CHAPTER III

RESULT

3.1 DNA extraction

Nuclear DNA of *A. cerana* was extracted from each *A. cerana* individual using the extraction protocol described in 2.8. High molecular weight DNA obtained was larger than 23.1 kb. The concentration of extracted DNA was about 1.0-1.5 μg per individual as estimated by comparing its intensity of EtBr-DNA complex with that of the known amount of λ/*Hind*III marker in 0.7% agarose gel electrophoresis (Figure 3.1). The DNA solution was diluted to final concentration of 20 ng/μl for subsequent used in the PCR reactions.

3.2 Optimization of $MgCl_2$ concentration for amplification of the ITS region in *A. cerana.*

In order to amplified ITS region of nuclear ribosomal RNA gene of *A. cerana* using primers of fungal ribosomal RNA genes, the optimization of $MgCl_2$ concentration for PCR reaction was then performed. The ITS region of *A. cerana* rRNA gene was routinely amplified by primer ITS4 and ITS5 using the standard condition described in 2.10.1 with $MgCl_2$ concentration varied from 1.0, 1.2, 1.5, 1.8 and 2.0 mM. As can be seen in Figure 3.2, the amplified product firstly appeared when the concentration of $MgCl_2$ is 1.0 mM. The more intense band was observed in a reaction containing 1.2 mM $MgCl_2$. From the result, the optimal $MgCl_2$ concentration was 1.2 mM.

Therefore, ITS region of *A. cerana* was amplified in the reaction mixture contain 1X PCR buffer, 200 μM each of dNTP, 0.2 μM each of primers, 1.2 mM $MgCl_2$, 30 ng total DNA and 0.5 unit *Taq* DNA polymerase.

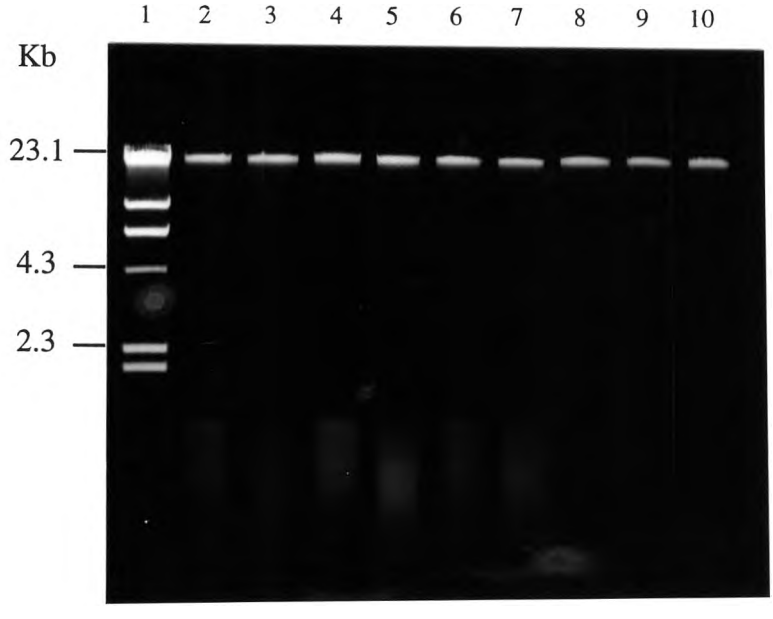

Figure 3.1 High molecular weight DNA of *A. cerana* extracted from the thorax of each *A. cerana* worker and subjected to 0.7% agarose gel electrophoresis at 100 V for 50 minutes.

Lane 1 λ/*Hind*III DNA standard

Lane 2-6 Total nuclear DNA isolated from *A. cerana*.

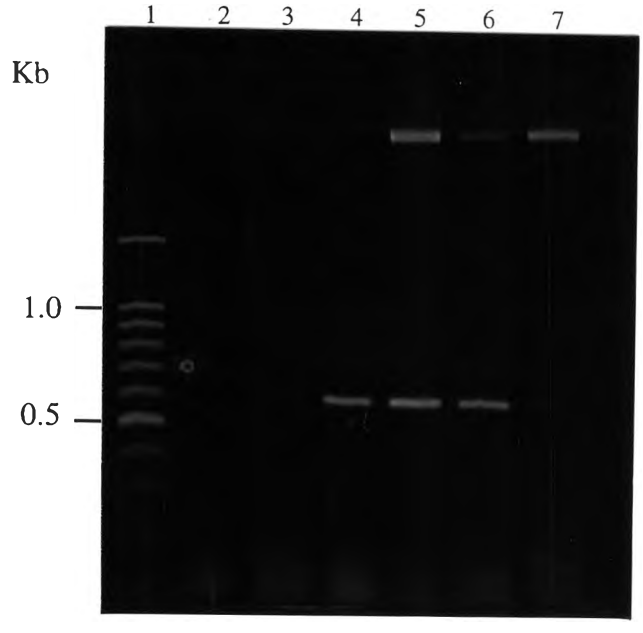

Figure 3.2 Optimization of $MgCl_2$ concentration used for amplification of ITS region in ribosomal RNA gene of *A. cerana*. The amplified products were analyzed by a 1.5 % agarose gel electrophoresis at 120 V for 1.5 hours and stained with ethidium bromide.

Lane 1 A 100 bp DNA ladder

Lane 2-6 The amplified products of ITS region resulted from amplification reactions containing 0.0, 1.0, 1.2, 1.5 ,1.8 and 2.0 mM of $MgCl_2$, respectively.

3.3 Characterization of the ITS amplified product

The ITS region of nuclear ribosomal RNA gene was amplified from five geographic samples of *A. cerana* in Thailand using primers ITS4 and ITS5. When the PCR reaction was completed, the ITS amplified product was electrophoresed through a 1.5 % agarose gel and stained with ethidium bromide. The ITS amplified products of each samples had different band patterns that the size varied from about 500 to 800 bp (Figure 3.3 and Table 3.1). A 580 amplified fragment was a common band which was found from most of the DNA samples used in the experiment. Therefore, it was chosen for further genetic study of *A. cerana* in Thailand by DNA sequencing.

3.4 DNA sequencing

The ITS region was sequenced from 21 individuals of *A. cerana* from five different geographic samples. Direct sequencing of ITS amplified products was done for three to five additional individuals from each of these samples (Table 3.1). After amplification, a 580 bp amplified band of each individuals was purified from agarose gel and used as the DNA template of sequencing as described in 2.11.

The DNA template of each i ndividual was sequenced using external primers (ITS4 and ITS5) and internal primers (ITS2 and ITS3) as described in 2.12. The 511 of 580 ITS nucleotide base were determined. An autoradiogram of partial ITS sequence derived from the primer ITS3 is shown in Figure 3.4. Comparing of these with previous GenBank deposited DNA sequences using BLAST (Basic Local Alignment Search Tool) at the website http://www.ncbi.nlm.nih.gov (Altschul *et al.*, 1990) indicated that the sequence obtained were certainly the ITS region of nuclear ribosomal clusters (Appendix 2).

Moreover, the nucleotide sequences from each samples were aligned by CLUSTALX as shown in Figure 3.5. Highly homology between sequence of Thai *A. cerana* was observed. There were 4 point mutations after alignment of all sequences.

A G-T transversion was found at the 40th position. Specimens from the South possessed a G while the remaining had a T instead. The T-C transitions were observed at positions 305 and 326. The Samui sample possessed a T at these positions while others had C base. Dissociation of *A. cerana* from the South and Samui could be done by these three point mutation. At the position 419, all specimen from the North, Central and North-East areas had a C whereas the South and Samui *A. cerana* possessed a G base. The average base frequencies of the ITS in *A. cerana* were approximately equal A: 22.9 % , T: 25.0 % , G: 25.7 % and C: 26.4 %.

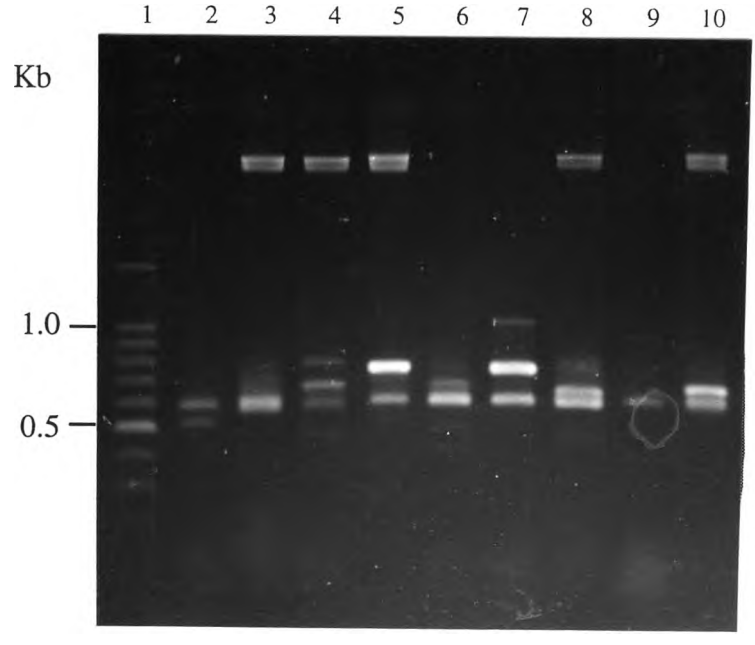

Figure3.3 The ITS amplified products were electrophoresed though a 1.5 % agarose gel at 120 V for 1 hours.

Lane 1 A 100 bp DNA ladder

Lane 2-11 The amplified products of ITS region of *A. cerana* from DNA samples of C11,C12, C14, N39, NE19, NE28, NE30, S22 and I30.

Table 3.1 The size of ITS amplified products of *A. cerana* from North (N), Central (C), North-East (NE), South (S) and Samui Island (I).

Sample	Size of ITS amplified product (bp)						
	500	580	600	650	700	780	800
N6			*	*			
N16**		*					
N18		*					
N21		*					
N24**		*					
N28**		*					
N30	*						
N38	*				*		
N39		*				*	
N40					*		
N48	*						
C1		*					
C7**		*		*			
C11	*	*					
C12**		*					
C14		*		*			*
C15**		*			*		
C16**		*					
C20		*					
C21**		*					
C26		*					
C28		*			*		
NE18		*		*			

** amplified product of 580 bp was sequenced

Table 3.1 (continued)

Sample	Size of ITS amplified product (bp)						
	500	580	600	650	700	780	800
NE19		*		*			
NE24**		*		*			
NE25		*		*			
NE26**		*		*			
NE28		*				*	
NE30**		*		*			
NE35**		*					
S22**		*					
S39		*			*		
S51		*					
S53		*					
S59**		*			*		
S65**		*			*		
S66**		*					
S70		*					
I16		*					
I18			*				
I21**		*	*				
I24		*					
I27		*	*				
I29**		*					
I30		*		*			
I32				*			
I33**		*					
I35**		*		*			

** amplified product of 580 bp was sequenced

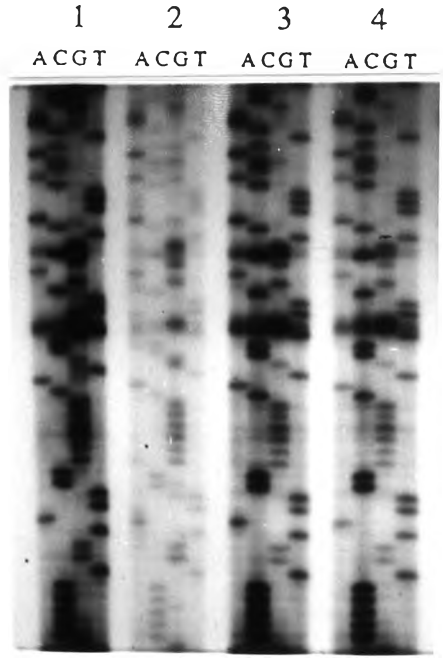

Figure 3.4 An autoradiogram of partial ITS sequence derived from primer ITS3. No sequence polymorphism was observed between geographically different samples.

Lane 1-4 Nucleotide sequences of a 580 amplified product from N28, NE24,NE26 and S59, respectively.

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                                                                                                                    60
N16             GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGGCTAACCACCGGGATGTTCAT
N24             GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGGCTAACCACCGGGATGTTCAT
N28             GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGGCTAACCACCGGGATGTTCAT
C7              GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGGCTAACCACCGGGATGTTCAT
C12             GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGGCTAACCACCGGGATGTTCAT
C15             GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGGCTAACCACCGGGATGTTCAT
C16             GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGGCTAACCACCGGGATGTTCAT
C21             GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGGCTAACCACCGGGATGTTCAT
NE24            GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGGCTAACCACCGGGATGTTCAT
NE26            GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGGCTAACCACCGGGATGTTCAT
NE30            GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGGCTAACCACCGGGATGTTCAT
NE35            GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGGCTAACCACCGGGATGTTCAT
S22             GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGTCTAACCACCGGGATGTTCAT
S39             GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGTCTAACCACCGGGATGTTCAT
S59             GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGTCTAACCACCGGGATGTTCAT
S65             GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGTCTAACCACCGGGATGTTCAT
S66             GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGTCTAACCACCGGGATGTTCAT
I21             GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGGCTAACCACCGGGATGTTCAT
I29             GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGGCTAACCACCGGGATGTTCAT
I33             GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGCCTAACCACCGGGATGTTCAT
I35             GTAGTGAACCTGCGGAGGGATCATTACAAGTGACCCCGGGCTAACCACCGGGATGTTCAT
                ************************************** ********************

                                                                                                                   120
N16             AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
N24             AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
N28             AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
C7              AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
C12             AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
C15             AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
C16             AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
C21             AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
NE24            AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
NE26            AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
NE30            AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
NE35            AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
S22             AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
S39             AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
S59             AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
S65             AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
S66             AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
I21             AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
I29             AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
I33             AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
I35             AACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGCGGGGGCTCC
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(A)

Figure 3.5 Alignment of nucleotide sequences of *A. cerana* in ITS region of nuclear ribosomal RNA gene of 21 honeybee samples using Clustal X (A). The diagram show position of four point mutations of these ITS sequence (B).

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                                                                      180
N16         GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
N24         GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
N28         GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
C7          GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
C12         GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
C15         GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
C16         GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
C21         GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
NE24        GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
NE26        GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
NE30        GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
NE35        GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
S22         GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
S39         GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
S59         GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
S65         GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
S66         GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
I21         GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
I29         GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
I33         GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
I35         GGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATT
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                                                                      240
N16         AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
N24         AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
N28         AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
C7          AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
C12         AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
C15         AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
C16         AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
C21         AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
NE24        AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
NE26        AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
NE30        AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
NE35        AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
S22         AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
S39         AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
S59         AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
S65         AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
S66         AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
I21         AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
I29         AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
I33         AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
I35         AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA
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(A)

Figure 3.5 (continued)

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                                                                                                                    300
N16             TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
N24             TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
N28             TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
C7              TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
C12             TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
C15             TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
C16             TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
C21             TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
NE24            TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
NE26            TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
NE30            TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
NE35            TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
S22             TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
S39             TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
S59             TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
S65             TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
S66             TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
I21             TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
I29             TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
I33             TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
I35             TAAGTAATGTGAATTGCAGAATTCAGTCAATCATCGAATCTTTAACGCACATTGCGCCCC
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                                                                                                                    360
N16             CTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
N24             CTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
N28             CTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
C7              CTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
C12             CTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
C15             CTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
C16             CTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
C21             CTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
NE24            CTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
NE26            CTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
NE30            CTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
NE35            CTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
S22             CTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
S39             CTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
S59             CTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
S65             CTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
S66             CTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
I21             CTGGCATTCCGGGGGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
I29             CTGGCATTCCGGGGGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
I33             CTGGCATTCCGGGGGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
I35             CTGGCATTCCGGGGGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGT
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(A)

Figure 3.5 (continued)

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                                                                                                                    420
N16             ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTCG
N24             ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTCG
N28             ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTCG
C7              ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTCG
C12             ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTCG
C15             ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTCG
C16             ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTCG
C21             ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTCG
NE24            ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTCG
NE26            ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTCG
NE30            ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTCG
NE35            ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTCG
S22             ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTGG
S39             ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTGG
S59             ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTGG
S65             ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTGG
S66             ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTGG
I21             ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTGG
I29             ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTGG
I33             ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTGG
I35             ATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCTTCTGTCCCCTGG
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                                                                                                                    480
N16             CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
N24             CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
N28             CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
C7              CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
C12             CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
C15             CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
C16             CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
C21             CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
NE24            CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
NE26            CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
NE30            CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
NE35            CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
S22             CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
S39             CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
S59             CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
S65             CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
S66             CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
I21             CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
I29             CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
I33             CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
I35             CGTTGTGGAAACTATTCGCTAAAGGGTGCTCGGGAGTACGCCGTAAAACCAAACCCATTT
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(A)

Figure 3.5 (continued)

```
                                                    511
N16             CTAAGGTTGACCTCGGATCAGGTAGGGATAC
N24             CTAAGGTTGACCTCGGATCAGGTAGGGATAC
N28             CTAAGGTTGACCTCGGATCAGGTAGGGATAC
C7              CTAAGGTTGACCTCGGATCAGGTAGGGATAC
C12             CTAAGGTTGACCTCGGATCAGGTAGGGATAC
C15             CTAAGGTTGACCTCGGATCAGGTAGGGATAC
C16             CTAAGGTTGACCTCGGATCAGGTAGGGATAC
C21             CTAAGGTTGACCTCGGATCAGGTAGGGATAC
NE24            CTAAGGTTGACCTCGGATCAGGTAGGGATAC
NE26            CTAAGGTTGACCTCGGATCAGGTAGGGATAC
NE30            CTAAGGTTGACCTCGGATCAGGTAGGGATAC
NE35            CTAAGGTTGACCTCGGATCAGGTAGGGATAC
S22             CTAAGGTTGACCTCGGATCAGGTAGGGATAC
S39             CTAAGGTTGACCTCGGATCAGGTAGGGATAC
S59             CTAAGGTTGACCTCGGATCAGGTAGGGATAC
S65             CTAAGGTTGACCTCGGATCAGGTAGGGATAC
S66             CTAAGGTTGACCTCGGATCAGGTAGGGATAC
I21             CTAAGGTTGACCTCGGATCAGGTAGGGATAC
I29             CTAAGGTTGACCTCGGATCAGGTAGGGATAC
I33             CTAAGGTTGACCTCGGATCAGGTAGGGATAC
I35             CTAAGGTTGACCTCGGATCAGGTAGGGATAC
                ********************************
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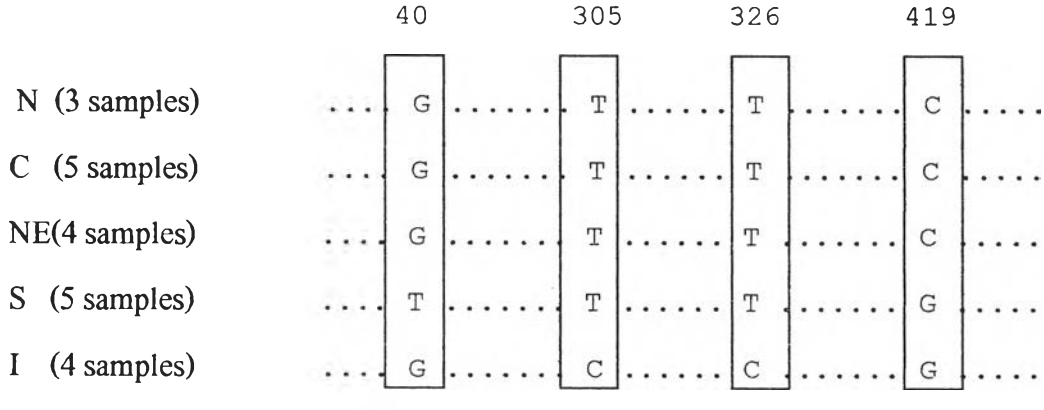
(A)



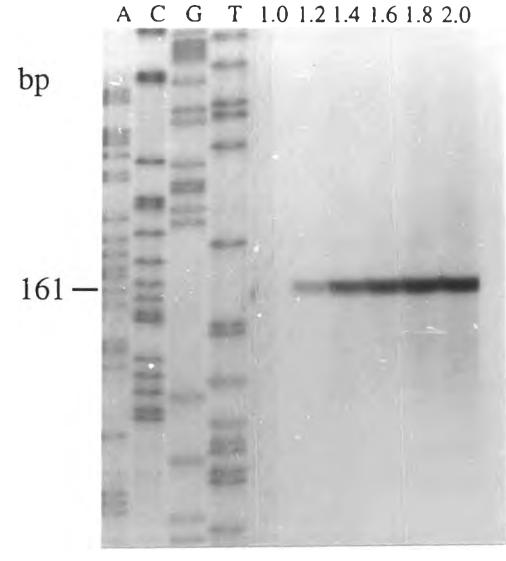
(B)

Figure 3.5 (continued)

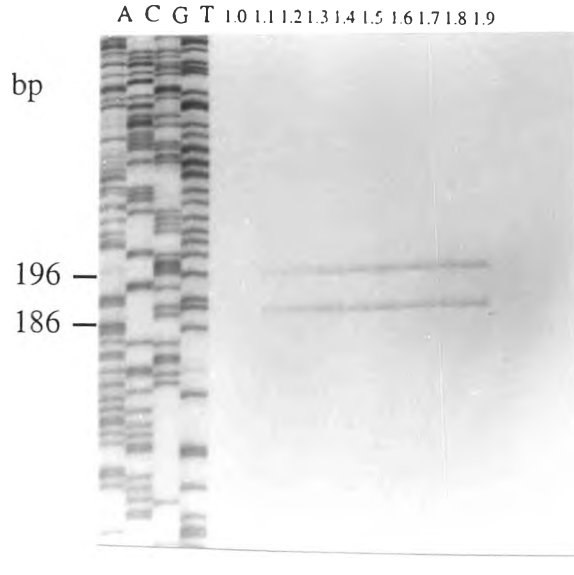
3.5 Optimization of $MgCl_2$ concentration for amplification of microsatellite loci in *A. cerana*.

In this study, 13 microsatellite loci were selected from *A. mellifera* microsatellite loci, so the heterospecific microsatellite DNA of *A. cerana* was amplified by PCR reaction using conditions previously worked well for *A. mellifera* (Estoup *et al.*, 1995) as described in 2.10.2. The $MgCl_2$ concentration for each *A. mellifera* microsatellite locus was optimized to be used for *A. cerana* and was varied from 1.0-1.9 mM for locus A7, A8, A14, A24, A29, A35, A43, A79, A81, A88 and A113 and from 0.6-2.5 mM for locus A28 and A107. The optimal $MgCl_2$ concentration was chosen from that provide the most intense band of PCR product (Table 3.2 and Figure 3.6).

From the amplification results, five of the thirteen microsatellite loci (A7, A29, A35, A43 and A79) tested was not amplified in *A. cerana*. Nonspecific amplified products were observed in locus A35 at 55-57 ^{O}C annealing temperature and no amplified products were obtained in locus A7, A29, A43 and A79 at PCR reactions containing 1.0-1.9 mM $MgCl_2$ at 52 ^{O}C annealing temperature. For others loci, eight microsatellite loci (A8, A14, A24, A28, A81, A88, A107 and 113) were able to amplify microsatellites in *A. cerana* population investigated.



A8



A113

Figure 3.6 The optimal $MgCl_2$ concentration for microsatellite loci (A8 and A113) detected by polyacrylamide gel electrophoresis. The size standard is a M13 sequencing marker.

Table 3.2 PCR conditions of microsatellite primer used to screen polymorphic loci in *A. cerana*

Locus	$MgCl_2$, mM	Annealing Temps, ^{O}C	No. Alleles Observed	Size of allele (bp)
A7	1.0-1.9	52	None	-
A8	1.4	55	2	160, 165
A14	1.5	58	1	180
A24	1.0	58	3	95, 96, 97
A28	1.6	55	24	108-132
A29	1.0-1.9	52	None	-
A35	1.2	55-57	Nonspecific products	-
A43	1.0-1.9	52	None	-
A79	1.0-1.9	52	None	-
A81	1.2	52	1	132
A88	1.2	57	1	137
A107	1.2	55-57	10	155-169
A113	1.6	58	3	182, 186, 196

3.6 Characterization of the amplified product of eight microsatellite loci in *A. cerana*

Eight microsatellite loci (A8, A14, A24, A28, A81, A88, A107 and 113) were tested for study about genetic differentiation of *A. cerana* population in Thailand by 40-50 individuals *A. cerana* DNA from each of five geographic populations (North, North-East, Central, South and Samui Island). The microsatellite products were identified on the 6% denaturing polyacrylamide gel with M13 standard sequencing marker as a size standard as described in 2.10.2.

The locus A14, A81 and A88 were fixed for 180, 132 and 137 bp, respectively. Only two alleles were observed with locus A8 (160, 165 bp). In addition three alleles were observed with locus A24 (95, 96, 97 bp) and A113 (182, 186, 196 bp)(Figure 3.9). A large number of alleles were found at locus A28 and A107 (Figure 3.7 and 3.8). The results of amplification of each locus are shown in table 3.2.

Based on the amplification success and observed number of alleles, three microsatellite loci, A28, A107 and A113, were chosen for further analysis of genetic diversity and differentiation of *A. cerana* in Thailand.

3.7 Genetic variation in Thai *A. cerana*

The A28, A107 and A113 microsatellite loci were polymorphic and gave microsatellite products in all investigated samples. For amplification of microsatellite loci, its products appeared as the single (homozygote) or double (heterozygote) groups of stutter bands. The actual allele size was determined from the most intense band within a group of stutter bands and assigned microsatellite product sizes in base pair length (bp) by comparison with a M13 sequencing marker.

All 265 individual colonies of *A. cerana* from five geographic samples (North : 47, Central : 54, North-East : 54, South : 71 and Samui Island : 39 colonies) were genetically typed using three microsatellite loci (A28, A107 and A113). When all investigated samples were amplified with primer A28, A107 and A113, they could

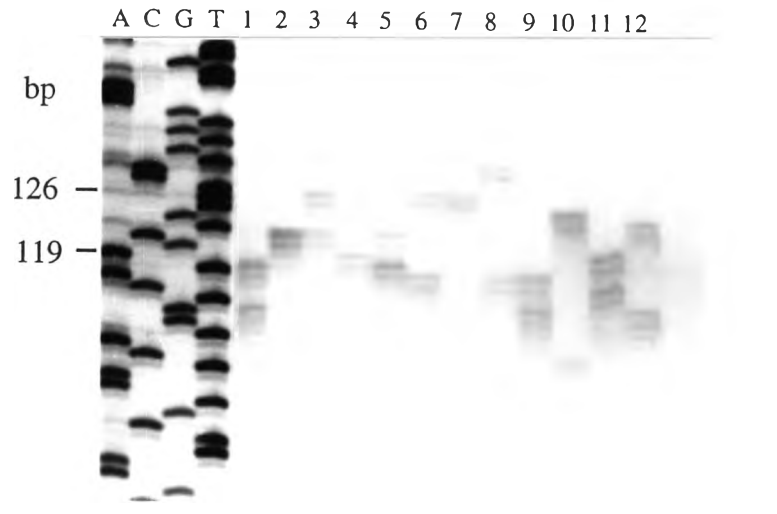


Figure 3.7 Microsatellite patterns of *A. cerana* induviduals at locus A28 (lanes 1-12). A M13 sequencing marker was used as a standard.

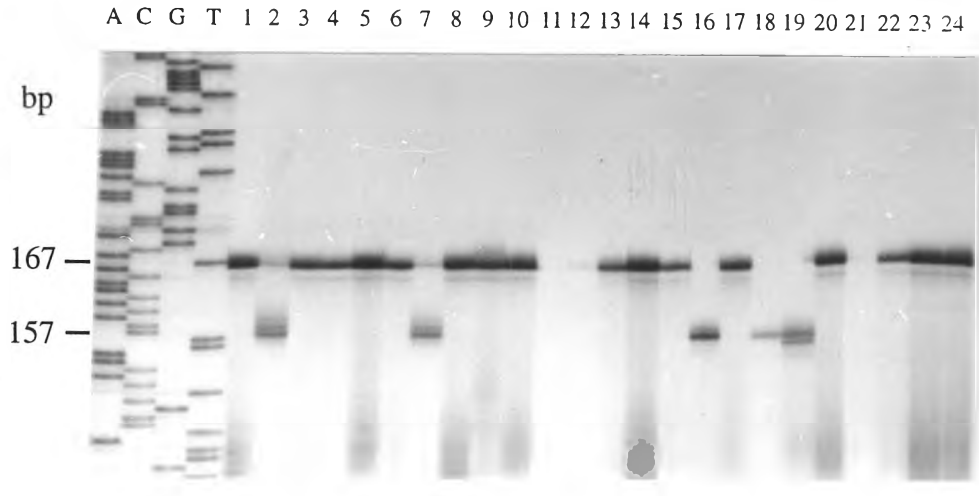


Figure 3.8 Microsatellite patterns of *A. cerana* induviduals at locus A107 (lanes 1-24). A M13 sequencing marker was used as a standard.

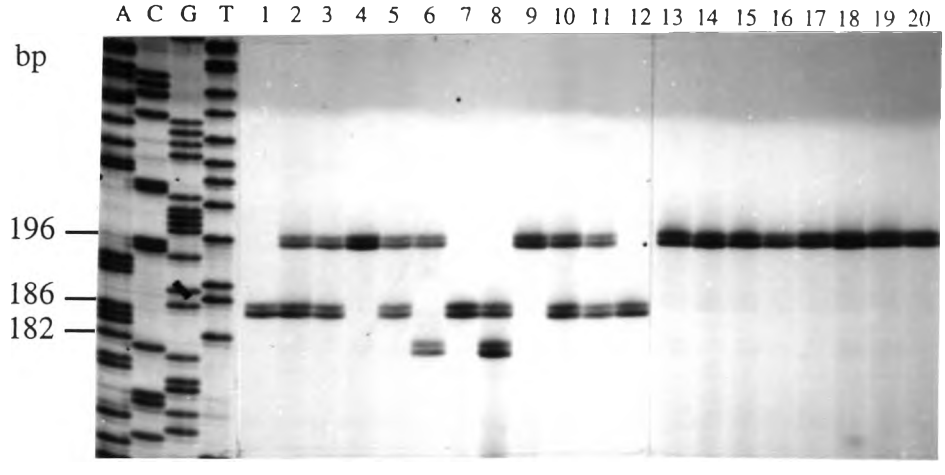


Figure 3.9 Microsatellite patterns of *A. cerana* induviduals at locus A113 (lanes1-20). A M13 sequencing marker was used as a standard.

be successfully amplify to 97 %, 97 % and 95 % , respectively. A total of 24 alleles was observed at locus A28 with allele size between 108-132 bp. Only a 118 bp allele showed highly allele frequencies in North (0.344), Samui Island (0.289), Central (0.240), South (0.125) and North-East (0.102), respectively. For a 124 bp allele, the highest allele frequency was 0.316 found in the Samui Island sample.

For A107, a total of 10 alleles (155, 156, 157, 158, 159, 161, 165, 167 and 169 bp) was observed. A 167 bp allele was commonly distributed in all geographic samples with the allele frequencies greater than 0.80 in each geographic sample. At Samui Island, only a 167 bp allele was found so its frequency was 1.00.

The lowest polymorphic locus A113 showed three alleles (182, 186 and 196 bp). A 182 bp allele was found only in North and Central samples with relatively low frequencies. Additionally, a higher frequency was observed at a 186 bp allele at the frequency of 0.750 in North followed by 0.692 in North-East , 0.606 in Central and 0.582 in South. This alleles was available at extremely low frequency (0.014) in the Samui Island. Unlike the mainland samples, the highest allele frequency in this sample was found at a 196 bp with 0.986 in frequency.

The allele frequency distribution varied markedly for the three microsatellite loci assayed and showed in Figure 3.10, 3.11 and 3.12 and Table 3.3.

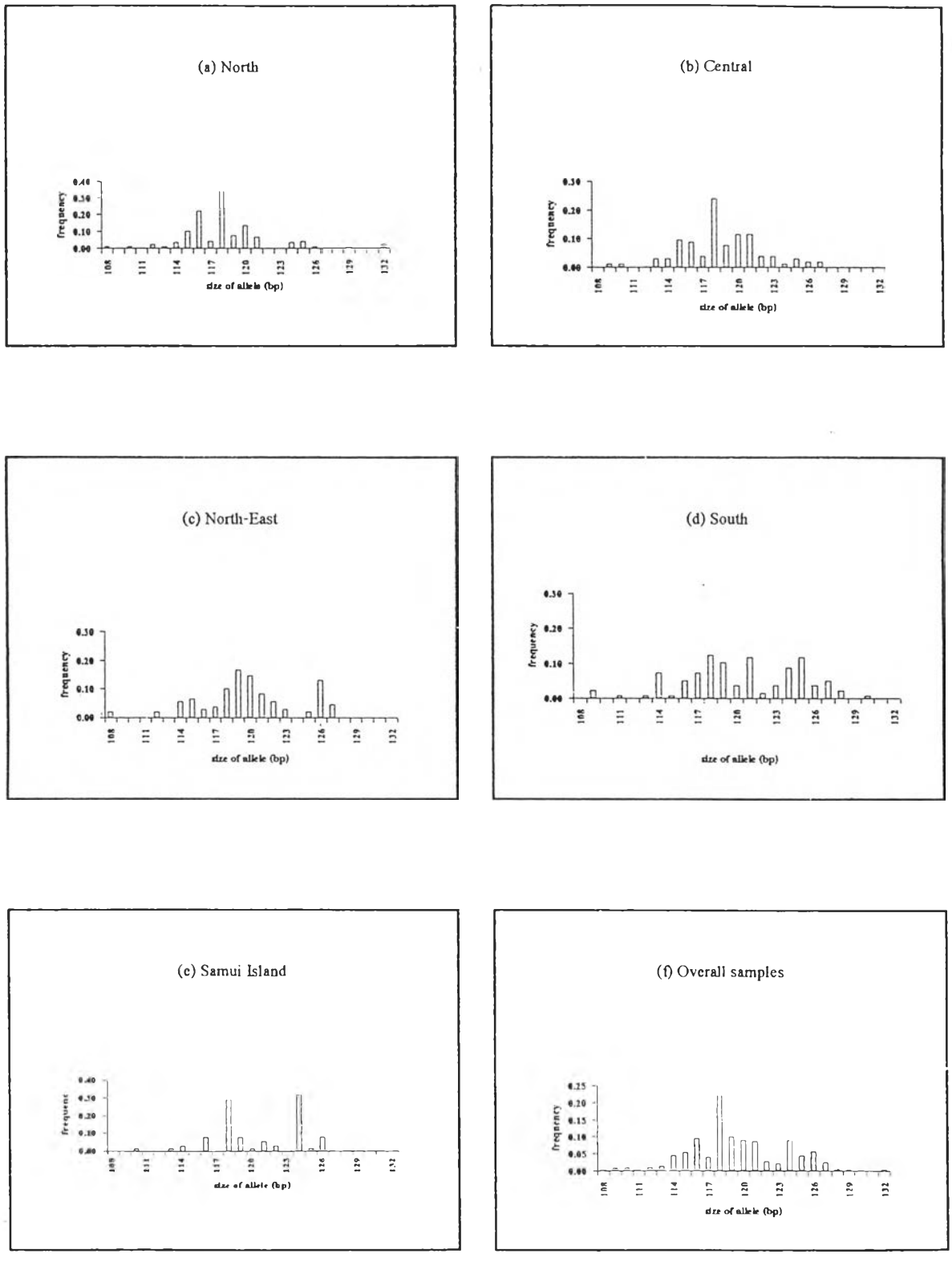


Figure 3.10 Allele frequency distributions at the microsatellite locus A28 from North (n=45), Central (n=52), North-East (n=54), South (n=68), Samui Island (n=38) and Overall samples (n=257).

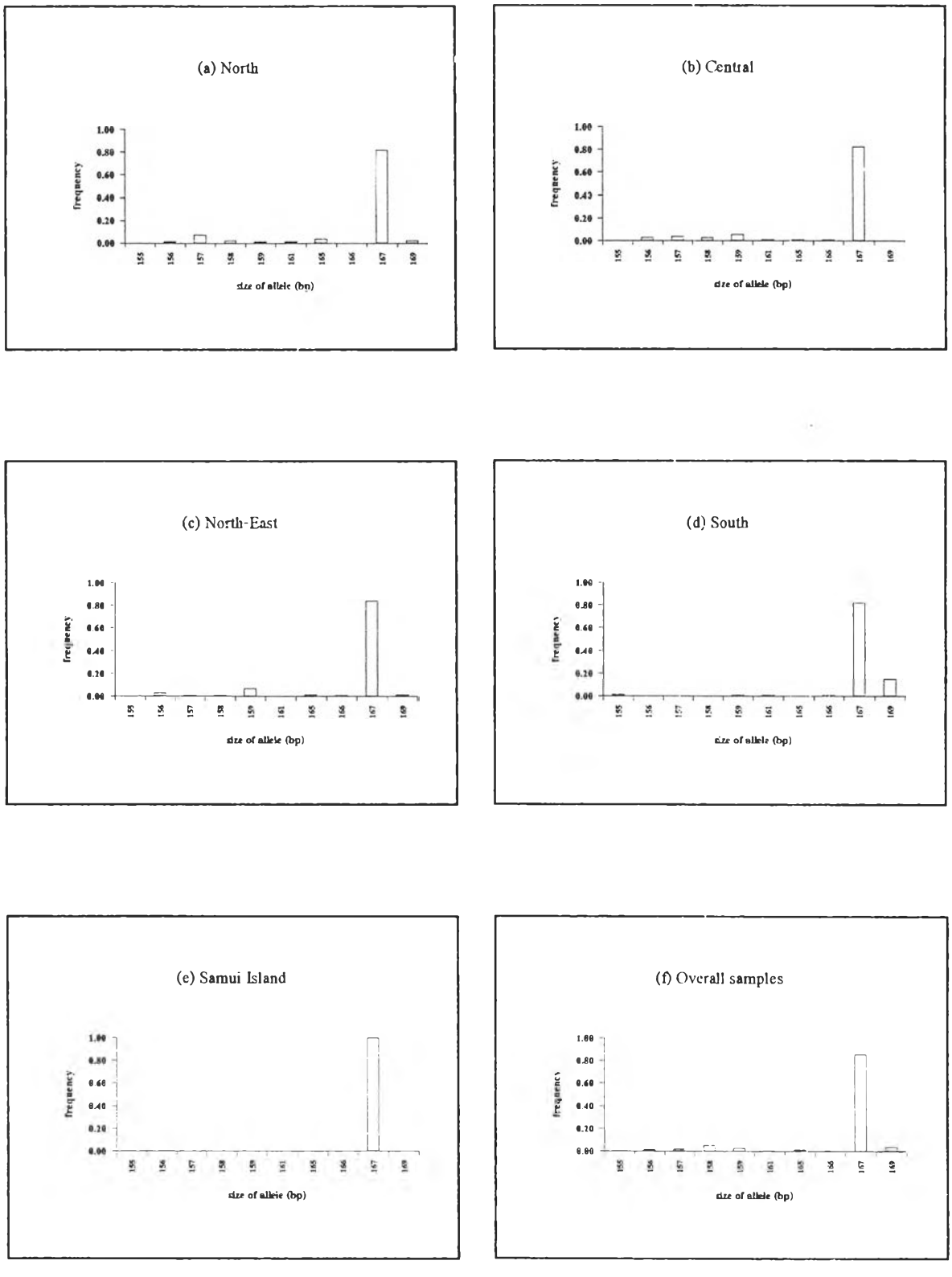


Figure 3.11 Allele frequency distributions at the microsatellite locus A107 from North(n=43), Central (n=54), North-East (n=54), South (n=68), Samui Island (n=38) and Overall samples (n=257).

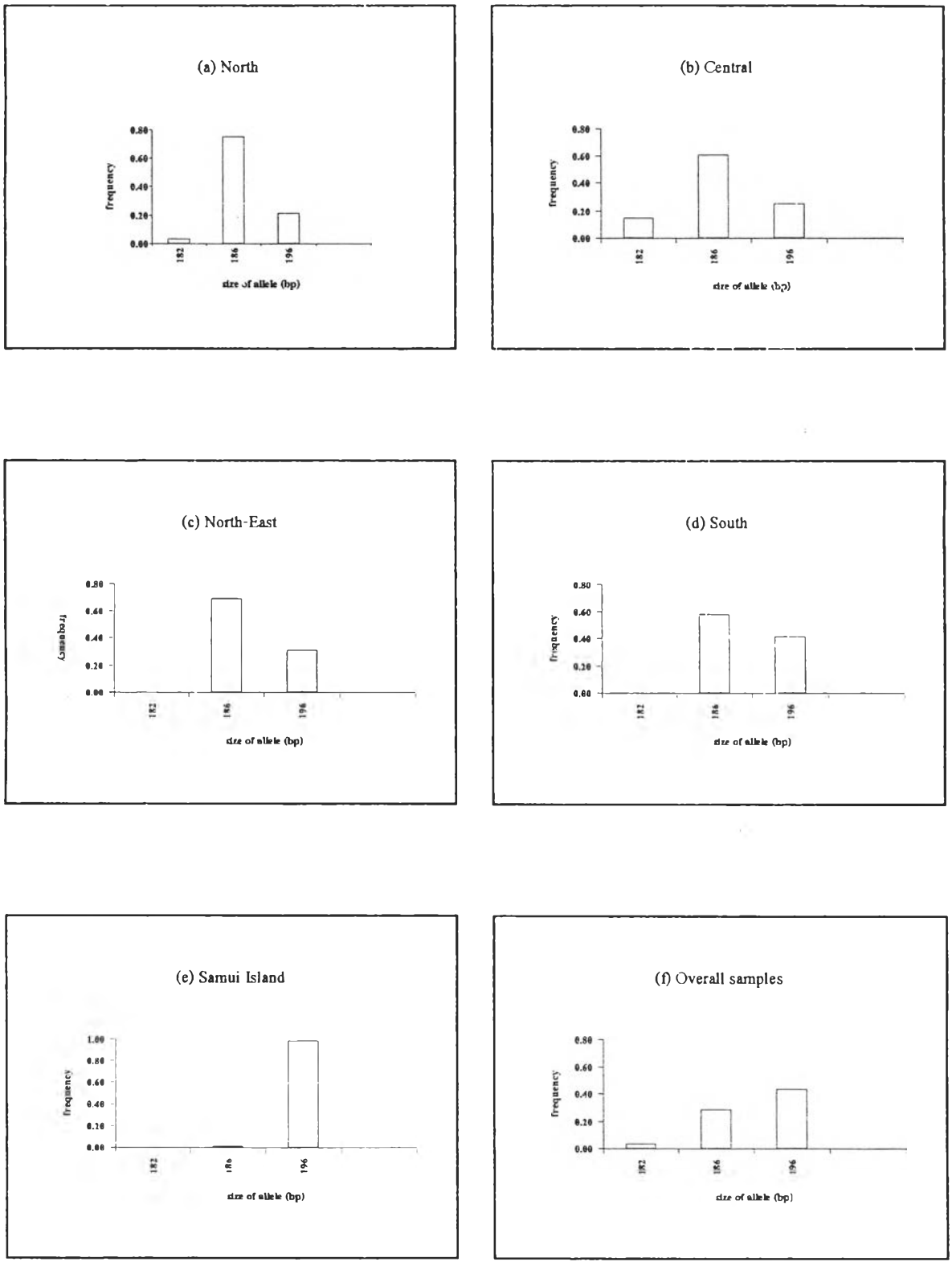


Figure 3.12 Allele frequency distributions at the microsatellite locus A113 from North(n=44), Central (n=52), North-East (n=52), South (n=67), Samui Island (n=37) and Overall samples (n=252).

Table 3.3 Allele frequencies, number of allele, observed and expected heterozygosity of three microsatellite loci in five samples of *A. cerana* in Thailand.

Locus	Allele	North	Central	North-East	South	Samui Island
A28	(bp)	(N=45)	(N=52)	(N=54)	(N=68)	(N=38)
	108	0.011	-	0.019	-	-
	109	-	0.010	-	0.022	-
	110	0.011	0.010	-	-	0.013
	111	-	-	-	0.007	-
	112	0.022	-	0.019	-	-
	113	0.011	0.029	-	0.007	0.013
	114	0.033	0.029	0.056	0.074	0.026
	115	0.100	0.096	0.065	0.007	-
	116	0.22	0.087	0.028	0.051	0.079
	117	0.044	0.038	0.037	0.074	-
	118	0.344	0.240	0.102	0.125	0.289
	119	0.078	0.077	0.167	0.103	0.079
	120	0.133	0.115	0.148	0.037	0.013
	121	0.067	0.115	0.083	0.118	0.053
	122	-	0.038	0.056	0.015	0.026
	123	-	0.038	0.028	0.037	-
	124	0.033	0.010	-	0.088	0.316
	125	0.044	0.029	0.019	0.118	0.013
	126	0.011	0.019	0.130	0.037	0.079
	127	-	0.019	0.046	0.051	-
	128	-	-	-	0.022	-

Table 3.3 (continued)

Locus A28	Allele (bp)	North (N=45)	Central (N=52)	North-East (N=54)	South (N=68)	Samui Island (N=38)
	129	0.011	-	-	-	-
	130	-	-	-	0.007	-
	132	0.022	-	-	-	-
Number of alleles		17	17	15	19	12
Observed heterozygosity		0.578	0.558	0.667	0.676	0.526
Expected heterozygosity		0.844	0.894	0.908	0.924	0.804

Locus A107	Allele (bp)	North (N=43)	Central (N=54)	North-East (N=54)	South (N=68)	Samui Island (N=38)
	155	-	-	-	0.015	-
	156	0.012	0.028	0.028	-	-
	157	0.070	0.037	0.009	-	-
	158	0.023	0.028	0.009	-	-
	159	0.012	0.056	0.074	0.007	-
	161	0.012	0.009	-	0.007	-
	165	0.035	0.009	0.019	-	-
	166	-	0.009	0.009	0.007	-
	167	0.814	0.824	0.833	0.816	1.000
	169	0.023	-	0.019	0.147	-
Number of alleles		8	8	8	6	1
Observed heterozygosity		0.538	0.167	0.259	0.279	0.000
Expected heterozygosity		0.334	0.317	0.301	0.314	0.000

Table 3.3 (continued)

Locus A113	Allele (bp)	North (N=44)	Central (N=52)	North-East (N=52)	South (N=67)	Samui Island (N=37)
	182	0.034	0.144	-	-	-
	186	0.750	0.606	0.692	0.582	0.014
	196	0.216	0.250	0.308	0.418	0.986
Number of alleles		3	3	2	2	2
Observed heterozygosity		0.477	0.512	0.269	0.418	0.027
Expected heterozygosity		0.394	0.555	0.430	0.490	0.027

N = Number of sample examined

Table 3.4 The number of allele per locus and heterozygosity averaged overall loci.

Sample	Mean number of Allele per locus	Mean of Heterozygosity	
		Observed (*Ho* ± SD)	Expected (*He* ± SD)
North	9.3 ± 1.90	0.41 ± 0.072	0.52 ± 0.314
Central	9.3 ± 1.90	0.42 ± 0.302	0.59 ± 0.309
North-East	8.3 ± 1.84	0.40 ± 0.301	0.55 ± 0.353
South	9.0 ± 2.42	0.46 ± 0.243	0.58 ± 0.338
Samui Island	5.0 ± 2.22	0.18 ± 0.509	0.28 ± 0.638

The direct count heterozygosity (*Ho*) and the expected heterozygosity (*He*) of five geographic samples for all three loci were shown in Table 3.4. The average observed heterozygosities ranged from 0.18 (Samui Island samples) to 0.46 (South samples) indicating a low genetic variation levels in *A. cerana*.

The number of alleles detected per polymorphic locus in all *A. cerana* samples was 3 for locus A113, 10 for locus A107 and 24 for locus A28. The lowest mean number of alleles per locus per sample was 5.0 ± 2.22 for Samui Island and the highest of this was 9.3 ± 1.90 for North and Central (Table 3.4).

The *A. cerana* sample from Samui Island showed a low averaged calculated heterozygosity (0.18 ± 0.509) because the monomorphic allele (167 bp) was observed at locus A107. Difference between observed and expected heterozygosity was observed for all loci in all samples which the observed heterozygositie were lower than the expected values.

Allele frequencies at 3 microsatellite loci in each pair of the *A. cerana* samples were used to calculated genetic distance based on Cavalli-Sforza and Edwards chord distance as showed by Table 3.5. The lowest genetic distance was found between North and Central samples (0.0200) whereas the highest was observed between North-East and Samui Island samples (0.0944). In addition, high level of genetic distance was observed among Samui Island and other samples (North, Central, North-East and South) with genetic distance ranged from 0.0690 (Samui Island and South) to 0.0944 (Samui Island and North-East).

The neighbor-joining tree based on chord distance showed that overall *A. cerana* are grouped into three different groups consisting of 1) North, Central and North-East, 2) South and 3) Samui Island (Figure 3.13).

Geographic heterogeneity of allele frequencies among *A. cerana* populations in Thailand was shown in Table 3.6. Significant differences in distribution of allele frequencies was observed for overall populations ($p<0.001$). The allele distribution frequencies of *A. cerana* from South was different from other samples for overall loci

except North-East at the locus A113 (p=0.104). The North-East could not separated from Central at locus A107 (p=0.691) and from North at locus A107 (0.068) and locus A113 (p=0.059). On the other hand, the geographic homogeneity between North and Central was found at overall loci (p=0.154, p=0.336 and p=0.018 for A28, A107 and A113, respectively).

Intraspecifically geographic differentiation of *A. cerana* in Thailand was further supported by *F*-statistics (*Fst*). The *Fst* values of each pair of samples were –0.0005 to 0.0810, -0.0085 to 0.1124 and 0.0153 to 0.7147 for locus A28, A107 and A113, respectively (Table 3.7). *Fst* between North and Central sample was negative value at locus A28 and A107. Between Central and North-East, *Fst* was –0.0089 at locus A107. The *Fst* values for overall loci were 0.03286, 0.03213 and 0.26580 for locus A28, A107 and A113, respectively and the *Fst* for overall loci was 0.10661 (Table 3.8). The mutilocus *Fst* values for overall loci (A28, A107 and A113) was significantly larger than zero for the five geographic populations of *A. cerana* in Thailand indicated a significant degree of genetic differentiation within this species.

Table 3.5 Cavalli-Sforza and Edwards chord distance between the five geographic samples of *A. cerana* in Thailand.

	North	Central	North-East	South	Samui Island
North	-				
Central	0.0200	-			
North-East	0.0307	0.0252	-		
South	0.0436	0.0418	0.0331	-	
Samui-Island	0.0929	0.0892	0.0944	0.0690	-

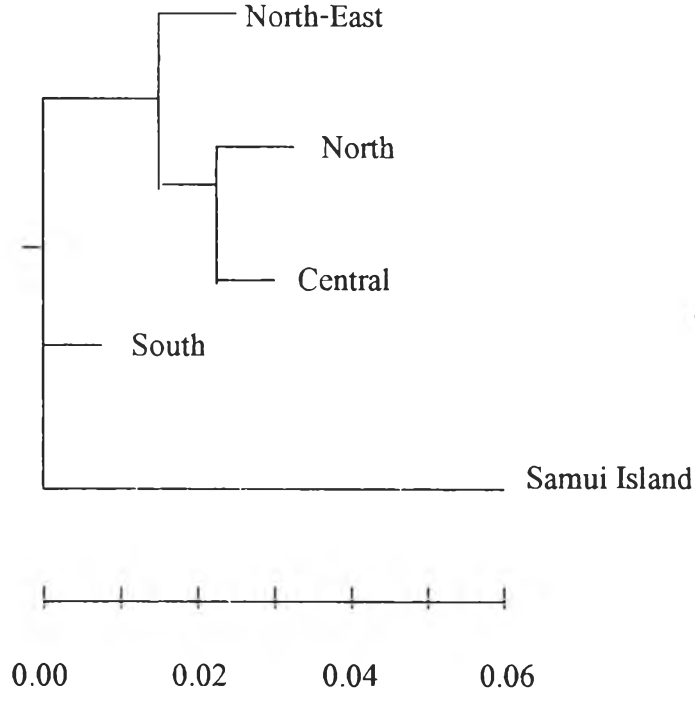


Figure 3.13 A neighbor-joining tree illustrating relationships among 5 geographic populations of *A. cerana* in Thailand based on Cavalli-Sforza and Edwards chord distance.

Table 3.6 Geographic heterogeneity analysis of five geographic samples of *A. cerana* in Thailand using three microsatellite loci (A28, A107 and A113).

Sample	P-value [a]		
	A28	A107	A113
North – Central	0.154[ns]	0.336[ns]	0.018[ns]
North – North-East	< 0.001	0.068[ns]	0.059[ns]
North – South	< 0.001	< 0.001	< 0.001
North – Samui Island	< 0.001	0.002	< 0.001
Central – North-East	0.003	0.691[ns]	< 0.001
Central – South	< 0.001	< 0.001	< 0.001
Central – Samui Island	< 0.001	0.009[ns]	< 0.001
North-East – South	< 0.001	< 0.001	0.104[ns]
North-East – Samui Island	< 0.001	0.009[ns]	< 0.001
South – Samui Island	< 0.001	< 0.001	< 0.001

a = significant levels was adjusted using a sequential Bonferroni technique.

ns = not significant.

Table 3.7 *F*-statistics for microsatellite analysis of each pair of five geographic samples of *A. cerana* in Thailand.

Sample	Locus A28		Locus A107		Locus A113	
	Fst	P-value	*Fst*	P-value	*Fst*	P-value
North – Central	-0.0005	0.4372	-0.0085	0.7823	0.0247	0.0428
North – North-East	0.0375	0.0003	0.0003	0.3496	0.0038	0.2552
North – South	0.0361	0.0001	0.0221	0.0494	0.0636	0.0044
North – Samui	0.0533	0.0004	0.0904	0.0011	0.7147	0.0001
Central – North-East	0.0148	0.0172	-0.0089	0.9485	0.0201	0.0798
Central – South	0.0159	0.0076	0.0314	0.0137	0.0369	0.0166
Central – Samui	0.0554	0.0001	0.0763	0.0018	0.5738	0.0001
North-East – South	0.0183	0.0017	0.0269	0.0186	0.0153	0.1469
North-East – Samui	0.0810	0.0001	0.0791	0.0006	0.6336	0.0001
South – Samui	0.0471	0.0001	0.11235	0.0002	0.4945	0.0001

χ^2 : infinity, D.f. : 6

Table 3.8 *F*-statistics for microsatellite analysis of five geographic populations of *A. cerana* in Thailand.

Locus	*Fst*	P-value	S.E.
A28	0.03286	0.00001	0.00000
A107	0.03213	0.00022	0.00007
A113	0.26580	0.00001	0.00000
Overall	0.10661	-	-

χ^2 : infinity, D.f. : 6