การแพร่กระจายของประชากรผึ้งโพรง *Apis cerana* กลุ่มภาคเหนือและใต้ ในบริเวณรอยต่อของจังหวัดประจวบคีรีขันธ์และจังหวัดชุมพรโดยใช้ดีเอ็นเอเครื่องหมาย

นาย วชิระ สุขถาวรเจริญพร

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DISTRIBUTION OF NORTHERN AND SOUTHERN HONEYBEES *Apis cerana* POPULATIONS IN BORDERLINE REGION AT PRACHUAP KHIRI KHAN AND CHUMPHON PROVINCES USING DNA MARKER

MR. WACHIRA SUKTAWONJAREARNPON

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biochemistry

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วชิระ สุขถาวรเจริญพร: การแพร่กระจายของประชากรผึ้งโพรง *Apis cerana* กลุ่มภาคเหนือและใต้ในบริเวณรอยต่อของจังหวัดประจวบคีรีขันธ์และจังหวัดชุมพร โดยใช้ดีเอ็นเอเครื่องหมาย. (Distribution of northern and southern honeybees *Apis cerana* populations in borderline region at Prachuap khiri khan and Chumphon provinces using DNA markers) อ. ที่ปรึกษา : รศ.ดร.ศิริพร สิทธิประณีต, 100 หน้า. ISBN 974-03-0579-2.

การตรวจสอบการแพร่กระจายของผึ้งโพรง *Apis cerana* กลุ่มภาคเหนือและใต้ ในบริเวณรอยต่อของจังหวัดประจวบคีรีขันธ์และจังหวัดชุมพร โดยใช้เทคนิค PCR-RFLP บนไมโทคอนเดรียลดีเอ็นเอ บริเวณยีน srRNA, ยีน lrRNA และบริเวณระหว่างยีน COI-COII ด้วยเรสทริกชันเอนไซม์ *Dra*I พบว่ามีรูปแบบของดีเอ็นเอเป็น 3, 4 และ 8 รูปแบบ ตามลำดับ เมื่อรวมรูปแบบของแถบดีเอ็นเอที่ได้จากแต่ละตัวอย่างเข้าด้วยกัน จะให้รูปแบบรวมเป็น 11 รูปแบบ และเป็น 12 รูปแบบ เมื่อนำผล PCR-RFLP จากตัวอย่างในประเทศจีน (ยูนนาน) และประเทศเวียดนาม (ฮานอย) ร่วมด้วย เมื่อคำนวณค่า Genetic distance และสร้างความสัมพันธ์เชิงวิวัฒนาการตามแบบ UPGMA จะสามารถแบ่งกลุ่มตัวอย่างได้เป็น 2 กลุ่ม คือ กลุ่มผึ้งโพรงตอนเหนือและกลุ่มผึ้งโพรงตอนใต้ โดยผึ้ง 2 กลุ่มนี้มีอาณาเขตที่ซ้อนทับกันอยู่ในเขตอำเภอบางสะพาน, บางสะพานน้อย จังหวัดประจวบคีรีขันธ์ และอำเภอท่าแซะ, ประทิว จังหวัดชุมพร และเมื่อนำข้อมูลจากตัวอย่างในประเทศจีนและประเทศเวียดนามมาร่วมวิเคราะห์จะพบว่า กลุ่มผึ้งโพรงตอนเหนือมีพันธุกรรมใกล้เคียงกับผึ้งโพรงในประเทศเวียดนามมากกว่าประเทศจีน ชี้ให้เห็นว่า ผึ้งโพรงตอนเหนือน่าจะมาจากการกระจายเข้ามาของผึ้งเวียดนาม

จากการรวมรูปแบบของแถบดีเอ็นเอพบว่า มีผึ้งโพรง 1 ตัวอย่างจากจังหวัดประจวบคีรีขันธ์ ซึ่งมีสัณฐานคล้ายกับผึ้งโพรงทั่วไปแต่มีรูปแบบรวมเป็น CED ซึ่งมีความแตกต่างทางพันธุกรรมกับตัวอย่างผึ้งโพรงอื่นๆ มาก น่าสงสัยว่าเป็นผึ้งต่างสปีชีส์กัน จึงควรมีการศึกษาเพิ่มเติมเพื่อทราบสถานะทางอนุกรมวิธานที่แน่ชัดต่อไป

การวิเคราะห์ด้วย microsatellite DNA ในตัวอย่างผึ้งโพรง 4 บริเวณของประเทศไทย คือ 1. ภาคกลาง, 2. ภาคใต้, 3. จังหวัดประจวบคีรีขันธ์ และ 4. จังหวัดชุมพร โดยใช้ microsatellite primer ของ *A. mellifera* ที่ตำแหน่ง A28, A107 และ A113 พบว่า ที่ตำแหน่ง A28 และ A107 มีความหลากหลายสูงกว่า A113 แต่การหาขนาดค่อนข้างยุ่งยากและอาจผิดพลาดได้ จึงวิเคราะห์ผล microsatellite ที่ตำแหน่ง A113 เพียงตำแหน่งเดียว พบว่า มีจำนวนอัลลีลทั้งหมด 4 อัลลีล มีค่า heterozygosity ตั้งแต่ 0.451-0.550 และผึ้งโพรงทั้ง 4 บริเวณของประเทศไทยไม่สามารถแบ่งกลุ่มประชากรออกจากกันได้ แสดงให้เห็นถึง gene flow ที่มีอิทธิพลจากการผสมพันธุ์ข้ามบริเวณของผึ้งเพศผู้

ภาควิชา……ชีวเคมี…… ลายมือชื่อนิสิต .....

สาขาวิชา……..ชีวเคมี…… ลายมือชื่ออาจารย์ที่ปรึกษา .....

ปีการศึกษา……2544…… ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....-.....

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KEY WORD: HONEYBEES / *Apis cerana* / DISTRIBUTION / BORDERLINE REGION / PCR-RFLP / MICROSATELLITE

WACHIRA SUKTAWONJAREARNPON : DISTRIBUTION OF NORTHERN AND SOUTHERN HONEYBEES *Apis cerana* POPULATIONS IN BORDERLINE REGION AT PRACHUAP KHIRI KHAN AND CHUMPHON PROVINCES USING DNA MARKERS. THESIS ADVISOR : ASSOC.PROF. SIRIPORN SITTIPRANEED, Ph.D., 100 pp. ISBN 974-03-0579-2.

PCR-RFLP of three mtDNA regions (srRNA gene, lrRNA gene and intergenic COI-COII regions) were used to investigated the distribution of northern and southern honeybees *Apis cerana* populations. Three, four and eight haplotypes were obtained from *Dra*I digestion of PCR-amplified and eleven composite haplotypes were generated. Tweleve composite haplotypes were generated when sample used for PCR-RFLP analysis was covered four geographic locations; 1) Prachuap Khiri Khan 2) Chumphon 3) Yunnan and 4) Hanoi. A UPGMA phenogram based on genetic distance allocated *A. cerana* in borderline region in to 2 distinct groups: northern and southern. Their distribution areas had overlapped in Amphur Bang saphan, Bang saphan noi, Tha sae and Pa thiu. And the northern population might be colonized by Vietnam honeybees.

Only one colony from Prachuap Khiri Khan had the composite haplotype CED. A CED was extremely different from all samples, although the morphological was similarly. It was suspected to be different species. Further study need to classify their actual taxonomic status.

Microsatellite DNA analysis of 4 geographic samples (1. Central, 2. South, 3. Prachuap Khiri Khan and 4. Chumphon) was performed by using *A. mellifera* microsatellite primers. Three microsatellite loci (A28, A107 and A113) showed polymorphic. PCR products of loci A28 and A107 were very difficult to accurately score because of their stutter bands. The heterozygosities of *A. cerana* were estimated from microsatellite loci A113 was 0.451- 0.550. The analysis of geographic differentiation indicated no differentiation of four geographics of *A. cerana*.

Department Biochemistry	Student's signature
Field of study Biochemistry	Advisor's signature
Academic year 2001	Co-advisor's signature -

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ABBREVIATIONS

A, T, C, G	=	nucleotide containing the base adenine, thymine, guanine and cytosine, respectively
bp	=	base pair
°C	=	degree celcius
Ci	=	curie
DNA	=	deoxyribonucleic acid
dNTPs	=	deoxyribonucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
ddNTPs	=	dideoxyribonucleotide triphosphates (ddATP, ddTTP, ddGTP, ddCTP)
EDTA	=	ethylenediamine tetraacetic acid
HCl	=	hydrochloric acid
ITS	=	internal transcribed spacer
kb	=	kilobase
KCl	=	potassium chloride
$MgCl_2$	=	magnesium chloride
ml	=	millilitre
mM	=	millimolar
mtDNA	=	mitochondrial DNA
ng	=	nanogram
PCR	=	polymerase chain reaction
RFLP	=	restriction fragment length polymorphism
RNA	=	rivonucleic acid
rpm	=	revolution per minute
SDS	=	sodium dodecyl sulfate

TEMED	=	N, N, N’, N’-tetramethylethy lenediamine
Tris	=	tris (hydroxy methyl) aminomethane
UV	=	ultraviolet
v	=	volume
V	=	volt
W	=	watt
μg	=	microgram
μl	=	microlitre
μM	=	micromolar

CHAPTER I

INTRODUCTION

Honeybees are social insects which they are great economic importance. Honeybees produce important hive products such as honey, beeswax, royal jelly and pollen that provide both nutritious food and cash income. Honey can used for making candies, syrup, cosmetics and some is bottled for grocery stores. In Thailand most honey goes to pharmaceutical factories for making Thai medicines. Moreover, bee pollination helps increasing crop yield and improving quality of seed and fruit. Beekeeping and the use of bee products have a long history in Thailand (Wongsiri *et al.*, 1989).

The honeybees in Thailand

There are five species of honeybees in Thaland. Four native species are 1) the giant or rock honeybees, *Apis dorsata* 2) the dwarf honeybees, *Apis florea* 3) the small dwarf honeybees, *Apis andreniformis* and 4) the eastern honeybees, *Apis cerana*. The imported species is the western honeybees, *Apis mellifera*. The former three native species are open-nesting species which they are difficult for beekeeping industry. Only *Apis mellifera* and *Apis cerana* could be kept and managed in hive for commercial beekeeping. All honeybees are similar in morphology, social biology, nest architecture, aging behaviors, and performed some behavior such as a complex "dance" to signal direction and distance to food source (Smith, 1991).

Apis cerana

The social structure of *A. cerana* colonies are composed of one queen, large number over several thousand of workers and a few hundred of drones. The queen and the workers are heterozygotes (diploid 2n = 32) grown from fertilized eggs and the drones are hemizygotes (haploid individuals) from unfertilized eggs (Wongsiri *et al*., 1989). The queen can release queen pheromone from mandibular gland with is composed of 9-Oxodectrans 2-eonic acid (9O2) and 9-Hydroxydec-2-eonic acid (9H2)(Wongsiri *et al*., 1989). These two compounds can inhibit development of worker's ovaries and regulate the social activity. During early stage of worker (nurse bees), they consume large quantities of pollen and their pharyngeal glands are fully active to produce royal jelly. The royal jelly was feed for young larvae and the queen. Another phase of life, the workers are taking and storing honey and pollen. Both honey and pollen are brought to the hive (Wongsiri *et al*., 1989). They are also ventilating the hive by rapid fanning of the wings, producing wax for making combs and removing dead bees and debris. In their final stage of life, workers serve foragers, flying far and wide to search for and bring nectar, pollen and water back to the colony. They live for only ten to twelve weeks (Punchihewa *et al*., 1990).

Mating distance of *A. cerana* drone in Sri Lanka seems not to exceed 500 m (Punchihewa *et al*., 1990). The flight range of *A. mellifera* drone is reported to vary between 1 and 5 km, and that of queens is between 1 and 2 km (Woyke, 1960; Taylor and Rowell, 1988). Whereas in isolated areas, the mating distance can range up to 16 km (Peer, 1957). However there are no data for the *A. cerana* in Thailand.

Distribution of *A. cerana*

Apis cerana, the "Eastern Honeybee" occurs in all of Asia, which can be found even above at 3000 m in the Himalaya and can be adapted to semi-desert like environment in the mountains of central of Afghanistan, the natural distribution area is shown in Figure 1.1. In the north, *A. cerana* occurs up to 46° northern latitude in Ussuria. In the east, it was found in the northern Island of Hokkaido. In Southeast Asia, *A. cerana* is restricted to the Malayan region, west of the Wallece line (Philippines–Celebes–Timor), occuring in different Island races. And the location of western colonies of *A. cerana* was found in west Afganistan. However, no overlapping or direct contact between distribution area of *A. cerana* and *A. mellifera* by the northern sector of border between Iran and Afghanistan. Two species occur only 600 km apart.

Intraspecific construction of *A. cerana* can be expected by using population genetics, which is essential in many areas such as comparing relation fitness of life history variants, determination of population size and structure, conservation program and improvement of a selective breeding program. Population genetics is an integral part of classical and molecular genetics for the purpose to understand evolutionary process. For evolutionary biology in general and population genetics in particular, the starting point is the consideration of genetic variation. Accordingly, study of population genetics using molecular markers, which are composed of genetic variation in groups of individuals, is necessary.

Traditional methods such as comparative anatomy, morphology and physiology have been used to evaluate genetic variability of various taxa, but these methods are not adequate for study genetic variations. Molecular techniques, which was based on polymorphisms in proteins or

DNA and was named “ molecular markers”, were developed during the past decade.

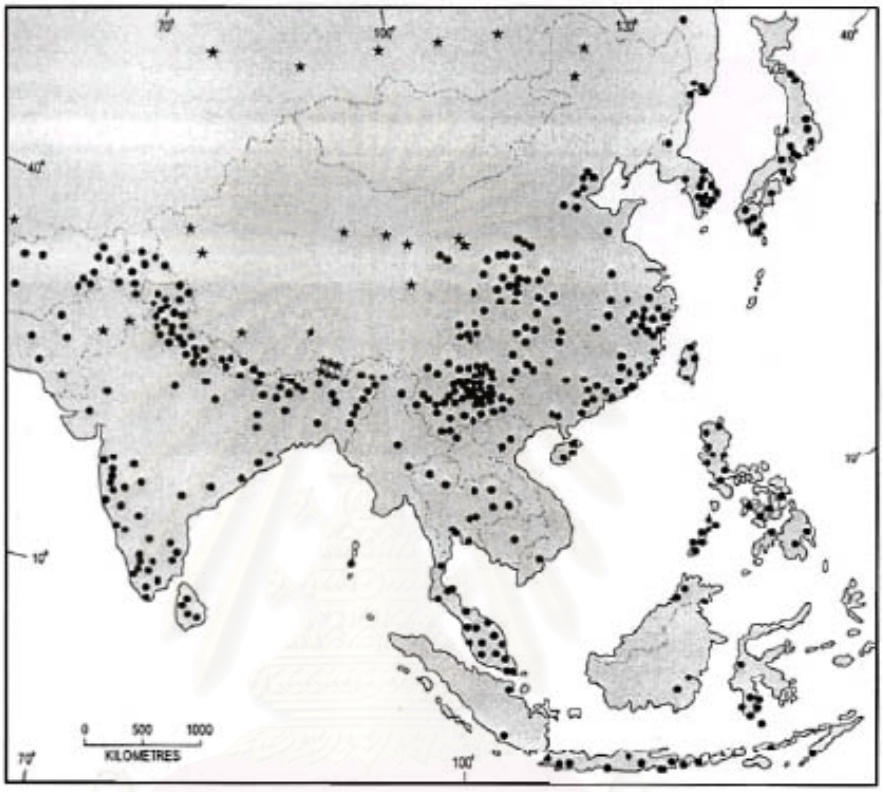

Figure 1.1 Known distribution of *A. cerana* = circles; reported absence = star (Hepburn *et al*., 2001)

Molecular markers for population genetics analysis

1. Protein marker

Allozymes

Allozyme refers to the enzyme that is produced by different alleles at the same chromosomal DNA locus (Park and Moran, 1994). The polymorphic allozyme can be detected by electrophoresis observed based on the properties of enzymes in which different net charges migrate at the

different rate through a gel matrix when exposed to an electric field. Homozygotes at a given locus typically yield one band while heterozygotes typically yield two, three or five bands, depending on the quaternary structure of the enzyme. The advantage of allozymes is the technique which is relatively inexpensive and straight forward once the basis procedures have been developed for a given species. Accordingly, allozyme analysis is the first technique that allows genetic diversity examination. Nevertheless, limitation of using allozymes are the strict requirement of fresh or frozen tissue, the need for more material than DNA methods, and the fact that protein loci evolves more slowly than non-coding DNA sequences. In other species, allozymes have proved to be very useful but have brought little additional information to our understanding of genetic diversity of honeybees. In the Hymenoptera, protein variation is limited (Graur, 1985) because of their sex-determination system. Therefore, allozyme analysis cannot make certain identifications, especially, of hybrids after several generations (Rinderer and Sylvester, 1981).

