INVESTIGATION OF GENETIC VARIATION AMONG THAI HONEYBEE *Apis cerana* USING MITOCHONDRIAL DNA CONTROL REGION

Mr. Suratep Pootong

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biochemistry
Department of Biochemistry
Graduate School
Chulalongkorn University
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การตรวจสอบความแตกต่างทางพันธุกรรมของผึ้งโพรงไทย *Apis cerana* โดยใช้บริเวณควบคุมของไมโทคอนเดรียลดีเอ็นเอ

นาย สุรเทพ ภู่ทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเคมี ภาควิชาชีวเคมี
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By	Mr. Suratep Pootong
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Thesis Co-advisor	Kanoktip Packdibamrung, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree.

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PCR-RFLP of control region of mtDNA was used to analyze genetic variation and population structure of 125 *Apis cerana* colonies from 6 geographically locations in Thailand : 1) North, 2) North/East, 3) Central, 4) South, 5) Samui Island and 6) Phuket Island. Primer AM8 and AM11, designed from *A. mellifera* mtDNA sequence, produced PCR product of 2,750 bp. Two, three and ten haplotypes were obtained from *Taq*I, *Rsa*I and *Hin*fI digestion of amplified control region, respectively. Eleven different composite haplotypes were generated. A UPGMA phenogram based on genetic distance among different composite haplotypes and nucleotide divergence between six populations predominately separated *A. cerana* into two evolutionary lineages, Northern area (North, North/East and Central) and Southern area (South, Samui Island and Phuket Island). The nucleotide divergence between the two evolutionary lineages were 3.8%. Geographic heterogeneity analysis based on a Monte Carlo simulation (Chi-square) and F-statistic could divide *A. cerana* in Thailand into three groups by further separated the Samui Island from Southern area.

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต .....
สาขาวิชา ชีวเคมี ลายมือชื่ออาจารย์ที่ปรึกษา .....
ปีการศึกษา 2541 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

สุรเทพ ภู่ทอง : การตรวจสอบความแตกต่างทางพันธุกรรมของผึ้งโพรงไทย *Apis cerana* โดยใช้บริเวณควบคุมของไมโทคอนเดรียลดีเอ็นเอ (INVESTIGATION OF GENETIC VARIATION AMONG THAI HONEYBEE *Apis cerana* USING MITOCHONDRIAL DNA CONTROL REGION) อ. ที่ปรึกษา: รศ. ดร. ศิริพร สิทธิประณีต, อ. ที่ปรึกษาร่วม: อ. ดร. กนกทิพย์ ภักดีบำรุง, 104 หน้า. ISBN 974-332-430-5.

การวิเคราะห์ความหลากหลายทางพันธุกรรมของไมโทคอนเดรียลดีเอ็นเอของผึ้งโพรงไทย *A. cerana* โดยใช้ PCR-RFLP บริเวณ control region ของตัวอย่างผึ้งโพรง 125 รัง จาก 6 กลุ่มประชากร ได้แก่ 1) ภาคเหนือ, 2) ภาคตะวันออกเฉียงเหนือ, 3) ภาคกลาง, 4) ภาคใต้, 5) เกาะสมุย และ 6) เกาะภูเก็ต การเพิ่มปริมาณดีเอ็นเอในบริเวณดังกล่าวด้วย PCR ทำโดยใช้ไพร์เมอร์ AM8 และ AM11 ซึ่งออกแบบจากลำดับดีเอ็นเอของผึ้งพันธุ์ *A. mellifera* ให้ผลิตภัณฑ์ PCR ของผึ้งโพรงขนาด 2,750 คู่เบส เมื่อนำมาตัดด้วยเอนไซม์ตัดจำเพาะ *Taq*I, *Rsa*I และ *Hin*fI ให้รูปแบบของแถบดีเอ็นเอจำนวน 2, 3 และ 10 รูปแบบตามลำดับ และมีรูปแบบรวมที่แตกต่างกัน 11 รูปแบบ จากค่า genetic distance และ nucleotide divergence นำมาสร้างความสัมพันธ์เชิงวิวัฒนาการสามารถจัดกลุ่มตัวอย่างผึ้งโพรงได้ 2 กลุ่มหลักได้แก่ กลุ่มทางตอนเหนือ (ภาคเหนือ, ภาคตะวันออกเฉียงเหนือ และภาคกลาง) และกลุ่มทางตอนใต้ (ภาคใต้, เกาะสมุย และเกาะภูเก็ต) โดยมีค่า nucleotide divergence ระหว่างกลุ่มเป็น 3.8% เมื่อวิเคราะห์ความแตกต่างระหว่างกลุ่มประชากรด้วย Monte Carlo simulation (Chi-square) และค่า Fst พบว่าสามารถแยกกลุ่มผึ้งโพรงจากเกาะสมุยออกจากกลุ่มตอนใต้ได้

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต สุรเทพ ภู่ทอง

สาขาวิชา ชีวเคมี ลายมือชื่ออาจารย์ที่ปรึกษา

ปีการศึกษา 2541 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

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ABBREVIATIONS

bp	Base pair
°C	Degree celcius
cm	Centrimeter
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
hr	Hour
µg	Microgram
µl	Microliter
ml	Milliliter
µM	Micromolar
mM	Millimolar
min	Minute
ng	Nanogram
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
TBE	Tris/borate/EDTA buffer
TE	Tris/EDTA buffer
TEMED	*N, N, N′, N′*- tetramethylethylenediamine
Tris-HCl	Tris hydrochloride buffer
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

The honeybee is one of the most importance insect. It increases crop plant production by mediation of pollination. When the honeybee gathers nectar her body becomes dusted with pollen. As she moves from flower to flower, the pollen passes from male to female stigma that causes of cross-pollination. The honeybee produces natural honey, beeswax, pollen, royal jelly and propolis which are of high economic value.

In United States, the annual value of honey produced in North America is estimated at about 25 million dollar (Anonymous, 1993) and estimated approach 57 billion dollars for the value of pollination (Southwick and Southwick, 1992). In Thailand during 1986 to 1997, the honey exported and earning in country is sharply increased up to 44 million baht. (Table 1.1)

The honeybees are social insects and live together in colonies. In one colony consists of one queen, serveral thousand of workers and a few hundred of drones. The queen is the mother of all members of the colony. The size of queen is the largest bee and each normal colony has only one. A normally mated queen is capable of laying two kinds of eggs, fertilized eggs from female bees arise, and unfertilized eggs which, through the phenomenon of parthenogenesis, produce males (Wongsiri and Deowanish, 1995). Both the workers and queen are heterozygous diploid reproduced from sexual fertilization, whereas the drone are the

Table 1.1 The import and export of natural honey of Thailand during 1986-Sept. 1998.

Year	Import		Export	
	Quantity (Ton)	Value (Million Baht)	Quantity (Ton)	Value (Million Baht)
1986	132	4.03	1222	21.02
1987	130	3.84	745	11.11
1988	125	4.42	1750	24.53
1989	146	5.59	704	9.29
1990	166	6.19	2432	1.11
1991	232	8.79	1206	16.96
1992	172	7.30	2407	32.39
1993	230	10.60	2108	28.30
1994	264	12.24	1894	26.94
1995	238	11.10	1908	29.37
1996	326	16.29	2656	44.01
1997	284	17.66	1996	32.94
1998 (Jan-Sept)	104	7.05	1053	27.19

Source: Thai Customs Department, Finance Ministry, Thailand.

future fathers. Their only task is to mate with the queen bee and have hemizygotes (haploid individuals) arising from unfertilized eggs.

The honeybees use their dance language for communication such as indicate distance of food to other bees in the colony. In 1961, Lindauer studies the dance language of vary honeybee species and found that *Apis florea* in performed the waggle dance on a horizontal surface, and straight run portion of the dance is pointed right in the direction foragers should fly when they leave the nest. The other honeybee species are performed the dance on the vertical face of a honey comb, and directional cues are presented with reference to the pull of gravity.

The honeybees in Thailand

In Thailand, five honeybee species are found. Four of them are the native species. The giant or rock honeybee, *A. dorsata;* the dwarf *(A. florea)* and small dwarf honeybee (*A. andreniformis*) are open-nesting species that build a single, exposed comb. The eastern honeybee (*A. cerana*) and the imported species, the western honeybee (*A. mellifera*) are nested in cavities where they construct multiple exposed comb. The eastern honeybee, *A. cerana,* has been used in domestic beekeeping in Thailand for along time. Mostly they are introduced from wild colonies and kept in domestic hives (Figure 1.1). For industrial beekeeping, *A. mellifera* is more sucessful than *A .cerana* because the imported bee is not aggressive, produces high quantity of honey products and lay eggs at higher rate than *A. cerana.* However, *A. cerana* has some better biological features such as resistance to honeybee mites, do not required sugar feeding and has better climatic adaptability. The disadvantages of *A. cerana* are their aggression,

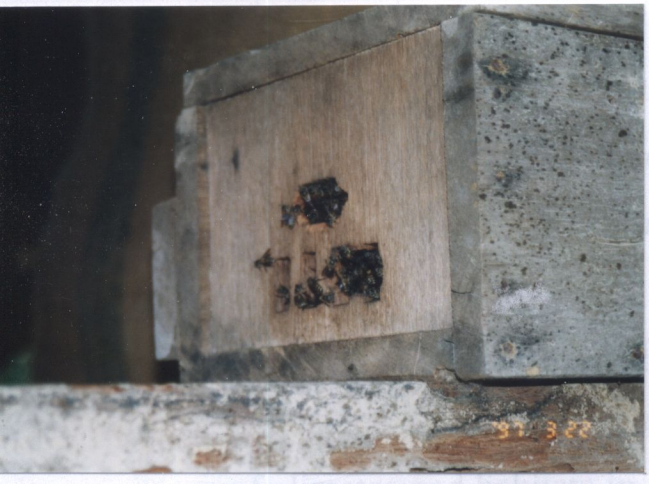


Figure 1.1 Beekeeping of *Apis cerana* inThailand

low quantity of honey production and slowly rate of egg laying.

The classification of *Apis cerana*

The honeybees are species belonging to the superfamily Apoidea, order Hymenoptera and genus *Apis* .The taxonomic definition of the honey bee is as follow (Borror *et al.*, 1976 and Gojmerac, 1980);

Kingdom	Metazoa
Phylum	Arthropoda
Class	Insecta
Order	Hymenoptera
Superfamily	Apoidea
Family	Apidae
Genus	*Apis*
Species	*cerana*

Scientific name: *Apis cerana Fabricius*, 1793.

The studied of *Apis cerana*

A. cerana, the Asian cavity-nesting bees, occurs over a wide range of climates and habitats in Asian. They have been subdivided into subspecies on the basis of morphological difference. In 1953, Maa placed these bees in their own subgenus, *Apis* (Stigmatapis) and recognized 11 species in this group. Maa's scheme was not widely accepted, and until recently, most authors used *A. cerana* to refer to all of these populations. Ruttner (1988) reexamined the morphometric information on the eastern cavity-nesting bees, which he considered as

one species, *A. cerana*. He grouped the *A. cerana* population into four subspecies; a northern subspecies, *A. cerana cerana* from Afghanistan, Pakistan, north India, China and north Viet Nam; a southern subspecies, *A.c. indica*, from south India, Sri Lanka, Bomgladesh, Burma, Malaysia, Thailand, Indonesia and the Philippines; a Japanese subspecies, *A. c. Japonica*; and Himalayan subspecies, *A. c. himalaya*. Limbipichai (1990) studied morphological characters (such as: proboscis, fore and hind wing, hind leg, third and sixth sternites etc.) of the eastern honeybee (*A. cerana*) in Thailand and the Malaysian peninsula. The analysis was performed by Multivariate Statistical Analysis Software (SAS) with two methods, the clustering analysis and the cononical discriminant analysis. The former method is capable to discriminate into two groups; Northern latitude bees, from Chiang Rai-Phetchaburi and Southern latitude bees, from Chumphon-Songkhla which include Samui Island bees. The last method is able to separate Samui Island bees from Southern latitude bees.

However, the morphometric parameters are subject to environmental effect, and their genetic basic is undefined (Daly, 1998). Thus, the discovery of numerous electrophoretic allozymes opened up a new field of molecular taxonomy. For allozyme analysis, the western honeybee, *Apis mellifera* has been the subject of a great number of allozyme studies such as; studies of the bees from different geographic regions, others have demonstrated the possibility of using allozymes for racial discrimination (Nunamaker *et al.*, 1984b; Daly, 1991). The studies of allozymes in Asian honeybee were done by Nunamaker, Wilson and Ahmad (1984). They used specimens of *A. cerana* , *A. dorsata* and *A. florea* from Pakistan and studied with two enzymes: malate

dehydrogenase (MDH) and esterase (EST). MDH produced one intense band for *A. florea* and one intense and faint band in both *A. dorsata* and *A. cerana*. While, for EST, *A. cerana* had two faint band which different to the other two species. In a brief report on EST by Tanabe and Tamaki (1985) indicated that *A. mellifera* and *A. cerana* had species specific differences. Unfortunately, the low level of allozyme polymorphism in social insects, especially honeybees (Parker and Owen, 1992) limits applied ability of this technique. This may be caused by the effect of haplo-diploid sex determination system in social insects (Pamilo *et al.*, 1978; Graur, 1985).

Recently, variation at the nuclear and mtDNA level have been effectively investigated to determine genetic variation and population structure among honeybee populations. The nuclear DNA of honeybee has a diploid chromosome number of 16 (Petrunkewitsch, 1901) and a total size of about 180 megabase pair (Jordan and Brosemer, 1974). In 1990, Hall analysed of introgressive hybridization between African and European honey bees using nuclear DNA of *A. mellifera* as a probe. This report showed paternal gene flow between African and European honeybee but suggested asymmetrics in levels of introgressive hybridization. In addition, the eastern honeybee, *A. cerana* which collected from five different areas in Thailand, have been studied the genetic variation using constructed DNA probe number 99 which containing repetitive sequence of *A. cerana*. After digested chomosomal DNA of *A. cerana* male (haploid) with *Hae*III. The Southern blot analysis showed six different RFLPs patterns of *A. cerana* from five different area in Thailand. (Uthaisang, 1993)

Mitochondrial DNA (mtDNA) has recently been focused on number of molecular genetic and evolution studies. (Wilson *et al.*, 1985; Tzagologg and Myer, 1986; Avise *et al.*, 1987; Moritz *et al.*, 1987). In multicellular animals, mtDNA is a small circular molecule of appoximately 16 kb carrying a set of highly conserved genes. (Wilson *et al.*, 1985). It generally encodes 13 proteins, two ribosomal RNAs, 22 tRNAs and control region. Animal mtDNA is typically (though not in variably) maternally inherited without recombination (Lansman *et al.*, 1983; Brown, 1985; Gyllensten *et al.*, 1985), effective haploid, conservative gene order and rapid rate of evolution more than single copy nuclear DNA about 5-10 fold (Brown and George, 1979). Different parts of mtDNA mutate at different rates. For example, the control region change very rapidly, both within and between species, whereas ribosomal RNA genes change slowly (Moritz *et al.*, 1987). Thus, analysis of mtDNA became a powerful tool in population and evolutionary genetics studies (Moritz, 1994).

The first published of mtDNA polymorphisms in honeybee mtDNA (Moritz *et al.*, 1986) was the comparison of mtDNA restriction fragment length polymorphisms (RFLPs) in Australian honeybees derived from three European subspecies: *A. m. carnica*, *ligustica* and *caucasica*. The Western European group ; *A. mellifera* and the African group containing *A. m. intermissa*, *A. m. scutellata*. The mtDNA were prepared and digested with 16 restriction enzymes (6 base pair cutter) and the fragment were labeled radioactively with ^{32}P. The nineteen different *A. mellifera* mtDNA haplotypes were observed and percent sequence divergence were calculated. A cluster analysis using UPGMA

revealed three main groups of *A. mellifera* haplotype; an eastern Mediterranean, a Western European group and an African group.

In 1991, Hall and Smith employed the PCR-RFLP to detected RFLPs of three major groups (African, west European and east European) of *A. mellifera*. Three polymorphic regions (IsRNA, CO-I and Inter-CO-I/CO-II) were amplified and digested with three restriction endonuclease (*Eco*RI, *Hin*cII and *Xba*I).The PCR-RFLP analysis showed that an *Eco*RI site founded in the large ribosomal subunit gene of east European bees, a *Hin*cII site in the cytochrome C oxidase subunit I gene of west European bee and an *Xba*I site in the inter-CO-I/CO-II region of east European. This result showed that there are three polymorphic regions specific to a different subspecies groups. In 1991, Smith classified *A. cerana* species by analysed mtDNAs. The samples of *A. cerana* were collected from Southern India; the Andaman Island and India, Northern and Southern Thailand, Malaysia, Northern Borneo, Indonesia, Leuzon Island and Japan. Mitochondrial DNAs were prepared and surveyed with the 6-base restriction enzymes, The estimation of percent sequence divergence from the resulting restriction fragment data indicated three main lineages of *A. cerana* mtDNA. First include the samples from southern India, Thailand, Malaysia, Borneo and Japan; second consist of the sample from the Andaman Islands; and a third consist of the sample from Luzon. In addition, the mtDNA variation of *A. cerana* was examined by RFLP analysis. Using ten restriction endonucleases, all geographically investigated sample could be allocated to 6 different groups composed of 1) Japan 2) Nepal, Vietnam and north-to-central Thailand 3) Korea-Tsushima 4)Taiwan 5) south Thailand and 6) The Philippines (Deowanish *et al.*, 1996)

In recent years, PCR-RFLP of 3 regions were analyzed by Sihanantavong (1997). Three mtDNA regions (intergenic region of CO-I and CO-II, lrRNA and sRNA) were amplified following by digestion the PCR products with restriction enzyme *Dra*I. From these PCR-RFLP analysis *A. cerana* in Thailand was divided into three populations, the Northern (North, North/East and Central), South and Samui Island and the UPGMA phenogram of population derived from PCR-RFLP data can divide *A. cerana* in Thailand into 2 lineage. In 1997, Songram employed the PCR-RFLP analysis of ATPase 6-ATPase 8 gene of *A. cerana* in Thailand with three restriction enzyme (*Ssp*I, *Taq*I and *Vsp*I). The UPGMA phenogram of populations derived from PCR-RFLP data allocated five geographic locations of *A. cerana* in Thailand into two evolution lineage (Northern, Southern) same Sihanantavong (1997). While, based on Monte Carlo simulation, five geographic locations of *A. cerana* in Thailand could be genetically divided into three groups included of the Northern, the South and the Samui Island.

In addition, there is control (D-loop) region, a part of the animal mitochondrial genome which encompasses the site of initiation of heavy strand replication as well as both heavy strand and light strand transcription (Chang *et al.*, 1987; Clayton, 1991a,b). This region is organized very differently, without an obvious control region. In sea urchins, this region is under 200 bp length. In fish, it tends to be very long and is often full of repeated sequences. In insect, it is called the AT- rich region and can also be long and full of repeated sequences (Hillis, Moritz and Mable, 1996). The control region is the most rapid evolution (Brown, 1985) which more than another part on mtDNA approximate two to five fold (Aquadro, Kaplan and Risko, 1984).

Therefore, the control region is used as genetic marker for study of inter- and intra- specific taxonomy in animals. Norman, Moritz and Lympus (1994) used the control region as genetic marker for ecological studies of marine turtles. The control region was amplified and digested with *Mse*I and sequenced this PCR product. They found that only 2 of 12 different Indo-Pacific rookeries serveyed could not be differentiated, indicating that the Indo-Pacific *C. mydon* include a number of genetically differentiated populations.The control region also used to study levels of inter- and intra- populational variation of the harbour porpoise, *Phocoena phocoena* on interoceanic (Rosel, Dizon and Haygood, 1995).

In 1992, Crozier and Crozier showed the complete sequence of honeybee (*A. mellifera*) mtDNA. Two non-coding region were illustrated, the first region is the small region between the $tRNA^{Leu}$ and CO-II genes which is 92.2% AT. Cornuet, Garnery and Solignae (1991) argued that this region may contain an origin of replication. However, it is unclear that the gap between the $tRNA^{Leu}$ and CO-II gene represents essential functions of the region because in other bees if this region is often reduced and sometimes absent. The other region is the large region which situates between the small rRNA and $tRNA^{Glu}$ genes which is 96.0% AT and lacks any apparent signals for the initiation of replication such as those of vertebrate.

The genetic variation study of control region of *A. cerana* has been limited. The aim of this study was investigation of genetic variation among Thai honeybee, *A. cerana* from six geographic area in Thailand (North, North-East, Central, South, Samui Island and Phuket

Island) using PCR-RFLP analysis of control region . The results will provide information on the biology and geographic variation of *A. cerana* in Thailand, a basic of further selection and breeding for strain improvement.

CHAPTER 2

MATERIALS AND METHODS

2.1 Instruments

- Autoclave, NA-32, Manufacturing corporation, Japan.
- Automatic micropipette, pipetman; P_2, P_{20}, P_{100}, P_{200}, P_{1000} Gilson Medical Electrics S.A., France.
- Camera, Pentax Syer A.
- High speed microcentrifuge, Kubota 1300, Kubota corporation, Japan.
- Horizontal gel electrophoresis apparatus (8.5 × 12.5 cm).
- Incubator, BM 600, Memert GmbH, W., Germany.
- Shaker, S03, Stuart Scientific, Great Britain.
- PCR, Gene Amp System 2400, Perkin Elmer, USA.
- PCR workstation, HP-036, C.B. Scientific Co., California.
- pH/ION meter, PHM 95, Radiometer, Copenhagen.
- Power supply, Power PAG 300, Bio-Rad, USA.
- Standard cassette (14 × 17 inch) Okamoto, Japan.
- UV transilluminators: 2011 MA erovue, San Gabrial, USA.

2.2. Chemicals

- Agarose, Seakem LE, FMC Bioproducts, USA.
- Agarose, Metaphore, FMC Bioproducts, USA.
- Boric acid, BDH Laboratory suppliers, England.
- Deoxynucleotide (dATP, dTTP, dCTP, dGTP) Promega coporation, Medison, Wisconsin, USA.
- Chloroform, BDH Laboratory suppliers, England.

- Developer and Fixer, Eastman Kodak company Rochester, USA.
- Ethidium bromide, Sigma chemical company, USA.
- Lamda phage DNA, New England Biology company, USA.
-Oligonucleotide primers, Biosynthesis and Biotechnology Service Unit.
-OmiBase™ DNA Cycle Sequencing System Kit, Promega coporation, Medison, Wisconsin, USA.

2.3 Inventory supplies

- Black and White print film, Tri-Xpan 400, Eastman Kodak company, Rochester, USA.
- Whatman paper 3M, Whatman International Ltd., Maid stone, England.
- X-ray film, X-omat XK-1, Eastman Kodak company Rochester, USA.
- X-ray film, Fuji medical, Fuji photo film Co., LTD, Japan.

2.4 Standard markers

- 10 bp DNA ladder (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 bp), Promega, Madison, USA
- 100 bp DNA ladder (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 and 1500 bp), Promega, Madison, USA
- λ DNA digested with *Dra*I (0.53, 0.69, 1.07, 2.15, 3.03, 3.59, 6.03, 6.82, 7.83, 8.37 and 8.59 kb)
- λ DNA digested with *Hin*dIII (0.6, 2.0, 2.3, 4.3, 6.6, 9.4, and 23.1 kb)

2.5 Enzymes

- Proteinase K, Gibco BRL life Technologies, Inc., USA.
- Restriction endonucleases ;
  - *Ase*I, *Hin*fI, *Hin*dIII, *Ssp*I, *Rsa*I, *Nde*I (Biolabs)
  - *Dra*I (Pramacia)
  - *Alu*I, *Bfr*I, *Eco*RI, *Kpn*I, *Sau*3AI, *Sma*I, *Swa*I (Boehringer Manham).
  - *Taq* DNA polymerase, Perkin Elemer, Cetus, Norwalk, Connecticus.

2.6 Honeybees samples (*A. mellifera*, and *A. cerana*) and tissues preparation.

Samples of adult worker honeybees (*A. mellifera*) were collected from two hives, one from the North/East and the other from Bee Biology Research Unit, Chulalongkorn University. *A. cerana* adult workers were sampled from 177 colonies from 6 different geographic areas in Thailand. Number of sample obtained from the North, North/East, Central, South, Samui Island and Phuket Island was 28, 32, 36, 46, 29 and 6 colonies, respectively. About fifty honeybees from each colonies were immediately preserved in 95% ethanol or kept frozen in liquid nitrogen and were transported to the laboratory for further analysis.

2.7 Honeybees DNA extraction

Total DNA was individually extracted from each thorace using the modified method of Garnary *et al.*(1993), each thorace was homogenized in 1.5 ml eppendorf tubes containing 400 µl TEN buffer (100 mM NaCl, 100 mM EDTA, 0.1 M Tris-HCl, pH 8.0), then 20% SDS and proteinase K (10 mg/ml) was added. The mixture was incubated

at 55°C for 2 hr. Protein in the mixture was removed by extraction once with phenol/chloroform/isoamyl alcohol (25:24:1) and twice with chloroform/isoamyl alcohol (24:1). After spun at 6,000×g for 10 min, the supernatant (aqeous phase) was transferred to a new eppendrof and the DNA was precipitated by addition of 1/10 sample volumn of 3 M sodium acetate and 2 volumns of 95% ethanol and stored overnight at -20°C. The DNA was pelleted at 10,000×g and washed once with 75% ethanol and resuspened the DNA in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA).

2.8 Measurement of DNA concentration

Measurement of DNA concentration was done according to the principle that the intensity of fluorescence emitted by ethidium bromide molecules which were intercalated into the DNA was directly proportional to the total mass of DNA. The extracted DNA was subjected to 0.7% agarose gel electrophoresis. After finished, the gel was stained in ethidium bromide solution (2.5 μg/ml) and destained in distilled water. The fragments of DNA were visualized under UV light and the gel was photographed through a red filter using Kodax Tri-X-pan 400. The concentration and fragment size were determined by comparing the intensity of the ethidium bromide-DNA complex with those of the known concentration of standard markers.

Approximated 0.7% agarose gel was set up in 1X TBE buffer (89 mM Tri-HCl, pH 8.3; 89 mM boric acid; 2.5 mM EDTA) and solubilized by heating in microwave oven. The solubilizing gel was allowed to cool to 50°C and poured into a gel tray. After the gel was completely set up, the comb was carefully removed. Each DNA sample was mixed with one

fifth by volume of loading dye (0.1% bromphenol blue, 40% ficoll 400 and 0.5% SDS) and loaded into the well. The electrophoresis was performed in 1X TBE buffer at 100 volt for 2-3 hr. until bromphenol blue of tracking dye reached the end of the gel.

2.9 PCR amplification of control region

2.9.1 Primers

Control region of *A. cerana* mtDNA was amplified by PCR. Twelve oligonucleotides primers were designed by Oligo 4.0 program. Eight primers (H-strand primers) were derived from the ND_2 gene region of *A. mellifera* mtDNA sequence (Crozier and Crozier, 1993). Whereas four primers (L-strand primers) were from the region of small ribosomal gene. The designed primer sequences were in Table 2.1. The location of each oligonucleotide primers according to the physical mtDNA map of *A. mellifera* was shown in Figure 2.1. Oligonucleotides primers were synthesized by Bioservice Unit (BSU) of National Science and Technology Development Agency (NSTDA), Bangkok, Thailand.

2.9.2 Screening of specific primers for amplification of mtDNA control region.

The different primer combinations (H-strand and L-strand) were used to amplify mtDNA control region of *A.mellifera*. The amplification reaction were performed in 20 µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 0.001%(W/V) gelatin, 200 µM each of dATP, dCTP, dTTP and dGTP, 0.2 µM of each different primers, 50 ng of total DNA and 0.5 unit of

Table 2.1 The oligonucleotide primers used for PCR amplification of mtDNA control region

Primer designation	Primer sequence	Location on mtDNA of *A.mellifera*
H-strand primer		
AM-1	5′-CAG GGT ATG AAC CTG TTA GCT TTA TTT AG-3′	260-231
AM-2	5′-CAG GGT ATG AAC CTG TTA GC-3′	260-241
AM-3	5′-AAA ATA AAT AAA TCA GTG GTA-3′	543-523
AM-4	5′-AAT CTA ATT CTA ATG ATT GTA CCA AAT TC-3′	635-607
AM-5	5′-ATC TTG GGG TTT TAT TTG TGG-3′	670-650
AM-6	5′-TAT TCA AAA ATG AAA GGG GAA AGT TCC-3′	831-805
AM-7	5′-GAA TGA ATT AAA AAT TGT TGA ACA TGC TAG-3′	1044-1016
AM-8	5′-CTA TGA TAT ATA TAT TTA TTA TCT TTA TC-3′	1439-1411
L-strand primer		
AM- 9	5′-TTA TAA GTC AAG TTT AAC CGC TAT TGC-3′	15309-15335
AM-10	5′-CCG CTA TTG CTG GCG ACT CAT-3′	15326-15346
AM-11	5′-ACA ATT AAT CTA AAA AAC TAC AAC ATG-3′	15369-15495
AM-12	5′-ACT TTC ATT CAT TGT TTC AGA-3′	15380-15400

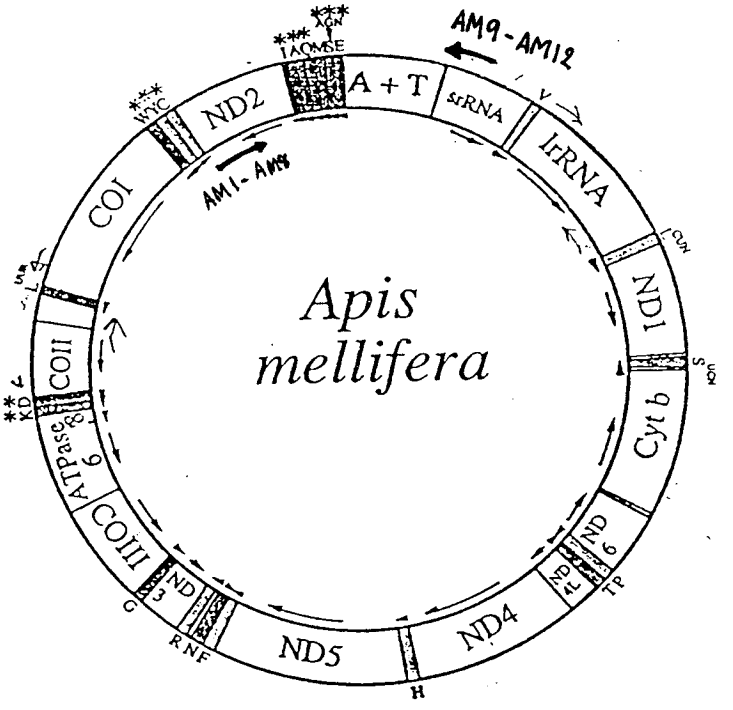


Figure 2.1 Location of oligonucleotide primers in *A. mellifera*. Descriptions of the primers are as in Table 2.1

Ampli *Taq* polymerase. Amplification were performed in a Gene Amp System 2,400 (Perkin Elmer) for pre-denaturation 2 min at 95°C, and then 35 cycles of denaturation at 94°C for 30 sec, annealing at 45°C for 30 sec, extension at 72°C for 1 min 50 sec to 2 min and the final extension was carried out at 72°C for 10 min. The amplification products were then electrophoretically analyzed by agarose gel electrophoresis.

2.9.3 PCR products analysis by agarose gel electrophoresis

The appearance of size of PCR products and their size were determined by 1.5% agarose gel electrophoresis with standard markers (λ/*Hin*dIII and 100 bp DNA marker). Primer pairs which gave single expected band of PCR product at the size were be selected for further amplification of the mtDNA control region of *A. cerana* DNA samples.

2.10 Amplification of control region of *A. cerana* DNA samples

The selected primer pairs (from 2.9.3) were used to amplify mtDNA control region of *A. cerana* DNA samples using the same PCR condition as in 2.9.2. The primer pair which could give PCR product were selected and then optimization of DNA template, $MgCl_2$ concentration and primer concentration were performed.

2.10.1 Optimization of the $MgCl_2$ concentration

A. cerana mtDNA was amplified using selected primer pair for control region. The amplified condition was as described in 2.9.2 excepted the magnesium ion concentration which was varied from 1.0 mM to 6.0 mM (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mM).

2.10.2 Optimization of DNA template concentration

A. cerana mtDNA was amplified using selected primer pair for control region. The amplified condition was as described in 2.9.2 the magnesium ion concentration from 2.10.1 excepted the DNA concentration which serial dilution (12.5, 25, 50 and 100 ng).

2.10.3 Optimization of the primer concentration

A. cerana mtDNA was amplified using selected primer pair for control region. The amplified condition was as described in 2.9.2 the magnesium ion concentration from 2.10.1 and DNA concentration as in 2.10.2 excepted the primer concentration which varied from 0.1 μM to 0.5 μM (0.1, 0.2, 0.3, 0.4 and 0.5 μM).

2.11 Characterization of PCR product by sequencing.

PCR products (control region) of *A. mellifera* and *A. cerana* were sequenced using inner primer (5′-AAA ATA AAT AAA TCA GTG GTA-3′) which designed from ND_2 gene of *A. mellifera ligustica* (Crozier and Crozier, 1992). The sequence of a deoxyribonucleic acid molecule of mtDNA ND_2 gene can be elucidated using enzymatic methods. This method is based on the ability of a DNA polymerase to extend a primer which 5′-end-labeled using T_4 polynucleotide kinase with [γ- ^{32}P] ATP or [γ- ^{33}P] ATP by one-step extension/termination reaction. The primer is hybridized to the template that is to be sequenced, until a chain terminating nucleotide is incorporated. Sequence determination is carried out as a set of four separate reaction, each of which contains all four deoxyribonucleotide triphosphates (dNTPs) supplemented with limiting amount of a different dideoxyribonucleotide triphosphate (ddNTPs) per reaction. Because ddNTPs lack the 3′OH

group necessary for chain reaction, the growing oligonucleotide is terminated selectively at G, A, T or C, depending on the respective dideoxy analog in the reaction. The relative concentrations of each of the dNTPs and ddNTPs can be adjusted to give a nested set of terminate chains over several hundred to a few thousand bases in length. The resulting fragments, each with a common origin but ending in a different nucleotide are separated according to size by high resolution denaturing polyacrylamide gel electrophoresis.

2.11.1 Template preparation

The PCR product for characterization was purified by QIAEX II agarose gel extraction. The PCR-product was electrophoresed in 1.5% agarose gel and the desired DNA band was excise from the agarose gel with a clean, sharp scalpel. The gel slice was weighted in 1.5 ml eppendrof and added 3 vol of buffer QX I to 1 volume of DNA fragment gel. The QIAEX II was resuspened by vortexing for 30 sec after addition of 10 µl QIAEX II to the sample. After , incubated at 50°C for 10 min to solubilize the agarose and bind the DNA. Mixed by vortexing every 2 min to keep QIAEX II in suspension. The sample was centrifuged for 30 sec and carefully removed supernatant with a pipet and washed a pellet with 500 µl of buffer QX I. The pellet was resuspended by vortexing, the sample was centrifuge for 30 sec and carefully removed all trace of supernatant with a pipet and washed the pellet twice with 500 µl of buffer PE. The pellet was collected by centrifuge and the supernatant was removed with a pipet. After air-dried the pellet until it became white the DNA would be eluted by ultrapure water. Following, incubate at room temperature for 5 min and centrifuged for 30 sec. The supernatant was carefully transfered into a

clean tube. The supernatant containing the eluted DNA was collected for sequencing.

2.11.2 End-label the sequencing primer

The primer can be labelled at 5'end by using either [γ- ^{32}P] ATP or [γ- ^{33}P] ATP. In this studied [γ- ^{32}P] ATP with specific activity of 3,000 ci/mmol was used. The labelling reaction mixture was performed in 0.5 eppendrof which contained 20 ng of sequencing primer, 10 pmol of γ- labeled ATP, 1 µl of 10X buffer (500 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 50 mM DTT and 1.0 mM spermidine), T_4 polynucleotide kinase 5 unit and nuclease-free water to final volume of 10 µl. The mixture was incubated at 37°C for 10 min and then the kinase was inactivated at 90°C for 2 min. This volume (10 µl) is appropriate to label enough primer for 6 sets of double-stranded sequencing reactions. These end-labeled primers could be stored at -20°C as long as one month and still generate clear sequence data.

2.11.3 Extension and termination reactions

For each set of sequencing reactions, four 0.5 ml eppendrof was labeled (G, A, T, C) and 2 µl of the appropriate d/ddNTPs was added. After mixing each tube was capped and stored on ice or at 4 °C until need. Primer template mix consisted of template DNA 50 ng, 5 µl of DNA sequencing 5X buffer (250 mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$), 1.5 µl of labeled primer and nuclease-free water to final volume of 16 µl. One microliter of OmmiBase™ Sequencing Enzyme Mix (10 U/µl) was added to the primer/template mix. After mixed briefly by pipetting, 4 µl of the enzyme/primer/template mix was added into the d/ddNTPs mix

tube and placed the reaction tube in a Perkin Elmer Cetus DNA Thermal Cycler (Model 2400). The extension are performed for 95 °C for 2 min, 30 cycles at 95°C for 30 sec, 45°C for 30 sec, 70°C for 1 min and final extension at 70°C for 7 min then stored at 4°C. After the cycling program had been completed, 3 µl of DNA sequencing stop solution (10mM NaOH, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol) was added each tube. Immediately before loading on a sequencing gel, the reactions was heated at 70 °C for 2 min.

2.11.4 Preparation of the polyacrylamide gel

The polyacrylamide gel was set between two glass plates, the shorter glass plate and the longer glass plate. The shorter glass plate should be siliclonized to available water repellent and both plates were cleaned with water followed by 95% ethanol. The spacer was placed on both side of the longer plate and then cover with the shorter plate with the siliconized side facing down. Both sides and the bottom were sealed with sealing tape. The plates were optionally clamped on both sides with clips and ready for gel setting. Six percent denaturing acrylamide gel (460 g urea, 150 ml 40% (w/v) acrylamide, 100 ml 10X TBE and made up to 1 lite with deionized water) was prepared and could be stored in the refrigerator for a few week. For sequencing, gel mix (6%) of 60 ml was transfered to breaker and add immediately 150 µl and 42 µl of 10% ammonium persulfate and TEMED, respectively. Quickly mixed and poured the gel plates, avoid air bubble, let the solution flow continuously down along one side of the plate. After full the plate with gel mixed, comb and clamp were inserted at both sides with clips. The gel was usually polymerized after let stand for another 15 min.

2.11.5 Polyacrylamide gel electrophoresis

The comb and the tape were removed from the bottom and both sides from the polymerize gel. Then the inside well was clean with water for a few times. After the gel plate was fitted with a sequencing gel box and 1X TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM Na_2EDTA; pH 8.3) was added into the chamber. Before loading the samples, the residual gel mix and urea was removed from the well using syring with needle. The samples was loaded and subjected the gel with constant 40 watts. After electrophoresis was completed the glass plates were separated and the gel was exposed to X-ray film for about 10 hr, at -80°C. The X-ray film was developed and fixed using the method recommended by the supplier.

2.12 Analysis mtDNA control region polymorphism

The amplified mtDNA control region from each individual workers of *A.cerana* was digested with a panel of 15 restriction enzymes which include 10 six-base cutters (*Ase*I, *Ssp*I, *Nde*I, *Hin*dIII, *Dra*I, *Bfr*I, *Eco*RI, *Kpn*I, *Sma*I and *Swa*I), 1 five-base cutter (*Hin*fI) and 4 four-base cutters (*Rsa*I, *Alu*I, *Sau*3AI and *Taq*I). The reaction mixture of 20 µl containing about 500 ng of DNA, 10 units of each restriction endonucleases, 2 µl of 10X reaction buffer and adjusted the volumn to 20 µl by sterile deionized water. Then the reaction mixture was incubated at 37 °C for 2-3 hr and the reaction was stop by addition of one-fifth volume of a loading dye. The fragments of DNA were separated by electrophoresed in 1.5% agarose gel or 2-5% metaphor gel and stained in ethidium bromide. The DNA fragments on agarose and metaphor gel were visualized and photographed under UV light.

2.13 Data analysis

In statistical analysis, two input files was prepared and analyzed in Restriction Enzyme Analysis Package, REAP version 4.0. First, the composite haplotype file consists largely of a rectangular data matrix, with alphanumeric characters corresponding to alternative restriction phenotyped across OTUs for restriction enzymes; the corresponding enzyme profile input file is a tabular matrix (by enzyme) of the binary representations of those restriction phenotypes specified in the haplotype file. Second, the enzyme profile was generated from restriction pattern of each restriction enzyme with digested control region. The fragments size in restriction pattern were recorded in binary matrix for each haplotype. The character state '1' denoting the presence of a particular fragment and '0' the absence of a fragment.

2.13.1 Genetic distance

The nucleotide substitution per site between composite haplotype was calculated and showed in genetic distance (d-value) which using the formula.

$$d = - (2/r) \ln G$$

Where r is the number of recognized sequences of each restriction endonuclease

G is $[F(3-2 GI)]^{1/4}$ and repeatly calculated G = GI then $GI = F^{1/4}$ is the recommended to initial trial value

F is the similarity index between haplotypes was estimated by

$$F = 2n_{xy} /(n_x + n_y)$$

Where n_{xy} is the number of fragments shared by individuals x and y and n_x and n_y are the number of fragments scored for each individual.

2.13.2 Haplotype and nucleotide diversity

The haplotype and nucleotide diversity within population were calculated from combined haplotype frequency distributions for each population and d values among haplotypes

The haplotype diversity was calculated by

$$h = n (1- \Sigma x_i^2)/ (n-1)$$

Where n is the number of individuals investigated and x_i is the frequency of the i^{th} haplotype.

The nucleotide diversity (d) is an average number of nucleotide differences per site between two sequences which could be calculated using

$$d = (n/n-1) \Sigma x_i x_j d_{ij}$$

Where n is the number of individuals investigated and x_i and x_j are the frequencies of i and j genotype and d_{ij} is the number of nucleotide difference per site between the i^{th} and j^{th} haplotype.

The nucleotide diversity is more appropriate than of the haplotype diversity when compare the same DNA markers with difference in length because the haplotype diversity value vary enormously with the length if investigated genome.

2.13.3 Nucleotide divergence

The nucleotide divergence was estimated to nucleotide diversity between haplotypes in population X and Y (d_x and d_y) as

equation 2.13.2. The average nucleotide diversity between haplotypes from population x and y (d_{xy}) was estimated by

$$d_{xy} = \sum x_i x_j d_{ij}$$

Where d_{ij} is nucleotide substitution between the i^{th} and j^{th} haplotype from population x and y. Then the nucleotide divergence between two populations (d_a) is calculated from

$$d_a = d_{xy} - (d_x + d_y)/2$$

2.13.4 Chi-square analysis

The Chi-square (χ^2) analysis based on Monte carlo simulation which performed on composite haplotype frequencies to as certain the difference between population.

2.13.5 Fst analysis

The genotypic differentiation among population was analyzed by Fst value using GENEPOP(version 2)

2.13.6 Dendrograms

The genetic distance (d-value) among composite haplotypes and the nucleotide divergence between populations was used to construction phenograme using UPGMA in phylip version 3.57c.

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

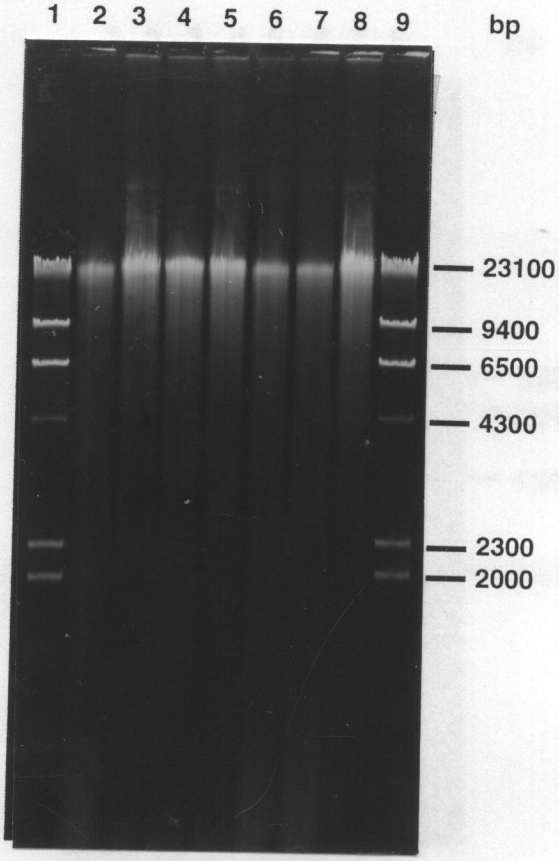


Figure 3.1 Total DNA extracted from *A. mellifera* and six *A. cerana* individuals

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

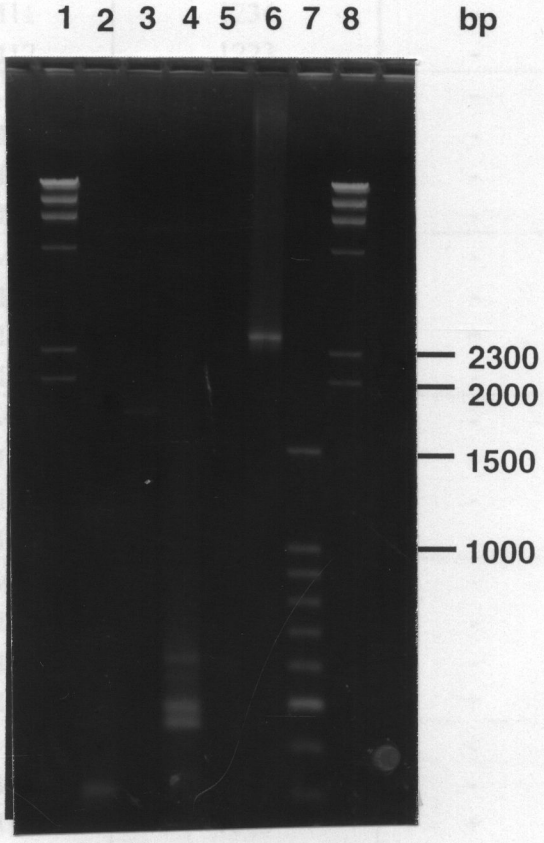


Figure 3.2 The resulting PCR products amplified from *A. mellifera* total DNA using different combining primers

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).

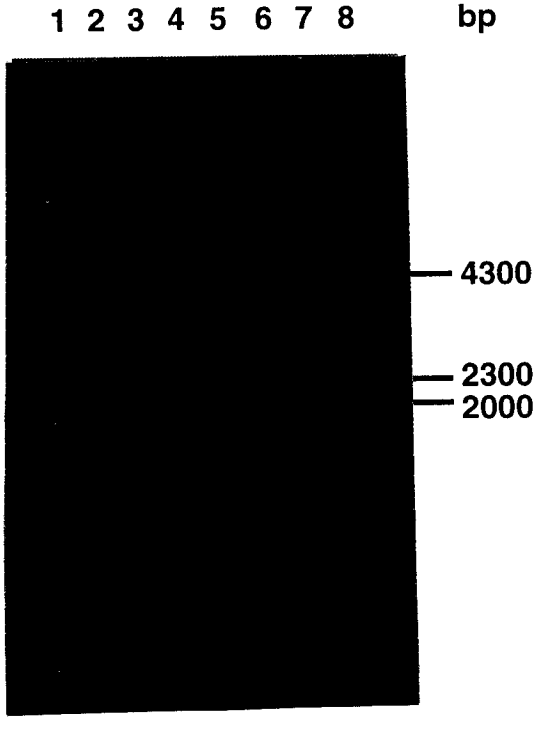


Figure 3.3 Optimization of $MgCl_2$ concentration used for amplification of the control region of *A. cerana*

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

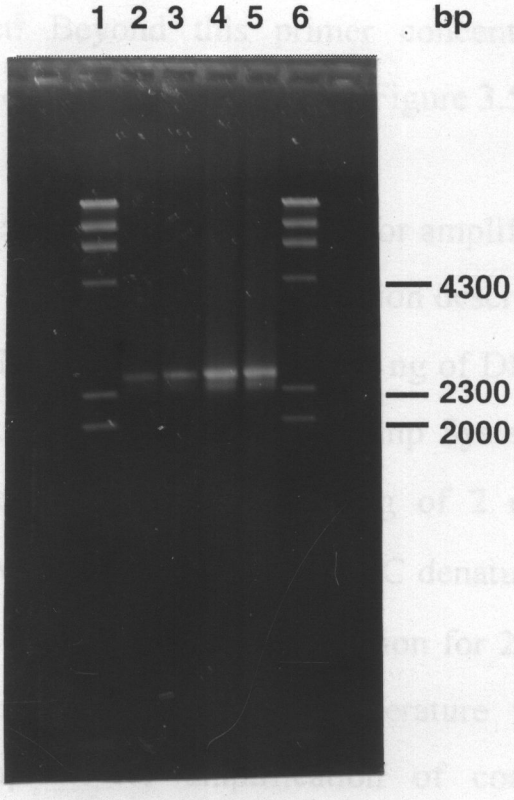


Figure 3.4 Optimization quantity of total DNA template used for amplification of the control region of *A. cerana*

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

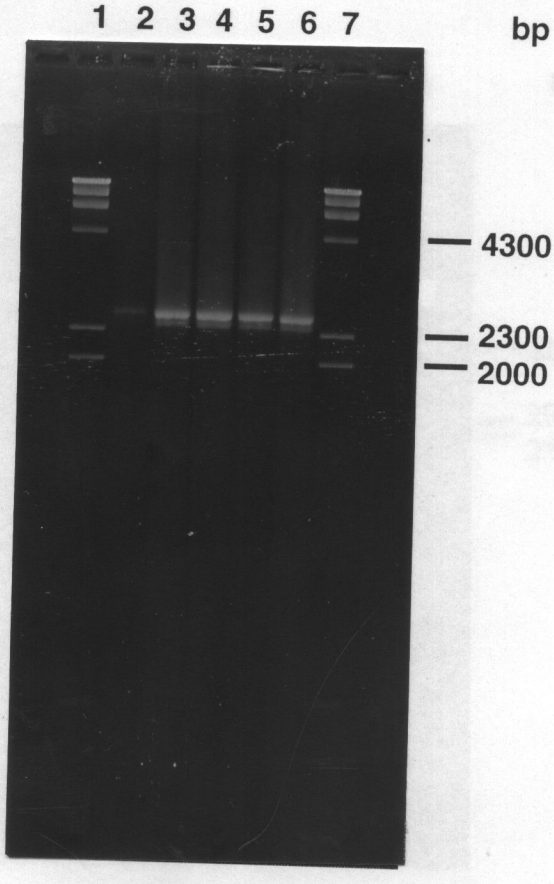


Figure 3.5 Optimization of primers concentration used for amplification of the control region of *A. cerana*

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

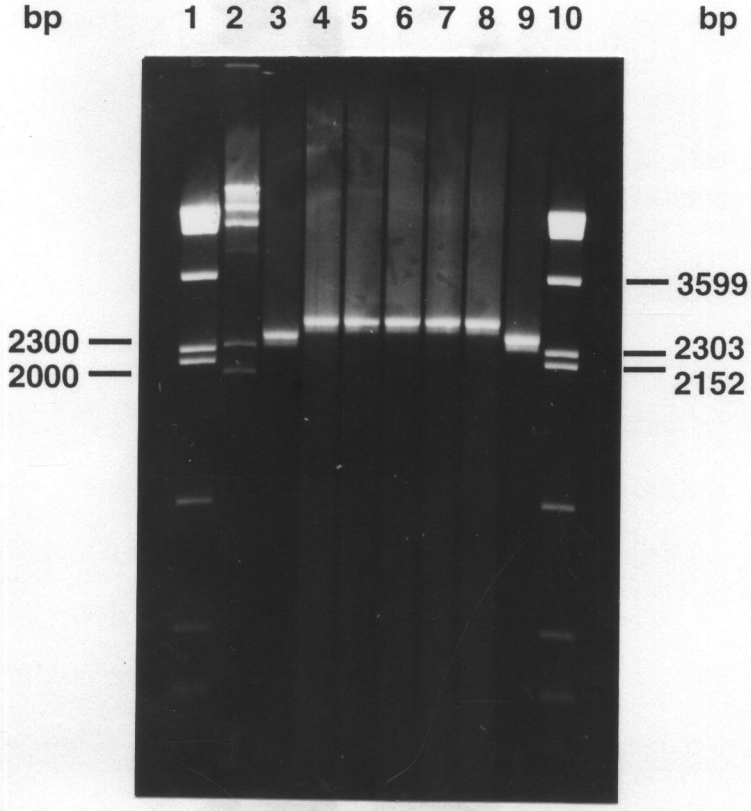
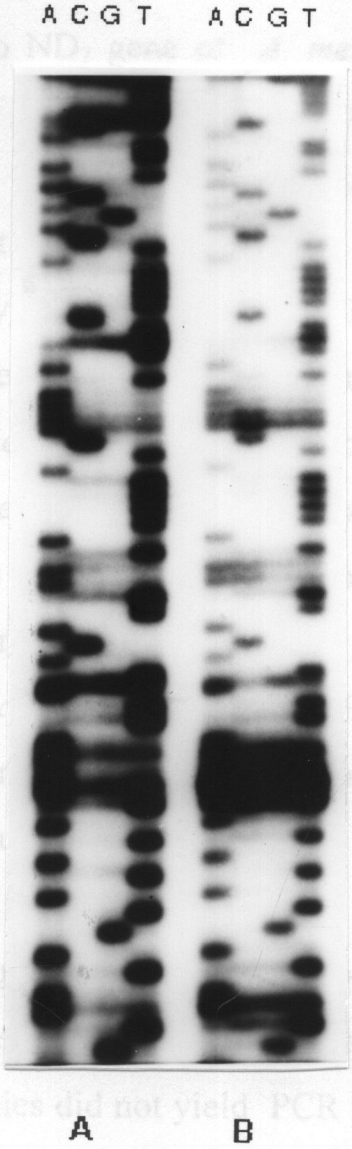


Figure 3.6 Control region from *A. mellifera* and *A. cerana* from six population in Thailand.

lanes 1,10 λ/*Dra*I DNA marker
lane 2 λ/*Hin*dIII 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.

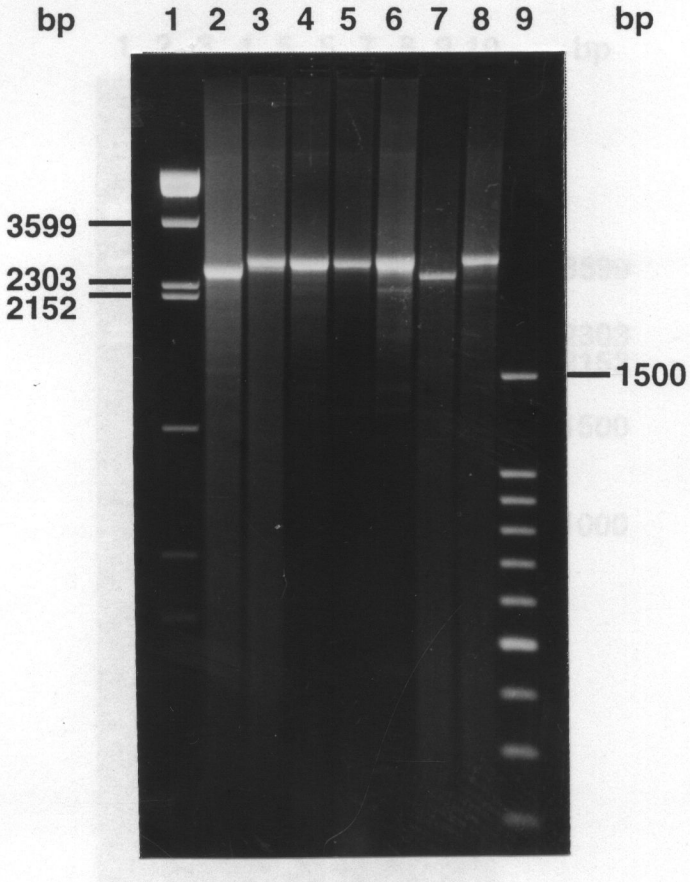


Figure 3.9 Agarose gel electrophoresis showing restriction fragment size of amplified control region of *A. cerana* digested with *Bfr*I. The results showed no restriction site for this restriction endonuclease

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)

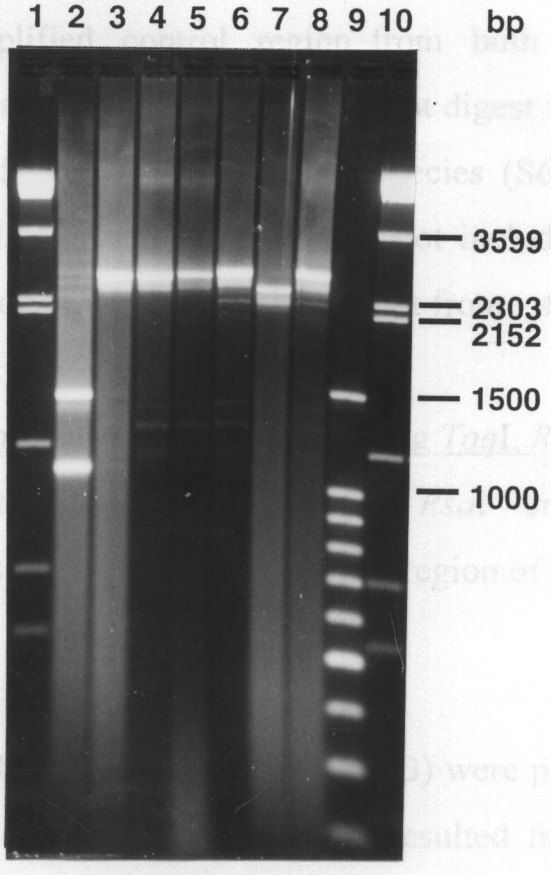


Figure 3.10 Agarose gel electrophoresis showing restriction patterns of the amplified control region of *A. mellifera* and *A. cerana* restricted with *Hin*dIII

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.

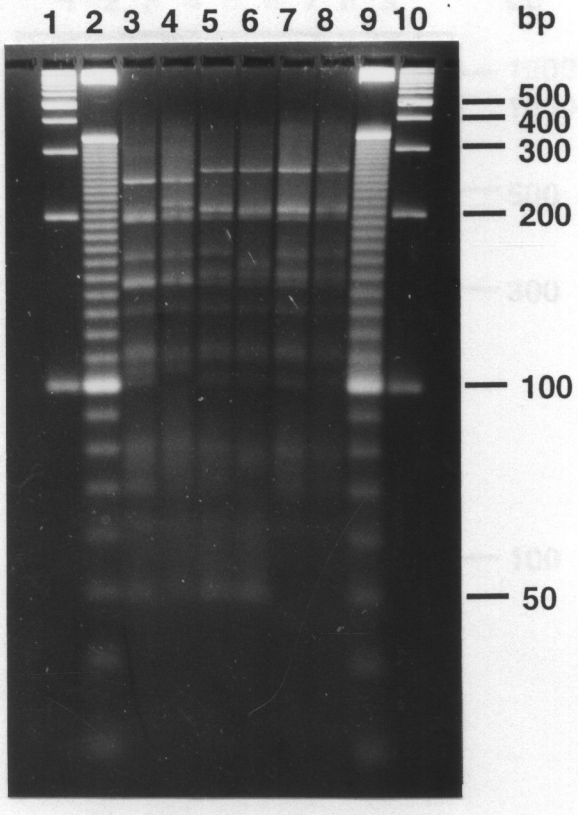


Figure 3.11 Agarose gel electrophoresis showing restriction patterns generated by digestion of control region of *A. cerana* with *Ase*I.

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

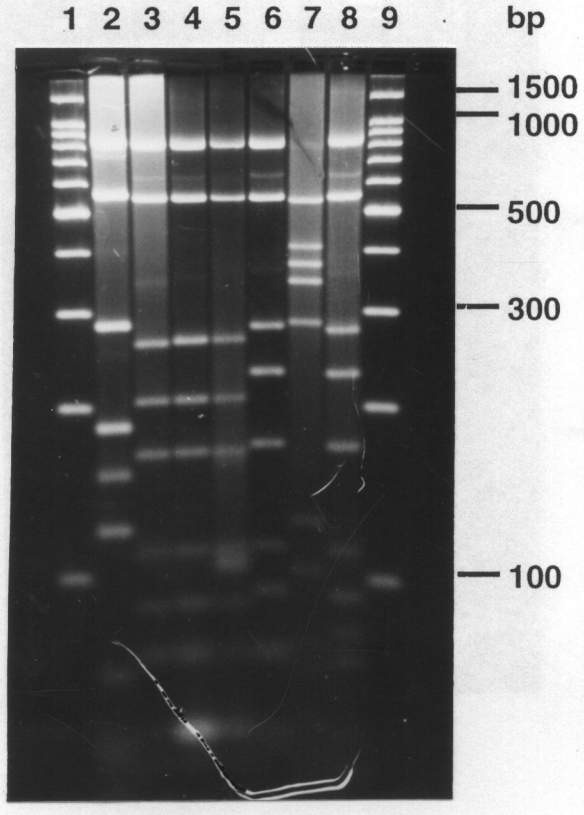


Figure 3.12 Agarose gel electrophoresis showing restriction patterns of amplified control region of *A. mellifera* and *A. cerana* digested with *Dra*I

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*

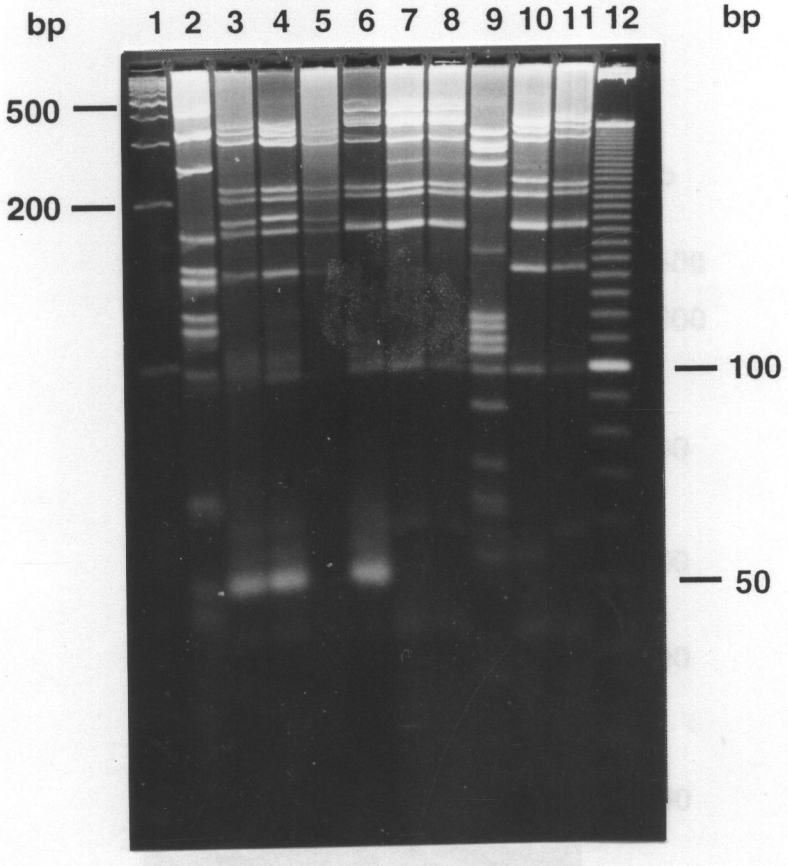


Figure 3.13 Agarose gel electrophoresis showing restriction patterns of amplified control region of *A. mellifera,* S60 and *A. cerana* digested with *Ssp*I

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

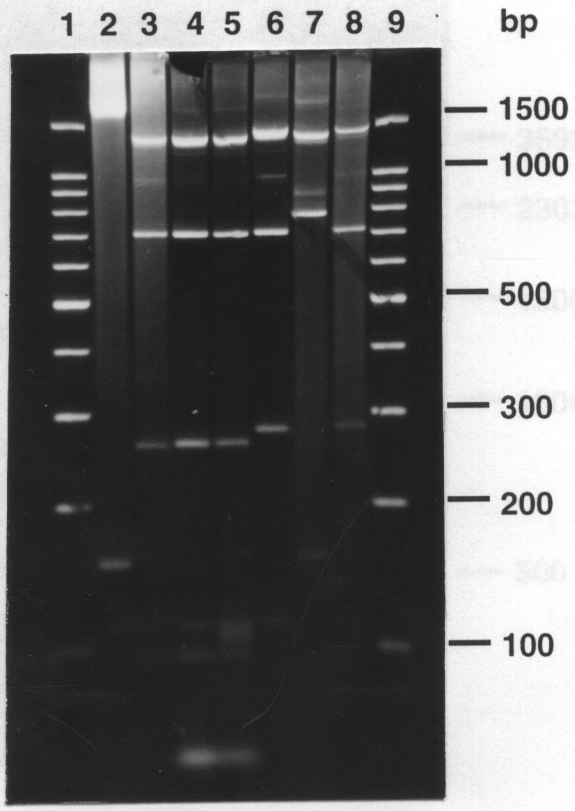


Figure 3.14 Agarose gel electrophoresis showing restriction patterns of amplified control region of *A. mellifera* and *A. cerana* digested with *Swa*I

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*

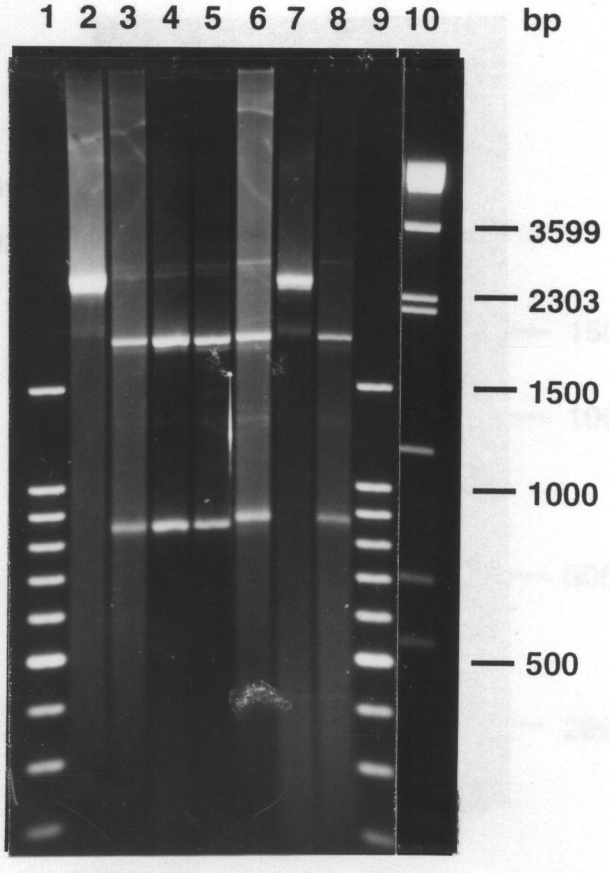


Figure 3.15 Agarose gel electrophoresis showing restriction patterns of amplified control region of *A. mellifera* and *A. cerana* digested with *Nde*I

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

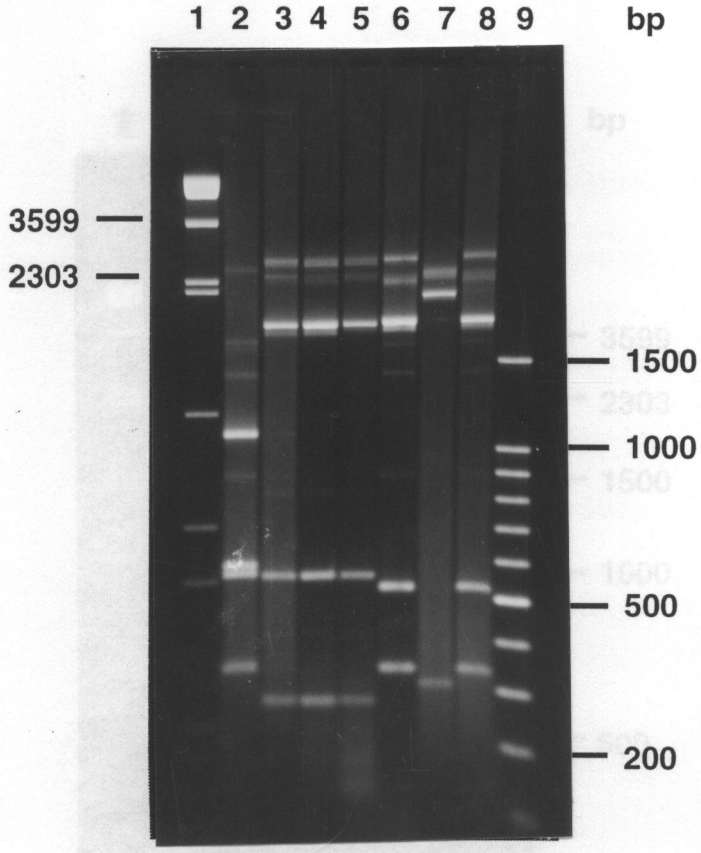


Figure 3.16 Agarose gel electrophoresis showing restriction patterns of amplified control region of *A. mellifera* and *A. cerana* digested with *Alu*I

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

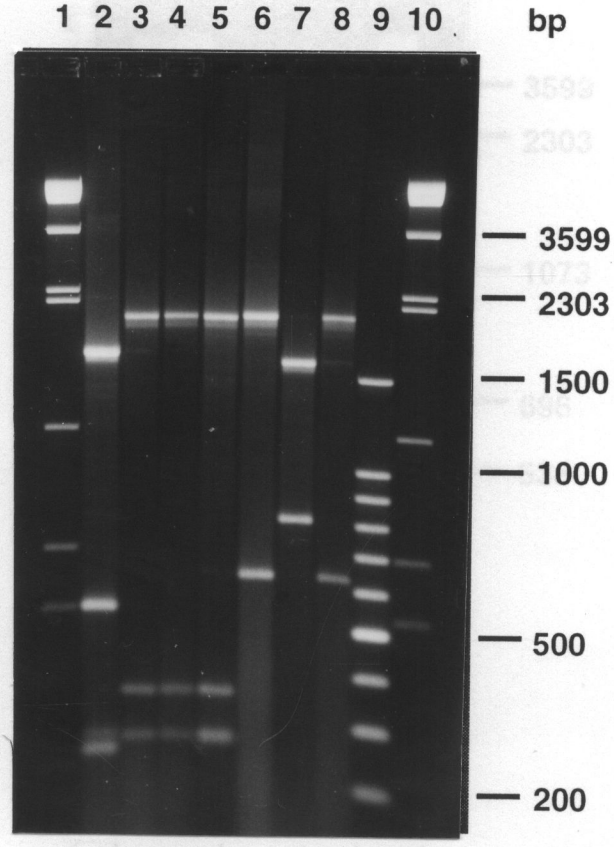


Figure 3.17 Agarose gel electrophoresis showing restriction patterns of amplified control region of *A. mellifera* and *A. cerana* digested with *Taq*I

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

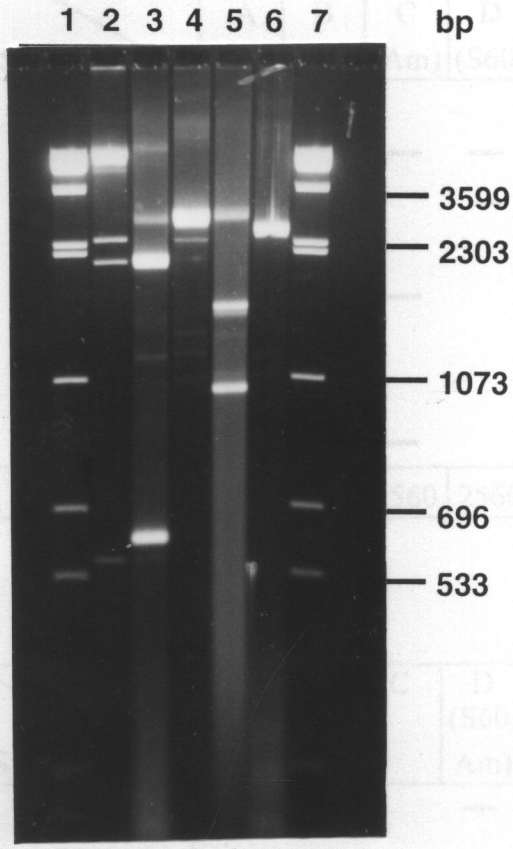


Figure 3.18 Agarose gel electrophoresis showing restriction patterns of amplified control region of *A. mellifera* and *A. cerana* digested with *Rsa*I

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

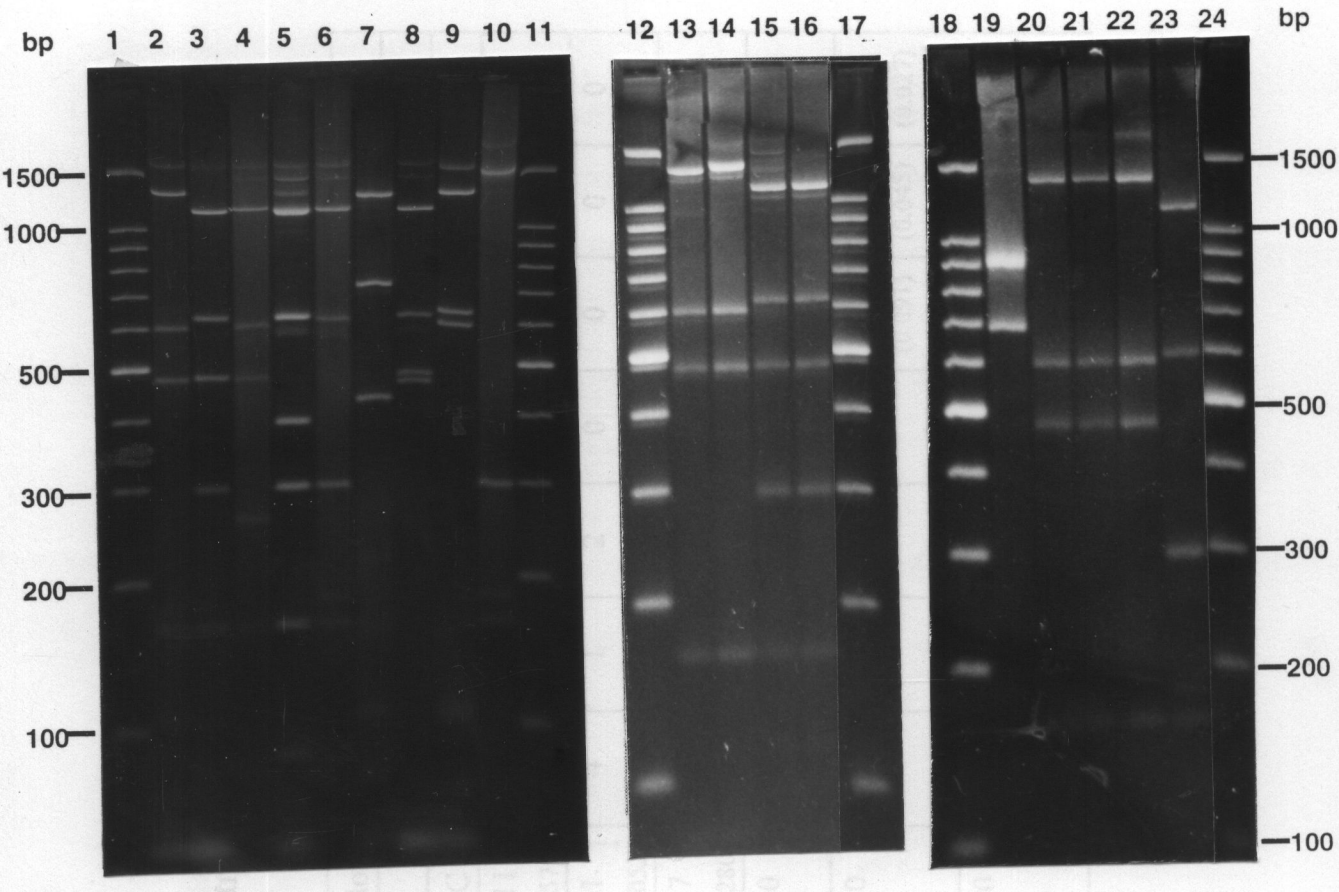


Figure 3.19 Agarose gel electrophoresis showing restriction patterns of amplified control region of *A. mellifera* and *A. cerana* digested with *Hin*fI

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

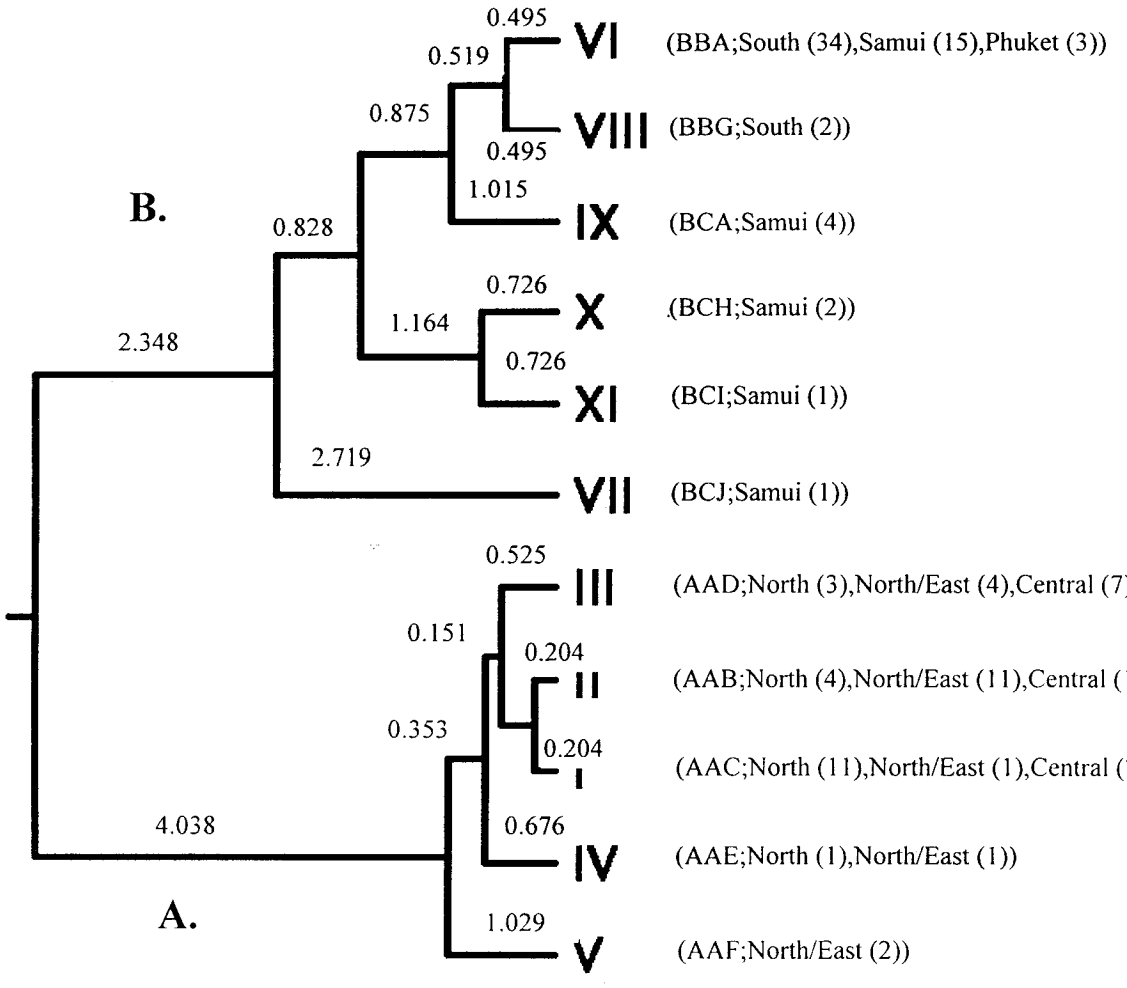


Figure 3.20 UPGMA phenogram showing relationship between 11 composite haplotypes of digested control region of *A . cerana* in Thailand based on nucleotide diversity listed in Table 3.5.

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 ($P<0.0028$)

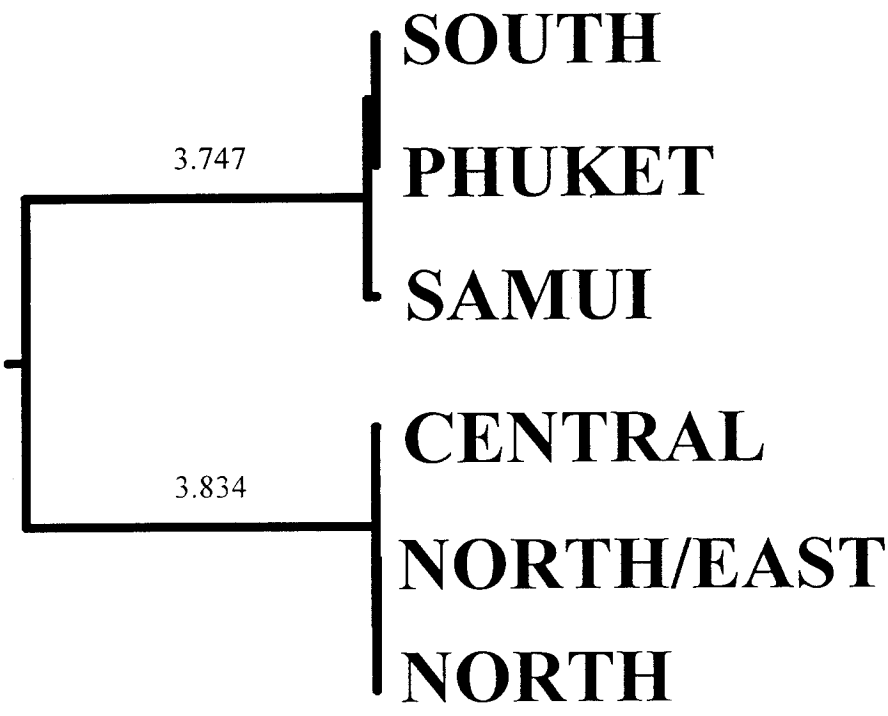


Figure 3.21 UPGMA phenogram showing relationship of six conspecific samples of *A. cerana* in Thailand based on percentage of nucleotide divergence.

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)	-

CHAPTER 4

DISCUSSION

Classification of related species can be performed based primarily on morphology. Moreover, behaviour and other biological characters (e.g. life history) are also useful for studies of evolutionary relationship at inter- and intraspecific levels. Ruttner (1988) dissociate the *Apis* group to 24 different species using morphometric method. Generally, *A. mellifera* is well studied in several biological and genetical aspects compared to other bee species. Genetic diversity of honeybees based on allozymes has been published. The variation at within and between populations of *A. mellifera* were well documented for discrimination of races (Nunamaker *et al.*, 1984a), for identification of the hybride zone between two races of *A. mellifera* (Sheppard and McPheron, 1986) and for studying of bee behaviour (Robinson and Page, 1988, 1989). Recently, molecular genetic markers from nuclear and mtDNA have been increasingly used because these provide wider information than did the allozymes alone. Introgressive hybridization between European and Africanized *A. mellifera* was found using restriction fragment length polymorphism (RFLP) of nuclear DNA (Hall, 1990). Genetic polymorphism based on RFLP, restriction cleavage maps and length polymorphism of mtDNA in *Apis* has been reported and possibly more popular than that of nuclear DNA. The reason to explain this is maternal transmission of mitochondrial genome resulting in much easier sampling strategy when this extrachromosomal DNA is used in these taxa. Therefore, only one individual can represent the maternal lineage of a colony. In contrast, more number of individuals within a colony need to be investigated when nuclear marker are used.

Biogeography of *A. cerana* in Asia was illustrated by Smith and Hagen, 1996 who sequences the non-coding intergenic region of mtDNA from 110 colonies of *A. cerana* originating from large different geographic regions. Two major forms of *A. cerana*, a western form (India, Sri Lanka and Andaman Islands) and an eastern form (Nepal, Thailand, Malaysia, Indonesia, Philippines, Hong Kong, Korea and Japan). The latter can be further divided to two separated group constituting of the Sundaland found in penisular Malaysia, Borneo, Java, Bali, Lom bok, Timor and Flores and the Philippines group found in Luzon, Mindanao and Sangihe. Both groups of haplotypes were found in the Island of Sulawesi, suggesting that this Island was colonized by both the Philippines and Sundaland (Smith and Hagen, 1996). Additionally, mtDNA polymorphism of *A. cerana* was reported from that originating from Japan, Korea, Taiwan, Vietnam, Thailand, Nepal and the Philippines using conventional RFLP analysis. All geographically investigated samples could be allocated to 6 different groups composing of Japan (1), Nepal, Vietnam and North-to-Central Thailand (2), Korea-Tsushima (3), Taiwan (4), Southern Thailand (5) and Philippines (6) (Deowanish *et al.*, 1996). More recently, a large genetic discontinuity between Northern and Southern latitudes of *A. cerana* was reported using PCR-RFLP of mtDNA genes (Sihanantavong, 1997 and Songram, 1997). Both studies could dissociate the Samui sample from the South *A. cerana* based on significant differences in allele distribution frequencies (χ^2 analysis) and population subdivision estimate (Fst) indicating that intraspecific genetic differentiation of *A. cerana* in Thailand did exist and was not resulted from artifacts of sampling errors.

The control region (D-loop) of animal mtDNA is a large non-coding region area containing specific region for initiation of replication and transcription of mitochondrial genome. Generally, base substitutions (transitions and transversions), length polymorphism (insertions and deletions) of this region is greater than those in other regions. Length polymorphism of control region among different individuals within a species has been reported in several species. In insects, the control region is called the AT-rich region because it lacks of any apparent signals for replication and transcription (Desjardins and Morais, 1990; Saccone, Pesole and Sbisa, 1991). Although several mtDNA portion have been used for population genetic and systematic studies of *A. cerana*, the use of the AT-rich region for such purposes is limited. Results for the present study illustrated the possibility to use primer AM8-AM11 originating form *A. mellifera* to amplify the homologous locus in *A. cerana.* Although length polymorphism was observed at intraindividual level (heteroplasmy), interpretation of restriction patterns was not interfered by this phenomenon particularly when three enzymes (*Taq*I, *Rsa*I and *Hin*fI) were carefully chosen based on their simply scorable patterns.

Approximately seventy-one percent of all investigated colonies were successfully amplified by the polymerase chain reaction (PCR). The main amplification product from *A. cerana* was 2750 bp in length. This fragment was coexisted with a slightly smaller PCR product after gel fractionation in all investigated individuals. The homologous product from *A. mellifera* and unidentified *Apis* species (S60) showed the same size of PCR product implying their closed relationship. It is interesting to directly compare the whole amplification sequence of

A. cerana, A. mellifera and S60 with other local *Apis* species in Thailand for a better conclusion of the systematic status of the unidentified S60.

Sequencing of the most common amplification fragment of *A.cerana* was carried out. Based on the fact that the inner primer (5′-AAA ATA AAT AAA GCA GTG GTA-3′) was designed from mitochondrial ND_2 gene of *A. mellifera* , high similarity (>90%) between this and previously deposited ND_2 sequences in the GenBank comfirmed that the amplification product was the actual target fragment composing of the expected control region.

Additionally, digestion of this PCR product amplified from *A. mellifera* in the present study with various restriction enzymes exhibit expected restriction patterns and fragments inferred from a restriction map of *A. mellifera ligustica* reported by Crozier et al (1993). All evidences confirmed that the amplification fragment was the control region of *A. cerana* mitochondrial genome.

Fifteen restriction endonucleases (*Eco*RI, *Kpn*I, *Sma*I, *Sau*3AI, *Bfr*I, *Dra*I, *Ssp*I, *Ase*I, *Hin*fI, *Hin*dIII, *Nde*I, *Swa*I, *Alu*I, *Taq*I and *Rsa*I) could digest the PCR product. Three restriction enzyme (*Taq*I, *Rsa*I and *Hin*fI) were used to analysis the control region. Basically, their enzymes produced with discrete a coexisted digestion band and a fainter fragment with smaller molecular length. Presumably, the fainter band was originated from heteroplasmic product. Nevertheless, scoring of restriction patterns and fragments resulted from these three enzymes were not interfered by length heteroplasmy. Conversely, results from *Ssp*I, *Dra*I and *Ase*I did not include in analysis of genetic polymorphism. The

reason for this was that the sum of restriction fragments resulted from these enzymes was significantly smaller than the size of undigested fragments indication that several smaller DNA fragments may move out of the gels. Moreover, different restriction patterns could not be interrelated by simple loss and gain of restriction site. Therefore, it was not possible to eliminate effects of length heteroplasmy that interfere scoring of band sharing between compared specimens.

Phylogenetic relationship based on genetic distance (the number of nucleotide substitutions per site) indicated two different clades of composite haplotypes. While clade B was observed only in the South, Samui and Phuket samples, clade A genotypes were possessed by specimens from the North, North/East and Central *A. cerana.* No overlapping haplotype between the Northern and Southern latitudes of *A. cerana* was observed indicating large genetic difference between these two groups of *A. cerana* in Thailand. Moreover, clearly differentiation of Thai *A. cerana* should be resulted from restricted gene flow between these populations. Similarity, a UPGMA dendrogram based on nucleotide divergence between pairs of samples well allocated 6 investigated samples to two separated groups, Northern latitude and Southern latitude of *A. cerana.* This supported the results inferred from phylogeny of composite haplotypes as described earlier. The results in the present study were in agreement with those of Sihanantavong (1997) and Songram (1997).

The mtDNA diversity within *A. cerana* samples showed higher haplotype diversity of the Northern sample (0.6088-0.6629) than did the Southern *A. cerana* (0.0000-0.5032). Patterns of nucleotide diversity

within geographic samples also showed the same trend. Approximately equal distributions in frequencies of AAC, AAB and AAD in each of the Northern latitude *A. cerana* caused high genetic diversity within each geographic sample. On the other hand, the most common haplotype BBA was contributed by 91.9%, 68.2% and 100% of *A. cerana* originating from the South, Samui and Phuket, respectively. This reflected lower genetic polymorphism in the Southern latitude of *A.cerana*. It should be noted that only 3 samples from the Phuket Island was investigated and the sample size of this geographic location was unfortunately too small to draw several conclusions in this sample. Therefore, increasing of the sample size of *A. cerana* from the Phuket Island is necessarily required.

The average nucleotide divergence in this study was 4.647% which was greater than that previously reported by Sihanantavong (1997) and Songram (1997) at approximately 2 %. The contradictory results between these results should be primarily responded by the limited number of restriction endonucleases used in this and other experiments.

Considering levels of nucleotide divergence within each group (Northern and Southern), the divergence was lower than 0.2% whereas such levels were larger than 7% when comparing a geographic sample in one region to each of the other. This evidenced strong genetic differentiation of North/South *A. cerana* in Thailand. Nevertheless, it was not possible to compare this circumstance with data from previous publications of *A. cerana* from different locations. This due to lack of standard parameters indicating level of distance and differentiation (e.g. Fst and divergence) in these previously reported.

Genetic heterogeneity analysis using psudo Chi-square indicated significant different in allele distribution frequencies of *A. cerana* in Thailand ($P<0.0028$). Therefore, population subdivision was existance in this species. The results from all possible pairwise comparisons showed the same conclusions drawn from phylogenetic studies with the exception that *A. cerana* from the South had significant genetic heterogeneity compared to that from the Samui Island ($P=0.0014$). Therefore, the Samui *A. cerana* should be considered as different population. It should be noted that, the level of confidence in statistical analysis was further adjusted by the Sequential Bonferroni technique [0.05/(no. of population × no. of restriction enzyme)], for the presence of the significant level of genetic heterogeneity and subsequently, population differentiation analysis.

Likewise,Fst statistics showed overview comparable results with those from Chi-square analysis confirming genetic differentiation of *A. cerana* in Thailand. Restricted gene flow levels were observed between pair of geographic samples from different regions ($Fst>0.2$) whereas high gene flow levels were observed when compared pairs of samples within the same region. Nevertheless, the data on gene flow in *A. cerana* might have been underestimated as transferring of *A. cerana* from different location has been promoted by beekeepers.

Based on all analysis carried out by the present study, all investigated *A. cerana* could be differentiated into 3 different population composing of A (North, North/East and Central *A.cerana*), B (South and Phuket) and C (Samui). It is extremely interesting to test the performance in some economically important traits of *A. cerana* carrying BBA with

those carrying AAD, AAB and AAC haplotypes. The information obtained will be significantly useful for selective breeding programes of *A. cerana* in Thailand. Moreover, patterns of genetic differentiation in other local *Apis* species should not be overlooked and actually need to be classified by molecular genetic approaches.

The most important disadvantage to use the control region (D-loop) for population genetic and systematic studies was possibly due to the extensive length heteroplasmy found in this region producing difficulties for analysis of the results. Although, this phenomenon was previously found in the amplified ATPase6-ATPase8 region of *A. cerana* in Thailand. It was restricted to specimens originating from the South and Samui Island (Songram, 1997). In contrast, heteroplasmy in the control region was observed in all investigated specimens and did not relate to geographic origin of the experimental *A. cerana*. Moreover, the approach used for interspecific analysis among *Apis* species needs to be altered from RFLP to restriction cleavage maps because it was not possible to use band sharing method to compare the amplified products exhibiting length polymorphism between different species.

The results from this study indicated that mtDNA polymorphism analyzed by PCR-RFLP provided useful genetic information in *A.cerana.* The large genetic discontinuity in the continuously distributed species like *A. cerana* indicated that *A. cerana* in the Northern and Southern latitudes experienced restricted gene flow level. This basic information requests for a requirement of further study to test whether *A. cerana* from different populations exhibit different level of performance on

economically important phenotypes (e. g. disease resistance, more honey production).

CHAPTER 5

CONCLUSIONS

1. PCR-amplified control region of *A. mellifera* and all *A. cerana* using primer AM8-AM11 was 2560 and 2750 bp, respectively.

2. Digestion of the amplification product of *A. cerana* with *Taq*I, *Rsa*I and *Hin*fI generated 2, 3 and 10 restriction profile, respectively. A total of 11 composite haplotypes were observed.

3. UPGMA phenograms of composite haplotype and geographic samples indicated 2 genetically different lineages composed of Northern latitude (North, North/East, Central) and Southern latitude (South, Samui Island and Phuket Island) of *A. cerana* in Thailand.

4. Geographic heterogeneity and population differentiation analyzes could allocate six geographic location of *A. cerana* in Thailand to 3 groups composed of 1) North, Central and North/East 2) South and Phuket Island and 3) Samui Island.

REFERENCES

Anonymous. 1993. **Honey USDA agricultural statistics board, national agricultural statistics service**. Washington D C.

Aquadro, C. F., Kaplan, N., and Risko, K. J. 1984. An analysis of the dynamics of mammalian mitochondrial DNA sequence evolution. **Mol. Biol. Evol**. 1: 423-434.

Avise, J. C., et al. 1987. Intraspecific phytogeography: the mitochondrial DNA bridge between population genetics and systematics. **Ann. Rev. Ecol. Syst**. 489-522.

Borror, D. J., Delong, D.M., and Triplehorn, C. A. 1976. **An introduction to the study of insects**. New York: Rinehart and Winston.

Brown, W. M. 1985. The mitochondrial genome of animals. In MacIntyre, R. J. (ed.), **Monographs in Evolutionary Biology: Molecular Evolutionary Genetics Plenum**, 95-130. New York.

Brown, W. M., George, M., and Wilson, A. C. 1979. Rapid evolution of animal mitochondrial DNA. **Proc. Natl. Acad. Sci. USA**. 76: 1967-1971.

Chang, D. D., Fisher, R. P., and Clayton, D. A. 1987. Roles for a promotor and RNA processing in the synthesis of mitochondrial displacement-loop stands. **Biochim. Biophys. Acta**. 909: 85-91.

Clayton, D. A. 1991a. Nuclear gadgets in mitochondrial DNA replication and transcription. **Trends Biochem. Sci**. 16: 107-111.

Clayton, D. A. 1991b. Replication and transcription of vertebrate mitochondrial DNA. **Annu. Rev. Cell. Biol**. 7: 453-478.

Cornut, J. M., Garnery, L., and Solignac, M. 1991. Putative origin and function of the intergenic region between COI and COII of *Apis mellifera L.* mitochondrial DNA. **Genetics** 128: 393-403.

Crozier, R. H., and Crozier. Y. C. 1992. The mitochondrial genome of the honeybee *Apis mellifera*: complete sequence and genome organization. **Genetics** 133: 97-117.

Crozier, Y. C., Koulianos, S., and Crozier, R. H. 1991. **An improved test for Africanized honeybee mitochondrial DNA.** Experientia submitted.

Daly, H. V. 1988. **In Africanized honeybees and bee mites.** Horwood, chichester, U. K.

Deowanish, S., Nakamura, J., Matsuka, M., Kimura, K. 1996. MtDNA variation among subspecies of *Apis cerana* using restriction fragment length polymorphism. **Apidologie**. 27: 407-413.

Desjadins, P., and Morais, R. 1990. Sequence and gene organization of the chicken mtDNA genome. A novel gene order in higher vertebrates. **J. Mol. Biol.** 212: 599-634.

Eickwort, G. C. 1988. The origin of mites associated with honeybees. In Bowman, C. E., Needham, G. R., Page, R. E., and Delfinado-Baker, M. (ed.), **Africanized honeybees and bee mites,** 327-328. Ellis Horwood Limited, Chichester, U.K.

Ferris, S. D., Sage, R. D., and Wilson, A. C. 1982. Evidence from mtDNA sequences that common laboratory strains of mice are descended from a single female. **Nature (London).** 295: 163-165.

Garnery, L., Solignac, M., Celebrano, G., and Cornut, J. M. 1993. A simple test using PCR- amplified mitochondrial DNA to study the genetic structure of *Apis mellifera L.* **Experientia** 49: 1016-1021.

Gojmerac, W. L. 1980. **Bee, beekeeping, honey and pollination.** Westport: The AVJP publishing company.

Graur, D. 1985. Gene diversity in hymenoptera. **Evolution**. 39: 190-199.

Gyllensten, U., Wharton, D., and Wilson, A. C. 1985. Maternal inheritance of mitochondrial DNA during backcrossing of two species of mice. **J. Hered**. 76: 321-324.

Hall, H. G. 1986. DNA differences found between Africanized and European honeybees. **Proc. Natl. Acad. Sci. USA**. 83: 4874-4877.

Hall, H. G. 1988. Characterization of the African honeybee genotype by CAN restriction fragments. In Needham, G. R., Page, R. E., Delfinado-Baker, M.,and Bowman, C. E. (ed.), **In Africanized honeybees and bee mites**, 294-302. Ellis Horwood Limited, Chichester, U.K.

Hall, H. G. 1990. Parental analysis of introgressive hybridization between African and European honeybees using nuclear DNA RFLPs. **Genetics** 125: 611-621.

Hall, H. G., and Muralidharan, K. 1989. Evidence from mtDNA that African honeybees spread as continuous maternal lineages. **Nature**. 339: 211-213.

Hall, H. G., and Smith, D. R. 1991. Distinguishing African and European honeybees matrilines using amplified mitochondrial DNA. **Proc. Natl. Acad. Sci. USA.** 88: 4548-4552.

Jordan, R. A., and Brosemer, R. W. 1974. Characterization of DNA from three bee species. **J. Insect. Physiol**. 20: 2513-2520.

Lansman, R. A., Avise, J. C., and Hueltel, M. D. 1983. Critical experimental test of the possibility of "paternal linkage" of mtDNA. **Proc. Natl. Acad. Sci. USA**. 80: 1969-1971.

Limbipichai, K. 1990. **Morphometric studies on the eastern honeybee (*Apis cerana Fabricius*) in Thailand and Malaysian peninsula.** Master's Thesis, Department of Biology, Graduate School, Chulalongkorn University.

Lindauer,M. 1961. **Communication among social bees.** Harvard University Press: Cambridge ,MA.

Maa, T.C. 1953. An inquiry into the systematics of the tribus Apidini or honeybees (Hym.). **Treubia** 21: 525-640.

Moritz, C. 1994. Applications of mitochondrial DNA analysis in conservation: a critical review. **Molecular Ecology**. 3: 401-411.

Moritz, C. F., Dowling, T. E., and Brown, W. M. 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. **Ann. Rev. Ecol. Syst**. 18: 269-292.

Moritz, R. F. A., Hawkins, C. F., Crozier, R. H., and Mackinlay, A. G. 1986. A mitochondrial DNA polymorphism in honeybees (*Apis mellifera L.*). **Experientia** 42: 322-324.

Norman, J., Moritz, C., Limpus, C. J. 1994. Mitochondrial DNA control region polymorphisms: genetic markers for ecological studies of marine turtles. **Molecular Ecology** 3: 363-373.

Nunamuker, R. A., Wilson, W. T., and Ahmad, R. 1984a. Malate dehydrogenase and non-specific esterase isoenzymes of *A. florea* , *A. dorsata*, and *A. cerana* as detection by isoelectric focusing. **J. Kans. Entomol. Soc**. 57: 591-595.

Packer, L., and Owen, R. E. 1992. Variable enzyme systems in the Hymenoptera. **Biochem. Syst. Ecol.** 20: 1-7.

Pamilo, P., Varvio-Aho, S., and Pekkarinen, A. 1978. Low enzyme gene variability in Hymenoptera as a consequence of haplodiploidy. **Hereditas**. 88: 93-99.

Robinson, G. E., and Page, R. E. 1988. Genetic determination of guarding and undertaking in honeybee colonies. **Nature** 333: 356-358.

Robinson, G. E., and Page, R. E. 1989. Genetic determination of nectar foraging, pollen foraging, and nest-site scouting in honeybee colonies. **Behav. Ecol. Sociobiol.** 24: 317-323.

Rosel, P. E., Dizon, A. E., and Haygood, M. G. 1995. Variability of the mitochondrial control region in populations of the harbour porpoise, *Phocoena phocoena*, on interoceanic and regional scales. **Can. J. Fish.Aquat.Sci.** 52: 1210-1219.

Ruttner, F. 1988. **Biogeography and taxonomy of honeybees**. Springer: Berlin.

Saecone, S., Pesole, G., and Sbisa, E. 1991. The main regulatory region of mammalian mtDNA: Structure-function model and evolutionary pattern. **J. Mol. Evol.** 33: 83-91.

Sheppard, W. S., and McPheron, B. A. 1986. Genetic variation in honeybees from an area of racial hybridization in Western Czechoslovakia. **Apidologie** 17: 21-31.

Sihanuntavong, D. 1998. **Genetic variation in mitochondrial genes of honeybee *Apis cerana* in Thailand.** Master's Thesis, Department of Biotechnology, Graduate School, Chulalongkorn University.

Smith, D. R. 1991. Mitochondrial DNA and honeybee biogeography. In Smith, D. R. (ed.), **Diversity in the genus *Apis*,** 131-176. Oxford: Westview Press.

Smith, D. R., Hagen, R. H. 1996. The biogeography of *A. cerana* as revealed by mitochondrial DNA sequence data. **J. Kans. Entomol. Soc.** 69: 294-310.

Smith, D. R., Taylor, O. R., and Brown, W. M. 1989. Neotropical African bees have African mitochondrial DNA. **Nature**. 339: 213-215.

Songram, O. 1998. **Genetic variation of *Apis cerana* in Thailand inferred by PCR-RFLP analysis of the mitochondrial ATPase6-ATPase8 gene**. Master's Thesis, Department of Biochemistry, Graduate School, Chulalongkorn University.

Southwick, E. E., and Southwick, L. 1992. Estimating the economic value of honeybees (Hymenoptera: Apidae) as agricutural pollinators in the United States. **J. Econ. Entomol**. 85: 621-633.

Tanabe, Y., and Tamaki, Y. 1985. Biochemical genetic studies on *Apis mellifera* and *Apis cerana*. **Proc. 30 th. International beekeeping congress**, Nagoya, Japan. 152-154.

Tegelstrem, H. 1987. Transfer of mitochondrial DNA from the Northern red-backed vole (*Cleithrionomys rutilus*) to the bank vole (*C. glarcolus*). **J. Mol. Evol**. 24:218-227.

Tzageloff, A., and Myers, A. M. 1986. Genetic of mitochondrial biogenesis. **Ann. Rev. Biochem**. 55: 249-285.

Uthasang, W. 1994. **Preparation of DNA probe for the analysis of genetic variation in *Apis cerana***. Master's Thesis, Department of Biochemistry, Graduate School, Chulalongkorn University.

Wilson, A. C., et al. 1985. Mitochondrial DNA and two perspectives on evolutionary genetics. **Biol. J. Linn. Soc**. 26: 375-400.

Wongsiri, S., and Deowanish, S. 1995. Queen production of *A. cerana*. Bee biodiversity of honeybees in Thailand . **The Asiantic hive bee**. 91-96.

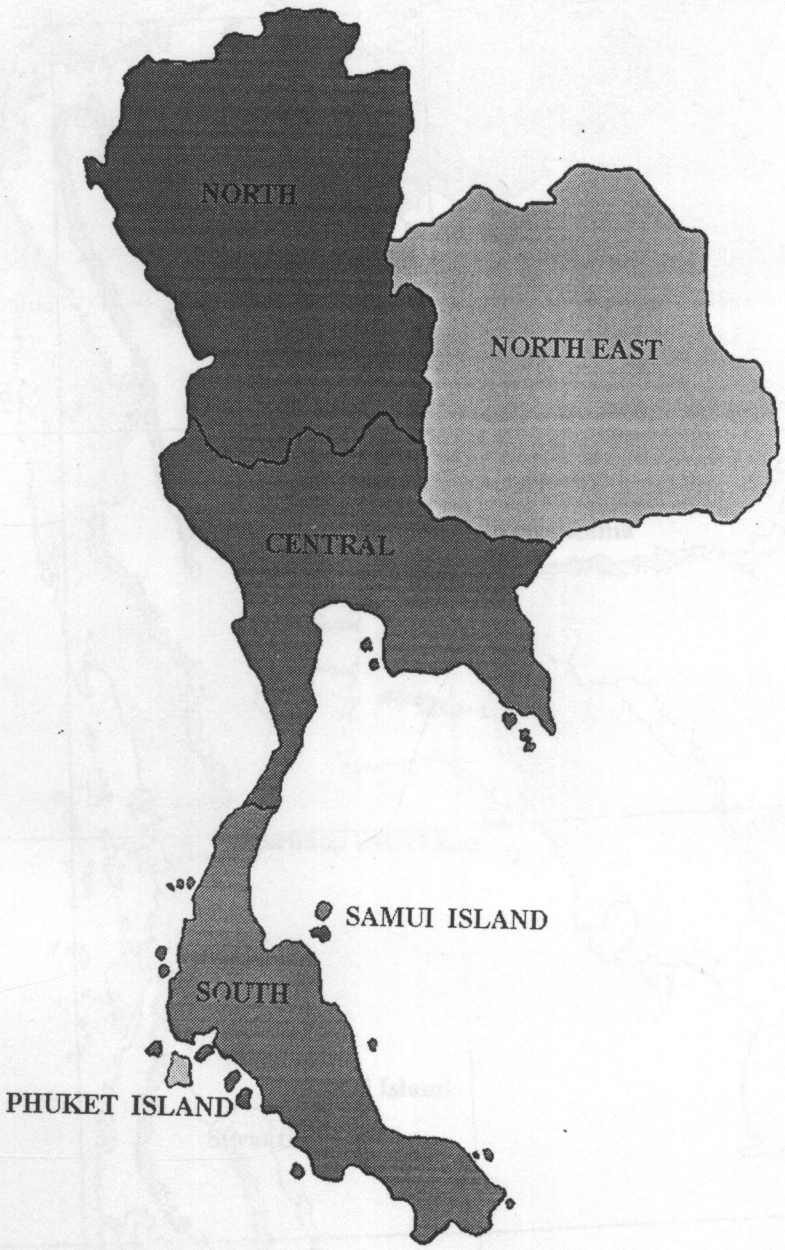
Wongsiri, S., and Tangkanasing, P. 1989. The resistance behavior of *Apis cerana* againt *Tropilaelaps clareae*. **Proceedings First Asia pacific Conference Entomology** 25-34.

Wright, J. W., Spolsky, C., and Brown, W. M. 1983. The origin of the parthenogenetic lizard chemidophone laredoensis inferred from mtDNA analysis. **Herpetologica**. 39: 410-416.

APPENDICES

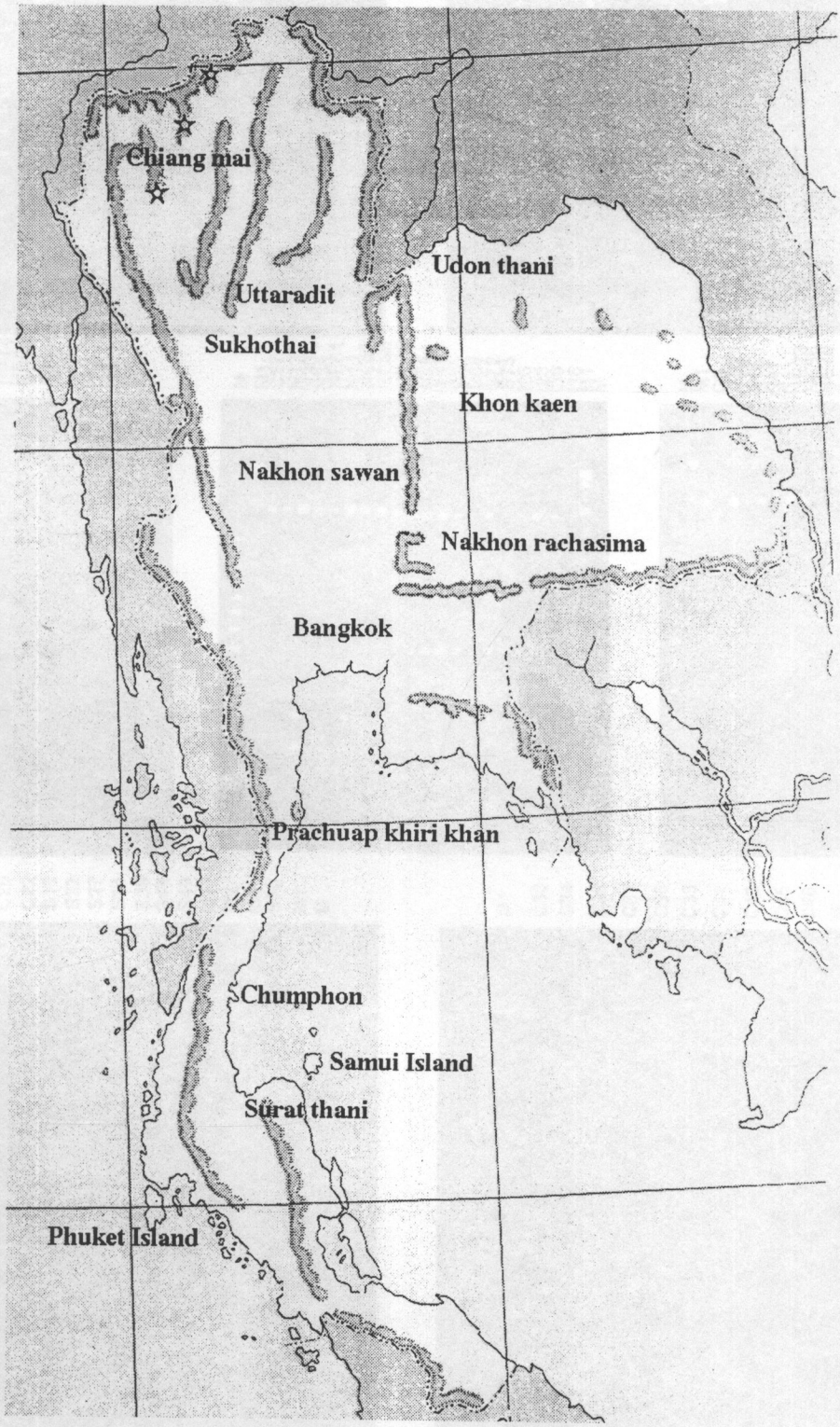
APPENDIX A

Thailand map indicate six locations sampling of honeybee *A. cerana*



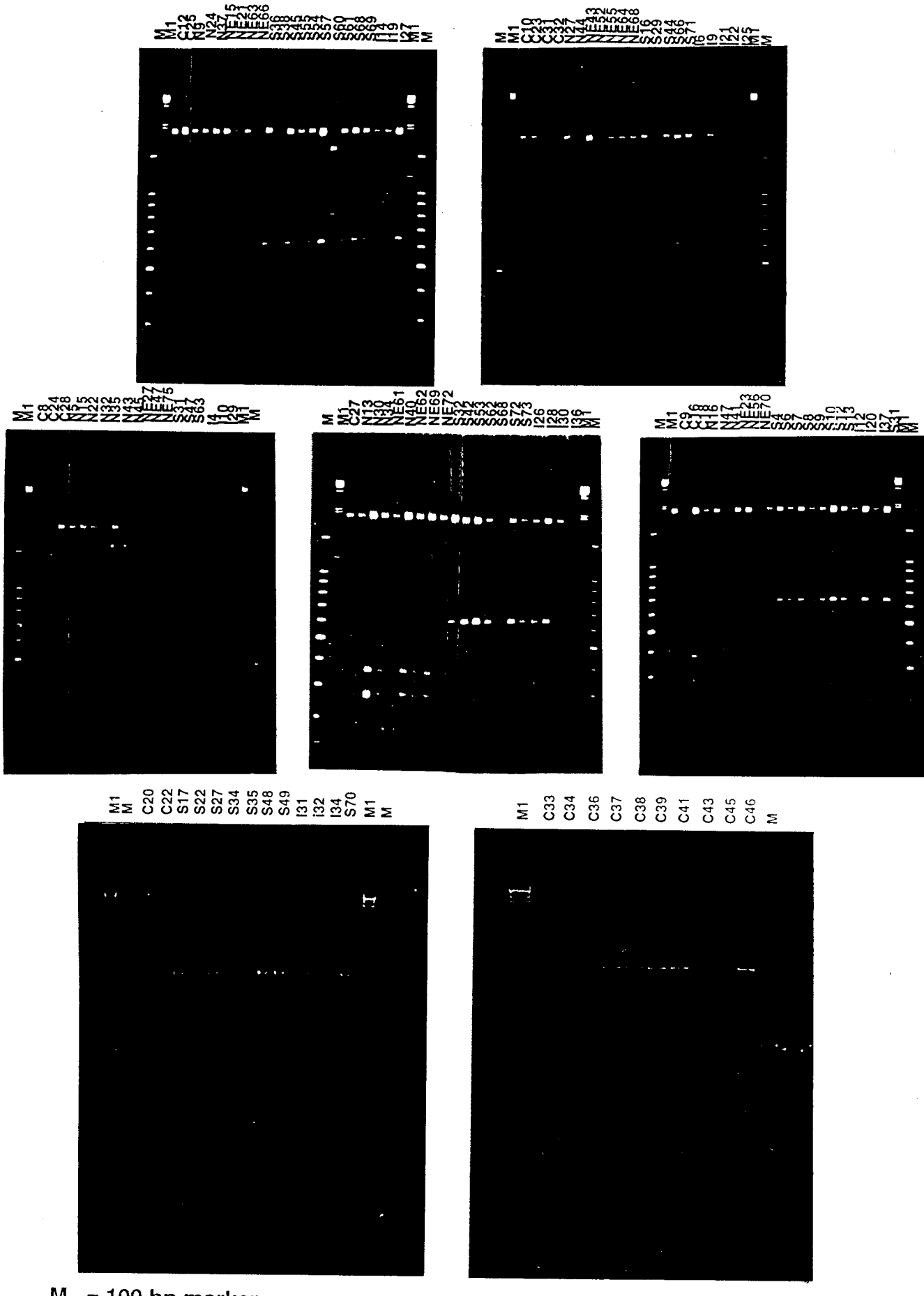
APPENDIX B

Thailand map indicate six geographic locations sampling of honeybee *A. cerana*

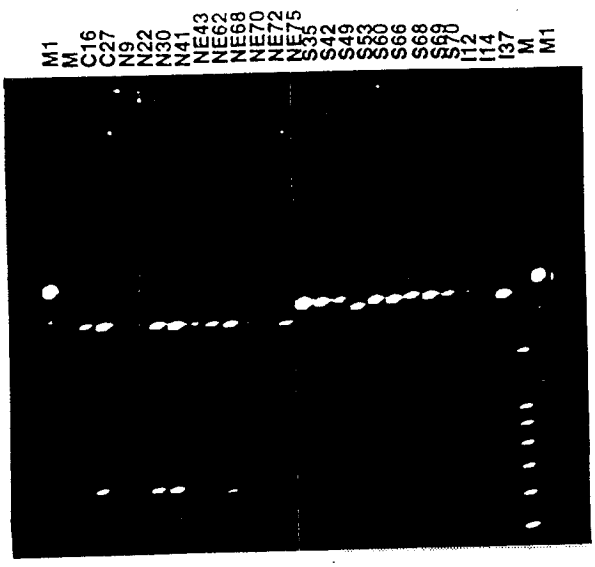


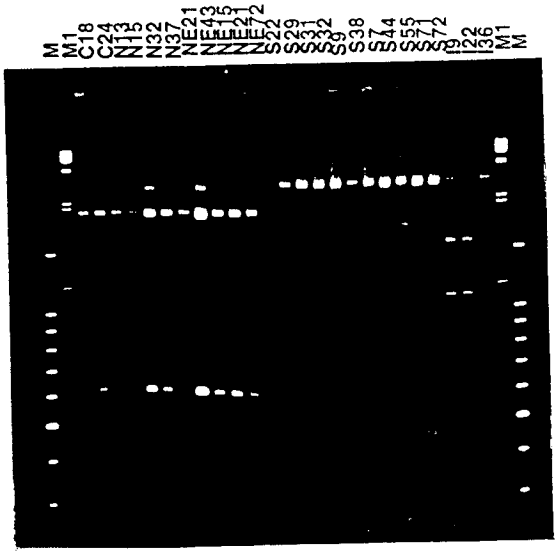
APPENDIX C

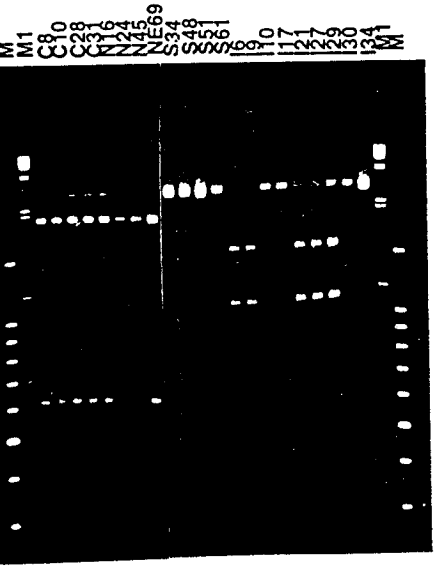
Sample of *A. cerana* control region digested by *Taq*I

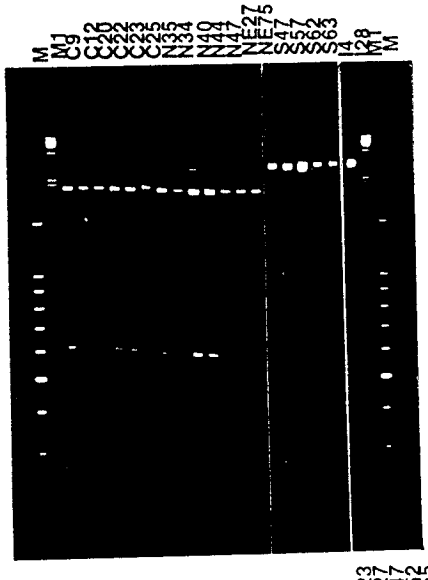


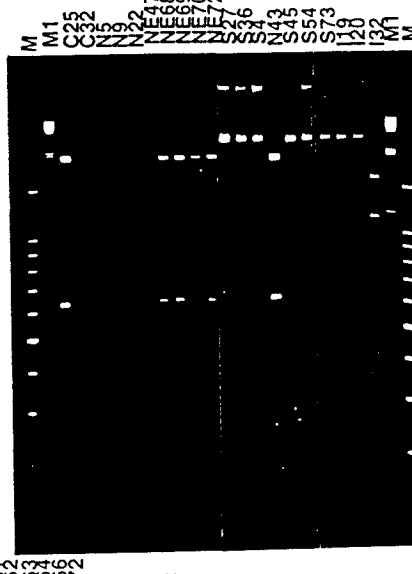
M = 100 bp marker
M1 = Lamda/*Dra*I

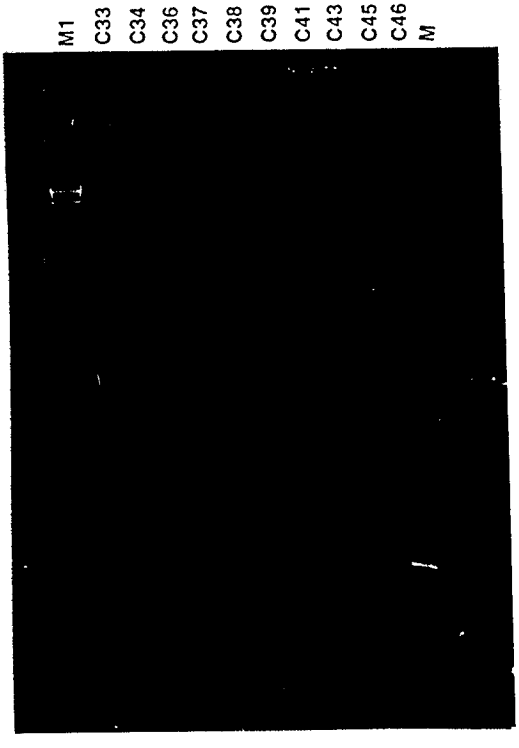


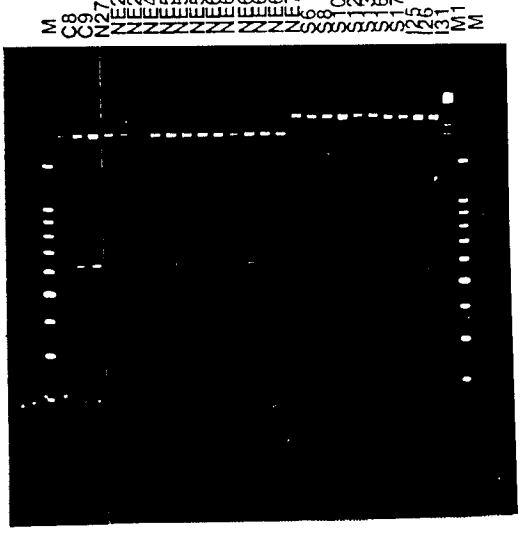




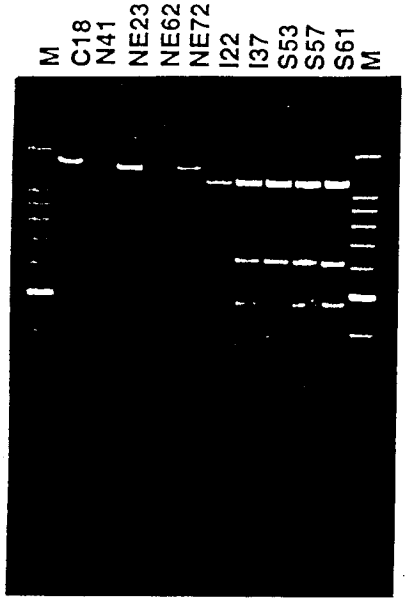


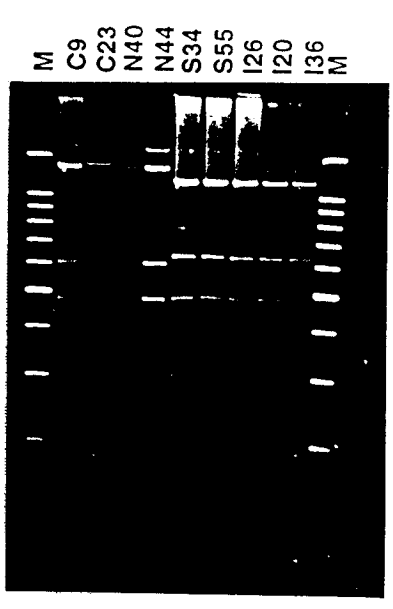


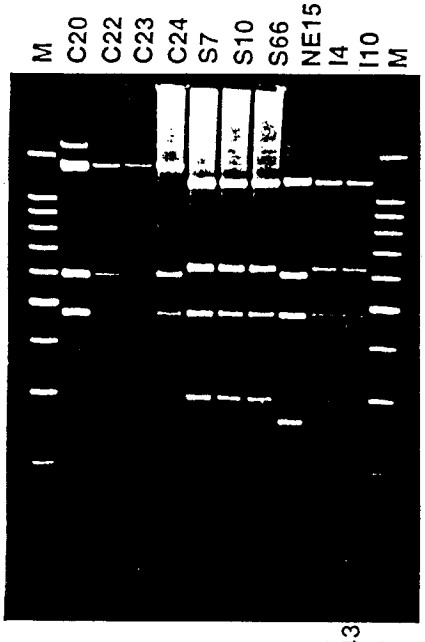


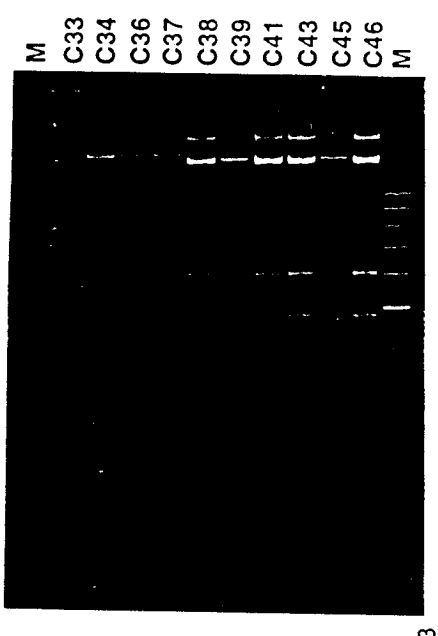


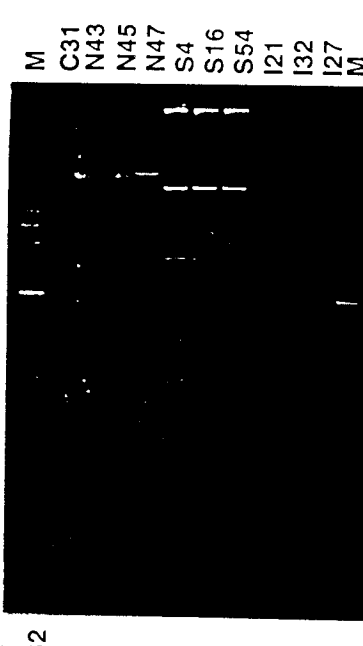
M = 100 bp marker
M1 = Lamda/ *Dra*I

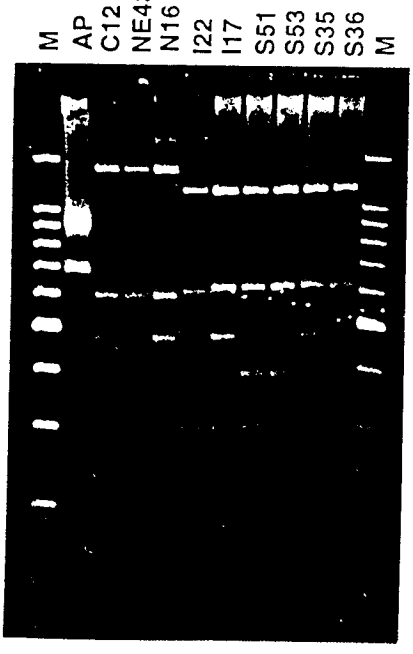


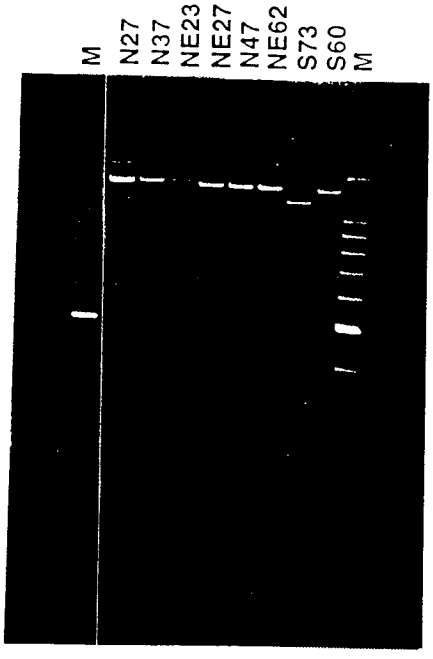












M = 100 bp marker

APPENDIX D

Collection area and composite haplotype of *A. cerana* from the North of Thailand

Code of colonies	Sampling area	Composite Haplotype		
		I	II	III
N01	Muang, Lamphun	ND	AAA	ND
N02	Muang, Uttaradit	AAA	AAA	ND
N05	Muang, Lamphun	AAA	ND	AAC
N06	Muang, Lamphun	AAA	AAA	ND
N07	San pa tong, Chiang mai	AAA	AAA	ND
N09	San pa tong, Chiang mai	AAA	AAA	AAC
N12	San pa tong, Chiang mai	ND	AAA	ND
N13	Hang dong, Chiang mai	AAA	AAA	AAC
N14	Muang, Uttaradit	BAA	AAA	ND
N15	Muang, Phitsanulok	AAA	AAA	AAB
N16	Muang, Phitsanulok	ADA	AAA	AAC
N17	Muang, Phitsanulok	AAA	AAA	ND
N19	Phayuha khiri, Nakhon sawan	AAA	AAA	ND
N20	Manorom, Chai nat	AAA	AAA	ND
N21	Wat sing, Chai nat	AAG	AAA	ND
N22	Muang, Chai nat	AAA	AAA	AAC
N23	Banphot phisai, Nakhon sawan	AAA	AAA	ND
N24	Banphot phisai, Nakhon sawan	ND	AAA	AAD
N25	Banphot phisai, Nakhon sawan	AAA	AAA	ND
N26	Muang, Kamphaeng phet	AAA	AAA	ND
N27	Ban Tak, Tak	AAA	AAA	AAC
N28	Ban Tak, Tak	AAA	ND	ND
N29	Sam ngao, Tak	AAA	ACA	ND
N30	Sam ngao, Tak	AAA	ND	AAD
N32	Thoen, Lampang	AAA	AAA	AAC
N34	Thoen, Lampang	ND	ND	AAD
N35	Mae tha, Lampoon	ND	ND	AAE
N36	Mae tha, Lampoon	AAA	AAA	ND
N37	Doi saket, Chiang mai	AAA	AAA	AAC
N38	Doi saket, Chiang mai	ND	AAA	ND
N39	Doi saket, Chiang mai	AAA	AAA	ND
N40	Doi saket, Chiang mai	ND	ND	AAC
N41	Wiang pa pao, Chiang mai	AAA	ACA	AAC

Code of colonies	Sampling area	Composite Haplotype		
		I	II	III
N42	Wiang pa pao, Chiang mai	AAA	AAA	ND
N43	Wiang pa pao, Chiang mai	AAA	AAA	AAB
N44	Ngao, Lampang	AAA	AAA	AAC
N45	Sungmen, Phrae	AAA	AAA	AAB
N46	Sungmen, Phrae	AAA	AAA	ND
N47	Sungmen, Phrae	ND	AAA	AAB
N48	Sungmen, Phrae	AAA	AAA	ND

Collection area and composite haplotype of *A. cerana* from the Central of Thailand

Code of colonies	Sampling area	Composite Haplotype		
		I	II	III
C01	Phrapradaeng, Samut prakan	ADA	AAA	ND
C02	Kamphaengsan, Nakhorn pathom	AAA	AAA	ND
C03	Dan chang, Suphan buri	AAA	ADA	ND
C04	Muang, Samut songkhram	AAA	AAA	ND
C05	Muang, Samut songkhram	AAA	AAA	ND
C06	Muang, Samut songkhram	AAH	AAA	ND
C07	Phrapradaeng, Samut prakan	ADA	AAA	ND
C08	Don tum, Nakhorn pathom	AAA	AAA	AAD
C09	Dan chang, Suphan buri	AAA	AAA	AAC
C10	Dan chang, Suphan buri	ADA	AAA	AAD
C11	Pong nam ron, Chanthaburi	AAA	AAA	ND
C12	Makham, Chanthaburi	AAA	AAA	AAC
C13	Makham, Chanthaburi	AAA	AAA	ND
C14	Makham, Chanthaburi	AAA	AAA	ND
C15	Muang, Trat	AAA	AAA	ND
C16	Khao saming, Trat	ND	AAA	AAC
C17	Khao saming, Trat	ND	AAA	ND
C18	Muang, Trat	AAA	AAA	AAD
C20	Sam roi yod, Prachuap khiri khan	ND	AAA	AAB
C21	Sam roi yod, Prachuap khiri khan	ND	AAA	ND
C22	Sam roi yod, Prachuap khiri khan	ND	AAA	AAB
C23	Thong pha phum, Kanchana buri	ND	AAA	AAC
C24	Muang, Phetchaburi	ND	AAA	AAC
C25	Maekrong,Samut songkhram	ND	AAA	AAB
C26	Maekrong,Samut songkhram	ND	AAA	ND
C27	Maekrong,Samut songkhram	ND	AAA	AAC
C28	Maekrong,Samut songkhram	ND	AAA	AAB
C29	Maekrong,Samut songkhram	ND	AAA	ND
C30	Muang, Samut sakhorn	ND	AAA	ND

Code of colonies	Sampling area	Composite Haplotype		
		I	II	III
C32	Lad lum kaew, Pratum tani	ND	ND	AAC
C33	Cha um, Phetchaburi	ND	ND	AAB
C34	Huaw hin, Prachuap khiri khan	ND	ND	AAD
C36	Pranburi, Prachuap khiri khan	ND	ND	AAD
C37	Kuyburi, Prachuap khiri khan	ND	ND	AAD
C38	Muang, Prachuap khiri khan	ND	ND	AAB
C39	Tubsakae, Prachuap khiri khan	ND	ND	AAB
C41	Bangsapan, Prachuap khiri khan	ND	ND	AAB
C43	Bangsapannoy, Prachuap khiri khan	ND	ND	AAB
C45	Bangsapannoy, Prachuap khiri khan	ND	ND	AAD
C46	Tubsakae, Prachuap khiri khan	ND	ND	AAB

Collection area and composite haplotype of *A. cerana* from the North/East of Thailand

Code of colonies	Sampling area	Composite Haplotype		
		I	II	III
NE02	Na haeo, Loai	ND	AAA	ND
NE03	Na haeo, Loai	AAA	AAA	ND
NE14	Muang, Khonkaen	AAA	AAA	ND
NE15	Muang, Khonkaen	ADA	AAA	AAE
NE16	Chum phae, Khonkaen	AAA	AAA	ND
NE17	Chum phae, Khonkaen	AAA	AAA	ND
NE18	Chum phae, Khonkaen	AAH	AAD	ND
NE19	Chum phae, Khonkaen	AAH	ND	ND
NE20	Ubol ratana, Khonkaen	AAA	ACA	ND
NE21	Non sang, Nong bua lamphu	AAA	AAA	AAF
NE22	Non sang, Nong bua lamphu	AAA	AAA	ND
NE23	Non sang, Nong bua lamphu	AAA	AAA	AAB
NE24	Nong wua so, Udon thani	AAA	AAA	ND
NE25	Muang, Udon thani	AAA	AAA	ND
NE26	Muang, Udon thani	AAA	AAA	ND
NE27	Muang, Udon thani	AAA	AAA	AAF
NE28	Muang, Nongkhai	AAA	AAA	ND
NE29	Muang, Nongkhai	AAA	AAA	ND
NE30	Nong han, Udon thani	AAA	AAA	ND
NE31	Phang khon, Sakonnakhon	AAA	AAA	ND
NE32	Nong bon nak, Nakhonratchasima	AAA	AAA	ND
NE33	Nong bon nak, Nakhonratchasima	AAA	AAA	ND
NE34	Nong bon nak, Nakhonratchasima	AAA	ND	ND
NE43	Praconechai, Burirum	ND	ND	AAC
NE47	Praconechai, Burirum	ND	ND	AAD
NE52	Rattanaburi, Surin	ND	ND	AAD
NE55	Prasat, Surin	ND	ND	AAB
NE56	Prasat, Surin	ND	ND	AAB
NE61	Nongbunnak, Nakonrachsima	ND	ND	AAB
NE62	Nongbunnak, Nakonrachsima	ND	ND	AAB
NE63	Nongbunnak, Nakonrachsima	ND	ND	AAD
NE64	Nongbunnak, Nakonrachsima	ND	ND	AAB
NE66	Nongbunnak, Nakonrachsima	ND	ND	AAD
NE68	Nongbunnak, Nakonrachsima	ND	ND	AAB
NE69	Nongbunnak, Nakonrachsima	ND	ND	AAB

Code of colonies	Sampling area	Composite Haplotype		
		I	II	III
NE70	Thawatchaburi, Roiet	ND	ND	AAB
NE72	Warin chamrab, Ubon ratchathani	ND	ND	AAB
NE75	Muang, Konkaen	ND	ND	AAB

Collection area and composite haplotype of *A. cerana* from the South of Thailand

Code of colonies	Sampling area	Composite Haplotype		
		I	II	III
S01	Thalang, Phuket	BBB	BBB	ND
S02	Muang, Chumphon	CED	BBB	ND
S03	Muang, Chumphon	BBB	BBB	ND
S04	Tha chana, Suratthani	ND	BBB	BBA
S06	Tha chana, Suratthani	BBB	BBB	BBA
S07	Tha chana, Suratthani	ND	BBB	BBA
S08	Tha chana, Suratthani	ND	BBB	BBA
S09	Tha chana, Suratthani	ND	BBB	BBA
S10	Tha chana, Suratthani	BBB	BBB	BBA
S11	Muang, Phuket	ND	BBB	ND
S12	Muang, Phuket	ND	BBB	BBA
S13	Muang, Phuket	BBB	BBB	BBA
S14	Thalang, Phuket	BBB	BBB	ND
S15	Thalang, Phuket	BBB	BBB	ND
S16	Muang, Phuket	BBB	BBB	BBA
S17	Sawi, Chumphon	BBB	BBB	BBA
S18	Sawi, Chumphon	BBB	BBB	ND
S20	Sawi, Chumphon	BBB	BBB	ND
S21	Muang, Chumphon	BBB	BBB	ND
S22	Muang, Chumphon	BBB	BBB	BBA
S23	Muang, Chumphon	BBB	BBB	ND
S24	Muang, Chumphon	ND	BBB	ND
S25	Kra buri, Ranong	BBB	BBB	ND
S27	Kapoe, Ranong	ND	BBB	BBA
S28	Kapoe, Ranong	BBB	BBB	ND
S29	Kapoe, Ranong	BBB	BBB	BBJ
S30	Kapoe, Ranong	BBB	BBB	ND
S31	Muang, Ranong	BBB	BBB	BBA
S32	Muang, Ranong	ND	BBB	BBA
S33	Khuraburi,Phangnga	BBB	BBB	ND
S34	Khuraburi,Phangnga	BBB	BBB	BBA
S35	Phanom, Suratthani	BBB	BBB	BBA
S36	Ao Luk, Krabi	BBB	BBB	BBA
S37	Ao Luk, Krabi	BBB	BBB	ND
S38	Ao Luk, Krabi	BBE	BBB	BBA
S39	Ao Luk, Krabi	ND	BBB	ND
S40	Ao Luk, Krabi	BBB	BBB	ND
S41	Muang, Krabi	ND	BBB	ND
S42	Muang, Krabi	BBB	BBB	BBA

Code of colonies	Sampling area	Composite Haplotype		
		I	II	III
S43	Muang, Krabi	BBB	BBB	ND
S44	Muang, Krabi	ND	BBB	BBA
S45	Muang, Krabi	ND	BBB	BBA
S46	Nua khlong, Krabi	ND	BBB	ND
S47	Nua khlong, Krabi	BBB	BBB	BBA
S48	Nua khlong, Krabi	BBB	BBB	BBA
S49	Nua khlong, Krabi	ND	BBB	BBA
S50	Khao phanom, Krabi	BBB	BBB	ND
S51	Thung yai, Nakhon si thammarat	BBC	BBB	BBG
S52	Thung yai, Nakhon si thammarat	BBB	BBB	ND
S53	Thung yai, Nakhon si thammarat	BBC	BBB	BBG
S54	Sikao, Trang	BBB	BBB	BBA
S55	Huai yot, Trang	ND	BBB	BBA
S56	Huai yot, Trang	BBB	BBB	ND
S57	Huai yot, Trang	BBB	BBB	BBA
S58	Huai yot, Trang	ND	BBB	ND
S59	Srinakarin, Phatthalung	BBF	BBB	ND
S60	Srinakarin, Phatthalung	CED	AEE	ND
S61	Srinakarin, Phatthalung	ND	BBB	BBA
S62	Pha bon, Phatthalung	BBB	BBB	BBA
S63	Pha bon, Phatthalung	BBB	BBB	BBA
S64	Muang, Songkhla	BBB	BBB	ND
S65	Muang, Songkhla	BBE	BBB	ND
S66	Muang, Songkhla	BBC	BBB	BBA
S67	Muang, Songkhla	ND	BBB	ND
S68	Muang, Songkhla	BBB	BBB	BBA
S69	Muang, Songkhla	BBB	BBB	BBA
S70	Chalerm pra, Nakhon si thammarat	BBB	BBB	BBA
S71	Tha sala, Nakhon si thammarat	BBB	BBB	BBA
S72	Tha sala, Nakhon si thammarat	ND	BBB	BBA
S73	Tha sala, Nakhon si thammarat	ND	ND	BBA

Collection area and composite haplotype of *A. cerana* from the Samui Island of Thailand

Code of colonies	Sampling area	Composite Haplotype		
		I	II	III
I04	Tham Bon Aungthong	ND	BBB	BBA
I06	Tham Bon Aungthong	ND	BBB	BCA
I09	Tham Bon Boput	BBB	BBB	BCA
I10	Tham Bon Maret	BBB	BBB	BBA
I12	Tham Bon Maret	BBB	BBB	BBA
I14	Tham Bon Limpanoi	BBB	BBB	BBA
I16	Tham Bon Maenam	BCB	BBC	ND
I17	Tham Bon Maenam	BBB	BBB	BBA
I19	Tham Bon Maenam	BBB	BBB	BBA
I20	Tham Bon Maenam	BBB	BBB	BBA
I21	Tham Bon Maenam	BCB	BBC	BCH
I22	Tham Bon Maenam	BCB	BBC	BCI
I23	Tham Bon Maenam	BCC	BBC	ND
I24	Tham Bon Maenam	BCB	BBC	ND
I25	Tham Bon Boput	BBB	BBB	BBA
I26	Tham Bon Boput	BBB	BBB	BBA
I27	Tham Bon Boput	BCC	BBC	BCA
I28	Tham Bon Boput	BBB	BBB	BBA
I29	Tham Bon Maret	BCC	BCC	BCA
I30	Tham Bon Maret	BCC	BBC	BBA
I31	Tham Bon Maret	BCC	BBC	BBA
I32	Tham Bon Maret	BCC	BBC	BCH
I33	Tham Bon Maret	BCC	BBC	ND
I34	Tham Bon Maret	ND	BBB	BBA
I35	Tham Bon Maret	BBB	BBB	ND
I36	Tham Bon Maret	BCC	BBC	BBA
I37	Tham Bon Boput	BBB	BBB	BBA

I = Composite haplotype of ATPase6-ATPase8 gene of mtDNA digested with *Taq*I, *Ssp*I and *Vsp*I (Songram, 1997)

II = Composite haplotype of sRNA gene, lrRNA gene and inter COI-COII of mtDNA digested with *Dra*I (Sihanuntavong, 1997)

III = Composite haplotype of mtDNA control region digested with *Taq*I, *Rsa*I and *Hin*fI (Pootong, 1998)

ND = non determined

APPENDIX E

AAATAATATA AAATAATTTT TAATATATAT ATATATATAT ATATATATAT ATTATAATTT
TTATTTATTC ATAGTATTTA ATATATAATT TTATTATTTA GTATAAAATT TTAAATATAA
AATTAACTAT TTTATATTAT AAATATTTTT TTTTATTAAT TAATTTAAAT ATTAATAATA
AATAATAAAA TGAGTTTTTT TTTTTTTTTG TTATATTTTA TTTATTTAAA ATAAAATATA
TATATATATA TATATAATAT TATATATATT TATAATTATA TAAATATATA TATATTATTT
TATTCTCTAA TTATATATTA ATTTATAAAT AATTATATAT ATATAAATAT TAATTTTATA
TTAATAAATA TATATATATA ATTATATATT GATTTATAAA TATTTATAAA ACCAATATTT
ATTAATTTAT TTTTTAAAAT TTAATAATTT TTATTAAATT AACTTAATTT TTTATACTAA
TTTAAAAAAT TACATAATAA CATATATGAA TAAATAAGCA TAATAATTAA TTTTTAATAT
TTTCTCTTAA ATTAAATAAT TTTAATAAAA AAACTTAAAT AAAAAATAAA AATTTTTAAA
AATAAAAACT TTAAAAATAA AAAATTTTAA AAAATAAAAA CTTTTTTAAA AAAATAAAAA
ACTTTTTTAA AAAATAAAAA CTTTTTAAAA AAATAAAAAT TTAAAAAATA AAAAATTTTT
AAAAAATAAA TAAAATAAAA AATAGTAACA TTATAAACAA TAAAAATAAT ATTTTCAATA
ACTAATATTA ATATTAATAT CTTATTTAAA AATATTTTAC TTAATTATTT ATATAGTTTA
AAAAAAACAT TATATTTTCA ATATAAAAAT AATTAAATTT AATTTATAAA TATAATTAAG
TCAAATTTAA TTTAAATAAT CTAAAAATTA TTTATTAATA AAGAAATATT AATAAATAAA
GCTTCTAACT TTAACTCTAG ATTCATAAAT AATCTATATT TCTTATTATA TAAATTAATT
TAGATAAATA TTAATTTTAA AATAATTATA TAATAAGCTA AATAAAGCTA ACAGGTTCAT
ACCCTGTCGA TAAATTAATA ATTTTTATAT AAATTATTAA ATTTATTTTA GTGTTTAAAG
CACATAAAAT TTTGAATTTT ATAGTATTAA CTAAATTAAT AAATTTGGAT ATTAGTTAAT
AAATAATAAC ATTTAAATTG CATTTAAAAA TTAATATTTT ATATATTATA TCTAAAAAAG
TAATATGTCT GATAAAAGAA ATATTTTGAT AAAATATTAA TGTATAATTT TATATATACT
ATTACTTATC TTCTTCATAA ATTTTAAATA CCACTGATTT ATTTATTTTT TAATTACTAT
TTTTGTATTA ATAATAAATT CCAATAATAT TTTTATTCAA TGAATATTAA TAGAATTTGG
TACAATCATT AGAATTAGAT TAATTAATAT TAAATCCACA AATAAAACCC CAAGATTAAT
TTATTATTCA GTATCAGTAA TTTCAAGAAT TTTTTTATTC TTTATAATTA TTGTATACTT
ATCATCCATT AGATTTACTA AAACAGATAC TTTTAATTTT ATAGTTCAAA TAATATTTTT
TTTAAAAATT GGAACTTTCC CCTTTCATTT TTGAATAATT TATTCTTATG AAATAATAAA
TTGAAAGCAA ATTTTTTTAA TATCAACATT AATTAAATTT ATTCCAATTT ATATAATAGT
TTCAATAACT AAAATTAATT CATGAACATT ATATTTTTTA ATTACAAATA GATTATATAT
TTCATTTTAT GCTAATAAAT TTTACACTCT AAAAAAATTA CTAGCATGTT CAACAATTTT
TAATTCATTC TATTTTATTT TTATTTTAGA ATTAAATAAA AATATATTTA TTGCTATAAT
TATTTTATAT TCATTTAATT ATTTTTTATT AATTAGATTC TTAAATAAAT TTAATATTCA
AAATTTTAAT TTTATATTTT ACAATAAATA TCAAATATAT ACATTCTTAA CATTAATATT
TAATTATTCA ATATATCCAA TTTTTCTTTC ATTTGTAATT AAATGAAATC TAATTTTTAT
AATAGTAAGA GTTAAAGCTT ATAATTGAAT TTTATTTCTT TTAATAATTT CTAGAATATT
AATAATTTGA AATTATATTA TTATTTTAAA ACGTGTATTT TTAAAAATAA ATTTTTATAA
AAATAATTTC ATTGAT

Control region sequence of *A. mellifera* (Crozier and Crozier, 1992)

APPENDIX F

Comparisons of restricted fragment size (base pair) by *Nde*I, *Swa*I, *Taq*I, *Hin*fI and *Hin*dIII in control region between sequence of *A. mellifera ligustica* (Crozier and Crozier, 1992) and amplified control region of *A. mellifera* (A. mell)

Enzyme	Template DNA	Restriction site	Size of fragment	Total
*Nde*I, *Swa*I	*A. mellifera*	0	-	2226
	A. mell	0	-	2560
*Taq*I	*A. mellifera*	1	1076, 1150	2226
	A. mell	1	1750,810	2560
*Hin*fI	*A. mellifera*	2	976, 969, 281	2226
	A. mell	3	800, 800, 680, 280	2560
*Hin*dIII	*A. mellifera*	2	1156, 948, 122	2226
	A. mell	2	1070, 1404, 86	2560

APPENDIX G

Blast result of *A. mellifera* in ND_2 gene

Sequences producing significant alignments: (bits) Value

gb|L06178|AMFGENOM Apis mellifera ligustica complete mitochondr... 161 3e-38
gb|U35757|AMU35757 Apis mellifera meda NADH dehydrogenase subun... 64 5e-09
gb|U35752|AMU35752 Apis mellifera ligustica NADH dehydrogenase ... 64 5e-09
gb|U35748|AMU35748 Apis mellifera carnica NADH dehydrogenase su... 64 5e-09
gb|U35760|AMU35760 Apis mellifera mellifera NADH dehydrogenase ... 64 5e-09
gb|U35754|AMU35754 Apis mellifera ligustica NADH dehydrogenase ... 64 5e-09
gb|U35758|AMU35758 Apis mellifera mellifera NADH dehydrogenase ... 64 5e-09

gb|L06178|AMFGENOM Apis mellifera ligustica complete mitochondrial genome
Length = 16343

Score = 161 bits (81), Expect = 3e-38
Identities = 87/90 (96%)
Strand = Plus / Plus

Query: 11 aaaagtaatatgtctgataaaagaaatatttgataaaatattaatgtataatttatat 70
Sbjct: 430 aaaagtaatatgtctgataaaagaaatatttgataaaatattaatgtataatttatat 489

Query: 71 atactattacttatcttcnnnataaattt 100
Sbjct: 490 atactattacttatcttcttcataaaattt 519

gb|U35757|AMU35757 Apis mellifera meda NADH dehydrogenase subunit 2 (ND2) gene, mitochondrial gene encoding mitochondrial protein.
partial cds
Length = 656

Score = 63.9 bits (32), Expect = 5e-09
Identities = 38/41 (92%)
Strand = Plus / Plus

Query: 60 taattttatatatactattacttatcttcnnnataaattt 100
Sbjct: 1 taattttatatatactattacttatcttcttcataaattt 41

gb|U35752|AMU35752 Apis mellifera ligustica NADH dehydrogenase subunit 2 (ND2) gene, mitochondrial gene encoding mitochondrial protein.
partial cds
Length = 656

Score = 63.9 bits (32), Expect = 5e-09
Identities = 38/41 (92%)
Strand = Plus / Plus

Query: 60 taattttatatatactattacttatcttcnnnataaattt 100
Sbjct: 1 taattttatatatactattacttatcttcttcataaattt 41

gb|U35748|AMU35748 Apis mellifera carnica NADH dehydrogenase subunit 2 (ND2) gene,

Blast result of *A. mellifera* in ND_2 gene (continue)

mitochondrial gene encoding mitochondrial protein.
partial cds
Length = 656

Score = 63.9 bits (32), Expect = 5e-09
Identities = 38/41 (92%)
Strand = Plus / Plus

Query: 60 taattttatatatactattacttatcttcnnnataaatttt 100
Sbjct: 1 taattttatatatactattacttatcttcttcataaatttt 41

gb|U35760|AMU35760 Apis mellifera mellifera NADH dehydrogenase subunit 2 (ND2) gene, mitochondrial gene encoding mitochondrial protein.
partial cds
Length = 656

Score = 63.9 bits (32), Expect = 5e-09
Identities = 38/41 (92%)
Strand = Plus / Plus

Query: 60 taattttatatatactattacttatcttcnnnataaatttt 100
Sbjct: 1 taattttatatatactattacttatcttcttcataaatttt 41

gb|U35754|AMU35754 Apis mellifera ligustica NADH dehydrogenase subunit 2 (ND2) gene, mitochondrial gene encoding mitochondrial protein.
partial cds
Length = 656

Score = 63.9 bits (32), Expect = 5e-09
Identities = 38/41 (92%)
Strand = Plus / Plus

Query: 60 taattttatatatactattacttatcttcnnnataaatttt 100
Sbjct: 1 taattttatatatactattacttatcttcttcataaatttt 41

gb|U35756|AMU35756 Apis mellifera mellifera NADH dehydrogenase subunit 2 (ND2) gene, mitochondrial gene encoding mitochondrial protein.
partial cds
Length = 656

Score = 63.9 bits (32), Expect = 5e-09
Identities = 38/41 (92%)
Strand = Plus / Plus

Query: 60 taattttatatatactattacttatcttcnnnataaatttt 100
Sbjct: 1 taattttatatatactattacttatcttcttcataaatttt 41

BIOGRAPHY

Mr. Suratep Pootong was born on March 17, 1972. He graduated with the Bachelor degree of Science in Biochemistry from Chulalongkorn University in 1994. He was enrolled in Master degree of Science, department of Biochemistry, Chulalongkorn University in 1996.