CHAPTER 3

RESULTS

3.1 DNA isolation

High molecular weight DNA (>23 kb) was routinely obtained from the extraction protocol described in 2.7. The amount of total DNA obtained was estimated by comparison of its intensity in a 0.7% agarose gel electrophoresis with that of λ/*Hin*dIII. Generally, 1-2 μg of high molecular weight DNA per individual was obtained (Figure 3.1). This amount of extract DNA was more than enough to be used in the subsequent PCR reactions.

3.2 Optimization in the PCR conditions

Based on the fact that primers used in the present study was originally designed from *A. mellifera* sequences, it was then necessary to optimize the PCR conditions to obtain reproducible amplification results. These were composed of the amount of template DNA, Mg^{2+} concentration, and primer concentration per reaction.

3.2.1 Optimization of DNA template

A series of two fold diluted total DNA (12.5, 25, 50 and 100 ng) were examined using the condition mentioned in 2.9.1 (Figure 3.2). All template concentrations yielded a single discrete band at 825 bp without any non-specific amplification products. Nevertheless, the

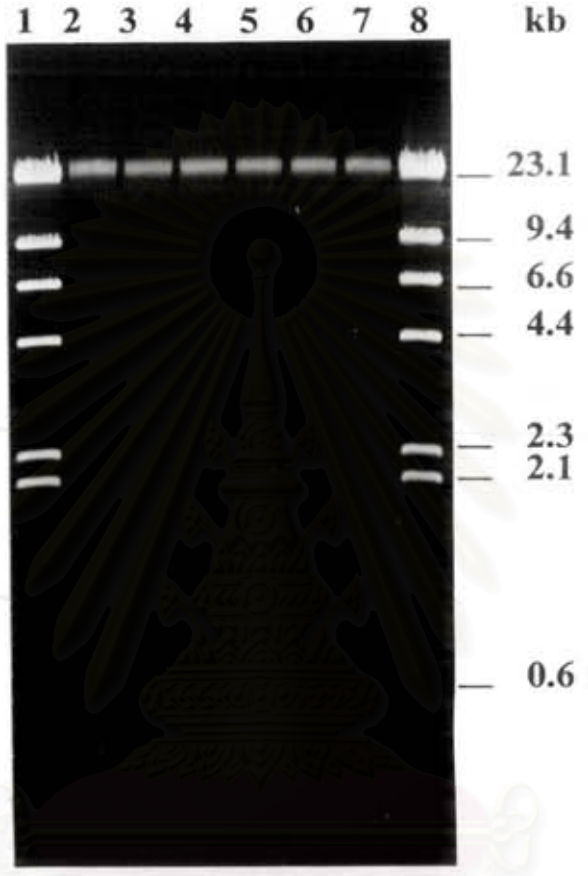


Figure 3.1 Ethidium bromide staining of 0.7% agarose gel showing the quality of individual extraced DNA

Lanes 1,8 Size standard: 0.5 µg of λ/ *Hin*dIII
Lane 2 *A. mellifera*
Lanes 3-7 *A. cerana* from North, North-East, Central region, South and the Samui Island, respectively

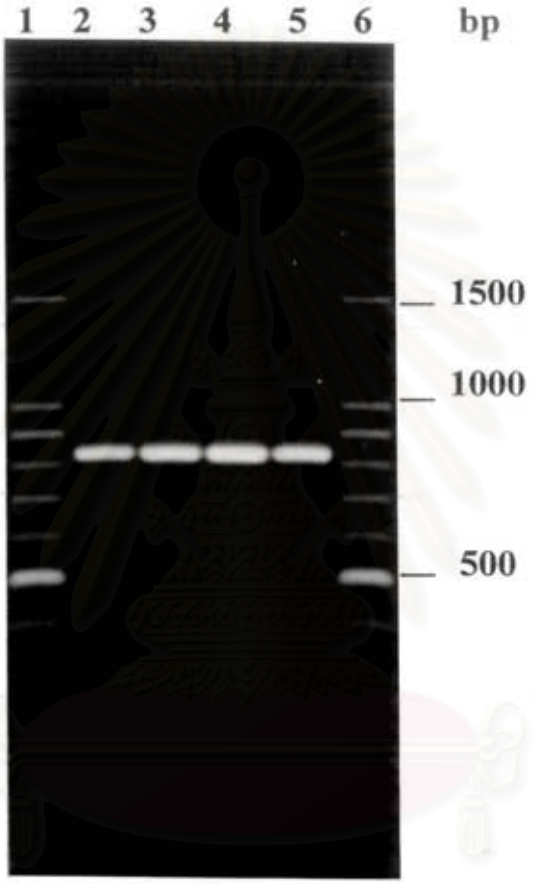


Figure 3.2 Effect of DNA template concentration in PCR amplification. Various amount of *A. cerana* total DNA were used as template under the PCR condition described in 2.9.1. The ATPase6-ATPase8 primers were F(TTTAATTCCTCAAATAATAC) and R(TTAATTTGATTCAGAGAAAT).

Lanes 1,6 A 100 bp DNA ladder
Lanes 2-5 The PCR product resulted from 12.5, 25, 50 and 100 ng template, respectively

resulting PCR product concentration from 25 ng of DNA template was usually higher than that from a reaction including 12.5 ng template therefore the optimal DNA concentration was evaluated to be 25 ng.

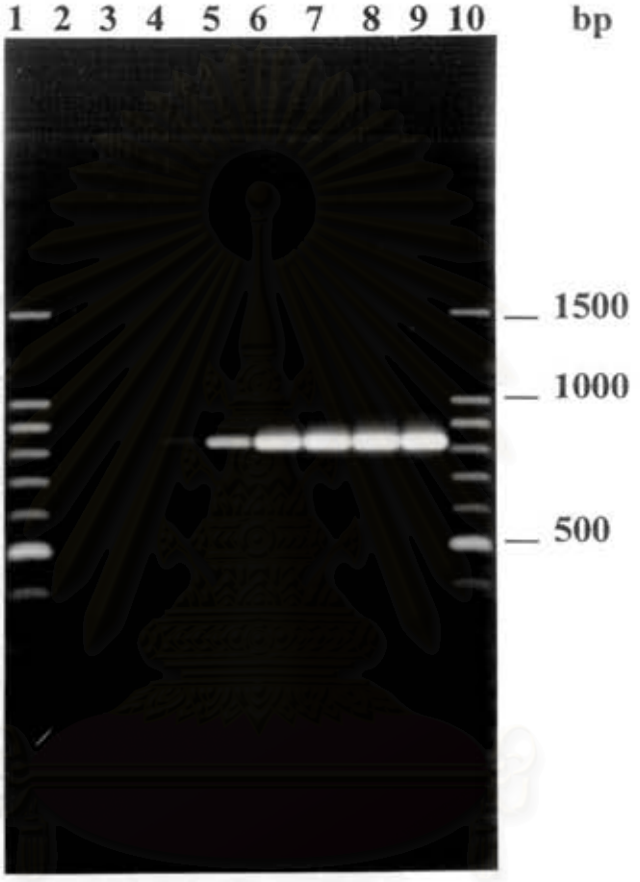
3.2.2 Optimization of Mg^{2+} concentration

ATPase6-ATPase8 gene of *A. cerana* mtDNA was amplified using the condition described previously with the exception that the $MgCl_2$ concentration was varied from 1.0-4.0 mM. As can be seen in Figure 3.3, the amplification product firstly appeared at a reaction containing 1.5 mM. The amount of amplification product consistently increased until 2.5 mM $MgCl_2$. As a result, the optimal $MgCl_2$ concentration at 2.5 mM was then chosen.

3.2.3 Optimization of primers concentrations

ATPase6-ATPase8 gene of *A. cerana* mtDNA was *in vitro* amplified using the condition that mentioned earlier with only the exception that $MgCl_2$ was added to a final concentration of 2.5 mM. The concentration for each primer were optimized (from 0.1-0.5 μM). It was found that the intense band was observed at a reaction containing 0.2 μM of each primer. Increasing of primer concentration over such a value did not increase the resulting product (Figure 3.4).

Therefore, the optimal conditions for amplification of ATPase6-ATPase8 were 1xPCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 2.5 mM $MgCl_2$, 200 μM each of dNTP, 0.2 μM each of primers, 25 ng total DNA and 0.125 unit *Taq* DNA polymerase.



Figuer 3.3 Optimization of Mg^{2+} concentration in PCR amplified ATPase6-ATPase8 gene region. *A. cerana* total DNA(25ng) was *in vitro* amplified with various Mg^{2+} concentration ranging from 1.0-4.0 μM with a 0.5 μM increment.

Lanes 1,10 A 100 bp DNA ladder
Lane 2 Negative control
Lanes 3-9 The PCR product resulted from amplification reaction containing 1.0-4.0 μM of Mg^{2+} respectively.

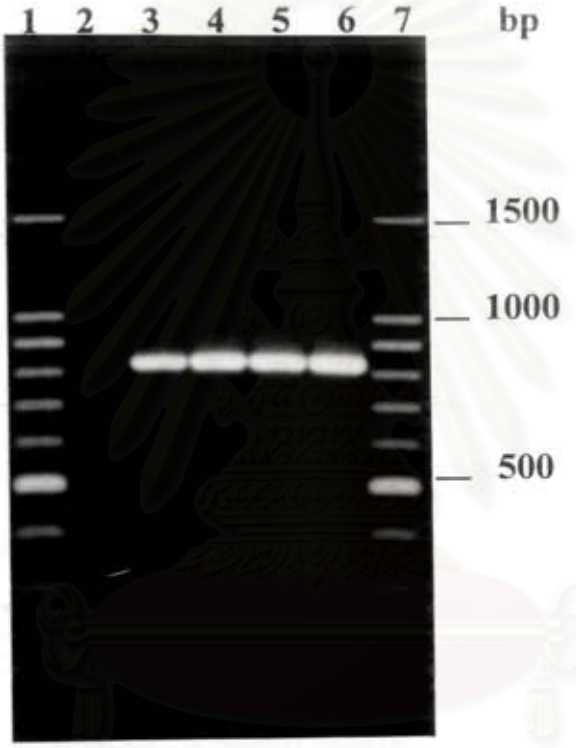


Figure 3.4 Optimization of ATPase6-ATPase8 primer concentration. Amplification of this gene region was carried out in the presence of 25 ng template and 2.5 mM $MgCl_2$ final concentration.

Lanes 1,7 A 100 bp DNA ladder

Lanes 2-6 The PCR product resulted from amplification of ATPase6-ATPase8 in the presence of 0.1, 0.2, 0.3, 0.4 and 0.5 μM each of primers, respectively.

The PCR profile was partially optimized. It was found that a profile consisting of a predenaturation at 95°C for 2 min followed by 35 cycles of a 94°C denaturation step for 30 sec, a 50°C annealing step for 30 sec and 72°C extension step for 2 min. Final extension was carried out at 72°C for 10 min. Amplification of ATPase6-ATPase8 gene using either homo or heterospecific DNA template provided equally reproducible and specific results (Figure 3.5).

3.3 Characterization of the amplification product

The ATPase6-ATPase8 amplified product from *A. cerana* and *A. mellifera* were characterized by running through a 1.5% agarose gel and then stained with ethidium bromide. Typically, only one amplification band were observed but some samples exhibited multiple band patterns.

A 825 amplification fragment was observed in all *A. mellifera* individuals. This fragment was also found in all *A. cerana* originating from North, North-East, Central region. Interestingly, eighty-three percent of *A. cerana* collected from the South of Thailand showed length heteroplasmy of ATPase6-ATPase8 gene portion (Table 3.1). Specimens originating from the Samui Island also showed a high level of length heteroplasmy as 60%. This basic datum indicated that *A. cerana* from the Southern region (South and the Samui Island) were difference from the Northern region (North, North-East and Central region).

As mentioned above that more than one amplification bands were observed in *A. cerana* collected from the South and the Samui Island. Two to four bands were observed in *A. cerana* from the South (83%) and

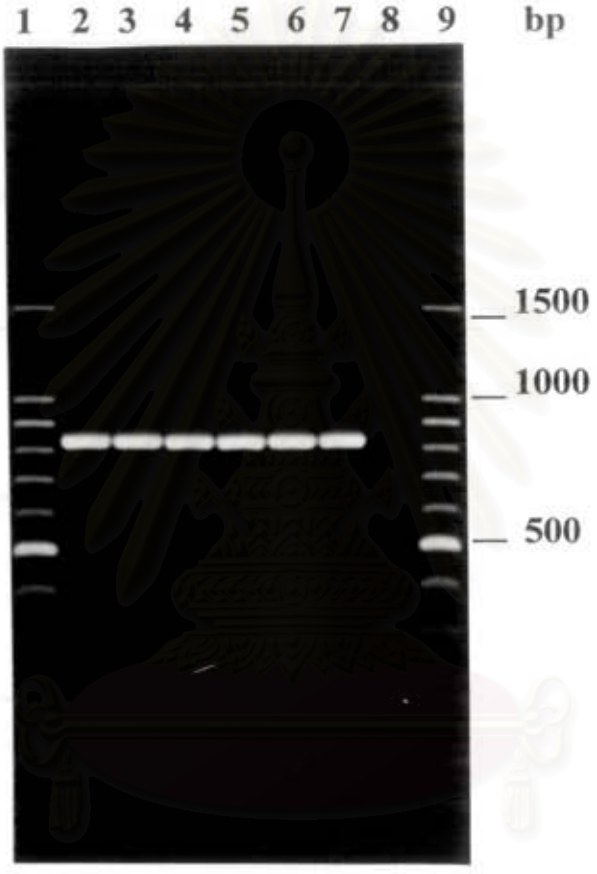


Figure 3.5 Comparison on amplification of ATPase6-ATPase8 from homo and heterospecific individuals

Lanes 1,9	A 100 bp DNA ladder
Lane 2	The PCR product using 25 ng of *A. mellifera* DNA
Lanes 3-7	The resulting product using a 25 ng DNA template from *A.cerana* of 5 geographic populations; North, North-East, Central region, South and the Samui Island, respectively
Lane 8	Negative control

Table 3.1 Geographic distribution of heteroplasmic individuals in ATPase6-ATPase8 of *A. cerana*

Region	Number of individuals (colonies)	Number heteroplasmic individual (%)
The North	36	none
The North-East	30	none
The Central region	31	none
The South	72	60 (83%)
The Samui Island	30	18 (60%)
Totals	199	78 (39%)

the Samui Island (60%). These band were comprised of 825, 900, 925, and 950 bp long, respectively (Figure 3.6). They were named as band 1, 2, 3 and 4 according to the molecular sizes from the smallest to the largest fragment. These four amplified bands were then sequenced with external primer of F (TTTAATTCCTCAAATAATAC) to verified that they are homologous. From the sequencing data of four amplified bands (~58-65 bp from the 5'end), it was found that band 2, 3, 4 which differed in sized were similar in their sequences (Figure 3.7) whereas a 825 bp fragment in heteroplasmic samples was identical to a homologous fragment in normal specimens from five of each samples (Figure 3.8). The sequences of these four amplified bands after alignment indicated approximately 81% similarity in average (Figure 3.9a). It should be noted, however, that heteroplasmy was only observed in the South and the Samui Island *A. cerana*. Alignment the sequence of these fragments to these of ATPase6-ATPase8 gene of *A. mellifera* mtDNA showed approximately 82% similarity (Figure 3.9b). A small deletion of 3 bp (or a small insertion on the opposite direction) was observed in *A. cerana* sequence. The most common ATPase6-ATPase8 band (825 bp) had 83% similarity to that from *A. mellifera* (Figure 3.9c).

Since a band at 825 bp was a common band which was found in all sample. Therefore, it was further used for RFLP analysis.

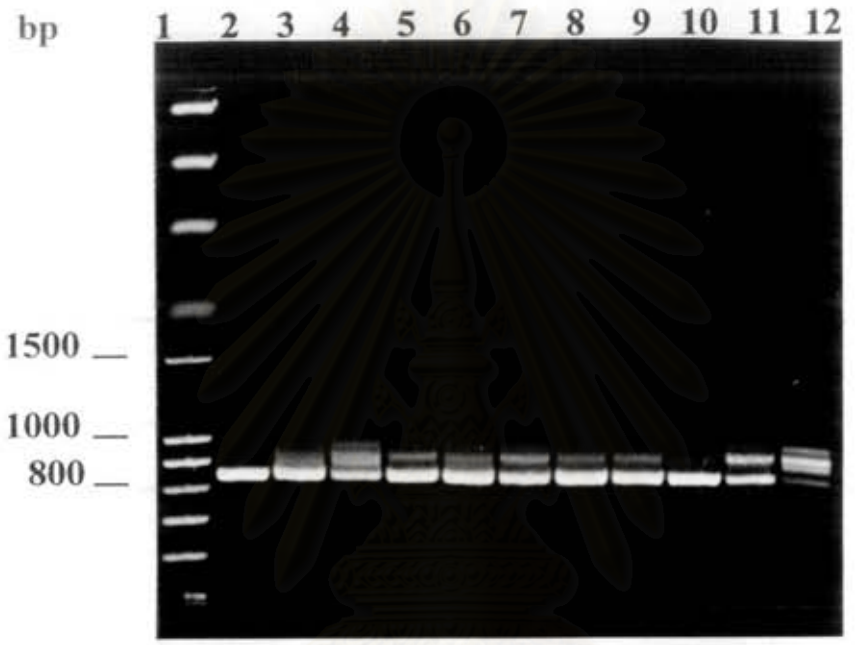


Figure 3.6 Heterplasmy in ATPase6-ATPase8 gene of *A. cerana*

Lane 1 A 100 bp DNA ladder
Lane 2 An amplified ATPas6-ATPase8 of homoplasmic *A. cerana*
Lanes 3-8 An amplified ATPas6-ATPase8 of heteroplasmic *A. cerana*

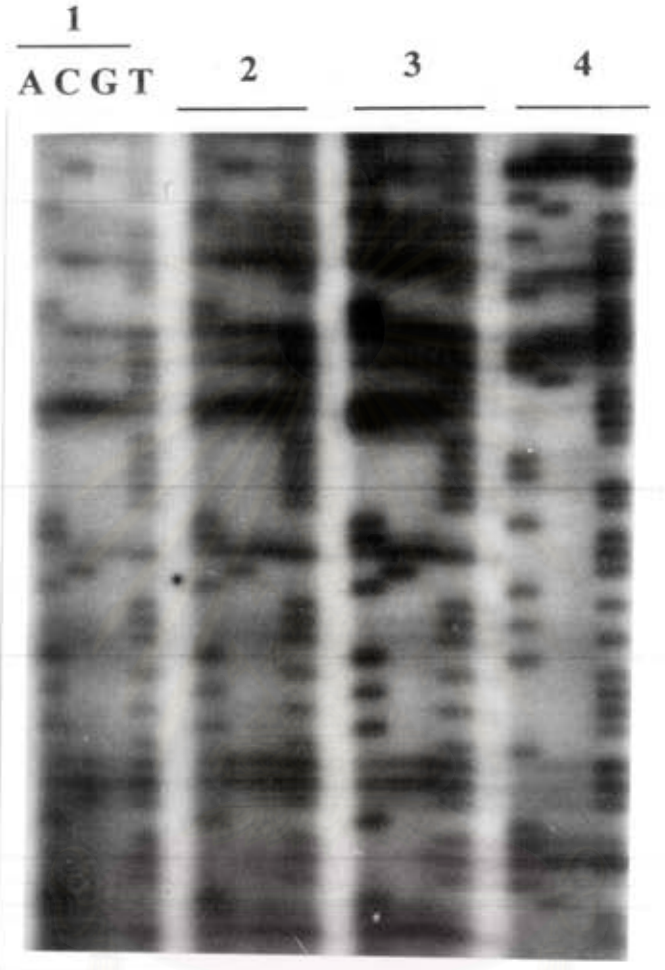


Figure 3.7 Comparisons of DNA sequences identified from PCR amplified of 4 heteroplasmic bands with a F primer (TTTAATTCCTCAAATAATAC) observed in South and the Samui Island *A. cerana*

Lane 1 DNA sequences from the 5′end of a 900 amplified fragment (band 2)
Lane 2 DNA sequences from the 5′end of a 925 amplified fragment (band 3)
Lane 3 DNA sequences from the 5′end of a 950 amplified fragment (band 4)
Lane 4 DNA sequences from the 5′end of a 825 amplified fragment (band 1)

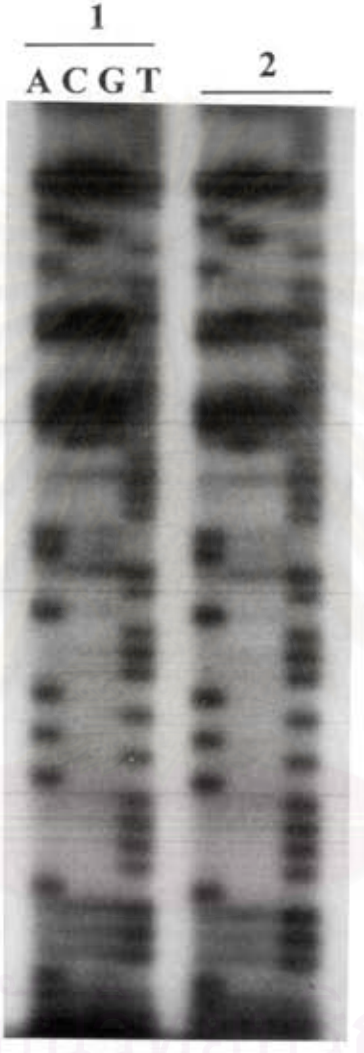


Figure 3.8 DNA sequences of amplified 825 bp ATPase6-ATPase8 of a representative of normal (1) and a heteroplasmic specimens (2)

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Band3 ----AATTATAATTTATTTTATATATTTACTAATTTTTAATTTTTAATTCATTATACTAATT------
Band4 ----AATTATAATTTATTTTATATATTTACTAATTTTTAATTTTTAATTCATTATACTAATT------
Band2 ----AATTATAATTTATTTTATATATTTACTAATTTTTAATTTTTAATTCATTATACTAATT------
Band1 AATTATTCATAATTTATTTTATATATTTATTAATTTTCAATTT---ATTTATCATACTTTTTAATTCA
        * * ********************** ******* *****   *** ** *****  **
```

(a)

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Band3 ----------AATTATAATTTATTTTATATATTTACTAAT
Band4 ----------AATTATAATTTATTTTATATATTTACTAAT
Band2 ----------AATTATAATTTATTTTATATATTTACTAAT
A.mel CTATAAAATGATTTTTAATTTATTTTATTTATTTATTAAT
Band1 ------AATTATTCATAATTTATTTTATATATTTATTAAT
               * *  ************* ****** ****

Band3 TTTTAATTTTTAATTCATTATACTAATT------
Band4 TTTTAATTTTTAATTCATTATACTAATT------
Band2 TTTTAATTTTTAATTCATTATACTAATT------
A.mel TTTTTATTT---ATTCATTATATTAATTAATTCA
Band1 TTTCAATTT---ATTTATCATACTTTTTAATTCA
      ***  ****   *** ** *** *  **
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(b)

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A.mel1   CTATAAAATGATTTTTAATTTATTTTATTTATTTATTAAT
A.cerana ------AATTATTCATAATTTATTTTATATATTTATTAAT
               *** ***  ************* ***********

A.mel1   TTTTTATTTATTCATTATATTAATTAATTCA
A.cerana TTTCAATTTATTTATCATACTTTTTAATTCA
         ***  ******* ** *** *  ********
```

(c)

Figure 3.9 The multiple sequence alignment using CLUSTAL X (1.64b)

(a) comparison of nucleotide sequences among the 5′ end of each four bands heteroplasmy of ATPase6-ATPase8 gene of *A. cerana*

(b) comparison of nucleotide sequences from the 5′ end of each band observed in *A. cerana* specimens showed heteroplasmy in ATPase6-ATPase8 gene and the 5′ end of ATPase6-ATPase8 gene of *A. mellifera*

(c) comparison of nucleotide sequences from the 5′ end of ATPase6-ATPase8 gene of *A. cerana* and that of ATPase6-ATPase8 gene of *A. mellifera*

3.4 Analysis of the amplification product by Restriction fragment length polymorphism (RFLP)

Three restriction enzymes (*Ssp*I, *Taq*I, *Vsp*I) used in this study were selected based on an initial survey of a representative of 12 different colonies of *A. cerana*. These *A. cerana* individuals were previously analyzed for their genetic diversity using PCR-RFLP of lrRNA and shown polymorphic patterns (Sihanuntavong, personal communication). A total of 33 restriction endonucleases were screened whether they gave polymorphic results. This was carried out by electrophoreses of each restriction enzyme digested products through 3-6% metaphor agarose gel.

Five restriction enzymes (*Acs*I, *Alu*I, *Dra*I, *Eco*RI and *Sau*3AI) produced a monomorphic pattern in all except two individuals from the South (S02 and S60) (Figure 3.10-3.14). Although *Tru*9I digested the ATPase6-ATPase8 amplified DNA into several fragments, the resulting products were too small to be unambigously scored. There were only two individuals from the South (S02 and S60) that contained a *Hin*fI site in the ATPase6-ATPase8 gene (Figure 3.15).

Three restriction endonuclease including *Ssp*I, *Taq*I and *Vsp*I, yield scoreble polymorphic patterns whereas the remaining restriction endonuclease (*Bam*HI, *Bfr*I, *Bgl*I, *Bgl*II, *Bst*EII, *Bst*NI, *Cla*I, *Hae*III, *Hin*cII, *Hin*dIII, *Kpn*I, *Mlu*NI, *Nde*I, *Pst*I, *Pvu*II, *Rsa*I, *Sal*I, *Sca*I, *Sma*I, *Swa*I, *Tha*I, *Xba*I and *Xho*I) did not digest the PCR-amplified product.

The ATPase6-ATPase8 gene of *A. mellifera* were *in vitro* amplified using the same condition for *A. cerana*. The amplified product of three *A. mellifera* individuals was singly digested with a battery of restriction enzymes (*Dra*I, *Sau*3AI, *Ssp*I, *Taq*I and *Vsp*I). The results

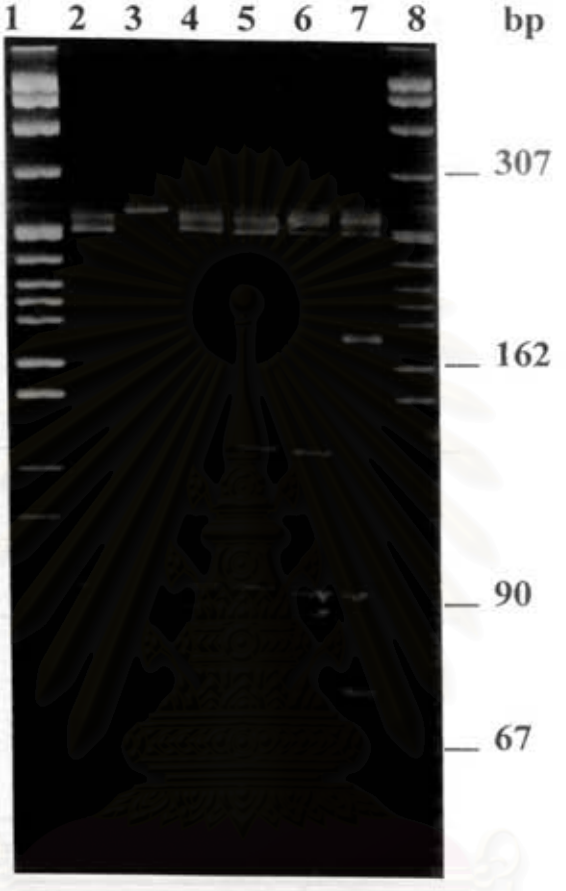

Figure 3.10 A 6% metaphor agarose gel illustrating the restriction patterns obtained from digestion of amplified ATPase6-ATPase8 with *Acs*I

Lanes 1,8 *Msp*I digested pBR322 standard marker
Lanes 2-4 Examples of restriction patterns of *A. cerana* from a representative of North, South and the Samui Island, respectively
Lanes 5-6 A restriction patterns observed from S02 and S60, respectively
Lane7 A restriction pattern of *A. mellifera*

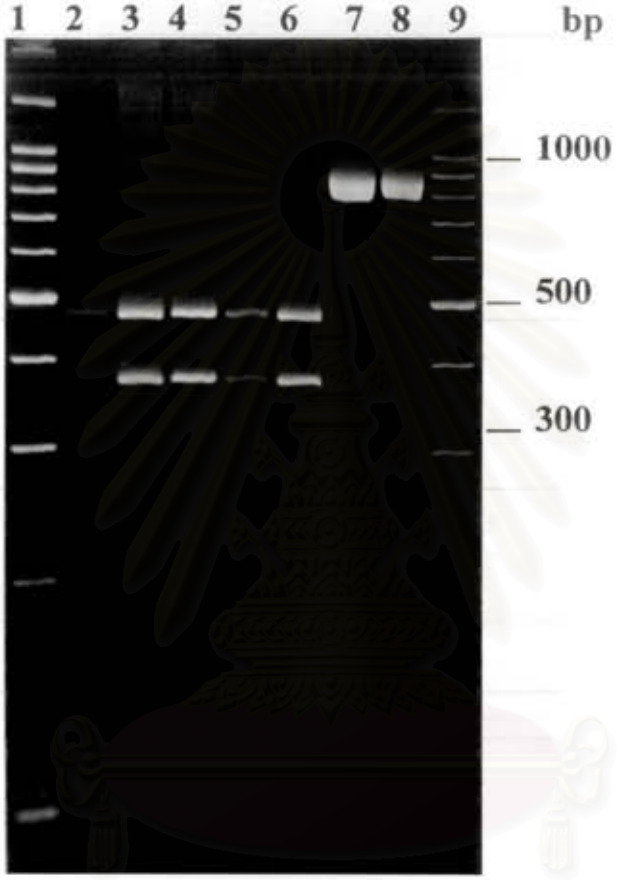

Figure 3.11 A 3% metaphor agarose gel illustrating the restriction patterns obtained from digestion of amplified ATPase6-ATPase8 gene with *Alu*I

I anes 1,9 A 100 bp DNA ladder
Lanes 2-6 Examples of restriction patterns of *A. cerana* from a representative of North, South and the Samui Island, respectively
Lane 7 A restriction patterns observed from S02 or S60
Lane 8 A restriction pattern of *A. mellifera*

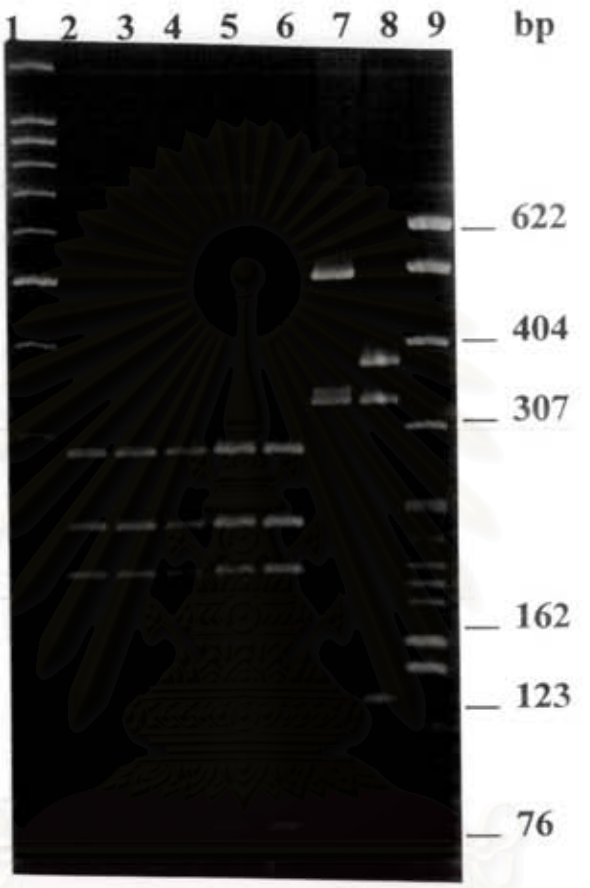

Figure 3.12 A 3% metaphor agarose gel illustrating the restriction patterns obtained from digestion of amplified ATPase6-ATPase8 gene with *Dra*I

Lanes 1 A 100 bp DNA ladder
Lanes 2-6 Examples of restriction patterns of *A. cerana* from a representative of North, South and the Samui Island, respectively
Lane 7 A restriction patterns observed from S02 or S60
Lane 8 A restriction pattern of *A. mellifera*
Lane 9 *Msp*I digested pBR322 standard marker

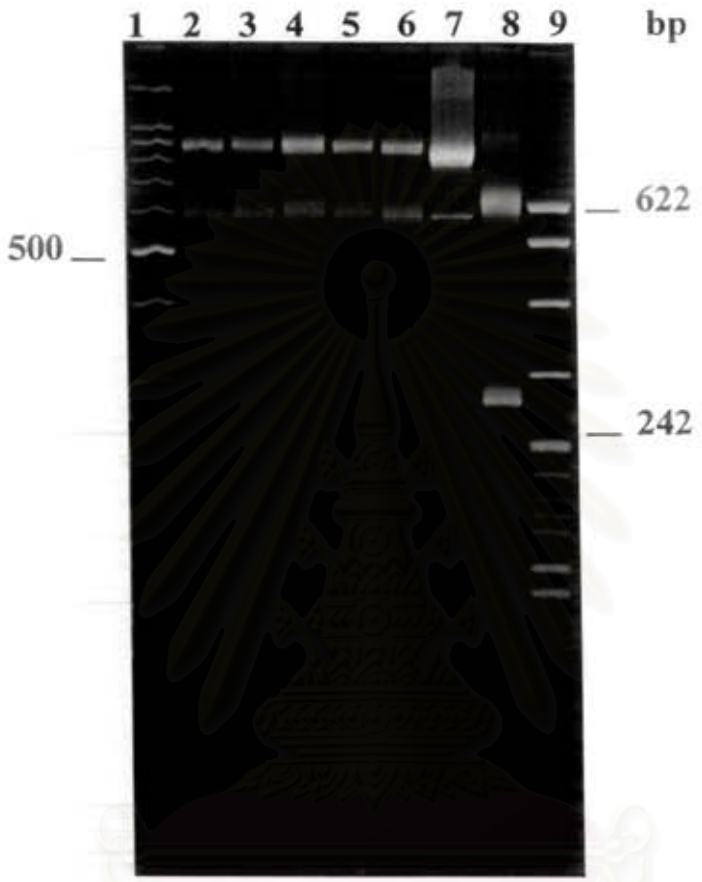

Figure 3.13 A 3% metaphor agarose gel illustrating the restriction patterns obtained from digestion of amplified ATPase6-ATPase8 gene with *Eco*RI

Lanes 1 A 100 bp DNA ladder
Lanes 2-6 Examples of restriction patterns of *A. cerana* from a representative of North, South and the Samui Island, respectively
Lane 7 A restriction patterns observed from S02 or S60
Lane 8 A restriction pattern of *A. mellifera*
Lane 9 *Msp*I digested pBR322 standard marker

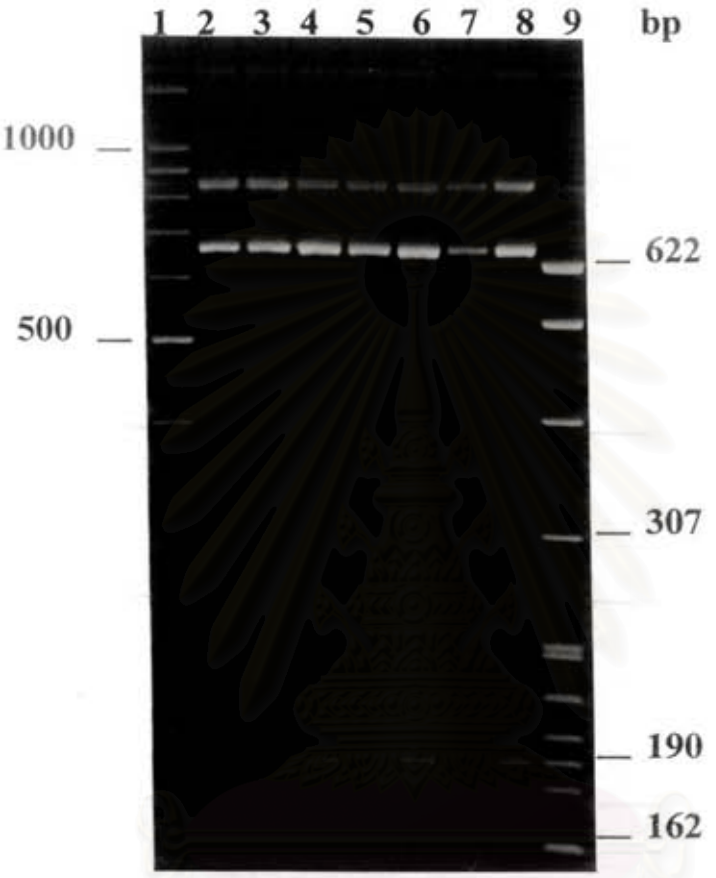

Figure 3.14 A 3% metaphor agarose gel illustrating the restriction patterns obtained from digestion of amplified ATPase6-ATPase8 gene with *Sau*3AI

Lanes 1 A 100 bp DNA ladder
Lanes 2-6 Examples of restriction patterns of *A. cerana* from a representative of North, South and the Samui Islan respectively
Lane 7 A restriction patterns observed from S02 or S60
Lane 8 A restriction pattern of *A. mellifera*
Lane 9 *Msp*I digested pBR322 standard marker

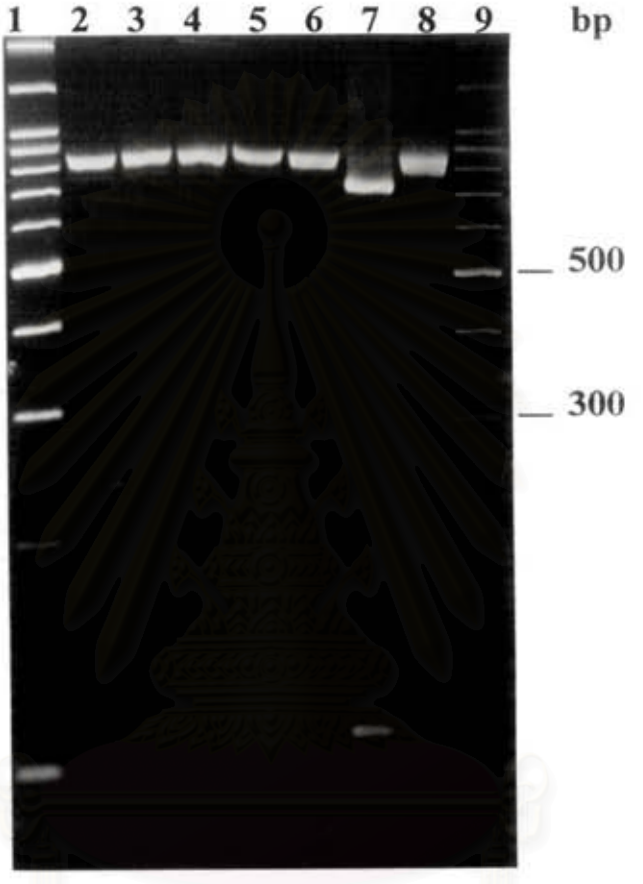

Figure 3.15 A 3% metaphor agarose gel illustrating the restriction patterns obtained from digestion of amplified ATPase6-ATPase8 gene with *Hin*fI

Lanes 1,9 A 100 bp DNA ladder
Lanes 2-6 Examples of restriction patterns of *A. cerana* from a representative of North, North-East, Central, South and the Samui Island, respectively
Lane 7 A restriction patterns observed from S02 or S60
Lane 8 A restriction pattern of *A. mellifera*

illustrated an occurrence of restriction site of amplified ATPase6-ATPase8 gene of *A. mellifera* allowing direct comparison of the homologous gene at interspecific level (Appendix 5). From this result could be suggested that the amplified segment of *A. mellifera* was really of ATPase6-ATPase8 gene. In data analysis, *A. mellifera* was used as outgroup.

3.5 Digestion of the amplification product of *A. cerana* by *Taq*I, *Ssp*I and *Vsp*I

The amplified product from a total number of 181 individuals from five different geographic locations including thirty-six, thirty, thirty-one, fifty-four and thirty individuals from North, North-East, Central region, South and the Samui Island, respectively were characterized using three restriction endonucleases (*Taq*I, *Ssp*I and *Vsp*I). As can be seen from Figure 3.16, 3.17 and 3.18; the suitable percentage of metaphor agarose used to fractionate the digestion products was 4% for *Vsp*I and 3% for both *Ssp*I and *Taq*I. Serial concentrations between 0.5-10 units per reaction was examined for the most appropriate concentration that yielded consistently digestible results. It was found that 5 units of restriction endonuclease were sufficient to gave reliable digestion result.

Restriction fragment patterns were given a single letter code (A,B,C,...etc) in the order of frequency in which they were observed.

Restriction fragment sizes generated from *Taq*I, *Ssp*I and *Vsp*I digestion (Figure 3.16- 3.18) of each haplotype are illustrated in Table

3.2 whereas the most parsimonious networks between single restriction profile was proposed as can be seen from Figure 3.19-3.21.

3.5.1 PCR-RFLP analysis by *Taq*I

Only two restriction patterns haplotype A and B were observed from digestion of amplified product with *Taq*I (Figure 3.16). A restriction pattern of *A. mellifera* could not discriminated from these of *A. cerana* with this enzyme. A single restriction site loss (or gain) related the interconnection of haplotypes A and B (Figure 3.19). Distribution of each haplotype was geographically related (Table 3.3) For instance, all investigated *A. cerana* samples in the North and Central population occupied haplotype A. Ninety-seven per cent of *A. cerana* from North-East had such a haplotype. On the other hand, haplotype B was predominate in the South (96%) and the Samui Island (100%).

3.5.2 PCR-RFLP analysis by *Ssp*I

*Ssp*I produced five polymorphic patterns as shown in Figure 3.17. The haplotype A was found with the highest frequency and related to three haplotypes, B, C and D, single site loss (or gain in the opposite direction) but the interconnection between A-E required two restriction site loss (or alternatively, gaining of two restriction sites) (Figure 3.20). Distribution of each haplotype are showed in Table 3.3, haplotype A was the most common haplotype found only in the Northern; the Northern is referred to North, North-East and Central sample (94% of North, 97%of North-East and 93.5% of Central) and haplotype B was the

other common haplotype found only in the Southern; the Southern is referred to the South and the Samui Island samples (96% of South and 100% of the Samui Island). Haplotype C was a variant found with a low frequencies in North (5.5%) and in North-East (3%) while haplotype D was only found in one individual of Central region (3.25%). Haplotype E was observed in two individuals, S02 and S60, of South.

3.5.3 PCR-RFLP analysis by *Vsp*I

Six different fragment patterns were observed when amplified ATPase6-ATPase8 was restricted with *Vsp*I (Figure 3.18). The haplotype A was the most common haplotype related to haplotype B by at least 5 mutation steps probably including three sites losses and two gains. The variants like haplotype C and D could be interrelated to the most common haplotype by a single mutation step (either loss or gain). Individuals occupied haplotype E and F was still connected to A if at least 6 and 5 mutation steps were proposed. At this stage, it was suspected that these to specimens were not *A. cerana* though they are morphologically identical to such a species. Further studies need to be carried out to verified this suspicion. PCR-RFLP analysis of all *A. cerana* samples are showed in Table 3.3. As similar to previous result, haplotype A was geographically related to the Northern (found in 96%,97% and 100% in North, North-East and Central, respectively). Likewise, haplotype B was only found in the Southern population. Haplotype C was a population specific genotype for the Samui Island occurring with relatively high frequency of 0.5.

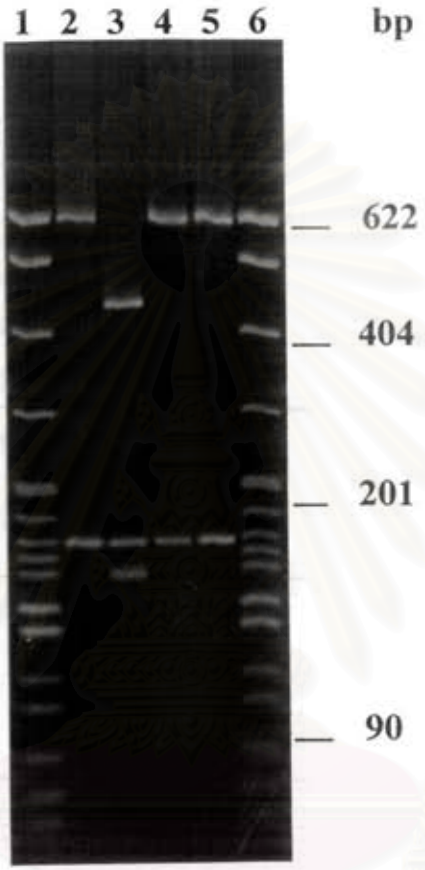

Figure 3.16 A 3% metaphor agarose gel showing restriction patterns of amplified ATPase6-ATPase8 gene with *Taq*I

Lanes 1,6 *Msp*I digested pBR322 standard marker
Lanes 2,3 Restriction patterns haplotype A and B, respectively
Lane 4 A restriction pattern observed from S02 or S60
Lane 5 A restriction pattern of *A. mellifera*

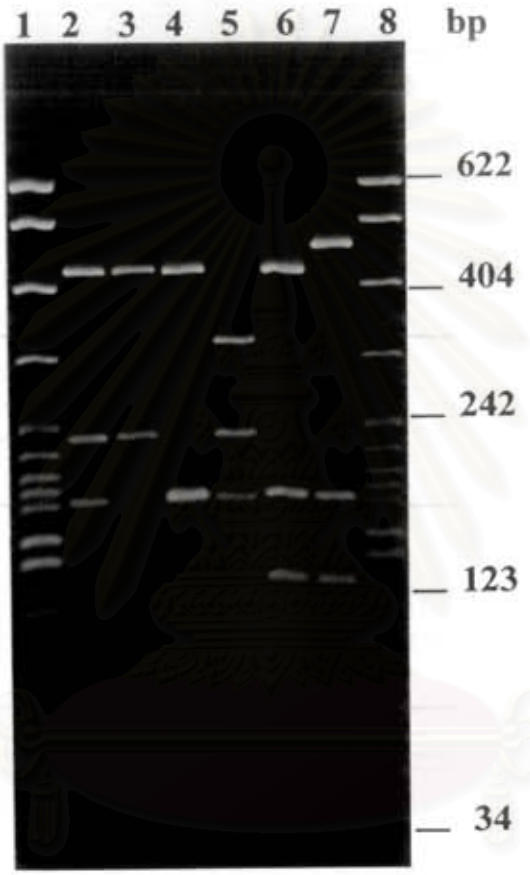

Figure 3.17 A 3% metaphor agarose gel showing restriction patterns of amplified ATPase6-ATPase8 gene with *Ssp*I

Lanes 1,8 *Msp*I digested pBR322 standard marker
Lanes 2-6 Restriction patterns haplotype A, B, C,D and E, respectively
Lane 7 A restriction pattern of *A. mellifera*(haplotype F)

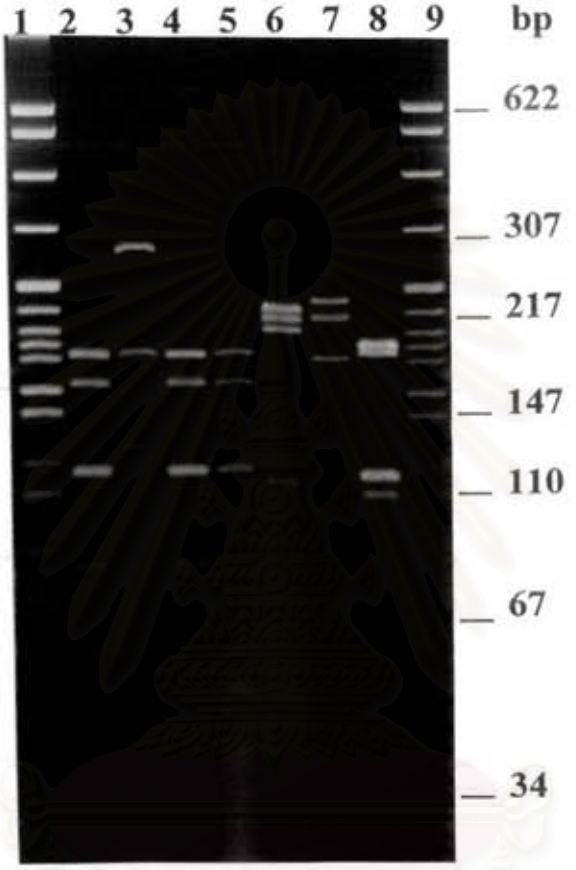

Figure 3.18 A 4% metaphor agarose gel showing restriction patterns of amplified ATPase6-ATPase8 gene with *Vsp*I

Lanes 1, 9	*Msp*I digested pBR322 standard marker
Lanes 2-7	Restriction patterns haplotype A, B, C, D, E, and F, respectively
Lane 8	A restriction of *A. mellifera* (haplotype G)

Table 3.2 Restricted fragment size (in base pairs) generated from digestion of amplified ATPase6-ATPase8 gene of *A. cerana* with *Taq*I, *Ssp*I and *Vsp*I. The digestion profiles resulted from digestion of the homologous gene with the same restriction enzymes are also included.

*Taq*I				*Ssp*I						*A. mel*	*Vsp*I							*A. mel*
	A	B	*A. mel*		A	B	C	D	E	F		A	B	C	D	E	F	G
620	—		—	465						—	275		—					
440		—		420	—	—	—		—		227						—	
205	—		—	315				—			217					—		
180		—		225	—	—		—			210					—	—	
Σ	82	825	825	180	—		---	—	—	—	200					—		
	5			125					—	—	195							—
				105				—			188							—
				100		—					185	—	—	—	—		—	
				80		—					165	—		—	—			
				55						—	125		—					
				50					—		115	—		---	---			—
				45			—				113					—	—	
				Σ	825	825	825	825	825	825	110				—			—
											80	—	—	—				
											55	—	—	—	—	—	—	—
											45	—			—			
											40		—	—				
											35	—	—	—	—		—	—
											30	—	—	—		—		
											20							—
											5			(---)				
											Σ	825	825	825	825	825	825	825

— = One fragment, ---- = Doublet with identical molecular length and (---) = Missing fragment

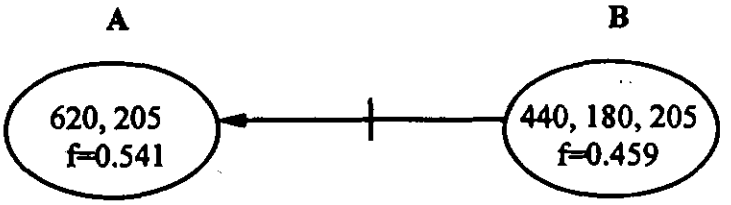

Figure 3.19 The most parsimonious network illustrating relationship between two haplotypes of *Taq*I digested ATPase6-ATPase8 gene in *A. cerana* mtDNA. The arrow indicate the direction of restriction site loss. Vertical bar indicates a single point mutation between haplotype A and B.

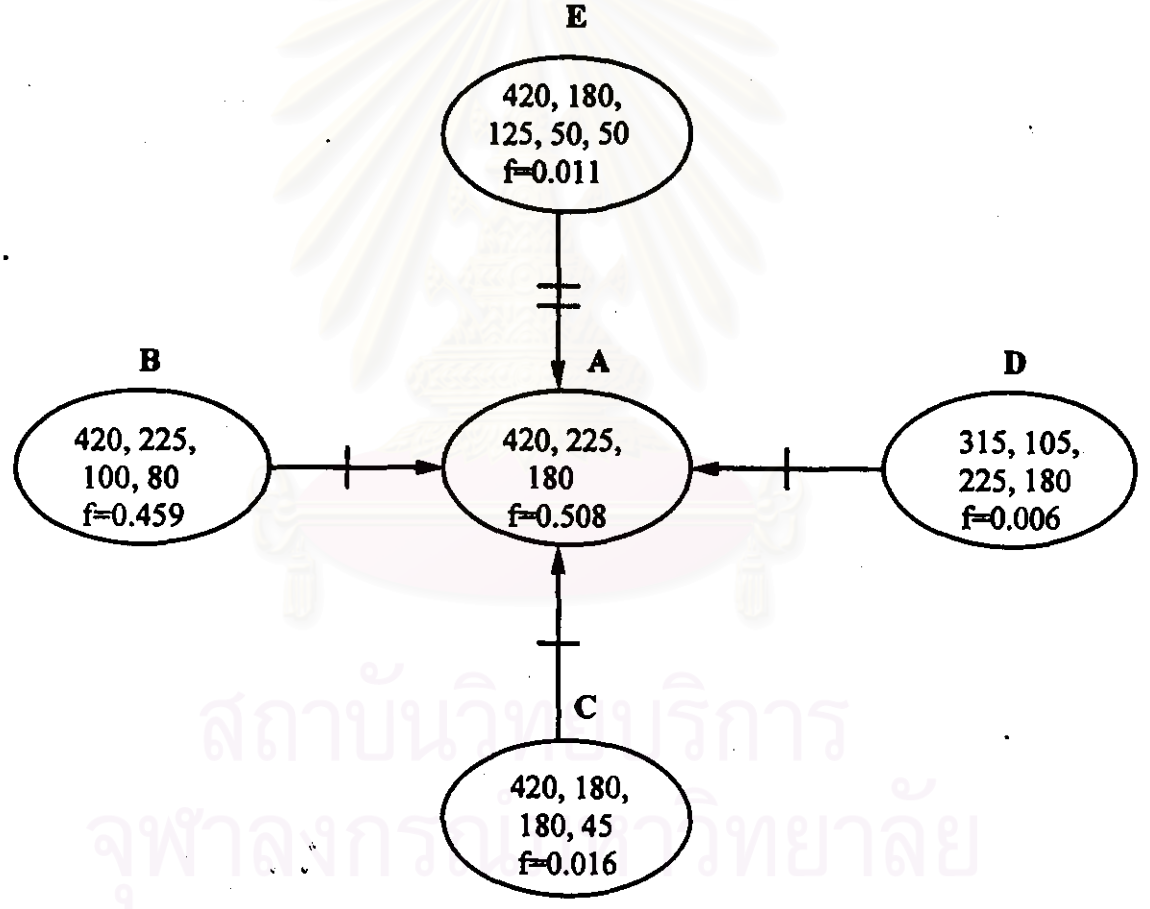

Figure 3.20 The most parsimonious network illustrating relationship between five haplotypes of *Ssp*I digested ATPase6-ATPase8 gene in *A. cerana* mtDNA. The arrows indicate the direction of restriction site loss. Vertical and horizontal bars indicates the minimum number of point mutation steps required to interrelate between each pair of haplotypes.

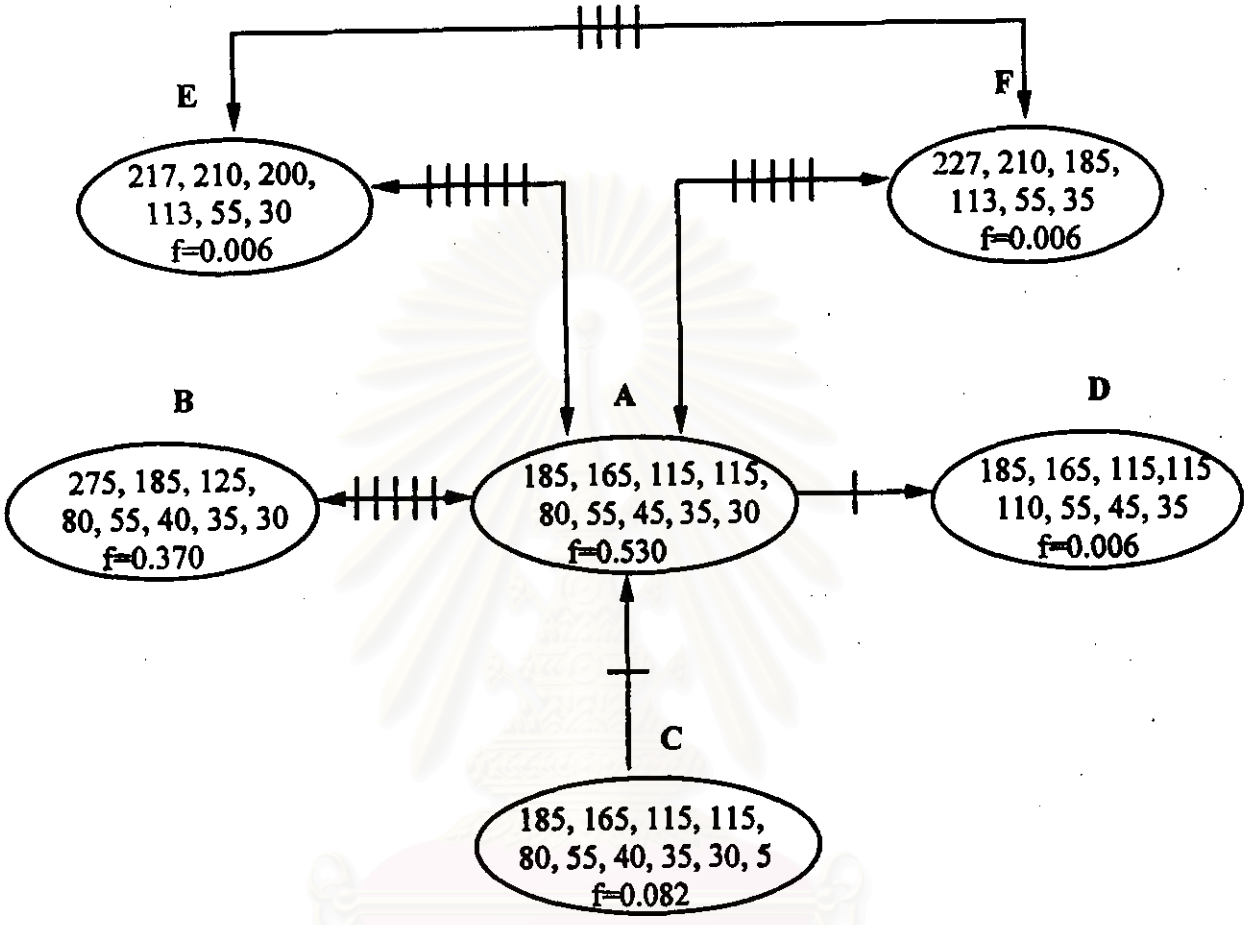

Figure 3.21 The most parsimonious network illustrating relationship between six haplotypes of *Vsp*I digested ATPase6-ATPase8 gene in *A. cerana* mtDNA. The arrows indicate the direction of restriction site loss. Vertical and horizontal bars indicates the number of point mutation steps required to interrelate between each pair of haplotypes. Bidirectional arrows indicate that such two haplotypes require both losses and gains of restriction sites.

Table 3.3 Summary of single enzyme haplotype frequencies generated from 3 restriction endonuclease in five geographic areas of *A. cerana* samples

Enzyme/ Haplotype	Sample site				
	North (n=36)	North-East (n=30)	Central (n=31)	South (n=54)	Samui Island (n=30)
*Taq*I					
A	1.0000	0.9667	1.0000	0.0370	0.0000
B	0.0000	0.0333	0.0000	0.9630	1.0000
*Ssp*I					
A	0.9444	0.9667	0.9354	0.0000	0.0000
B	0.0000	0.0000	0.0323	0.9630	1.0000
C	0.0556	0.0333	0.0000	0.0000	0.0000
D	0.0000	0.0000	0.0323	0.0000	0.0000
E	0.0000	0.0000	0.0000	0.0370	0.0000
*Vsp*I					
A	1.0000	0.9667	1.0000	0.0000	0.0000
B	0.0000	0.0000	0.0000	0.9630	0.5000
C	0.0000	0.0000	0.0000	0.0000	0.5000
D	0.0000	0.0333	0.0000	0.0000	0.0000
E	0.0000	0.0000	0.0000	0.0185	0.0000
F	0.0000	0.0000	0.0000	0.0185	0.0000

3.6 Geographic distributions of composite haplotypes in *A. cerana*

A restriction fragment pattern of each *A. cerana* individual from the single enzyme digestion was combined to generate composite haplotypes. The composite haplotypes for each individual (colony) were designed by 3 letter code representing the fragment pattern for three restriction endonucleases, *Taq*I, *Ssp*I and *Vsp*I, respectively. A total of ten composite haplotypes were observed from 181 investigated individuals (Table 3.4). Haplotype I (AAA) was the most common haplotype and region- specific found only in the Northern population. Haplotype II (ACA), III (AAD), IV (BAA), V (ADA) and VI (ABA) were variants found in relatively low frequencies in the Northern populations (North, North-East and Central). The most important information found in the present study was that all haplotypes found in the Northern populations did not appear in specimens from the Southern populations (South and Samui Island) and vice verse. This strongly indicated population differentiation of *A. cerana* in Thailand. There were four composite haplotypes found in the South and the Samui Island samples. Of which, haplotype VII (BBB) was the most common haplotype in such areas. The interconnected between AAA and BBB need 7 points mutation step. This also implied that *A. cerana* in Thailand could be divided into at least 2 lineages.

Some composite haplotypes (AAD, BAA, ADA, ABA, BBC, AEF, AEE) were population-specific. All but BBC were occurred with low frequencies which the BBC haplotype were found in 50% of *A. cerana* from the Samui Island. Only one composite haplotype was found in *A. mellifera* (BFG).

Table 3.4 Geographic distribution frequency of 10 composite haplotypes among five geographic locations of Thai honeybee *A. cerana*

Haplotype		Distribution frequency					Total
		North	North-East	Central	South	Samui	
I	AAA	34(0.944)	27(0.901)	29(0.936)			90(0.497)
II	ACA	2(0.056)	1(0.033)				3(0.017)
III	AAD		1(0.033)				1(0.0055)
IV	BAA		1(0.033)				1(0.0055)
V	ADA			1(0.032)			1(0.0055)
VI	ABA			1(0.032)			1(0.0055)
VII	BBB				52(0.962)	15(0.500)	67(0.370)
VIII	AEF				1(0.019)		1(0.0055)
IX	AEE				1(0.019)		1(0.0055)
X	BBC					15(0.500)	15(0.083)
Total		36(1.000)	30(1.000)	31(1.000)	54(1.000)	30(1.000)	181(1.000)

Geographic heterogeneity analysis based on a Monte Carlo simulation was examined the significant different in composite haplotype distribution frequencies among 5 geographic locations of *A. cerana*. As can be seen in Table 3.5, highly significant in haplotype frequencies were observed implying the existence of population subtraction within *A. cerana* ($P<0.0001$). All comparisons, with the exception of pairwise comparison within the Northern groups (North, North-East and Central) also showed statistically significant different (all at $P<0.0001$). These results illustrated strong genetic population differentiation of the Northern (North, North-East and Central) and the Southern (South and Samui Island) *A. cerana* in Thailand.

Table 3.5 Analysis of geographic heterogeneity in haplotype frequency distributions generated from Restriction Fragment Length Polymorphism (RFLP) of *A. cerana* using a Monte Carlo simulation for ten thousand times

	P-value
North & North-East	P=0.3110
North & Central region	P=0.1463
North & South	P<0.0001
North & the Samui Island	P<0.0001
North-East & Central region	P=0.4788
North-East & South	P<0.0001
North-East & the Samui Island	P<0.0001
Central region & South	P<0.0001
Central region & the Samui Island	P<0.0001
South & the Samui Island	P<0.0001
North, North-East, Central region & South	P<0.0001
North, North-East, Central region & the Samui Island	P<0.0001
SSouth & each 5 geographic areas of *A. cerana*	P<0.0001
SSouth & *A. mellifera*	P<0.0001

3.7 Data analysis

Genetic distance among 11 composite haplotypes of *A. cerana* and *A. mellifera* (AFG) ranging between 0.0 to 0.1087 (Table 3.6). The values were used to construct a dendrogram using unweighted pair group method of analysis (UPGMA) approach (Figure 3.22). The phenogram indicated that the 10 composite haplotypes of *A. cerana* can be allocated into 3 groups. Group 1 contained of 6 composite haplotypes (ABA, AAA, ADA, ACA, AAD and BAA) which were found in the Northern (North, North-East and Central) (Table 3.4). Group 2 consisted of 2 composite haplotypes (BBB and BBC) which were only observed in South and the Samui Island (Table 3.4). The last group was composed of 2 composite haplotypes (AEF and AEE) from 2 colonies (S02 and S60) from the South (Table 3.4).

Haplotype and nucleotide diversity within population (Table 3.7) were generally low in all geographic areas except that of the Samui Island. The average haplotype diversity within population of *A. cerana* was 0.2000. This value in *A. mellifera* was 0.000 indicating monomorphic haplotype in this species. However the number of *A. mellifera* individuals used in the present study was only three so that a large sample size needs to be investigated before premature conclusion on genetic diversity level in this species is drawn. The highest nucleotide diversity was observed in the Samui Island (0.667%) following by South (0.448%), North-East (0.157%), Central (0.084%) and North (0.070%), respectively.

Table 3.6 Genetic distance among 10 composite haplotypes of *A. cerana* and one composite haplotype of *A. mellifera* (AFG)

	AAA	ACA	BAA	AAD	ADA	ABA	BBB	AEF	AEE	BBC	AFG
AAA	-										
ACA	0.0065	-									
BAA	0.0099	0.0159	-								
AAD	0.0070	0.0147	0.0172	-							
ADA	0.0065	0.0135	0.0159	0.0147	-						
ABA	0.0065	0.0135	0.0159	0.0147	0.0135	-					
BBB	0.0373	0.0476	0.0267	0.0533	0.0476	0.0261	-				
AEF	0.0440	0.0466	0.0533	0.0414	0.0593	0.0593	0.0655	-			
AEE	0.0389	0.0412	0.0476	0.0466	0.0515	0.0515	0.0576	0.0092	-		
BBC	0.0224	0.0296	0.0128	0.0322	0.0296	0.0146	0.0128	0.0701	0.0617	-	
AFG	0.0467	0.0492	0.0555	0.0340	0.0492	0.0619	0.1080	0.0567	0.0733	0.0723	-

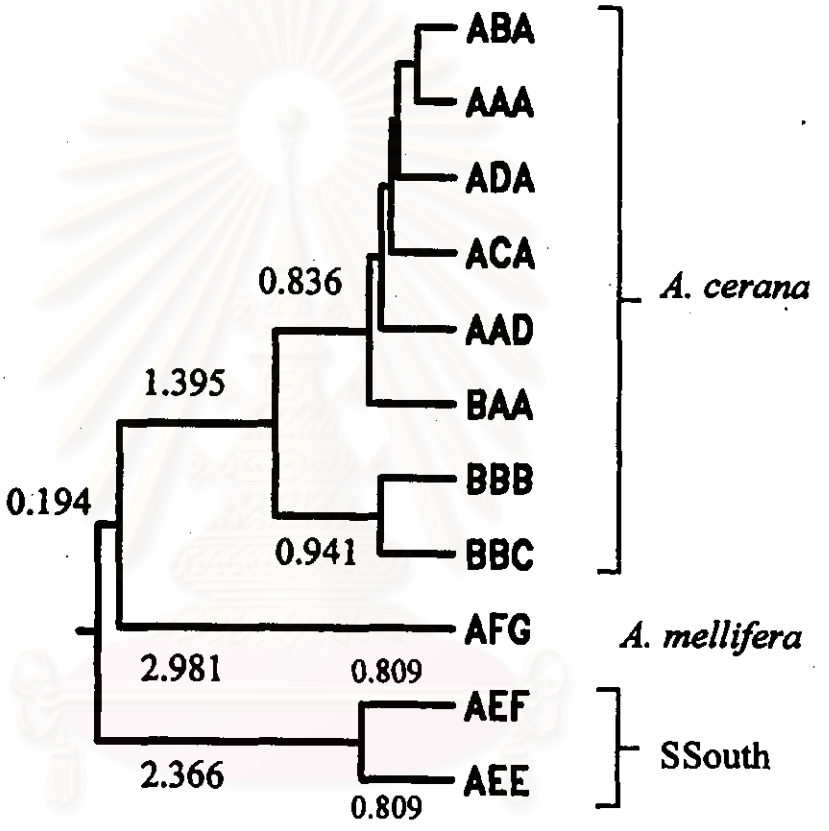

Figure 3.22 A UPGMA dendrogram showing relationships of composite haplotypes calculated from genetic distance

Table 3.7 Haplotype and nucleotide diversity of *A. cerana* from five geographic locations in Thailand

Population	Haplotype diversity (±SE)	Nucleotide diversity (%)
North	0.106 (0.048)	0.070
North-East	0.189 (0.067)	0.157
Central region	0.125 (0.056)	0.084
South	0.073 (0.034)	0.448
Samui Island	0.508 (0.012)	0.667
A. mellifera	0.000 (0.000)	0.000
Mean (for *A. cerana* only)	0.200 (0.043)	0.285

As can be seen from Table 3.8, nucleotide diversity between population of each pairwise comparison was high. The average of between population diversity was 2.1588% which was much greater than the average nucleotide diversity within population (0.2852%) (Table 3.7) indicating strong population differentiation in this taxon.

Nucleotide divergence between almost all of the pairwise comparison was high (Table 3.9). Relatively low values were found in the comparisons within the Northern (North, North-East and Central) population (0.0000%-0.0016%) whereas the remaining comparisons show much higher nucleotide divergence (0.31137%-3.75787%). The nucleotide divergence between *A. mellifera* and *A. cerana* from the Northern (North, North-East and Central) locations were between 4.58424%-4.68261% whereas those of *A. mellifera* and *A. cerana* from the Southern (South and Samui Island) locations were 8.68561% and 10.8069% when compared to the Samui Island and South populations, respectively. A UPGMA phenogram based on the nucleotide divergence is shown in Figure 3.23. These phenogram allocated the 5 geographic locations of *A. cerana* into 2 distinct groups composing of group 1 (North, North-East and Central) and group 2 (South and the Samui Island).

Table 3.8 Pairwise comparison between population nucleotide diversity (in per cent) among 5 geographic locations of *A. cerana*. Nucleotide diversity from *A. mellifera* is also illustrated.

	North	North-East	Central region	South	the Samui Island	*A. mellifera*
North	-					
North-East	0.1120	-				
Central region	0.0780	0.1203	-			
South	3.8066	3.8030	3.7516	-		
the Samui Island	3.0398	3.0297	2.9891	0.8573	-	
A. mellifera	5.0534	5.0962	5.1510	7.3704	7.6050	-
Average	2.1588±0.00273					
Minimum	0.0780					
Maximum	3.8066					

Table 3.9 Pairwise comparison of estimated nucleotide divergence (in per cent) among the 5 geographic locations of *A. cerana* in Thailand. Nucleotide divergence from *A. mellifera* is also illustrated.

	North	North-East	Central	South	Samui	*A. mel*
North	-					
North-East	0.00000	-				
Central	0.00116	0.00010	-			
South	3.75787	3.70953	3.69087	-		
Samui	2.67122	2.61774	2.61360	0.31137	-	
A. mel	4.64593	4.58424	4.68261	10.8069	8.68561	-

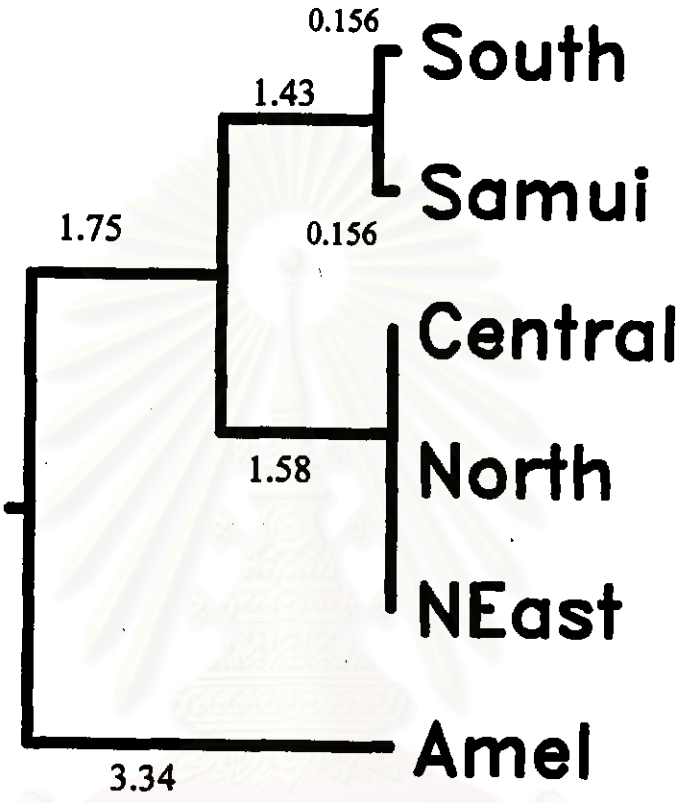

Figure 3.23 A UPGMA phenogram of the five geographic locations of *A. cerana* based on nucleotide divergence

Based on restriction patterns obtained from three restriction enzyme digestion, sample S02 and S60 from the South showed large genetic differences from other *A. cerana* samples. In addition, estimated genetic distance from mitochondrial haplotypes between S02 and S60 was low (d=0.0092) in contrast to between S02 or S60 with other (d>0.01) (Table 3.6). Furthermore, the nucleotide divergence between SSouth and each of *A. cerana* populations were high (Table 3.10). It was then suspected that these two individuals were not *A. cerana* therefor they are further separated from the South (named as SSouth = some of the South). The data were then reanalyzed. A UPGMA phenogram based on the nucleotide divergence is shown in Figure 3.24. The results showed that genetic distance between SSouth and other *A. cerana* group were enormously larger than between SSouth with the other *Apis* species like *A. mellifera*. This should not be overlooked and actually need to be verified the evolutionary status of *A. cerana* in Thailand.

Table 3.10 Pairwise comparisons of nucleotide divergence (in per cent) among the 5 geographic locations of *A. cerana* in Thailand

	North	North-East	Central	South	Samui	SSouth	*A. mel*
North	-						
North-East	0.0000	-					
Central	0.0012	0.0001	-				
South	3.7578	3.7095	3.6908	-			
Samui	2.6712	2.6177	2.6136	0.3113	-		
Ssouth	4.2093	4.2087	4.3000	6.5613	6.4623	-	
A. mel	4.6459	4.5842	4.6826	10.806	8.6856	5.6927	-

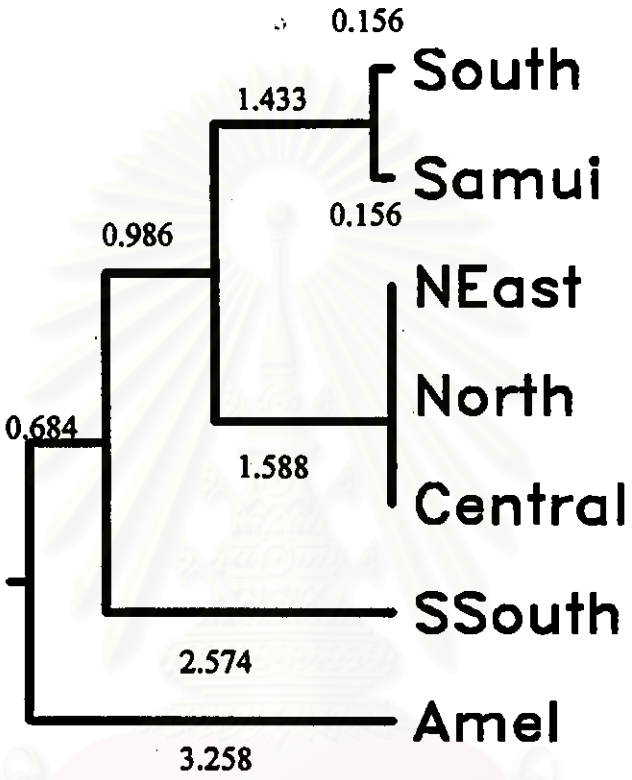

Figure 3.24 A UPGMA phenogram of the five geographic areas of *A. cerana* and SSouth based on nucleotide divergence