2. DNA marker

DNA markers have several advantages over allozyme studies. DNA is found in nearly all cells of all organisms and it can be recovered from both living and dead tissues. Besides, the tissues can be easily stored under field conditions such as stored in 95% ethanol and in many cases only nanograms are needed for analysis when amplified by polymerase chain reaction (PCR). The molecule is so stable that recognizable sequences can remain intact for hundreds of millions of year (Cano *et al*., 1993). Then, analysis of polymorphisms at the DNA level is the direct

approach to study genetic variation at both inter- and intraspecific levels. The information form genotypes and allele frequencies are assumed as primarily influenced by mutation, gene flow and genetic drift rather than by selection. DNA can be classified into two broad catagories: extrachromosomal (organelle) DNA and chromosomal (nuclear) DNA.

Mitochondrial DNA (mtDNA)

Mitochondrial DNA is an organelle DNA found in mitochondria. Most animal mtDNA occurs as a single circular molecule generally about 16,000-20,000 bp and contain 5-10 copies in each mitochondria. MtDNA composed of 12 or 13 proteins, two ribosomal RNAs (rRNAs) and 22 transfer RNAs (tRNAs) and noncoding region containing the origin of replication. The genes that code for protein are three subunits of cytochrome c oxidase (COI, II and III), seven subunits of NADH dehydrogenase (ND1-6 and ND4L), cytochrome b, and two subunits of ATP synthetase (ATPase6 and 8) (Crozier and Crozier, 1993) (Figure 4). Unlike the nuclear DNA, mtDNA are maternally inherited except in some species (e.g. *Mytilus edulis*, *M. galloprovencialis*) whose contribution of paternal mitochondria is observed (Gyllensten *et al*., 1991; Margoulas and Zouros, 1993). Basically, the mutation rate of mtDNA is much more rapidly than that of single-copy nuclear genes reflecting its potential to used for determination of intraspecific genetic variation among geographically different populations (Brown *et al*., 1979; Lynch and Jarrell, 1993).

The studies on mtDNA variation can be identified from DNA sequences, restriction fragments (RFLP) obtained either by hybridizing total DNA with known mitochondrial probes, or by PCR-RFLP

amplifying mtDNA with PCR and separating the cleaved restriction fragments by electrophoresis).

Restriction endonucleases are enzymes, which bind to specific sequence of DNA base pair and cut the DNA strand. Most commercially available restriction enzymes recognize particular sequences of four, five or six base pairs. Each restriction site can be mapped on the mtDNA Mutation occurring at a restriction site (either by substitutions or deletions) prevents or allows an investigated enzyme to cleave at such position and thus produces different numbers of DNA fragments from investigated individuals. Although RFLP of the whole mitochondrial genome is useful for studies of genetic variation in *A. cerana* population, this technique requires large amount of pure mtDNA. Furthermore, the procedures are more complicated with using radioisotope for visualization of the results. As a result, RFLP analysis of whole mitochondrial genome is tedious and time consuming. And presently, polymerase chain reaction (PCR) by Millis et al. (1987) has opened a new approach for molecular genetic studies. PCR is a method for an *in vitro* amplification of specific DNA seequences by the simultaneous primer extension of complementary strands of DNA. This technique can be synthesised millions copies of DNA from DNA target by activity of thermostable DNA polymerase (usually *Taq* DNA polymerase) within a few hours. The amplification reaction consists of three steps; denaturation of double stranded DNA template at high temperature, annealing to allow primers to form hybrid molecules at optimal temperature, and extension at working temperature of DNA polymerase. Therefore PCR-RFLP is an alternative technique with powerful means to study genetic variation in *Apis* species.

Nuclear DNA

Nuclear genomes are much larger than mtDNA , ranging form $<10^6$ bp in some bacteria to $>10^{11}$ bp in some plants. Diploid organisms have two copies of each genetic region (locus) on homologous pairs of chromosomes. Approximately 70% of the mammalian genome contains non repetitive DNA (Albert et al., 1983). Repetitive DNA consists of a core sequence that is repeated in varying degrees. They may be made up of coding segments such as ribosomal RNA (rRNA) genes,or non coding tandem repeated units. Variation number of tandem repeats (VNTR) are characterized by a core sequence which consists of a number of identical repeated sequences. They can be divided into three catagories; satellite, minisatellite and microsatellite based on the repeat length.

Microsatellites are short tandem repeated sequence (1-6 bp) which include more than about 70 repeat units and interspersed throughout the genome. Microsatellites are highly abundant randomly dispersed in eukaryote genomes. It was estimated that one microsatellite locus can be found every 10 kb in eukaryotic genomes. Microsatellite mutations are changes in repeat numbers caused by an intramolecular mutation mechanism called DNA slippage. The most common mutations are changes of a single repeat unit, which allows microsatellite mutations to be interpreted as a very good approximation of stepwise mutation process. The mutation rate of microsatellite is high, approximately 10^{-2} to 10^{-4} per generation (Tautz, 1989; Weber and Wong, 1993). The allelic state of microsatellites is scored by polymerase chain reaction (PCR) performed between two fixed sequence motifs flanking the tandem repeat region. In addition to genomic library or database screening, it is also

possible to detect microsatellite loci by using conserved primer sequences from closely related species. Rubinsztein *et al*., (1995) and Garza, Slatkin and Freimer (1995) used this approach to study genetic polymorphism of chimpanzees with human primers and Moritz *et al*., (1995) studied microsatellite variation in *A. dorsata* using locus specific primers developed from *A. mellifera* primer. Therefore, microsatellite loci constitute powerful nuclear marker, increasingly used for identify testing population studies, linkage analysis and genetic mapping.

After amplification, the products are fractionated for their length polymorphisms using polyacrylamide gel with either non-radioactive or radioactive method. Generally, detection of the products by autoradiography (labeled 5' end of the primers. Electrophoresed of the products and exposed the gel with the x-ray films) is more sensitive and gives clear results. Non-radioactive detection is composed of ethidium bromide, silver stain and fluorescence. In a non-radioactive detection, silver stain is the best. Recently, silver stain can detect nucleic acids (down to 1 pg DNA/mm^2 band cross-section) with minimum background staining. The advantages of silver stain are high sensitivity, low costs, rapid and reliable results.

The previous studied of *A. cerana*

Ruttner (1988) summarized morphometic information and grouped the *A. cerana* populations into four subspecies, whose range are shown in Figure 1.2 : 1) a northern subspecies, *A. cerana cerana*, from Afghanistan, Pakistan, north India, China, north Viet Nam; 2) a southern subspecies, *A. cerana indica*, from south India, Sri Lan Ka, Bangladesh, Burma, Malaysia, Thailand, Indonesia and the Phillippines; 3) a Japanese

subspecies, *A. cerana* japonica, from Japan and Korea and 4) a *Himalaya* subspecies, *A. cerana himalaya,* from Nepal, Thailand (including the moutains in Thailand; Chiang-Mai) and probably south-western China. In recent year, Hepbern et al., (2001) according to their morphometric information grouped the *A. cerana* population into eight subspecies which more than Ruttner summarized four subspecies: 1) *A. cerana skorikovi*, from Tibet; 2) *A. cerana abaensis*, from central China; 3) *A. cerana hainanensis*, from Hainan island; and 4) *A. cerana philippina*, from the Philippines. The geographic distributions of these species are shown in Figure 1.3.

Figure 1.2 Area of distribution of *Apis cerana* F. (dotted line) and gap to the area of *Apis mellifera* L. (broken line)
(1) *A. c. cerana*; (2) *A.c.indica*; (3) *A. c. Himalaya*;
(4) *A. c. japonica* (Ruttner, 1988)

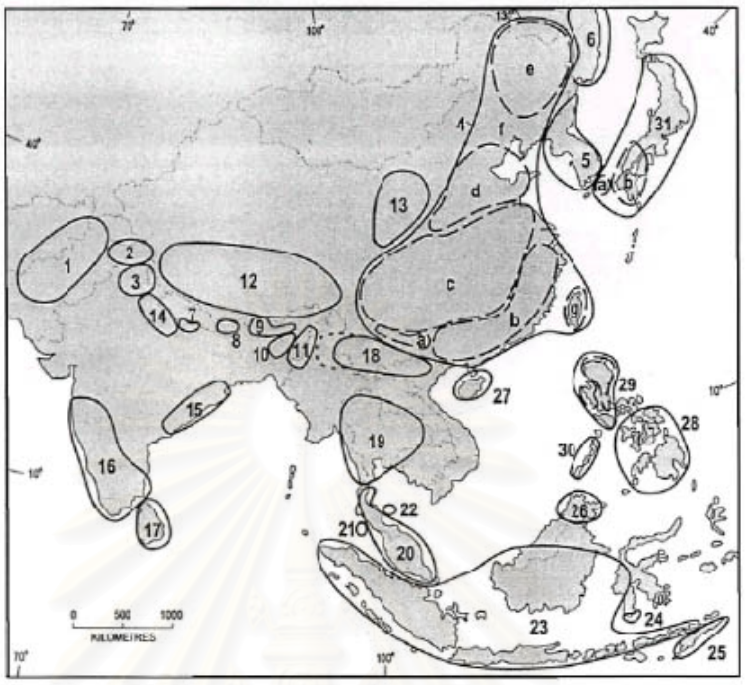

Figure 1.3 Distributional areas of putatively distinct subspecies, biometric groups and/or ecotypes of *A. cerana*. *A. cerana cerana*: 1. Eastern Afghanistan and northern Pakistan: 2. Kashmir: 3. Himachal Pradesh: 4. China (with biotypes/ecotypes a = Yunnan, b = Guangdong-Goungxi, c = Hunan, d = northern, e = Changbei Shan, f = unspecified, g = Taiwan): 5. Korea: 6. Ussuria. *A. cerana himalayana*: 7. Nepal Terai plains: 8. Nepal midlands: 9. Himalayas: 10. Brahmaputra: 11. Manipur, Mizoram and Nagaland. *A. cerana skorikovi*: 12. Tibet. *A. cerana abaensis*: 13. Central China. *A. cerana indica*: 14. Uuar Pradesh: 15. Orissa: 16. Southern India: 17. Sri Lanka: 18. Yunnan and possibly northern Myanmar: 19. Northern Thailand: 20. Southern Thailand and continental Malaysia: 21. Phuket Island: 22. Samui Island: 23. Sumatra, Java, Borneo, Lombok, Bali, Flores and most of Sulawesi: 24. Southern Sulawesi: 25.

Timor: 26. Sabah. *A. cerana hainanensis*: 27. Hainan Island. *A. cerana philippina*: 28. Cisayas and Mindanao: 29. Luzon: 30. Palawan. *A. cerana japonica*: 31. Japan. (Hepburn *et al*.,2001)

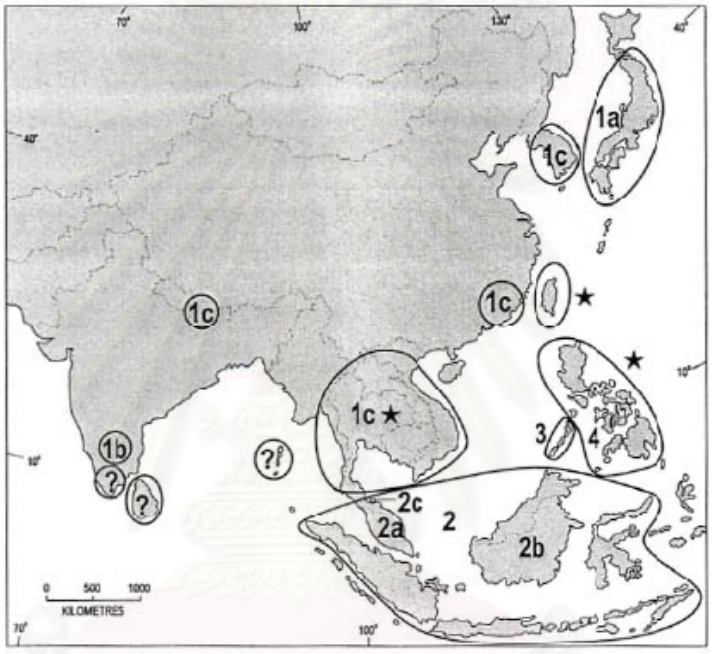

Figure 1.4 Geographical distribution of major mtDNA groups and subgroups *for A. cerana*. Stars indicate areas of high mtDNA diversity. mtDNA groups are indicated as follows: group1 consists of mainland and Asia with subgroups 1a = Japan, 1b = southern India, 1c = Himalayan region, Indochina peninsula, southeastern China and Korea: group2 consists of Sundaland region of peninsula Thailand, Malaysia and Indonesia: group3 comprises Palawan (Philippines) and group4 comprises the occanic island of the Philippines. (Hepburn *et al*.,2001)

For mtDNA analysis, Smith (1991) estimated of percent sequence divergence from the result of restriction fragment data indicated three main lineages of *A. cerana*: 1) mainland (Japan, Thailand, Malaysia, Borneo and south of India), 2) Andaman Islands and 3) Luzon from the Philippines. Moreover, Hepbern *et al*., (2001) collected reports of genetic variation and distribution of *A. cerana*, which was detected by using restriction fragment length polymorphisms and DNA sequence of non-coding region data. This study divided four major groups of *A. cerana*: 1) mainland Asia with subgroups 1a = Japan, 1b = southern India and 1c = Himalayan region, Indochina peninsula, south eastern China and Korea; 2) Sundaland (including southern or peninsular Thailand and the island of Samui); 3) Palawan (the Philippines); and 4) the oceanic islands of the Philippines (Figure 1.4).

In Thailand, *A. cerana* was obtained highly mtDNA diversity. From DNA sequence of the non-coding region data could geographically divide *A. cerana* in Thailand into two populations: 1) northern population (honeybees collected from Chiangmai to Chunthaburi) and 2) southern population (honeybees collected from Prachuap Khiri Khan to Phuket) (Bugharuang, 1996). From PCR-RFLP of some mtDNA regions, (Songram, 1997) *A. cerana* could be genetically divided into three groups included of 1) northern (north, northeast and central); 2) southern and 3) Samui Island. The same as Sangram's results, Sihanuntavong (1999) also genetically allocated *A. cerana* into 3 groups.

Furthermore, genetic variation of *A. cerana* in Thailand was studies using mictosatellite analysis (Laoaroon, 2001). *A. cerana* was allocated to 4 different groups; 1) northern (north and central), 2) northeastern, 3) southern and 4) Samui Island.

From above studies, population differentiation between southern and Samui Island can be explained by changes in sea level during the Pleistocene epoch (Dall *et al*., 1990). The most recent rise in sea level in this area occurred approximately 5000 years ago (Pianka, 1994).

Two populations of *A. cerana* in Thailand (northern and southern populations) were allocated different from each other. Two hypotheses were possible. First, mutation and natural selection paralleled with migration to different areas might cause the splitting of *A. cerana* from one population to two populations. Or another, two groups of *A. cerana*, which had different genetics, had colonized in each population area (northern and southern area). To study which hypothesis is most likely, this thesis is aimed to study the distribution of two populations of *A. cerana* in contact zone (the borderline region of northern and southern populations) using PCR-RFLP on 3 mtDNA regions, including srRNA gene, lrRNA gene and intergenic COI-COII regions. If the hypothesis of intergradation is correct, intermediate haplotypes between composite haplotypes of northern and southern populations will be found in the contact zone. But, if the hypothesis of colonization is correct, the common haplotype of northern and southern populations will be the same as common haplotype of other areas, which colonized *A. cerana* in Thailand. Also, gene flow of both male and female will be studied by using microsatellite at locus A28, A107 and A113, respectively.

CHAPTER II

MATERIALS AND METHODS

2.1 Instruments

-Autoclave HA-30 (Hirayama Manufacturing Co., Japan)

-Automatic micropipette P2, P20, P100, P200, P1000 (Gilson Medical Eletronics S.A., France)

-Camera Pentax super A (Asahi Opt. Co., Japan)

-Eletrophoresis apparatus

: Horizontal gel electrophoresis apparatus (9 x 12 cm gel)

: Vertical gel electrophoresis apparatus for sequencing (Hoefer, England)

-(-20°C) Freezer (Krungthai Ltd., Thailand)

-Gel dryer Model 583 (BIO-RAD Laboratories, USA)

-High speed microcentrifuge MC-15A (Tomy-Seiko, Japan)

-Heating Block 1761G-26 (Sybron Thermermolyne Co., USA.)

-Incubator BM-600 (Memmert GambH, Germany)

-Light box 2859 SHANDON (Shandon Scienctific Co., Ltd., England)

-Magnetic stirrer M21/1 (Franz Morat KG GambH, Germany)

-Microwave oven TRX 1500 (Turbora International Co., Ltd., Korea)

-Power supply (BIO-RAD Laboratories, USA.)

: Power Pac 300

: Power Pac 3000

-Shaking water bath Model G76 (New Brunswich Scientific Co., Inc., USA.)

-Thermal cycler : Gene Amp PCR system 2400 (Perkin Elmer Cetus, USA.)

-UV transilluminator 2011 Microwave (San Gabriel California, USA.)

2.2 Inventory supplies

-Black and White print film TriX-pan 400 (Eastman Kodak Company, USA.)

-Filter paper Whatman 3 mm (Whatman International Ltd., England)

-Microcentrifuge tubes 0.5 and 1.5 ml (Axygen Hayward, USA.)

-Mirror for sequencing 30 x 40 cm (Hoefer Inc., England)

-Pipette tips 10, 200 and 1000 µl (Axygen Hayward, USA.)

-Thin-wall microcentrifuge tubes 0.2 ml (Axygen Hayward, USA.)

2.3Chemicals

-Absolute ethanol (Merck, Germany)

-Acrylamide (Merck, Germany)

-Agarose gel (FMC Bioproducts, USA.)

: Seakem LE Agarose

: Metaphor Agarose

-Ammonium persulfate (Promega, USA.)

-25 base pair DNA ladder (Promega Co., USA.)

-100 base pair DNA ladder (Promega Co., USA)

-Boric acid (Merck, Germany)

-Bromophenol blue (Merck, Germany)

-Chloroform (Merck, Germany)

-Ethidium bromide (Sigma Chemical Company, USA.)

-Ethylene diamine tetraacetic acid, disodium salt dihydrate (Fluka, Switzerland)

-Ficoll 400 (Sigma Chemical Company, USA.)

-Formaldehyde (BDH, England)

-Formamide (Fluka, Switzerland)

-Gene Amp PCR core reagents (Perkin Elmer Cetus, USA.)

: 10x PCR buffer (100mM Tris-HCl pH 8.3, 500mM KCl)

: 25mM $MgCl_2$

-Glacial acetic acid (BDH, England)

-Hydrochloric acid (Merck, Germany)

-Isoamyl alcohol (Merck, Germany)

-N,N-methylene-bis-acrylamide(Sigma Chemical Company, USA.)

-N,N,N',N'-tetramethylenediamine (Sigma Chemical Company, USA.)

-pBR322/*Msp*I digest (Biolabs, USA.)

-Phenol crystal (Fluka, Switzerland)

-Silver nitrate (BDH, England)

-Silver Sequence Kit (Promega Co., USA.)

: 5x DNA sequencing buffer (250mM Tris-HCl pH 9.0, 10mM $MgCl_2$)

: d /ddNTP nucleotide mixes

: DNA sequencing stop solution (10mM NaOH, 95%

formamide, 0.05% bromophenol blue and 0.05% xylene cyanol)

: 1 μg/μl pGEM -3zf(+) control DNA

: 24 mer pUC/M13 forward primer

-Sodium acetate (Merck, Germany)

-Sodium chloride (Merck, Germany)

-Sodium carbonate anhydrous (AJAX, Australia)

-Sodium dodecyl sulfate : SDS (Sigma Chemical Company, USA.)

-Sodium thiosulfate (Sigma Chemical Company, USA.)

-TEMED

-Tris-(Hydroxy methyl)-aminomethane (Fluka, Switzerland)

-Urea (Fluka, Switzerland)

-Xylene cyanol (Sigma, USA.)

2.4 Enzymes

-Ampli *Taq* DNA polymerase (Perkin Elmer, USA.)

-Proteinase K (GibcoBRL life Technologies, Inc., USA.)

-Restriction endonucleases : *Dra*I (Promega Co., USA.)

2.5 Primers

Oligonucleotides (Bioservice Unit, Thailand or Biosynthesis, Inc., USA. or Vetrogen, Inc., Canada)

2.6 Samples collections

Adult honeybees worker *A. cerana* from natural colonies or unmanage beekeeping's cases in Thailand were individually collected by immediately preserved in 95% ethanol at ambient temperature for

1-5 days during transportation and then stored at 4°C for later use. The sampling areas were 1) Prachup Khiri Khan province and 2) Chumphon province (Table 2.1). The number of colonies per population was between 40-50 samples.

Table 2.1 Sampling areas and sample sizes of *A. cerana* used in this study

Sampling area	sample size (N)
Prachuap Khiri Khan province	
Hua hin	3
Pran buri	1
Kui buri	2
Muang	1
Thap sakae	8
Bang saphan	19
Bang saphan noi	17
Total	51
Chumphon province	
Tha sae	10
Pa thiu	9
Sawi	4
Muang	15
Total	38

Sampling area	sample size (N)
Central	
Nakorn pathom	2
Suphan buri	2
Samut songkhram	8
Samut phakan	1
Chunthaburi	3
Trat	4
Total	20
South	
Suratthani	5
Ranong	6
Phang nga	2
Krabi	3
Nakhon sri thammarat	1
Trang	1
Phatthalung	2
Total	20
China (Yunnan)	5
Vietnam (Hanoi)	1

2.7 DNA extraction

Total DNA was extracted from a thorax of each *A. cerana* individual using the modified method of Hall and Smith(1991). A thorax was transferred into a 1.5 ml microcentrifuge tube containing 500 µl of STE buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 mM EDTA) and briefly homogenized with a plastic pestle. The cells were lysed by adding 20% SDS to final concentration of 1% SDS. A

proteinase K solution (10mg/ml) was added to final concentration of 500 μg/ml and incubated at 65 °C for 2 hours. The DNA was extracted using a standard phenol-chloroform method. The extraction was carried out twice with an addition of an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1 V/V), mixed gently for 15 minutes and centrifuged at 7,000 x g for 10 minutes at room temperature.

The upper aqueous phase was carefully transferred to a new microcentrifuge tube and further extracted once with an equal volume of chloroform-isoamyl alcohol (24:1 V/V). After each extraction, the mixture was centrifuged at 7,000 x g for 10 minutes at room temperature. The upper aqueous phase was carefully transferred to a new microcentrifuge tube.

2.8 Amplification of mitochondrial DNA

Three mitochondrial DNA regions (ssRNA gene, lrRNA gene and intergenic COI-COII region) were amplified by PCR using conditions of Sihanuntavong (1999). Amplification reaction was performed in 25 μl reaction mixture containing 50 ng template DNA, 200 μM each of dNTPs (dATP, dCTP, dGTP and dTTP), 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 2.5-3.0 mM $MgCl_2$, 0.1 μM each of primers and 0.6 unit of Ampli*Taq* DNA polymerase (Perkin Elmer). The reaction was predenaturated at 94 °C for 1 minute following by 35 cycles of a denaturating step at 94 °C for 1 minute, annealing step for 1 minute and extension step at 72 °C for 2 minutes, the last extension at 72 °C for 10 minutes. Appropriate

$MgCl_2$ concentrations and annealing temperature for amplification of each region were illustrated in Table 2.2. Aliquots of the amplified DNA fragment were electrophoretically analyzed in agarose gel. The remaining solution of the successed amplification was then subjected to restriction analysis.

2.9 Agarose gel electrophoresis

The homogeniety of the amplified DNA is most conveniently assessed by electrophoresis through agarose gel. Because of the small size of the amplification products, an agarose specifically formulated to separation of small DNA fragments should be used.

Five microlitters of each the PCR products and DNA markers (λ/*Hin*dIII and 100bp DNA ladder) were loaded into 1.5% agarose gels. Electrophoresis was performed in 1x TBE buffer at constant voltage of 10 volt/cm until the faster migration dye (bromophenol blue) has traveled at least 2 cm from the bottom of the gel. The gel was stained with 2.5 μg/ml ethidium bromide solution for 5 minutes and destained in distilled water for 15 minutes. DNA fragments on agarose gel was visualized and photographed under UV light.

Table 2.2 Primer sequence, annealing temperatures, concentrations of primers and $MgCl_2$ used for PCR amplification of *A. cerana* mitochondrial genes

Gene	Sequence	Position	Annealing temperature (°C)	Primer conc. (μM)	MgCl conc. (mM)
16S	5' CTA TAG GGT CTT ATC GTC CC 3' 5' TTT TGT ACC TTT TGT ATC AGG GTT 3'	13708 14447	53	0.10	3.0
12S	5' AAA AAG CTT CAA ACT GGG ATT AGA TAC CCC 3' 5' TGA CTG CAG AGG GTG ACG GGC GGT GTG T 3'	14588 14206	53	0.10	3.0
inter COI-COII	5' TTG ATT TTT TGG TCA TCC AGA AGT 3' 5' CCA CAA ATT TCT GAA CAT TGA CC 3'	2492 4213	53	0.10	2.5

Primer sequences and their positions were inferred from *A. mellifera* (Crozier and Crozier, 1993).

2.10 Restriction pattern of amplified DNA fragments

An amplification products from each individual was singly digested with a *Dra*I restriction endonuclease according to the manufacturer's recommendations. The reaction mixture was carried out in 20 µl containing 500 ng of amplified products, 1x the recommended buffer, 5 unit of enzyme and sterile deionized water. The reaction was incubated at 37 °C for 2-3 hours. At the end period, one-fifth volume of a loading dye was added to stop the reaction. The resulting mixture was electrophoretically analyzed as soon as possible.

2.11 Metaphor gel electrophoresis

Digestion of amplification products with *Dra*I restriction endonucleases resulted in small DNA fragments, which their resolution in metaphor agarose gel was higher than in agarose gel. Therefore, metaphor agarose gel is more appropriate than agarose gel because high resolution is required in this study.

The horizontal gel was prepared by weighting out an enough amount of metaphor agarose to prepare 2.5-3.5 % gel (W/V), dissolving in 1x TBE buffer and heating to boiling in a microwave. The metaphor solution was incubated at 65 °C until all air bubbles was completely eliminated. The solution was then left at room temperature to 50 °C before pouring into the chamber set (8.5 x 12.5 cm). The total digestion volume was loaded into the gel. Electrophoresis was performed using 1x TBE buffer at 10 volt/cm. The gels were stained with ethidium bromide and photographed under UV light.

2.12 Amplification of microsatellite DNA

Three loci of microsatellite DNA (A28, A107 and A113) of *A. cerana* were amplified by PCR using *A. mellifera* microsatellite primers and using conditions modified from Laoaroon (2001). Amplification reaction was carried out in 10 μl of a mixture containing approximately 20 ng of genomic DNA isolated from each individual of *A. cerana*, 400 mM of each primer, 75 μM each dATP, dCTP, dGTP and dTTP, 1x PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl), 0.4 unit of *Taq* DNA polymerase (Ampli *Taq* DNA polymerase, Perkin Elmer) and an optimal concentration of $MgCl_2$. The reaction was predenaturated at 94 °C for 5 minutes following by 30 cycles of a denaturating step at 94 °C for 30 seconds, annealing step for 30 seconds at and optimal annealing temperature with performed in Table 2.3 and extension step at 72 °C for 30 seconds. The reaction was terminated by a last extention step at 72 °C for 10 minutes. After the amplification process was completed, the size of amplified microsatellite DNA was estimated using denaturating polyacrylamide gels electrophoresis.

Table 2.3 Primer sequences and PCR conditions for the 3 microsatellite loci used to screen for polymorphic loci in *A. cerana*. Primer sequences were taken from Estoup *et al.* (1994, 1995) and Oldroyd *et al.* (1997, 1998).

Locus	Core sequence	Primer sequence (5' to 3')	Mg Cl_2 (mM)	Annealing Temperature (°C)
A28	$(CCT)_3$ GCT$(CCT)_6$ $(CT)_5$ TT $(CT)_4$	GAAGAGCGTTGGTTGCGAGG GCCGTTCATGGTTACCACG	1.6	55
A107	$(GCTC)_2$ $(GCT)_2$ $(CT)_{23}$	CCGTGGGAGGTTTATTGTCG CCTTCGTAACGGATGACACC	1.2	55-57
A113	$(TC)_2$ C$(TC)_2$ TT$(TC)_5$ TT $(TC)_8$ TT$(TC)_5$	CTCGAATCGTGGCGTCC CCTGTATTTTGCAACCTCGC	1.6	58

-Standard marker of microsatellite allele

The sequencing marker was prepared using silver sequencing kit (Promega). The reaction mixture contained 4 μg of pGEM-3Zf(+), 3.2 μl of 5x DNA sequencing buffer (250 mM Tris-HCl pH 9.0, 10 mM $MgCl_2$), 3.6 μl of pUC/M13 forward primer (4.5 pmol), 3.4 μl of sterile water and 5 units of sequencing grade *Taq* DNA polymerase. The reaction mixture was complete by added 16 μl mixture in four microfuge tubes which each tube contained d/ddNTP mix. The reaction tube were placed in a thermal cycle that had been preheated to 95 °C for 2 minutes following by 60 amplification cycles of 95 °C for 30 seconds and 70 °C for 30 seconds. When the thermal cycling was completed, 3 μl of DNA sequencing step solution (95% formamide, 10 mM NaOH, 0.05% bromphenol blue, 0.05% xylene cyanol) was added into each tube. The sequencing marker was heated and loaded on the same gel of a sample.

2.13 Size estimation of amplified microsatellite DNA using denaturating polyacrylamide gels

Glass plates were thoroughly cleaned with detergent, rinsed with deionized water, air-dried and cleaned with 95% ethanol. The shorter glass plate was coated by binding solution (0.0118mM γ-methacryloxypropyltri-methoxysilane in 95% ethanol and 5% glacial acetic acid) and waited for 4-5 minutes. The short glass was carefully wiped with 95% ethanol to remove the remaining binding solution. Plates were placed together with spacer and their edges were sealed with sticky tape.

The amplified microsatellite DNA was mixed with loading dye buffer (95% formamide, 10 mM NaOH, 0.05% bromphenol blue, 0.05% xylene cyanol) at ratio 2 : 1. The mixing solution was heated at 95 °C for 10 minutes and immediately snap-cooled on ice. Nine and six microlitres of pGEM sequencing marker and amplified DNA samples was electrophoresis on 8% denaturating polyacrylamide gel. Electrophoresis was carried out with constant watt at 45 for 3, 4 and 4.5 hours for locus A28, A107 and A113, respectively.

-Silver Staining

At the end of electrophoresis, the plates were separated. The short plate containing a sequencing gel was placed in a fixed/stopped solution (10% acetic acid) to fix the DNA bands and to leach out urea which can block signal for silver staining if still remaining in the gel. This was carried out on orbital shaker for approximately 20 minutes at room temperature.

The gel was washed with deionized water 3 times for 2 minutes each and soaked by shaking for 30 minutes in a staining solution (0.6 mM of silver nitrate and 0.056% formaldehyde). Briefly rinsed with ultrapure water for 5-10 seconds before developing the gel in a cooled develolping solution (0.03 M sodium carbonate, 0.056% formaldehyde and 0.14 mM sodium thiosulfate) until microsatellite bands were clearly visualized. Fix the DNA bands with fixed/stopped solution of 5 minutes. The stained gel was briefly rinsed with deionized water to remove fixed/stopped solution. The gel was air-dried and could be kept permanently at room temperature until result

were confirmed. Finally, the gel was photographed using Fuji colour ISO100 films.

2.14 Data analysis

2.14.1 Restriction fragment data analysis

The different restriction patterns of each mtDNA region were assigned and given an alphabets name (A, B, C, ...) in the order of their frequencies. The fragment sizes of all restriction profiles were estimated in comparison with those of standard DNA markers. The composite haplotypes were generated from combination of restriction patterns of each region with an alphabetical arranging. Binary characters including present (1) or absent (0) of band in each haplotype were compared for further statistical analysis. All statistical analyses were carried out in Restriction Enzyme Analysis Package, REAP (Mc Elaroy, 1991).

2.14.1.1 Genetic distance

The relationship of composite haplotype was observed by genetic distance (d values) that could be estimated as the number of nucleotide substitution per site following the equation:

$$d = -(2/\gamma)\, ln\, G$$

Where γ is the number of recognized sequences at the restriction site and G is $[F\ (3\text{-}2GI)]^{1/4}$ and repeatly

calculated G = GI then GI = $F^{1/4}$ is 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 individual.

2.14.1.2 Haplotype and nucleotide diversity

Genetic diversity within populations was estimated from the haplotype and nucletide diversity. The former is a function of the frequency of different haplotypes in the same populations which can be illustrated as

$$h = n(1-\sum\chi_i^2) / (n-1)$$

Where *n* is the number of individuals investigated and χ_i is the frequency of the *i* th haplotype. Since this value varies enormously with the length of investigated genome while nucleotide diversity (*d*) is an average number of nucleotide differences per site between two sequences, which is more appropriate than the haplotype diversity for comparisons of the same DNA markers with difference in length. This estimate can be calculated using

$$d = (n / n\text{-}1) \sum \chi_i \chi_j d_{ij}$$

Where n is the number of individuals investigated and χ_i and χ_j are the frequencies of i and j genotype, respectively. d_{ij} is the number of nucleotide differences per site between the i^{th} and j^{th} haplotype.

2.14.1.3 Nucleotide divergence

The nucleotide divergence was used to investigated between population diversity which was calculated from nucleotide diversity between haplotypes in population X, Y (d_x , d_y) following equation in 2.14.1.2. The average nucleotide diversity between haplotypes from population X and Y (d_{xy}).

$$d_{xy} = \sum \chi_i \chi_j d_{ij}$$

Where d_{ij} is nucleotide substitution between the i^{th} and j^{th} haplotype from population X and Y, respectively. The nucleotide divergence between two populations (d_A) is then calculated from

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

2.14.1.4 Geographic heterogeneity analysis

Chi-square (χ^2) test was performed using Monte carlo simulation for analysis of geographic heterogeneity in

frequency distribution among population. This value was indicated the significant difference of the composite haplotype frequencies between population.

2.14.1.5 Phylogenetic reconstruction

The genetic distances (d values) between composite haplotypes and nucleotide divergence between populations (dA) were subjected to phylogenetic reconstruction using UPGMA in Phylip version 3.57c.

2.14.2 Microsatellite data analysis

Assumption

An electrophoretic pattern referred to a genotype of each *A.cerana* individuals was inferred from segregation of PCR product size for each microsatellite locus. Therefore, the genotypic state could be divided to homozygotic (single band) and heterozygotic states (double band). Nevertheless, the PCR products, appeared as stutter band, which are common for dinucleotide microsatellites and result from polymerase slippage during PCR amplification. Accordingly, scoring of a particular band can be carried out by making an assumption that an actual band of a given allele was the most intense band among the group of stutter band. The allele sizes were defined in base pair length (bp) by comparing their migration with pGEM sequencing marker. For each *A.cerana* individual was recorded to be

either homozygote or heterozygote. And the allelic stage was recorded from each individual for each locus.

2.14.2.1 Allele frequency and genetic variation

The frequency of a particular allele in a population for diploid organisms is given by

$$p = (2N_{AA} + N_{Aa}) / 2N$$

When p is the frequency of the A allele, N_{AA} and N_{Aa} are the number of homo- and heterozygotes for that allele and N is the number of individuals examined.

Genetic variation within populations was measured in terms of heterozygosity (H) which is the proportion of heterozygous individuals in all investigated samples, therefore observed heterozygosity can be estimated as

$$H = \sum (N_{Aa} / N) /n$$

When n is the number of investigated loci. When determined populations conform Hardy-Weinberg expectation, the unbiased estimated of heterozygosity (expected heterozygosity) can be calculated as

$$h = 1 - \sum p_i^2$$

When p_i is the frequency of i^{th} allele at a given locus, expected heterzygosity across all loci is the mean of *h* from each locus.

Practically, the number of allele per locus, allelic frequency, the proportion of homo- and heterozygotes individuals were estimated using GENEPOP version 2.0 (Raymond and Rousset, 1995)

2.14.2.2 Geographic heterogeneity analysis

The statistically significant difference in genotype frequencies between *A. cerana* from a pair of geographic sampling locations were tested using the exact test of differentiation using GENEPOP version 2.0. Results are expressed as the probability of homogeneity between compared populations or regions. To diminish type I error, level of significance was further adjusted using the sequential Bonferroni test (number of population X number of loci)

2.14.2.3 Estimation of population structure

F-statistics, F_{ST} is a standard parameter for measurement of population structure (or interpopulation diversity). It can be calculated using the exact test of genotypic differentiation of GENEPOP version 2.0.

CHAPTER III

RESULTS

3.1 DNA extraction

Total DNA from *A. cerana* individual was prepared using proteinase K/ phenol-chloroform extraction method. High molecular weight DNA larger than 23.1 kb was obtained . The concentration of extracted DNA was about 1.0-1.5 μg per individual as estimated by comparing its intensity of EtBr-DNA complex with that of the known amount of λ/*Hin*dIII marker in 0.7% agarose gel electrophoresis (Figure 3.1)

3.2 PCR amplification

In vitro DNA amplification of three regions on mitochondrial DNA (srRNA gene, lrRNA gene and intergenic COI-COII regions) were performed using primer designed from closely related species (*A.mellifera*) under optimal PCR condition for *A.cerana* (Sihanuntavong, 1999). After electrophoresis in 1% agarose gel and ethidium bromide staining, PCR product size was determined under UV light by comparing with λ/*Hin*dIII marker and 100 bp DNA ladder. For each region, all 95 individuals (colonies) (51 colonies of Prachuap Khiri Khan province, 38 colonies of Chumphon province, 5 colonies of Yunnan and 1 colony of Hanoi) were amplified to the same size. PCR-amplified srRNA gene, lrRNA gene and intergenic COI-COII region were 400, 700 and 1,710 bp, respectively. (Figure 3.2). Non-specific band and primer dimer were not found.

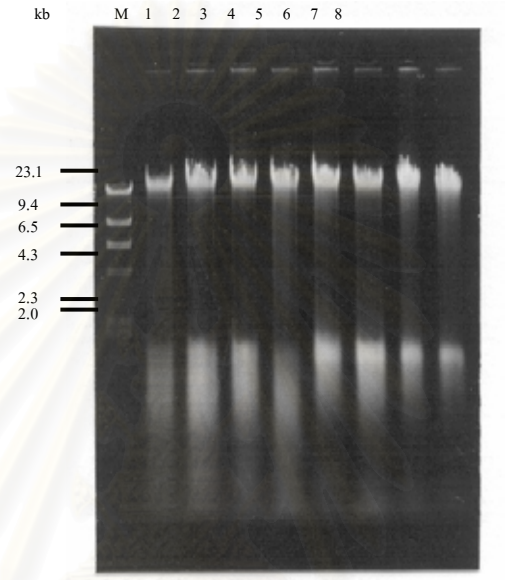

Figure 3.1 High molecular weight DNA extracted from thorax of *A. cerana*

lane M = □/*Hin*dIII standard DNA marker

lane 1-8 = total DNA from eight *A. cerana* individuals

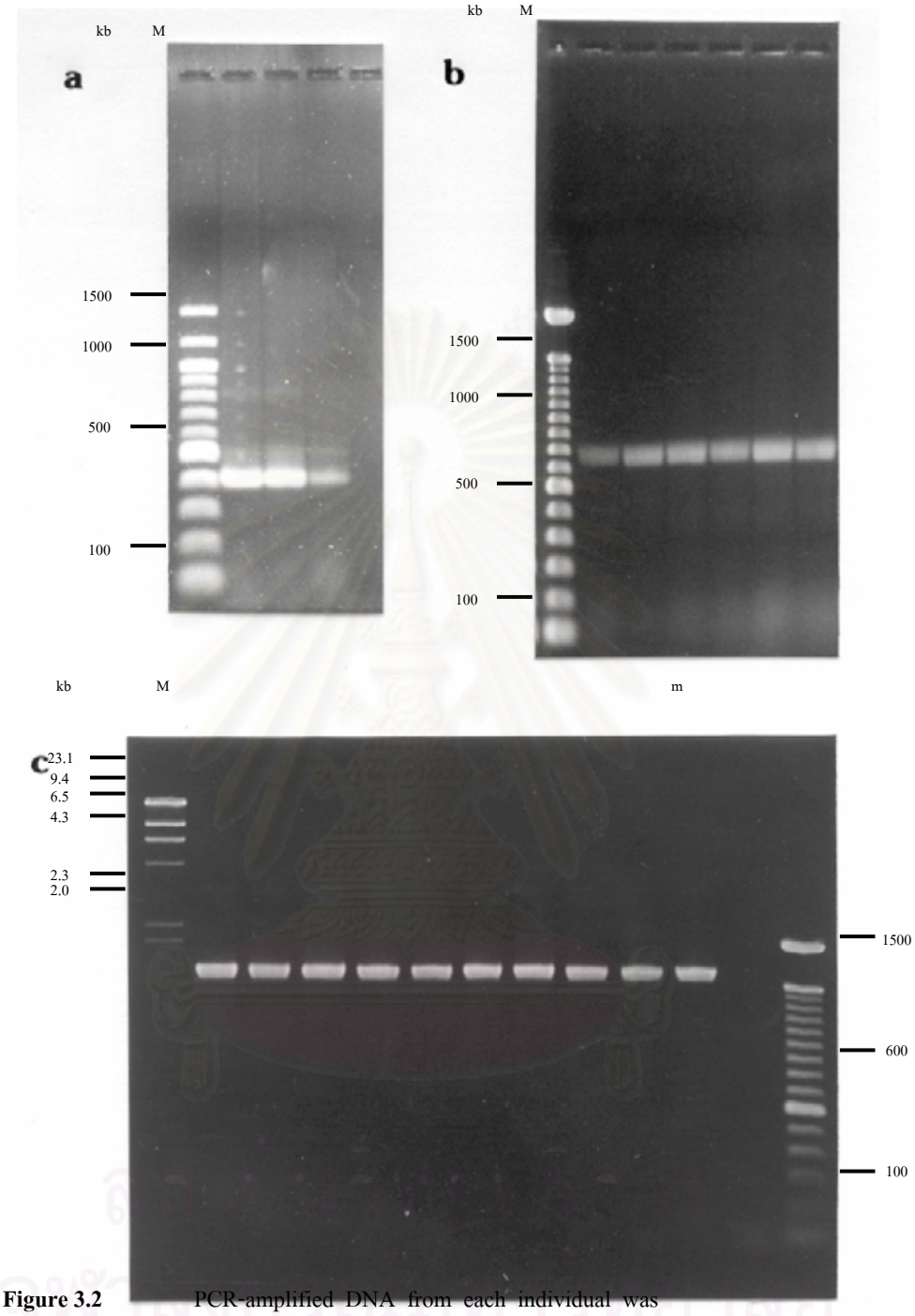

Figure 3.2 PCR-amplified DNA from each individual was electrophoresed: a. srRNA gene (400 bp); b. lrRNA gene (750 bp) and c. intergenic COI-COII regions (1710 bp)

lane M = □/*Hin*dIII standard DNA marker

lane m = 100 bp DNA ladder

3.3 Restriction analysis of srRNA gene, lrRNA gene and intergenic COI-COII regions

The amplified DNA of three regions was digested with *Dra*I restriction endonuclease. Three, four and eight digestion patterns were observed when the amplified srRNA, lrRNA and intergenic COI-COII were restricted, respectively. The digestion patterns of each region were presented in Table 3.1. srRNA was divided to 3 haplotypes (A, B and C) (Figure 3.3). Haplotype A was the most commonly found in Prachuap Khiri Khan province (46 colonies), Chumphon province (10 colonies), Yunnan (5 colonies) and Hanoi (1 colony). Haplotype B was mostly observed in Chumphon province (28 colonies) while only 5 colonies were from Prachuap Khiri Khan province. Haplotype C was least found. There was only one colony of Prachuap Khiri Khan occurred as haplotype C. The differences between 3 haplotypes were shown as step of point mutation in Figure 3.6. When haplotype A and C were compared to haplotype B (haplotype B was used as basic pattern), 320-bp-fragment of haplotype A was equal to 160-bp-fragment of haplotype B with double concentration. The 42- and 38-bp-fragments of haplotype B were combined to 80-bp-fragment of haplotype C. A single step of point mutation appeared among the change of haplotype B to A or C.

Four different haplotypes (A, B, D and E) were observed when lrRNA amplified gene was cut with *Dra*I (Figure 3.4). Thirty-four colonies of Prachuap Khiri Khan, 10 colonies of Chumphon, 5 colonies of Yunnan and 1 colony of Hanoi were haplotype A. Haplotype B was frequently distributed in Chumphon. Twenty-two colonies of Chumphon were found to be haplotype B while only 2 colonies of Prachuap Khiri Khan were. Four and five colonies of Prachuap Khiri Khan and

Chumphon were observed, as haplotype D, respectively. Haplotype E was rarely found; only one colony of Prachuap Khiri Khan was this haplotype. Table 3.1, *Dra*I digestion patterns of each haplotype were shown. A single step of point mutation occurred between haplotype A and B. The differences between haplotype D and A or B were 1 or 2 point mutation steps, respectively. Whereas, haplotype E differed from A and B by 2 and 1 point mutation steps, respectively. (Figure 3.6)

Higher variation was presented when PCR-amplified intergenic COI-COII region was cut with *Dra*I enzyme. There were 8 different haplotypes (A, B, C, D, E, F, G and H) (Figure 3.5). All haplotypes could be devided into 2 groups; the first was variants of haplotype A (A, D, G and H) and another was variants of haplotype B (B, C, E and F). Two or three steps of point mutation occurred within first group and one or two point mutation steps occurred within another group. The alternation between haplotype A and B was spaced by 3 point mutation steps. Pattern of *Dra*I digestion and steps of point mutation were illustrated in Table 3.1 and Figure 3.6, respectively. Haplotype A was predominant (45 colonies of Prachuap Khiri Khan, 8 colonies of Chumphon and 1 colony of Hanoi) whereas haplotype B was found in 24 colonies of Chumphon, 5 colonies of Yunnan and only 4 colonies of Prachuap Khiri Khan. The others (C, D, E, F, G and H) were rare haplotypes. Haplotype C, E, F and H were observed in Chumphon of 2, 1, 1 and 2 colonies, respectively. Both haplotypes D and G were found only one colony for each haplotype in Prachuap Khiri Khan.

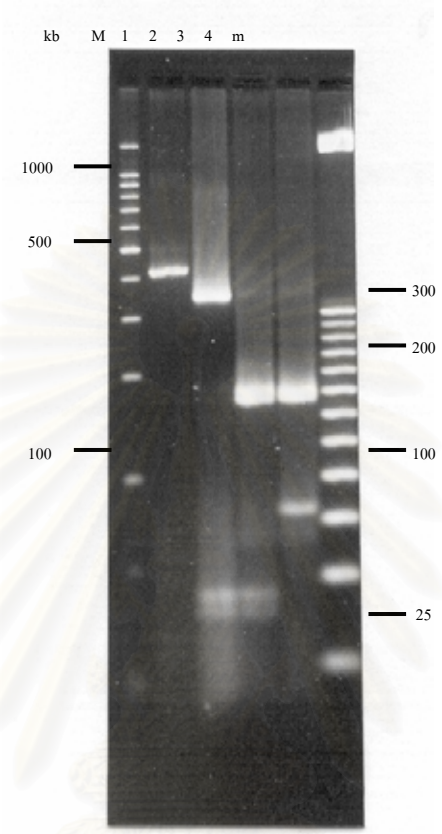

Figure 3.3 Three haplotypes were observed from *Dra*I digestion of amplified mitochondrial srRNA gene of *A. cerana* in Thailand.

lane M = 100 bp DNA ladder

lane m = 25 bp DNA ladder

lane 1-4 = Undigested DNA, haplotype A, B and C, respectively

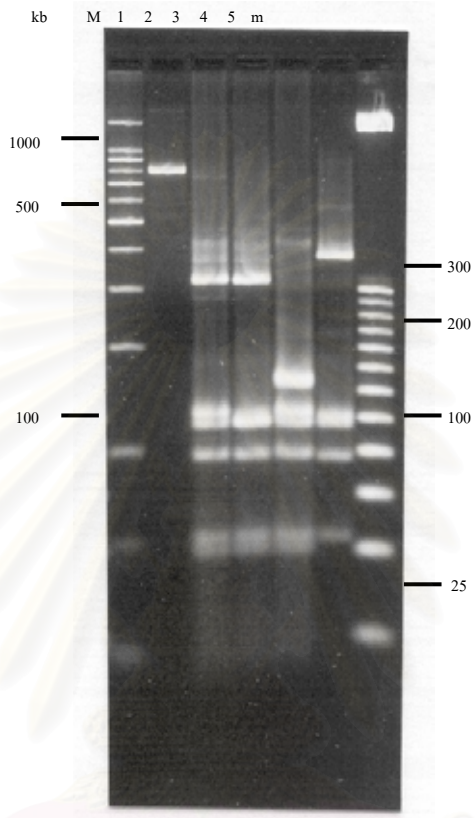

Figure 3.4 Four haplotypes were observed from *Dra*I digested of anplified mitochondrial lrRNA gene of *A. cerana* in Thailand.

lane M = 100 bp DNA ladder

lane m = 25 bp DNA ladder

lane 1-5 = Undigested DNA, haplotype A, B, D and E, respectively

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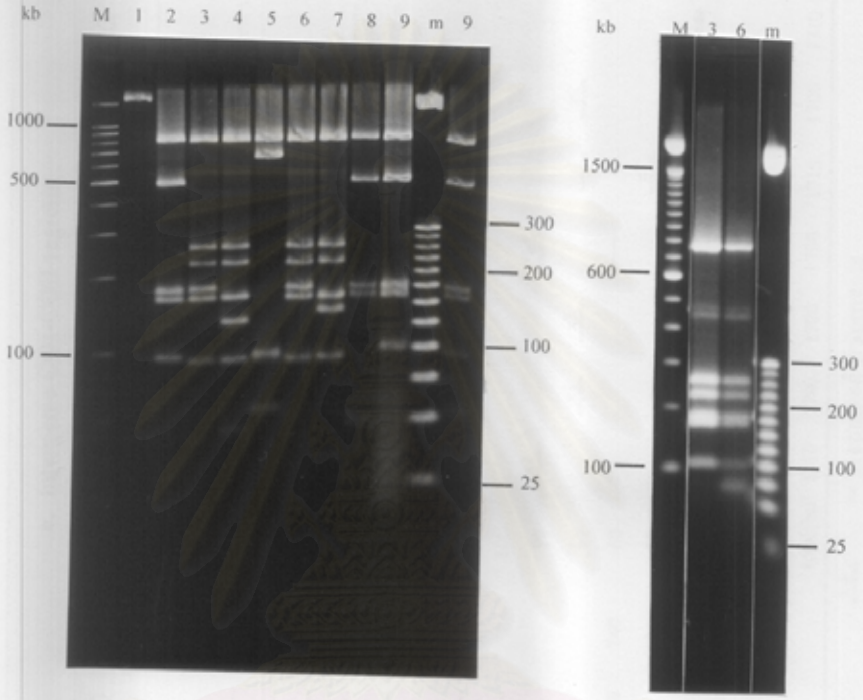


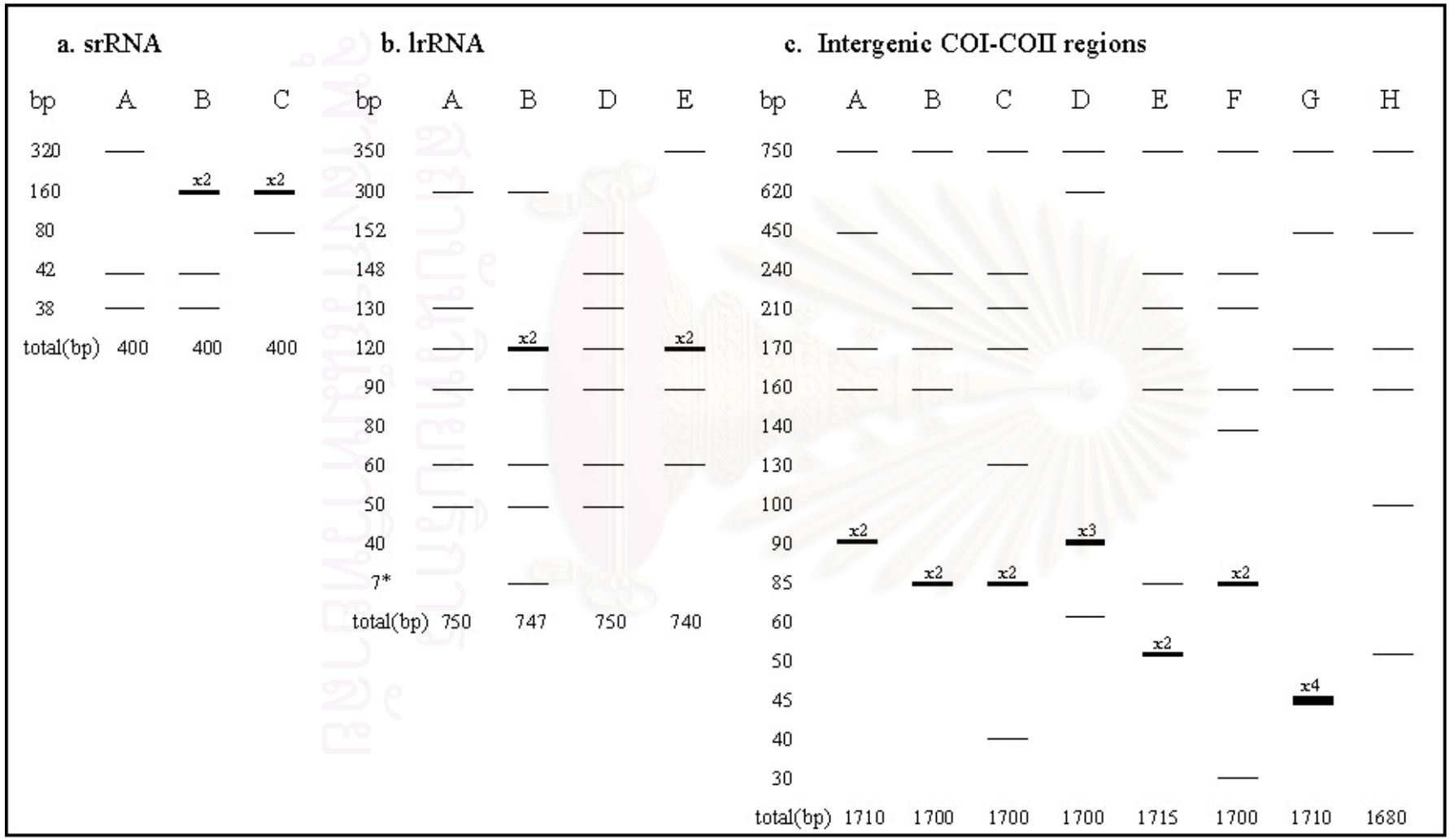
Figure 3.5 Eight haplotypes were observed from *Dra*I digested of amplified mitochondrial intergenic COI-COII regions of *A. cerana* in Thailand.

lane M = 100 bp DNA ladder

lane m = 25 bp DNA ladder

lane 1-9 = Undigested DNA, haplotype A, B, C, D, E, F, G and H, respectively

Table 3.1 Summary of restriction patterns of *Dra*I digested amplified DNA from three different regions in mitochondrial genome of *A.cerana*



—— = one fragment, $\overset{xn}{\text{——}}$ = Repeated fragments

* This fragment was not observed on the electrophoresis gel, but inferred from DNA sequencing data of this gene region

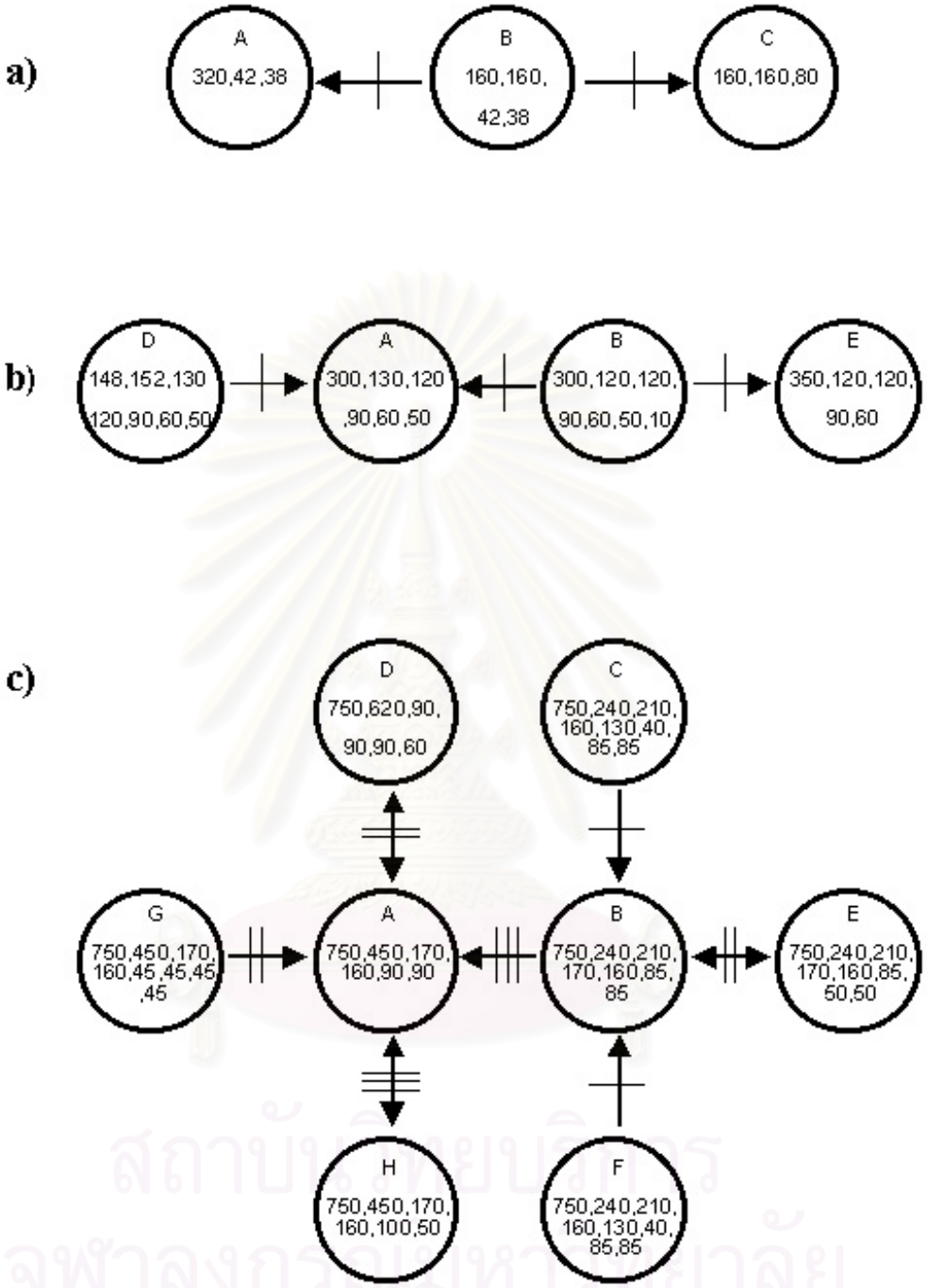


Figure 3.6 The most parsimonious networks based on *Dra*I digestion of PCR-amplified DNA of a) srRNA gene, b) lrRNA gene and c) intergenic COI-COII region of *A.cerana* in Thailand. Arrows indicate restriction site losses and not necessarily indicate evolutionary direction. Cross bars indicate the number of point mutation.

3.4 Distribution of composite haplotypes of *A.cerana*

The *Dra*I digestion patterns of three amplified regions were combined in each *A. cerana* to generate composite haplotype. The composite haplotype for each colony was designed by 3 letters representing the digestion pattern for srRNA haplotype, IrRNA haplotype and intergenic COI-COII haplotype, respectively. Twelve composite haplotypes were found in this study. The geographic distribution frequencies are shown in Table 3.2. The most common composite haplotype, AAA, was found in thirty-seven (39%) out of 95 individuals investigated. This composite haplotype was distributed in Prachuap Khiri Khan (31 colonies), Chumphon (5 colonies) and Hanoi (1 colony). The other common composite haplotype, BBB, was found in 22 (23%) out of 95 individuals investigated. Twenty and two individuals were observed in Chumphon and Prachuap Khiri Khan, respectively. Composite haplotype ADA (18%) was found in Prachuap Khiri Khan (14 individuals) Chumphon (3 individuals). Composite haplotype BAB (6%) was found in 4 and 2 individuals from Chumphon and Prachuap Khiri Khan, respectively. In addition, other 8 composite haplotypes were found in low frequencies at particular location; both AAG and CED were found only in one sample each from Prachuap Khiri Khan. Two individuals from Chumphon had composite haplotype ADH. BAC, BBC, BBE and BBF were found in one individual each in Chumphon and all of individuals from Yunnan (5 colonies) had AAB haplotype. Distribution of composite haplotypes within each location is shown in Figure 3.7. The genetic distances of these composite haplotypes are shown in Table 3.3. The values of genetic distances were ranged from 0.0047 to 0.0826. Distance

values were used to reconstruct a dendogram using unweighted pair group method.

Table 3.2 Geographic distribution frequency of 12 composite haplotypes in four geographic locations resulted from *Dra*I digestion of srRNA gene, lrRNA gene and intergenic COI-COII regions

Composite Haplotype	**Geographic distribution frequency (no. of individuals)**				**Total**
	Prachuap	**Chumphon**	**Yunnan**	**Hanoi**	
AAA	0.61 (31)	0.13 (5)		1.00 (1)	0.39 (37)
AAB			1.00 (5)		0.05 (5)
AAG	0.02 (1)				0.01 (1)
ADA	0.27 (14)	0.08 (3)			0.18 (17)
ADH		0.05 (2)			0.02 (2)
BAB	0.04 (2)	0.10 (4)			0.07 (6)
BAC		0.03 (1)			0.01 (1)
BBB	0.04 (2)	0.53 (20)			0.23 (22)
BBC		0.03 (1)			0.01 (1)
BBE		0.03 (1)			0.01 (1)
BBF		0.03 (1)			0.01 (1)
CED	0.02 (1)				0.01 (1)
Total	**1.00 (51)**	**1.00 (38)**	**1.00 (5)**	**1.00 (1)**	**1.00 (95)**

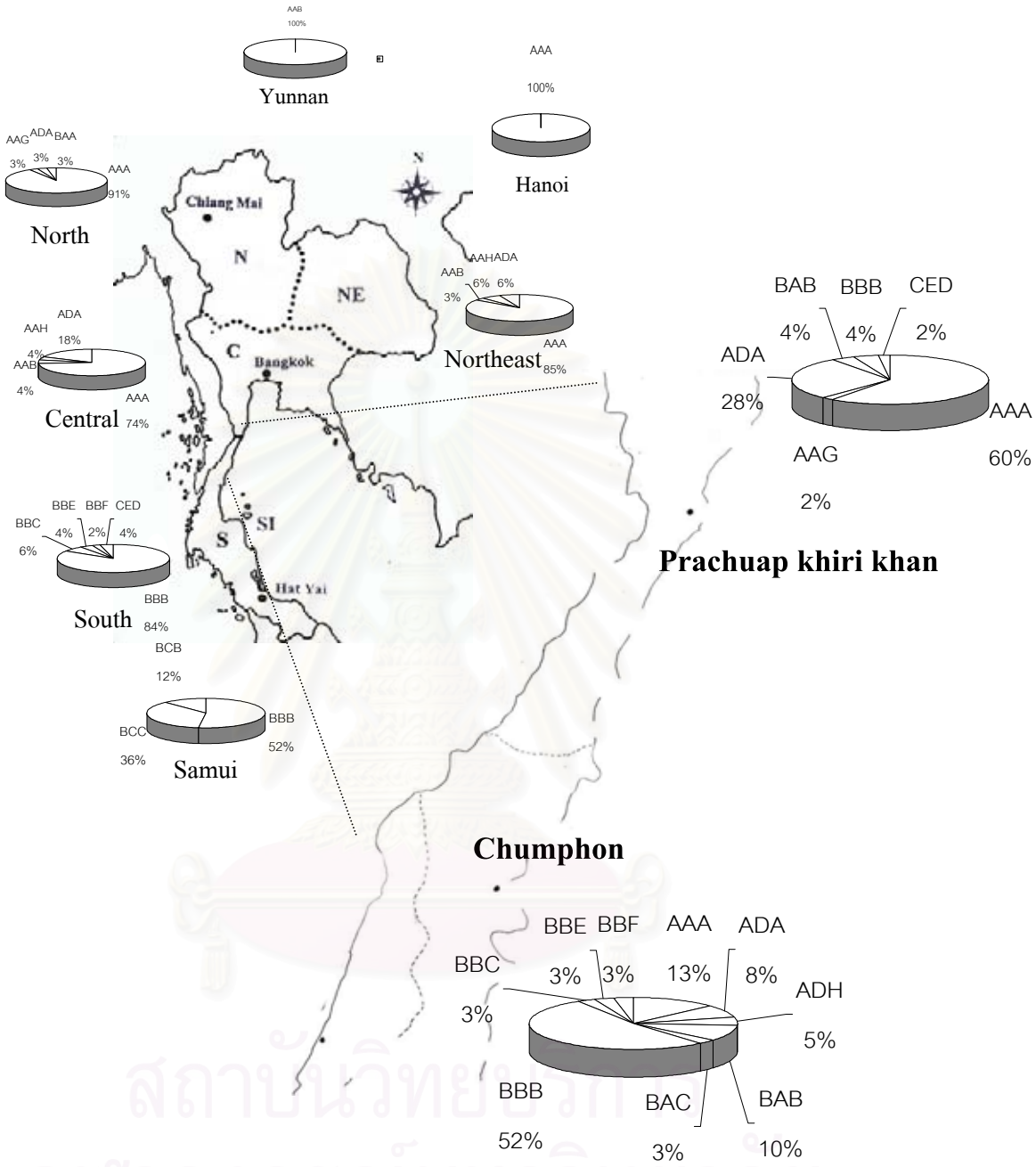


Figure 3.7 Distribution of mtDNA composite haplotypes within each sampling location of *A. cerana* in Thailand. Especially, Prachuap khiri khan and Chumphon provinces (North, North-east, Central, South and Samui datas referred to Sihanantavong, 1999)

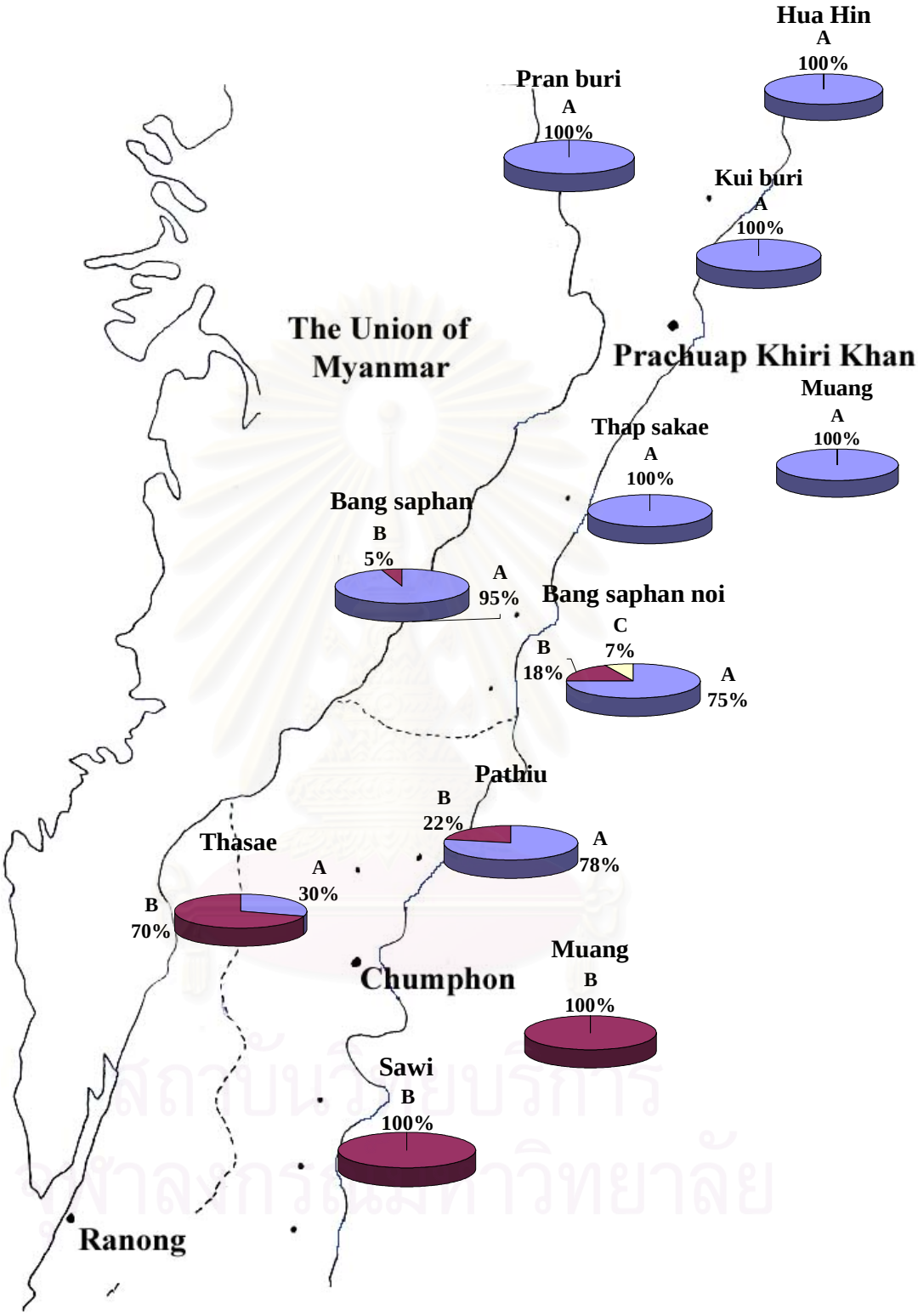


Figure 3.8 Distribution of mtDNA composite haplotypes within each sampling location of *A. cerana* in Prachuap khiri khan and Chumphon provinces.

Table 3.3 Estimate genetic distances among 12 composite haplotypes resulted from *Dra*I digested amplified DNA fragment of srRNA genes, lrRNA gene and intergenic COI-COII regions in *A. cerana* mitochondrial genome

	AAA	ADA	ADH	AAG	BBB	BBF	BBE	BBC	BAC	BAB	AAB	CED
AAA	-											
ADA	0.0057	-										
ADH	0.0145	0.0075	-									
AAG	0.0117	0.0181	0.0181	-								
BBB	0.0289	0.0371	0.0371	0.0325	-							
BBF	0.0371	0.0461	0.0461	0.0406	0.0047	-						
BBE	0.0307	0.0388	0.0325	0.0342	0.0047	0.0097	-					
BBC	0.0371	0.0461	0.0461	0.0406	0.0047	0.0062	0.0097	-				
BAC	0.0289	0.0371	0.0371	0.0325	0.1030	0.0118	0.0158	0.0047	-			
BAB	0.0214	0.0289	0.0289	0.0250	0.0050	0.0103	0.0103	0.0103	0.0050	-		
AAB	0.0145	0.0214	0.0214	0.0181	0.0110	0.0169	0.0169	0.0169	0.0110	0.0053	-	
CED	0.0523	0.0544	0.0804	0.0926	0.4880	0.0507	0.0507	0.0507	0.0585	0.0565	0.0804	-

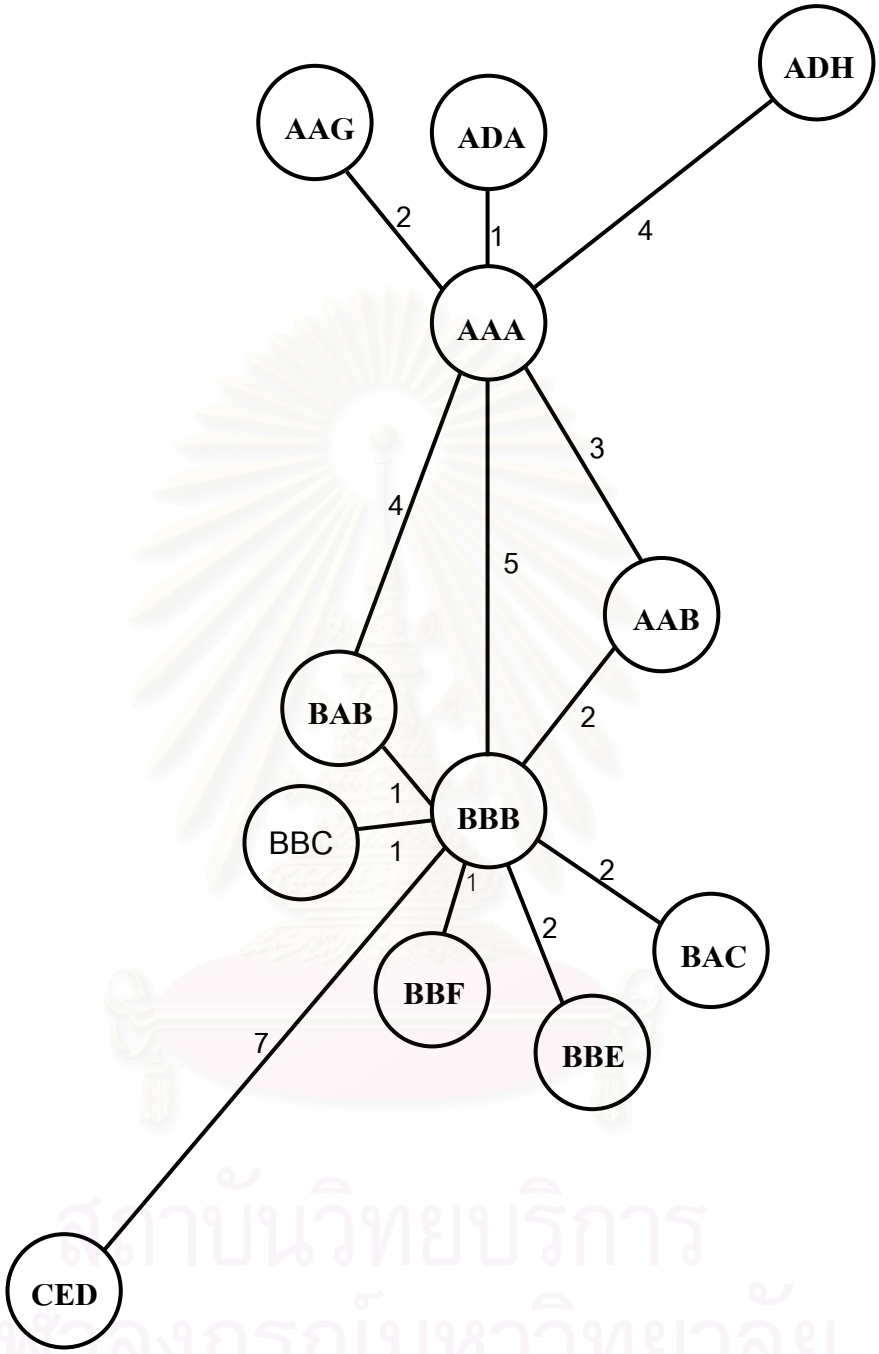


Figure 3.9 Phylogeographic pattern deduced from mtDNA composite haplotypes of *A. cerana* in borderline region between northern and southern populations in Thailand. Number along connected line indicated inferred mutation steps.

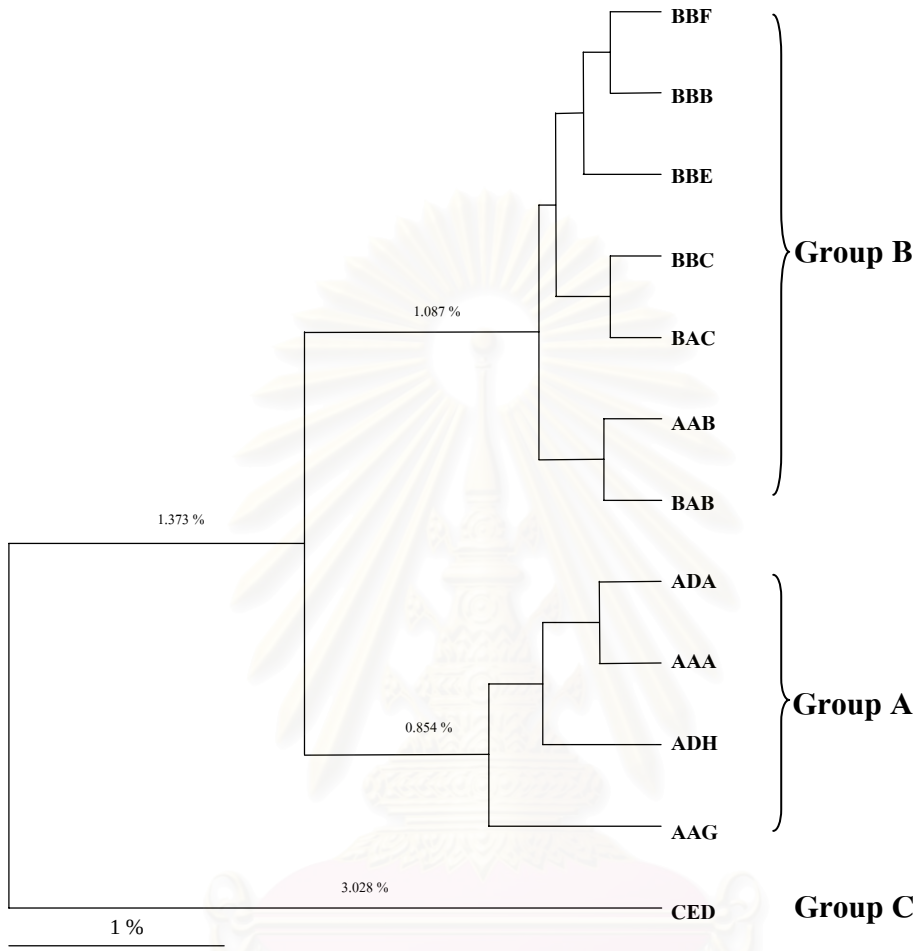


Figure 3.10 A UPGMA dendogram showing the relationships among twelve composite haplotypes based on *DraI* digestion of PCR amplified DNA of srRNA gene, lrRNA gene and intergenic COI-COII regions of *A. cerana* in borderline region between northern and southern populations

(UPGMA) (Figure 3.10). The phenogram indicated that the 12 composite haplotypes of *A. cerana* could be allocated into 3 groups (A, B and C). Group A was composed of ADA, AAA, ADH and AAG. Group B was composed of BBF, BBB, BBE, BBC, BAC, AAB and BAB. C group was composed of only one composite haplotype CED. Group A was mainly observed in Prachuap Khiri Khan and group B was in Chumphon, but the distribution of some composite haplotype (AAA, BBB, ADA and BAB) indicated the overlapping of these two groups in contact zone. The distance between haplotypes AAA and BBB was 5 point mutation steps. Among these mutation steps, the intermediate composite haplotypes, AAB and BAB, were observed. Distance between AAB and AAA was 3 mutation steps and distance between AAB and BBB was 2 mutation steps whereas distances from BAB to AAA and BBB were 4 and 1 mutation steps, respectively.

Haplotype and nucleotide diversity within population for 4 geographic locations were illustrated by Table 3.4. Bee samples from both Chumphon and Prachuap Khiri Khan showed high haplotype diversity of 0.7013 and 0.5624, respectively, whereas those of Yunnan and Hanoi were least (0.0000). The average haplotype diversity was 0.3159. The highest nucleotide diversity was observed in bee from Chumphon (1.49%) following by those from Prachuap Khiri Khan (0.85%), while those of Yunnan and Hanoi were 0%. However, percentage of nucleotide diversity of Yunnan and Hanoi could be changed because the individual numbers of both were only five and one colonies. The average nucleotide diversity was 0.59%.

According to the result in Table 3.5, nucleotide diversity of all pairwise comparisons were low. The average nucleotide diversity

Table 3.4 Haplotype and nucletide diversity within population for four geographic locations of *A. cerana* in Thailand

Population	haplotype diversity (h +/- S.E.)	Nucleotide diversity (%)
Prachuap	0.5624 +/- 0.05986	0.853
Chumphon	0.7013 +/- 0.07323	1.490
Yunnan	0.0000 +/- 0.00000	0.000
Hanoi	0.0000 +/- 0.00000	0.000
Average	0.3159 +/- 0.03407	0.589 +/- 0.0013

Table 3.5 Nucleotide diversity (above diagonal) and divergence (below diagonal) between populations for four geographic locations of *A. cerana*

	Prachuap	Chumphon	Yunnan	Hanoi
Prachuap	-	0.023580	0.017278	0.004795
Chumphon	0.011869	-	0.012682	0.022211
Yunnan	0.013014	0.005235	-	0.014512
Hanoi	0.000531	0.014764	0.014512	-

between two populations was 1.58%. The total nucleotide divergence of all populations was low (1% in average).

Monte Carlo simulation was used for geographic heterogeniety analysis. The significant difference in composite haplotype distribution frequencies among 4 geographic locations of *A. cerana* were shown in Table 3.6. High significance in haplotype frequencies were observed implying the existence of population subtraction within *A. cerana* ($P < 0.0001$) (Only pairwise comparisons with Hanoi individual were shown $P > 0.0001$).

Genotypic disequilibriums of 3 regions on mtDNA were resulted as significant values from 89 samples (Table 3.7). Testing in Prachuap Khiri Khan and Chumphon populations, there was no linkage disequilibrium detected. High significance between two populations were shown ($P = 0.0000$-0.00071). For all populations, genotypic disequilibrium of mtDNA among 3 regions was also highly significant ($P < 0.0001$).

3.5 Genetic variation of three microsatellites DNA

Microsatellite analysis using three microsatellite loci (A28, A107and A113) were performed in order to study genetic variation in the borderline region between northern and southern populations of *A. cerana* in Thailand. The amplified-PCR products, were identified in 8% denaturing polyacrylamide gel with pGEM sequencing marker. Even though highly polymorphic were observed at locus A28 and A107 (Figures 3.11 and 3.12), but these polymorphic loci (A28 and A107) were not used for analysis. Since they showed various stutter bands that were difficult to score both size and number of amplified products. Then, only

Table 3.6 Geographic heterogeneity analysis in distribution frequency of composite haplotype among four *A. cerana* locations based on *Dra*I digestion of srRNA gene, lrRNA gene and intergenic COI-COII region

	Prachuap	**Chumphon**	**Yunnan**	**Hanoi**
Prachuap	-			
Chumphon	P < 0.0001	-		
Yunnan	P < 0.0001	P < 0.0001	-	
Hanoi	P = 0.6732	P =0.2849	P = 0.0448	-

Total P < 0.0001

Table 3.7 Genotypic disequilibrium of srRNA gene, lrRNA gene and intergenic COI-COII regions of *A. cerana* in Thailand

	srRNA	lrRNA	Intergenic COI-COII
srRNA	-		
lrRNA	<0.001	-	
Intergenic COI-COII	<0.001	<0.001	-

Table 3.8 F-statistic among four *A. cerana* locations based on *Dra*I digestion of srRNA gene, lrRNA gene and intergenic COI-COII regions

Sample	sr RNA		lr RNA		intergenic COI-COII	
	Fst	P-value	Fst	P-value	Fst	P-value
Pruchuap-Chumphon	0.5946	<0.0001	0.3061	<0.0001	0.4979	<0.0001
Prachuap-Yunnan	-0.06643	1.0000	0.0899	0.2531	0.7963	<0.0001
Prachuap-Hanoi	-0.2738	1.0000	-0.0671	0.6346	-0.2681	1.0000
Chumphon-Yunnan	0.5737	0.0035	0.4341	0.0035	0.0715	0.2005
Chumphon-Hanoi	0.5207	0.0893	0.3552	0.1026	0.3892	0.0637
Yunnan-Hanoi	-	-	-	-	1.0000	0.0476

	Fst	P-value
srRNA	0.5729	<0.0001
lrRNA	0.3026	<0.0001
intergenic COI-COII	0.5154	<0.0001
All	0.45871	

χ^2 : infinity Df : 6

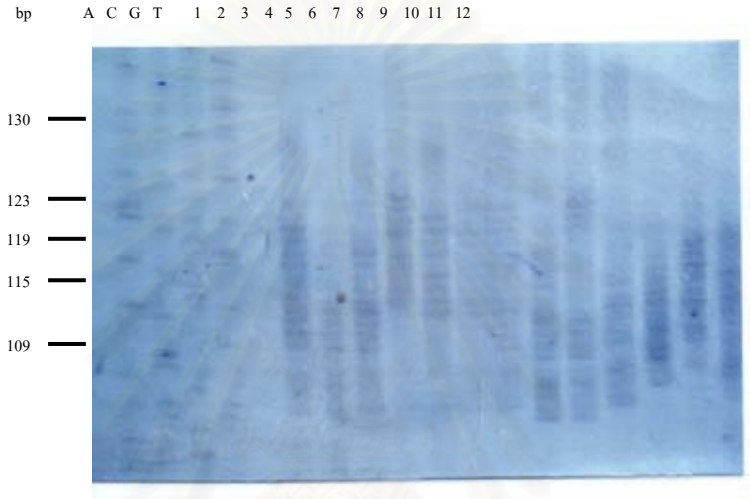


Figure 3.11 Microsatellite pattern of *A. cerana* individuals at locus A28 (lane 1-12).

pGEM sequencing marker was used as a standard

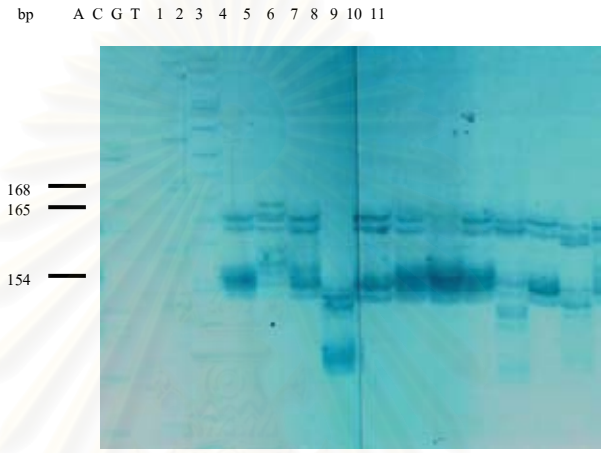


Figure 3.12 Microsatellite pattern of *A. cerana* individuals at locus A107 (lane 1-11)

pGEM sequencing marker was used as a standard

A113 locus was used for analysis (Figure 3.13).

Central and southern populations of *A. cerana* from flanking region of Prachuap Khiri Khan and Chumphon were also used in this analysis. Therefore, 4 geographic samples included 129 individual colonies in total (central: 20, southern: 20, Prachuap Khiri Khan: 51 and Chumphon: 38), were analyzed. The results showed that locus A113 had four alleles (175, 180, 185 and 195 bp). Only A175 bp allele was found in samples from southern population. A180 bp allele was found only in central and Prachuap Khiri Khan samples with relatively low frequencies. In contrast, A185 bp allele was observed in highest frequency. Its frequency was 0.625, 0.650, 0.706 and 0.684 for central, southern, Prachuap Khiri Khan and Chumphon samples, respectively. The frequency of A195 bp allele was 0.350, 0.325, 0.284 and 0.316 for central, southern, Prachuap Khiri Khan and Chumphon bee samples. All allele frequency distributions of microsatellite locus A113 were shown in Figure 3.14 and Table 3.9.

The observed heterozygosity (H_0) and expected heterozygosity (H_e) of microsatellite loci A113 of four geographies were shown in Table 3.9. The average observed heterozygosities ranged from 0.451 (Prachuap Khiri Khan samples) to 0.550 (central samples). These observed heterozygosities were almost equal to the expected heterozygosities.

Geographic heterogeneity of allele frequency of four geographic samples was shown in Table 3.10. No significance in distribution of alleles frequency was observed in all populations ($p = 0.6017$).

Intraspecifically geographic differentiations of four geographies of *A. cerana* were supported by F-statistics (*FST*). The average *FST* value was -0.01035. Genetic differentiation do not exist in these populations (Table 3.11).

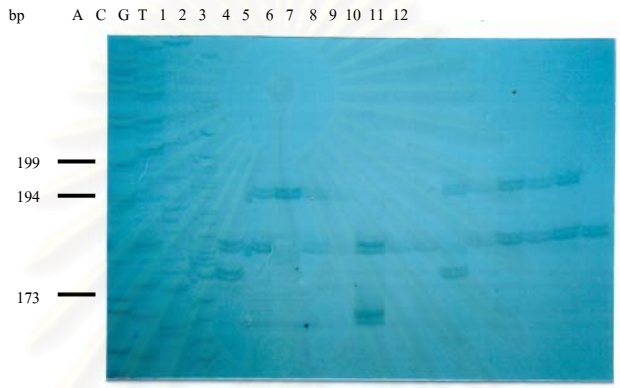


Figure 3.13 Microsatellite pattern of *A. cerana* individuals at locus A113 (lane 1-11)
pGEM sequencing marker was used as a standard

Table 3.9 Allele frequencies, number of allele, observed and expected heterozygosity of microsatellite A113 in four geographic samples of *A. cerana* in Thailand

Allele (bp)	Central (N = 20)	South (N = 20)	Prachuap (N = 51)	Chumphon (N = 38)
175		0.025		
180	0.025		0.010	
185	0.625	0.650	0.706	0.684
195	0.350	0.325	0.284	0.316
Number of alleles	3	3	3	2
Observed heterozygosity	0.550	0.500	0.451	0.474
Expected heterozygosity	0.486	0.471	0.425	0.434

N = Number of specimen examined

Table 3.10 Geographic heterogeneity analysis of four geographic samples of *A. cerana* in Thailand using microsatellite locus A113

	Central	South	Prachuap	Chumphon
Central	-			
South	$P = 1.000^{ns}$	-		
Prachuap	$P = 0.412^{ns}$	$P = 0.481^{ns}$	-	
Chumphon	$P = 0.375^{ns}$	$P = 0.434^{ns}$	$P = 0.851^{ns}$	-

ns = not significant

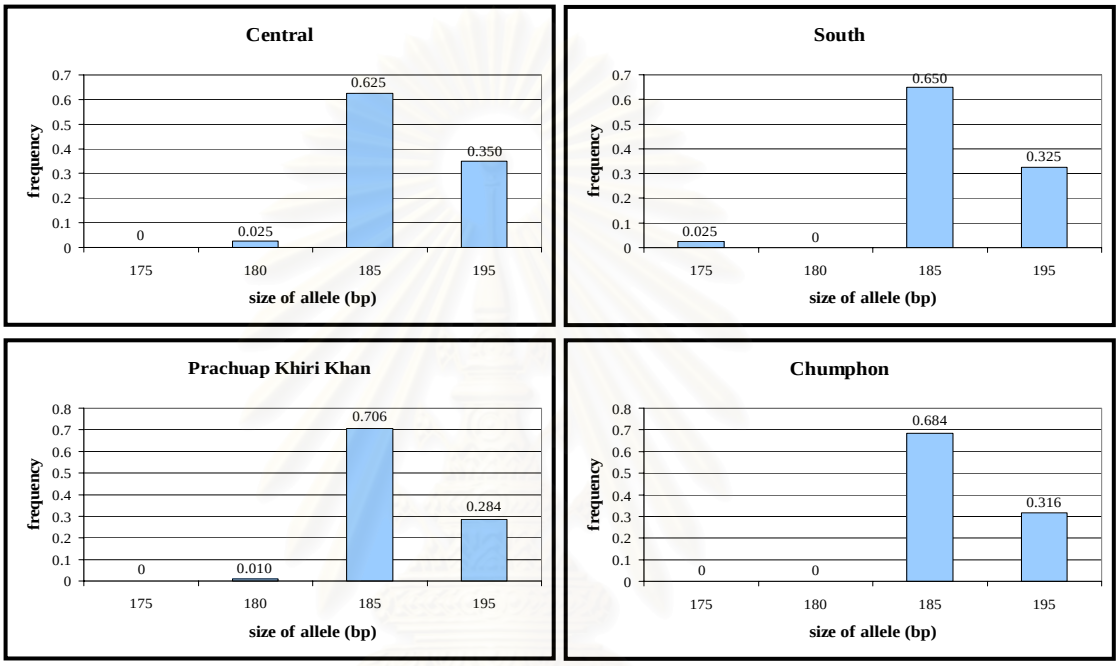


Figure 3.14 Allele frequency distributions at the microsatellite locus A113 from Central (n = 20), South (n = 20), Prachuap Khiri Khan (n = 51) and Chumphon (n = 38)

Table 3.11 F-statistics for microsatellite analysis loci A113 each pair of four geographic samples of *A. cerana* in Thailand

	Central	**South**	**Prachuap**	**Chumphon**
Central	-			
South	-0.0211 (P = 1.0000)	-		
Prachuap	-0.0036 (P = 0.454)	-0.0103 (P = 0.5986)	-	
Chumphon	-0.0117 (P = 0.5667)	-0.016 (P = 0.8288)	-0.0089 (P = 0.7292)	-

F-statistics for microsatellite analysis locus A113 of four geographic populations of *A. cerana* in Thailand. The average FST value across overall geographic samples was –0.0104 (P = 0.8287).

CHAPTER IV

DISCUSSION

Genetic variation of the honeybees, *A. cerana*, in Thailand was studied from several techniques such as morphometric (Sylvester, 1998), PCR-RFLP in mtDNA (Songram, 1996; Deowanish, 1997 and Sihanuntavong,1999), microsatellite (Sittipraneed, 2001) and DNA sequencing (Bugharuang, 1996 and Sihanuntavong, 2001). Generally, *A. cerana* in mailand of Thailand was divided into two different groups; the northern and the southern population by such techniques. Polymorphism in mtDNA was useful for analysis of genetic variation and population structure of *A. cerana* in Thailand. Recently, Sihanuntawong (1999) investigated genetic variations of three mtDNA regions (srRNA gene, lrRNA gene and intergenic COI-COII regions). Thirteen composite haplotypes, which were generated from three, five and eight haplotypes, were obtained from *Dra*I digestion of amplified products from srRNA gene, lrRNA gene and intergenic COI-COII regions, respectively. A UPGMA phenogram based on genetic distance among populations clearly allocated 5 geographic samples of *A. cerana* into two genetically distinctive groups: northern (north, northeast and central) and southern (south and Samui Island). However, only haplotype C of lrRNA gene was specifically found in Samui Island. When geographic heterogeneity was analyzed with a Monte Carlo Simulation, the results showed three distinctive groups where the Samui *A. cerana* could be further separated from the south.

Songram (1997) investigated genetic variation in *A. cerana* by using PCR-RFLP on ATPase6-ATPase8 mtDNA genes. Five geographic locations surveyed with three restriction endonucleases (*Taq*I, *Ssp*I and *Vsp*I) revealed two, five and six haplotypes, respectively. Ten composite haplotypes were then generated. A UPGMA phenogram and a Monte Carlo Simulation results divided *A. cerana* populations as same as Sihanuntavong results. However, some amplified products of ATPase6-ATPase8 genes from south and Samui Island samples were complicated for PCR-RFLP methods due to the heteroplasmy, which was neccessary to eluted a band at 825 bp from agarose gel electrophoresis.

Three regions (srRNA gene, lrRNA gene and intergenic COI-COII regions) in mtDNA were chosen for study distributions of two populations of *A. cerana* in the borderline region of northern and southern populations. Nevertheless, contribution of the male component on population genetic sense could not be inferred from mtDNA. As a result, it was necessary to investigate an agreement of population genetic information of two different sources of DNA markers.

Sittipraneed (2001) demonstrated genetic differentiation in *A. cerana* by 13 *A. mellifera* microsatellite loci using PCR. Three microsatellites (A28, A107 and A113) were shown to be polymorphic with number of alleles at each locus of 24, 10 and 3 alleles, respectively. The analysis of geographic heterogeneity and phylogenetic reconstruction using the Neighbor-Joining approach divided 5 geographic *A. cerana* samples to 4 different groups consisting of 1) northern (north and central); 2) north-eastern; 3) southern and 4) Samui Island. Microsatellite DNA loci A28, A107and A113 in nuclear genome were chosen a complete datas.

The amplified of srRNA gene, lrRNA gene and intergenic COI-COII regions were 400, 750 and 1710 bp. Three, four and eight haplotypes were found with *Dra*I digestion of amplified products from srRNA gene lrRNA and intergenic COI-COII regions, respectively. Each haplotype, which found in Sihanuntavong (1999), reported that except haplotype C of lrRNA gene was found in this investigation. Eleven composite haplotypes were occurred in 89 colonies and twelve complosite haplotypes were occurred when samples were added with 5 colonies and 1 colony from Yunnan and Hanoi. Among these twelve composite haplotypes, 9 composite haplotypes had already reported (Sihanuntavong, 1999) and 3 new composite haplotypes were observed in this study. Their genetic distance were used to reconstruct a dendogram using UPGMA, which was allocated *A. cerana* into 3 groups. Group A was composed of ADA, AAA, ADH and AAG. Group B was composed of BBF, BBB, BBE, BBC, AAB and BAB and group C was composed of CED. Generally, the composite haplotype of *A. cerana* in Prachuap Khiri Khan province was AAA. Whereas, composite haplotype BBB was mostly found in Chumphon. The difference between them was approximately 5 mutation steps. Sihanuntavong (1999) showed that the intermediate haplotype, AAB, which was far 2 and 3 mutation steps from AAA and BBB, respectively, was found in one sample each of central and north-east. Surprisingly, this intermediate composite haplotype AAB was found in Yunnan (5 samples or 100%) in this study. Six samples of a new intermediate haplotype, BAB, were found in borderline region of *A. cerana* northern and southern population (4 samples of Chumphon and 2 samples of Prachuap Khiri Khan). Restriction site difference from BAB to AAA and BBB were 4 and 1 mutation steps, respectively.

Referring to Sihanuntavong (1999), the distribution of group A of *A. cerana* was completely different from group B. That meant group A was northern population, group B was southern population. However, overlapping between group A and B was occurred in Prachuap Khiri Khan and Chumphon. Ninety-two percent of Prachuap Khiri Khan samples were group A and eight percent were group B. Twenty-six percent of Chumphon were group A, exceeding were group B.

The haplotype diversity values of Chumphon and Prachuap Khiri Khan were 0.7013 and 0.6524 and nucleotide diversity values were 0.0149 and 0.0085, respectively. Those values were higher than values of 5 geographic areas, which ranged from 0.1686 to 0.5967 and 0.0014 to 0.0048, investigated by Sihanuntavong. Concluding, the genetic diversity was maintained in contact area and the environment might effect to the genetic diversity. Haplotype diversity and nucleotide diversity of *A. cerana* in Yunnan and Hanoi were zero. Their genetic variations were under estimate because the number of sample was too small (5 colonies of Yunnan and a colony of Hanoi). Then, their sample number should be increased for determination of estimated genetic variations. Otherwise, geographic heterogeneities between Prachuap Khiri Khan, Chumphon and Yunnan were significant different ($P<0.0001$). Perhaps, the error was from sample, which had only one colony. In the opposite, each pairwise comparison with Hanoi was not significant different ($P = 0.02849$-0.6732) (Table 3.6). In addition, mtDNA results supported that *A. cerana* in borderline region had high genetic diversity values. And the highest diversity was in Chumphon where presented highest haplotype diversity and nucleotide diversity. Since amphur Bangsaphan, amphur Bangsaphan noi in Prachuap Khiri Khan province to amphur Tasae and amphur Pratue

in Chumphon province were presumed to be the contact zone because a little overlapping between group A and B of *A. cerana* was found. By the way, the hypothesis of colonization of 2 allopathic populations was acceptable if intermediate haplotype absented in this contact zone. The results showed the intermediate haplotype, which was defined by mutation steps between haplotype AAA and BBB, was least. Only six samples out of 95 samples were investigated to be intermediate haplotype BAB and other intermediate haplotypws were not found in contact zone. Besides, an intermediate haplotype AAB was observed in Yunnan where was above Thailand. Consequently, the mutation steps between haplotype AAA and BBB were definitely not from intergradation, but they were possibly from colonization. And group A honeybees might be colonized by Hanoi honeybees. Anyhow, Bugharuang (1996) analyzed genetic variation of *A. cerana* in Thailand by mtDNA sequencing on noncoding intergenic region and compared them with *A. cerana* from other countries. Southern population of Thailand was combined in the same group as Malaysia *A. cerana*. Possibly was group B honeybee colonized by *A. cerana* of Malaysia. The lower haplotype diversity in northern and southern honeybee populations might be continuously colonized by some haplotype of Hanoi and Malaysia honeybees.

Surveying the genetic variation in borderline region presented a colony of haplotype CED (group C) from Prachuap Khiri Khan. Its genetic distance to A or B was 3.03% sequence divergence. Thus, this surveying was similar to Sihanuntavong's and Songram's reports that surveyed genetic variation of *A. cerana* around Thailand (2 colonies from south had been found). All discovering above could be summarized group C to other species of *A. cerana*.

Microsatellite marker was additional chosen for this study because it previously showed high level of polymorphism in *A. mellifera*. Sittipraneed (2001) had analyzed genetic differentiation and population structure in Thailand honeybees, *A. cerana*, by 13 sets of microsatellite primer from *A. mellifera*. Three microsatellite loci (A28, A107 and A113) were selected for determination of genetic differentiation within five geographic samples of *A. cerana* in Thailand since they exhibited a greater number of alleles than any others. Geographic heterogeneity from microsatellite locus A113 could be separated any populations except north-central, north-northeast and northeast-south.

Four areas (central, south, Prachuap Khiri Khan and Chumphon) were chosen for studying with microsatellite. And since loci A28 and A107 had many stutter bands, the mistakes of analysis might be happened. Then, only one locus, A113, was analyzed. The most common genotype at this locus was 185-bp allele carrying high frequencies in all population. Moreover, observed heterozygosity of each population was high (0.451-0.550).

According to the results of each comparison, P-values were more than 0.05 and F_{ST} values were negative. They suggested genetic variations among four *A. cerana* populations were none and subpopulations were not occurred although the geographic heterogeneities of specimens from central and south populations used in this study were previously examined by Sittipraneed (2001) had shown differentiations. Sampling error and sampling areas, which were less number of colonies and collected near the contact zone, might cause no significant difference between central and south populations. Surprisingly, mtDNA and microsatellite markers were shown the contrary

results. Perhaps, a factor that possibly effected to high gene flow levels detected by microsatellite markers was crossed mating of male honeybees between Prachuap Khiri Khan and Chumphon provinces. Anyway, mtDNA markers shown the honeybees *A. cerana* was rather not migrate.

CHAPTER V

CONCLUSION

1. Contact zone of northern and southern honeybees (*A. cerana*) populations were Prachuap khiri khan (Amphur Bang saphan and Amphur Bang saphan noi) and Chumphon (Amphur pa thiu and Amphur Tha sae) provinces.
2. Composite haplotypes had overlapping distribution in contact zone.
3. High genetic diversity level of *A. cerana* in contact zone was observed from
   srRNAgene, lrRNAgene and intergenic COI-COII regions on mtDNA.
4. Northern and southern populations of *A. cerana* in Thailand might be colonized by Vietnam and Malaysia honeybees, respectively.
5. Microsatellite loci A113 could not divided Prachuap Khiri Khan and Chumphon honeybee *A. cerana* populations.

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APPENDIX A

Collection of *A. cerana* from North of Thailand

Code of colonies	Sampling area	Size of alleles (bp)			Composite haplotype		
		Locus A28	Locus A107	**Locus A113**	I	II	III
N1	Muang, Lamphun	118/125	167/167	186/186	ND	AAA	ND
N2	Muang, Uttaradit	118/125	167/167	186/186	AAA	AAA	ND
N3	Muang, Uttaradit	ND	167/167	186/196	ND	ND	ND
N5	Muang, Lamphun	115/115	165/167	186/186	AAA	ND	AAC
N6	Muang, Lamphun	125/125	167/167	186/186	AAA	AAA	ND
N7	San pa tong, Chiang mai	118/118	167/167	186/196	AAA	AAA	ND
N9	San pa tong, Chiang mai	118/118	167/167	186/186	AAA	AAA	AAC
N12	San pa tong, Chiang mai	115/118	167/167	186/196	ND	AAA	ND
N13	Hang ding, Chiang mai	118/121	167/167	186/186	AAA	AAA	AAC
N14	Muang, Uttaradit	ND	167/167	186/186	BAA	AAA	ND
N15	Muang, Phitsanulok	115/120	167/167	182/186	AAA	AAA	AAB
N16	Muang, Phitsanulok	118/125	167/167	186/196	ADA	AAA	AAC
N17	Muang, Phitsanulok	108/118	167/167	186/186	AAA	AAA	ND
N18	Phayuha khiri, Nakhon sawan	115/118	165/165	186/186	ND	ND	ND
N19	Phayuha khiri, Nakhon sawan	115/121	167/167	186/196	AAA	AAA	ND
N20	Manorom, Chai nat	113/120	167/167	186/186	AAA	AAA	ND
N21	Wat sing, Chai nat	116/116	ND	186/186	AAG	AAA	ND
N22	Muang, Chai nat	118/118	167/167	182/186	AAA	AAA	AAC
N23	Banphot phisai, Nakorn sawan	120/120	167/167	186/186	AAA	AAA	ND
N24	Banphot phisai, Nakorn sawan	118/118	167/167	186/186	ND	AAA	AAD
N25	Banphot phisai, Nakorn sawan	118/118	167/167	ND	AAA	AAA	ND
N26	Muang, Kamphaeng phet	117/120	167/167	186/196	AAA	AAA	ND
N27	Ban tak, Tak	118/118	167/167	186/196	AAA	AAA	AAC
N28	Ban tak, Tak	117/120	167/167	186/186	AAA	ND	ND
N29	Sam ngao, Tak	114/118	169/169	186/196	AAA	ACA	ND

Code of colonies	Sampling area	Size of alleles (bp)			Composite haplotype		
		Locus A28	Locus A107	Locus A113	I	II	III
N30	Sam ngao, Tak	118/120	158/161	182/186	AAA	ND	AAD
N31	Sam ngao, Tak	132/132	167/167	186/196	ND	ND	ND
N32	Thoen, Lampang	110/120	156/167	186/196	AAA	AAA	AAC
N33	Thoen, Lampang	117/119	ND	186/186	ND	ND	ND
N35	Mae tha, Lampang	112/120	167/167	186/186	ND	ND	AAE
N36	Mae tha, Lampang	121/121	167/167	186/196	AAA	AAA	ND
N37	Doi saket, Chiang mai	118/126	167/167	ND	AAA	AAA	AAC
N38	Doi saket, Chiang mai	119/119	167/167	186/186	ND	AAA	ND
N39	Doi saket, Chiang mai	121/129	157/167	186/186	AAA	AAA	ND
N40	Doi saket, Chiang mai	118/124	167/167	186/196	ND	ND	AAC
N41	Wiang pa pao, Chiang mai	118/120	167/167	186/196	AAA	ACA	AAC
N42	Wiang pa pao, Chiang mai	115/115	167/167	186/186	AAA	AAA	ND
N43	Wiang pa pao, Chiang mai	118/118	ND	ND	AAA	AAA	AAB
N44	Ngao, Lampang	114/119	158/167	186/186	AAA	AAA	AAC
N45	Sungmen, Phrae	112/117	157/167	186/196	AAA	AAA	AAB
N46	Sungmen, Phrae	119/119	167/167	186/186	AAA	AAA	ND
N47	Sungmen, Phrae	120/120	167/167	186/196	ND	AAA	AAB
N48	Sungmen, Phrae	114/119	159/167	186/186	AAA	AAA	ND

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

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

III = Composite haplotype of mtDNA control region digested with *Taq*I, *Rsa*I and *Hinf*I (Pootong, 1999)

ND = non determined

Collection of *A. cerana* from the Central of Thailand

Code of colonies	Sampling area	Size of alleles (bp)			Composite haplotype		
		Locus A28	Locus A107	Locus A113	I	II	III
C1	Phrapradaeng, Samut prakan	118/118	167/167	186/196	ADA	AAA	ND
C2	Kamphaengsan, Nakhorn pathom	115/115	165/167	ND	AAA	AAA	ND
C3	Dan chang, Suphan buri	118/121	167/167	182/186	AAA	ADA	ND
C4	Muang, Samut songkhram	115/115	158/167	182/186	AAA	AAA	ND
C5	Muang, Samut songkhram	118/118	167/167	186/186	AAA	AAA	ND
C6	Muang, Samut songkhram	120/120	158/158	186/196	AAH	AAA	ND
C7	Phrapradaeng, Samut prakan	118/118	167/167	186/186	ADA	AAA	ND
C8	Don tum, Nakhorn pathom	118/118	167/167	186/196	AAA	AAA	AAD
C9	Dan chang, Suphan buri	113/113	167/167	186/196	AAA	AAA	AAC
C10	Dan chang, Suphan buri	118/126	167/167	196/196	ADA	AAA	AAD
C11	Pong nam ron, Chanthaburi	117/117	167/167	196/196	AAA	AAA	ND
C12	Makham, Chanthaburi	118/118	167/167	182/196	AAA	AAA	AAC
C13	Makham, Chanthaburi	115/118	167/167	186/186	AAA	AAA	ND
C14	Makham, Chanthaburi	116/116	167/167	196/196	AAA	AAA	ND
C15	Muang, Trat	118/120	167/167	186/196	AAA	AAA	ND
C16	Khao saming Trat	120/120	167/167	186/196	ND	AAA	AAC
C17	Khao saming Trat	113/118	167/167	186/196	ND	AAA	ND
C18	Muang, Trat	115/121	161/166	186/186	AAA	AAA	AAD
C20	Sam roi yod, Prachuap khiri khan	120/124	157/157	186/196	ND	AAA	AAB
C21	Sam roi yod, Prachuap khiri khan	110/118	156/156	186/186	ND	AAA	ND
C25	Mae krong, Samut songkhram	118/122	167/167	182/186	ND	AAA	AAB
C26	Mae krong, Samut songkhram	114/114	167/167	196/196	ND	AAA	ND
C27	Mae krong, Samut songkhram	118/118	167/167	186/196	ND	AAA	AAC
C28	Mae krong, Samut songkhram	115/120	167/167	186/196	ND	AAA	AAB
C29	Mae krong, Samut songkhram	118/118	167/167	186/186	ND	AAA	ND
C30	Muang, Samut sakhorn	115/120	167/167	182/186	ND	AAA	ND
C33	Cha-am, Phetchaburi	118/118	167/167	186/186	ADA	ND	AAB
C34	Hua hin, Prachuap khiri khan	122/122	157/167	186/186	AAG	ND	AAD

Code of colonies	Sampling area	Size of alleles (bp)			Composite haplotype		
		Locus A28	Locus A107	Locus A113	I	II	III
C35	Hua hin, Prachuap khiri khan	116/119	167/167	186/186	AAA	ND	ND
C36	Pran buri, Prachuap khiri khan	121/121	167/167	186/186	AAA	ND	AAD
C37	Kui buri, Prachuap khiri khan	121/121	167/167	186/186	AAA	ND	AAD
C38	Muang, Prachuap khiri khan	114/120	167/167	186/186	ADA	ND	AAB
C39	Thap sakae, Prachuap khiri khan	119/125	157/167	186/196	ADA	ND	AAB
C40	Thap sakae, Prachuap khiri khan	121/125	167/167	182/186	AAA	ND	ND
C41	Bang saphan, Prachuap khiri khan	109/121	167/167	186/196	AAA	ND	AAB
C42	Bang saphan, Prachuap khiri khan	119/119	167/167	186/196	AAA	ND	ND
C43	Bang saphan, Prachuap khiri khan	ND	167/167	ND	ADA	ND	AAB
C44	Bang saphan, Prachuap khiri khan	121/125	167/167	186/186	ADA	ND	ND
C45	Bang saphan, Prachuap khiri khan	116/119	167/167	196/196	AAA	ND	AAD
C46	Thap sakae, Prachuap khiri khan	115/117	167/167	182/186	AAA	ND	AAB
C47	Kui buri, Prachuap khiri khan	ND	ND	185/195	AAA	ND	ND
C48	Thap sakae, Prachuap khiri khan	ND	ND	180/195	AAA	ND	ND
C49	Thap sakae, Prachuap khiri khan	ND	ND	185/195	ADA	ND	ND
C50	Bang saphan, Prachuap khiri khan	ND	ND	185/185	AAA	ND	ND
C51	Bang saphan, