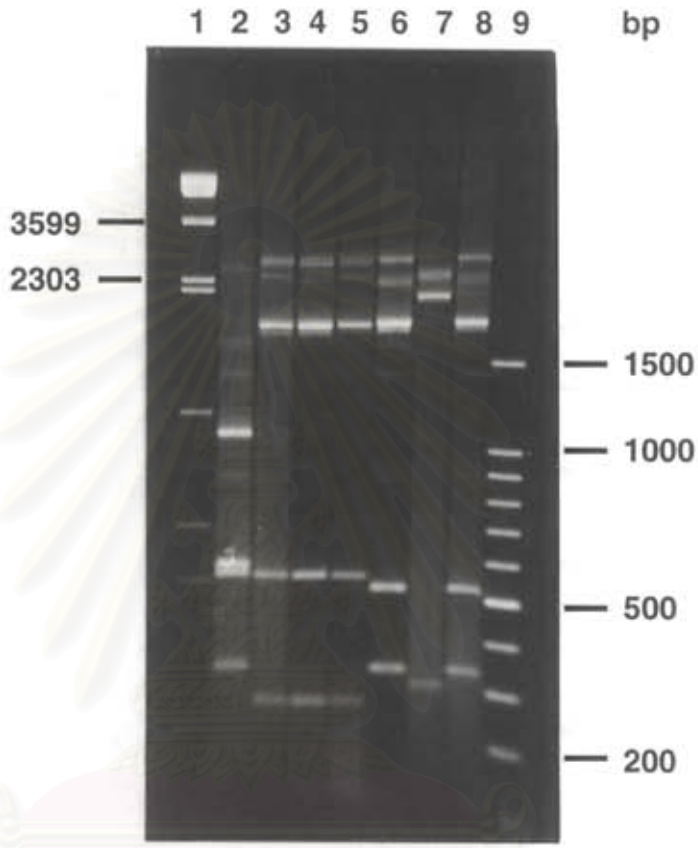


Figure 3.16 Agarose gel electrophoresis showing restriction patterns of amplified control region of *A. mellifera* and *A. cerana* digested with *Alu*I

lane 1 λ/*Dra*I DNA marker
lanes 2,7 Different of restriction profiles was observed in *A. mellifera* and the identified *Apis* species (S60), respectively.
lanes 3-6,8 Digested PCR product patterns from *A. cerana*
lane 9 100 bp DNA marker

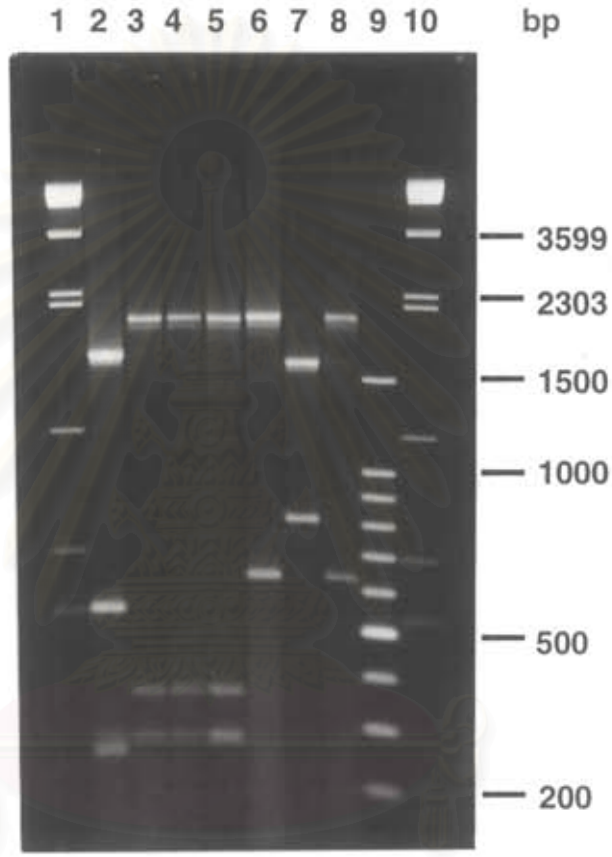


Figure 3.17 Agarose gel electrophoresis showing restriction patterns of amplified control region of *A. mellifera* and *A. cerana* digested with *Taq*I

lanes 1,10 λ/*Dra*I DNA marker
lanes 2,7 Restriction PCR product patterns observed in *A. mellifera* and unidentified *Apis* species (S60), respectively.
lanes 3-5 Restriction PCR product patterns of haplotype A from *A. cerana* Northern area
lanes 6,8 Restriction PCR product patterns of haplotype B from *A. cerana* Southern area
lane 9 100 bp DNA marker

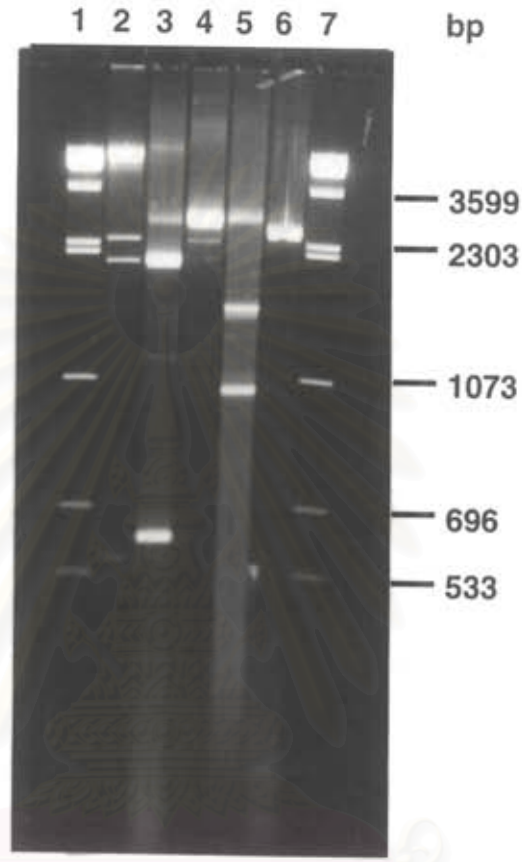


Figure 3.18 Agarose gel electrophoresis showing restriction patterns of amplified control region of *A. mellifera* and *A. cerana* digested with *Rsa*I

lanes 1,7 λ/*Dra*I DNA marker
lane 2 λ/*Hin*dIII DNA marker
lanes 3,5 Restriction PCR product patterns of haplotype A and C from *A. cerana*
lanes 4 Uncut PCR product pattern of haplotype B from *A. cerana*
lane 6 Uncut PCR product pattern from unidentified *Apis* species (S60)

Table 3.2 Restriction profiles resulted from digestion of amplified control region of Thai *A. cerana* by *Taq* I , *Rsa*I and *Hin*fI.

A. *Taq* I

Haplotype / Size (bp)	A	B	C (Am)	D (S60)
2100	—	—		
1750			—	—
810				—
650		—		
550			—	
370	—			
280	—			
260			—	
Total	2750	2750	2560	2560

B. *Rsa* I

Haplotype / Size(bp)	A	B	C	D (S60, Am)
2750		—		—
2560				
2130	—			
1600			—	
1150			—	
620	—			
Total	2750	2750	2560	2560

Table 3.2 (continued)

C. *Hinf* I

Haplotype / Size (bp)	A	B	C	D	E	F	G	H	I	J	K (Am)	L (S60)
>1500								—				
1380				—								
1350		—	—			—						—
1150	—				—		—		—	—		
800											....	
730												—
700											—	
630	—					—	—					
615									—	—		
600		—	—	—	—	—						
480	—	—	—	—	—					—		
475										—		
440												—
400							—					
300	—						—	—	—			
290					—							
190								—	—			
180	—	—	—	—	—		—	—	—			
110		—		—		—						
Total	2740	2720	2610	2750	2700	2690	2660	>2170	2435	2720	2300	2520

— One fragment

.... Doublet with identical molecular length

3.6.2 PCR-RFLP analysis by *Rsa*I

*Rsa*I generated three different restriction pattern; A, B and C, as illustrated by Table 3.2. Based on restriction site loss and gain, haplotype B was the intermediate haplotype between A and C (Figure 3.18). All *A. cerana* from the Northern area (North, North/East, Central) showed haplotype A (2 discrete bands of 2130 and 620 bp). A restrict site loss of A generated a single fragment of 2750 bp which is specifically found in South (100%) and the Samui Island (68%). The haplotype C was unique found only in 32% of the Samui sample. Gaining of a restriction site in B replaced that genotype with C if B was treated as the ancestral of C.

3.6.3 PCR-RFLP analysis by *Hin*fI

A total of 10 different patterns (A, B, C, D, E, F, G, H, I and J) were observed from *Hin*fI digestion of *A. cerana* control region (Figure 3.19). The haplotype B, C, D, E and F founded in North, North/East and Central. The haplotype C was the most common haplotype in North (58%) while haplotype B founded in North/East (58%). Three different haplotype B, C and D predominated haplotype in the Central (44%, 28% and 28%, respectively). The Southern area (South, Samui Island and Phuket Island), haplotype A are mainly observed in 92%, 86% and 100% , respectively and haplotype G, H, I and J distributed in low frequency rate throughly this region. Distribution of each haplotype are showed in Table 3.2. and 3.3.

3.7 Distribution frequency of composite haplotype among six population.

Eleven different composite haplotypes were generated from combination of three single haplotypes for each individual. Distribution

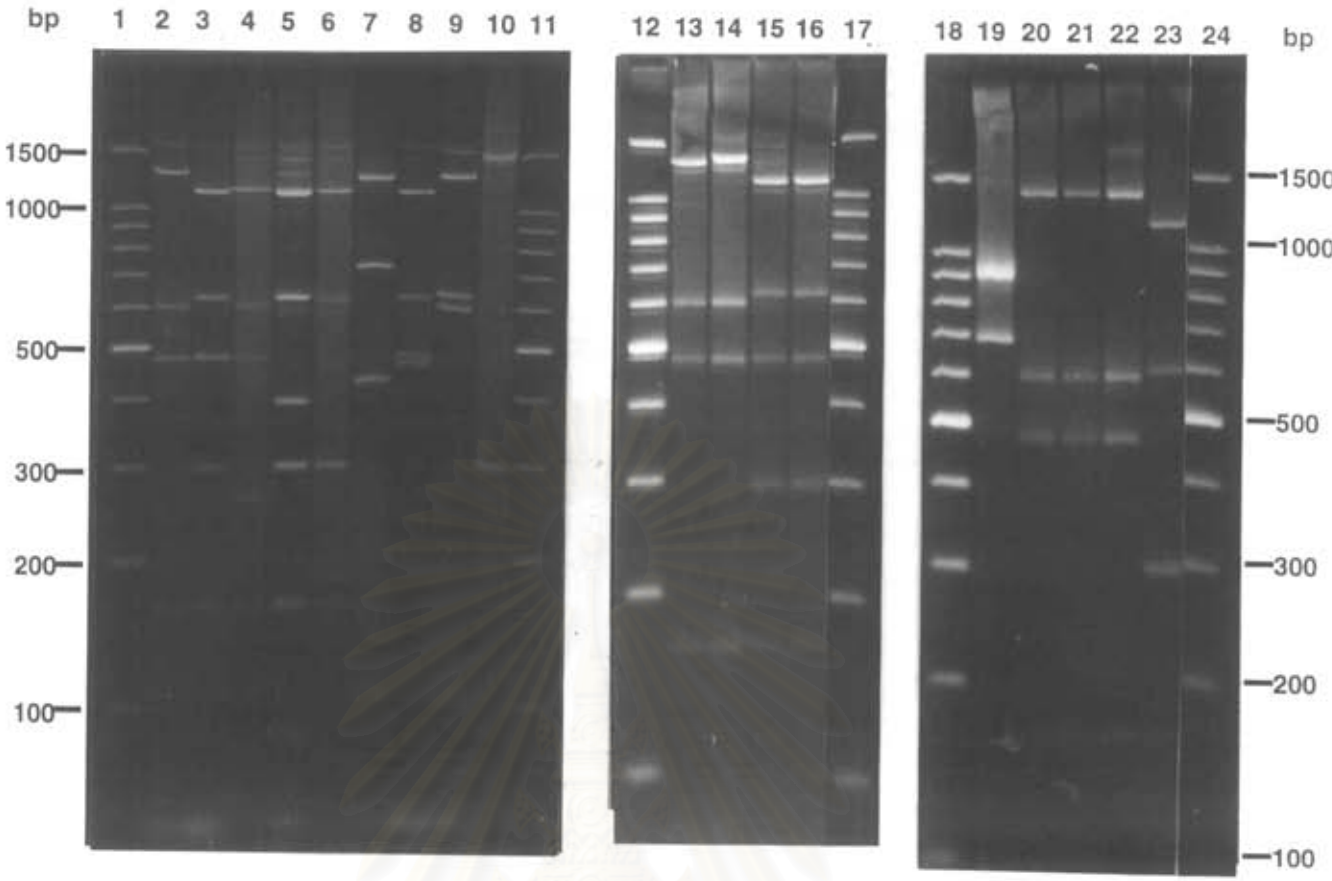


Figure 3.19 Agarose gel electrophoresis showing restriction patterns of amplified control region of *A. mellifera* and *A. cerana* digested with *Hin*fI

lanes 1,11,12,17,18,24 100 bp DNA marker
lanes 3,15,16,23 Restriction PCR product patterns of haplotype A from *A. cerana*
lane 2 Restriction PCR product patterns of haplotype B from *A. cerana*
lanes 13,14 Restriction PCR product patterns of haplotype C from *A. cerana*
lanes 20,21,22 Restriction PCR product patterns of haplotype D from *A. cerana*
lane 4 Restriction PCR product pattern of haplotype E from *A. cerana*
lane 9 Restriction PCR product pattern of haplotype F from *A. cerana*
lane 5 Restriction PCR product pattern of haplotype G from *A. cerana*
lane 10 Restriction PCR product pattern of haplotype H from *A. cerana*
lane 6 Restriction PCR product pattern of haplotype I from *A. cerana*
lane 8 Restriction PCR product pattern of haplotype J from *A. cerana*
lane 7 Restriction PCR product pattern of unidentified *Apis* species (S60)
lane 19 Restriction PCR product pattern of *A. mellifera*

Table 3.3 Haplotype distribution frequencies of digested control region of *A. cerana* with three restriction endonuclease (*Taq*I, *Rsa*I and *Hin*fI)

Population	Haplotype														
	*Taq*I		*Rsa*I			*Hin*fI									
	A	B	A	B	C	A	B	C	D	E	F	G	H	I	J
North (19)	19 (1)	0	19 (1)	0	0	0	4 (0.210)	11 (0.579)	3 (0.158)	1 (0.052)	0	0	0	0	0
North/East (19)	19 (1)	0	19 (1)	0	0	0	11 (0.579)	1 (0.052)	4 (0.210)	1 (0.052)	2 (0.105)	0	0	0	0
Central (25)	25 (1)	0	25 (1)	0	0	0	11 (0.440)	7 (0.280)	7 (0.280)	0	0	0	0	0	0
South (37)	0	37 (1)	0	37 (1)	0	34 (0.919)	0	0	0	0	0	2 (0.054)	0	0	0
Samui Island (22)	0	22 (1)	0	15 (0.681)	7 (0.318)	19 (0.864)	0	0	0	0	0	0	2 (0.091)	1 (0.045)	1 (0.027)
Phuket Island (3)	0	3 (1)	0	3 (1)	0	3 (1)	0	0	0	0	0	0	0	0	0

frequency of composite haplotypes in six geographically *A. cerana* samples in Thailand were shown by Table 3.4. No overlapping haplotype distribution was observed between the Northern (North, North/East and Central) and Southern (South and Samui Island) groups. Five composite haplotypes (AAC, AAB, AAD, AAE and AAF) was distributed in the former group. Of which, AAB was the most common haplotype in this group followed by AAC and AAD, respectively. The AAE was found in only one individual of *A. cerana* from the North and North/East whereas AAF was available at low frequency in the Central *A. cerana.* A total of six composite haplotypes were observed in the remaining samples; South, Samui and Phuket, where BBA was the most common genotypes for these areas. Approximately 92% of the South *A. cerana* carried this haplotype. A lower frequency of this genotype was observed in the Samui Island *A. cerana*. Due mainly to limited number of investigated individuals in Phuket Island (n=3), it was difficult to directly compare haplotype frequency of this geographic sample with others. The remaining five haplotypes found in the Southern part of Thailand were population specific but they were available in very low frequencies.

3.8 Data analysis

Genetic distance was calculated from restriction fragment sharing using REAP 4.0 (McElroy *et al.*, 1992) as illustrated by Table 3.5.The minimum genetic distance was observed between AAB and AAC (0.408%) whereas the height genetic distance was found between AAF and BCI (14.918%) . The average genetic distance for all possible comparison was 6.821%.

Table 3.4 Composite haplotype distribution of digested control region of *A. cerana* among different geographic samples.

Composite haplotype	Geographic samples						
	North (19)	North/East (19)	Central (25)	South (37)	Samui (22)	Phuket (3)	Total
I. AAC	11 (0.579)	1 (0.053)	7 (0.280)	-	-	-	19 (0.152)
II. AAB	4 (0.210)	11 (0.579)	11 (0.440)	-	-	-	26 (0.208)
III. AAD	3 (0.158)	4 (0.210)	7 (0.280)	-	-	-	14 (0.112)
IV. AAE	1 (0.053)	1 (0.053)	-	-	-	-	2 (0.016)
V. AAF	-	2 (0.105)	-	-	-	-	2 (0.016)
VI. BBA	-	-	-	34 (0.919)	15 (0.682)	3 (1)	52 (0.416)
VII. BBJ	-	-	-	1 (0.027)	-	-	1 (0.008)
VIII. BBG	-	-	-	2 (0.054)	-	-	2 (0.016)
IX. BCA	-	-	-	-	4 (0.182)	-	4 (0.032)
X. BCH	-	-	-	-	2 (0.091)	-	2 (0.016)
XI. BCI	-	-	-	-	1 (0.045)	-	1 (0.008)
Total	19	19	25	37	22	3	125

Table 3.5 Pairwise comparisons illustrating genetic distance substitution (the number of nucleotide substitution per site) between pairs of composite haplotypes found in Thai *A. cerana*.

Composite haplotype	AAC	AAB	AAD	AAE	AAF	BBA	BBJ	BBG	BCA	BCH	BCI
AAC	-										
AAB	0.00408	-									
AAD	0.01301	0.00800	-								
AAE	0.01699	0.01178	0.01178	-							
AAF	0.01893	0.01301	0.02316	0.02722	-						
BBA	0.07680	0.08162	0.08162	0.06535	0.10417	-					
BBJ	0.09896	0.10417	0.10417	0.08162	0.13864	0.02983	-				
BBG	0.10417	0.10914	0.10914	0.08623	0.10417	0.00991	0.04606	-			
BCA	0.08162	0.08623	0.08623	0.06965	0.10913	0.01452	0.05123	0.02607	-		
BCH	0.10417	0.10914	0.10914	0.11387	0.14404	0.05123	0.09349	0.05123	0.02607	-	
BCI	0.10914	0.11387	0.11387	0.09065	0.14918	0.03969	0.05123	0.03969	0.01893	0.01452	-

A dendrogram constructed from this value indicated two well separated haplotype lineages (or clades) (Figure 3.20). The clade A are compared of 6 haplotypes (VI, VII, VIII, IX, X and XI). All of this haplotypes carried by *A. cerana* originating from the South, Samui Island and Phuket Island. Likewise, the clade B consisted of the remaining five haplotypes which was found in specimens from the North, North/East and Central of Thailand. A large genetic distance of 6.386% was observed between these clades.

Disregarding the Phuket Island the haplotype and nucleotide diversity (Table 3.6) within samples were between 0.154±0.056 (South) to 0.663±0.026 (Central). The reason to explain limited diversity in the South and the Samui Island implying restricted gene flow between this geographic sample and others. High genetic diversity (both haplotype and nucleotide levels) in the North, North/East and Central indicated high gene flow exchanged between each of these samples. The average haplotype and nucleotide diversity was 0.425±0.013 and 0.580±0.000, respectively.

The nucleotide diversity and nucleotide divergence (Table 3.7) between six conspecific samples were calculated. Basically, nucleotide diversity between samples was greater than that within population implying differentiation of *A. cerana* in Thailand. The highest nucleotide diversity between a pair of population was 8.8% observed between

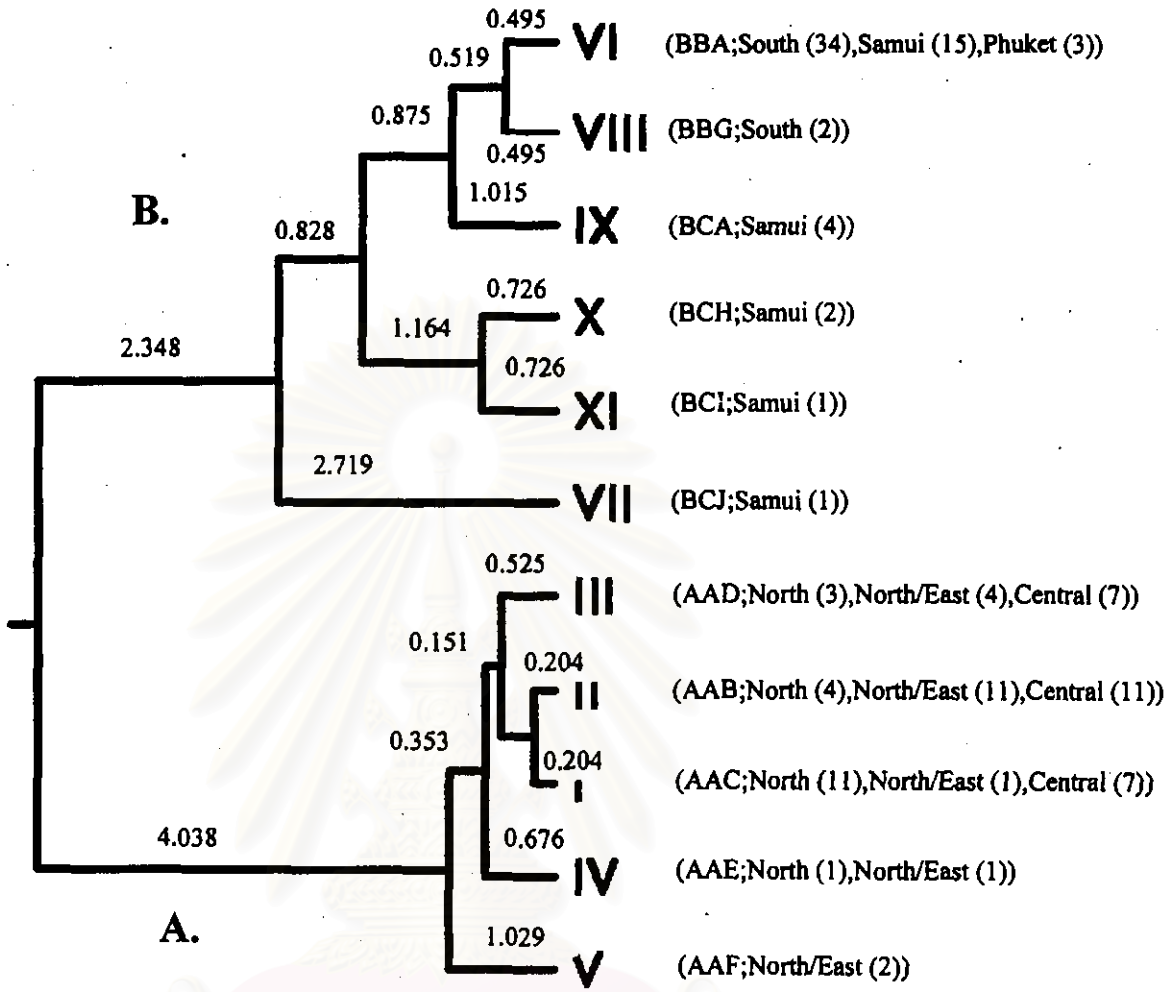


Figure 3.20 UPGMA phenogram showing relationship between 11 composite haplotypes of digested control region of *A . cerana* in Thailand based on nucleotide diversity listed in Table 3.5.

Table 3.6 Haplotype and nucleotide diversity with six geographic samples of *A. cerana* in Thailand resulted from digestion of the control region with three restriction enzymes (*Taq*I, *Rsa*I and *Hin*fI)

Population	Haplotype diversity ± SE	Nucleotide diversity (%)
North	0.609 ± 0.068	0.57
North/East	0.620± 0.072	0.71
Central	0.663 ± 0.026	0.52
South	0.154± 0.056	0.27
Samui Island	0.503 ± 0.078	0.14
Phuket Island	0.000± 0.000	0.00
Average	0.425 ± 0.013	0.58 ± 0.0004

Table 3.7 Percentage of nucleotide diversity (above diagonal) and nucleotide divergence (below diagonal) between of six conspecific samples of *A. cerana* in Thailand based on digestion of amplified control region with three restriction enzymes (*Taq*I, *Rsa*I and *Hin*fI) .

Population	North	North/East	Central	South	Samui	Phuket
North	-	0.910	0.134	8.027	8.288	7.797
North/East	0.108	-	1.043	8.509	8.795	8.288
Central	0.023	0.015	-	8.236	8.483	8.003
South	7.585	7.997	7.841	-	0.629	0.569
Samui Island	7.285	7.725	7.529	0.191	-	0.745
Phuket Island	7.513	7.935	7.766	0.0005	0.192	-
Average	4.647 ± 0.0099 (Nucleotide divergence)			5.231± 0.0099 (Nucleotide diversity)		

North/East and the Samui samples whereas the lowest diversity was 0.13% between North and Central. The average nucleotide diversity averaged overall sample was 5.231±0.010% .

Large genetic differences were observed between each member of the Northern and that of the Southern area (Table 3.7). The highest nucleotide divergence was found among North/East and South population (7.9%). The average nucleotide divergence between all possible comparisons of investigated *A. cerana* was 4.647±0.010%. A UPGMA dendrogram constructed from percentage of nucleotide divergence well allocated all investigated samples to 2 groups; A (North, North/East and Central) and B (South, Samui Island and Phuket Island) with the divergence of 7.581% (Figure 3.21).

Geographic heterogeneity analysis indicated significant in allele distribution of haplotypes in Thai *A.cerana* showing that genetic subdivisions do exist in this species ($P<0.0028$). As can be seen from Table 3.8A , 10 of 15 possible comparisons were statistically different. Comparisons between North-Central, North/East-Central, North-North/East, South-Phuket and Samui-Phuket did not show significant differents. On the basic of there results, North, Central and North/ East as well as South and Phuket Island were pooled and reanalyzed against the remaining samples (Table 3.8B). Moreover, geographic heterogeneity between the South and Samui Island was statistically supported by this analysis. All comparisons, showed statistically significant differences illustrating strong genetic population differentiation between Northern area and Southern area ($P<0.0028$)

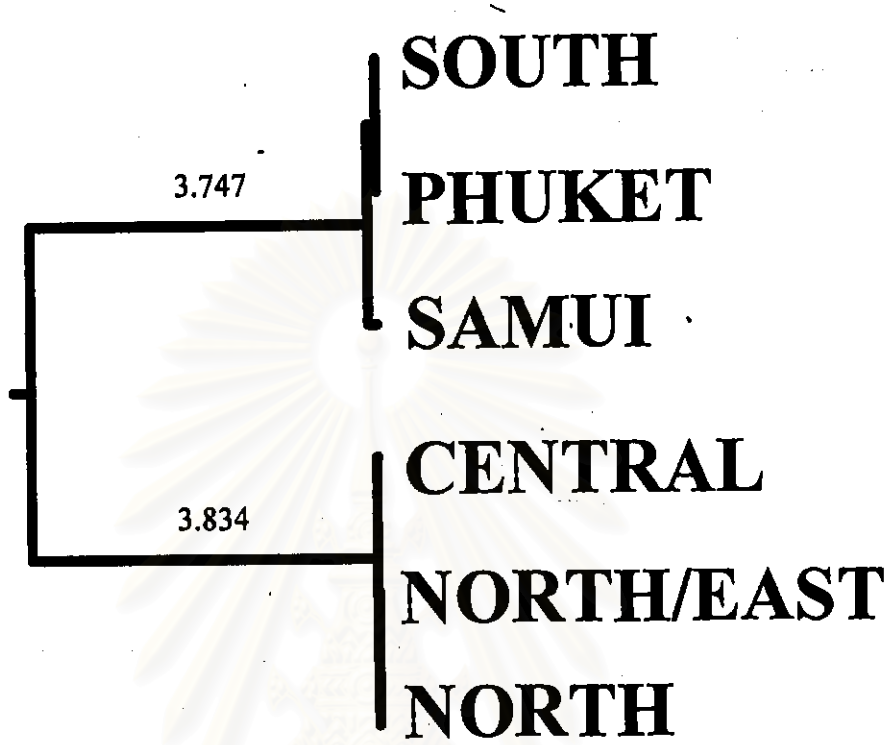


Figure 3.21 UPGMA phenogram showing relationship of six conspecific samples of *A. cerana* in Thailand based on percentage of nucleotide divergence.

Table 3.8A. Analysis of geographic heterogeneity using a Monte Carlo simulation in composite haplotype distribution frequency between six population honeybee *A. cerana* in Thailand.

Population	North	North/East	Central	South	Samui	Phuket
North	-					
North/East	P=0.0034^{ns}	-				
Central	P=0.0865^{ns}	P=0.0751^{ns}	-			
South	P=0.0000	P=0.0000	P=0.0000	-		
Samui Island	P=0.0000	P=0.0000	P=0.0000	P=0.0014	-	
Phuket Island	P=0.0011	P=0.0012	P=0.0002	P=1.0000^{ns}	P=0.7351^{ns}	-

ns = Not significant

Table 3.8B. Analysis of geographic heterogeneity using a Monte Carlo simulation in composite haplotype distribution frequency between pooled samples Northern (North-Central-North/East), Southern (South-Phuket Island) and Samui Island of honeybee *A. cerana* in Thailand.

Population	Northern	Southern	Samui Island
Northern	-		
Southern	P=0.0000	-	
Samui Island	P=0.0000	P=0.0000	-

Population differentiation was also examined using F-statistics. This estimate assumes the infinite allele model and selective neutrally. The Fst value between six geographic sample ranged between -0.17043-0.65814 (Table 3.9A). All comparisons, with the exception of that between North-Central (P=0.11331), North-North/East (P=0.00315), South-Phuket Island (P=1.0000) and Samui -Phuket Islands (P=0.60139) showed significant intraspecific genetic (P<0.0028) the North, Central and North/East were pooled differentiation as was South and Phuket. The data was reanalyzed (Table 3.9B). A large genetic discontinuity between Northern and Southern were observed. Unlike the results from Monte Carlo analysis, the Fst did not indicate differentiation between the South and Samui samples (P=0.00299)

Table 3.9A Population differentiation analysis of six geographic samples using F-statistics

Population	North	North/East	Central	South	Samui	Phuket
North	-					
North/East	0.18859 (P=0.00315^{ns})	-				
Central	0.04949 (P=0.11331^{ns})	0.05708 (P=0.04007^{ns})	-			
South	0.61842 (P=0.00000)	0.65814 (P=0.00000)	0.61702 (P=0.00000)	-		
Samui Island	0.38097 (P=0.00000)	0.42597 (P=0.00000)	0.40166 (P=0.00000)	0.12493 (P=0.00455^{ns})	-	
Phuket Island	0.45030 (P=0.00586)	0.50589 (P=0.00068)	0.47211 (P=0.00032)	-0.17043 (P=1.00000^{ns})	-0.03697 (P=0.60139^{ns})	-

ns = Not significant

Table 3.9B Population differentiation analysis of pooled samples Nothern (North-Central and North/East), Southern (South-Phuket Island) and Samui Island using F-statistics

Population	Northern	Southern	Samui Island
Northern	-		
Southern	0.56166 (P=0.00000)	-	
Samui Island	0.37088 (P=0.00000)	0.13587 (P=0.00299)	-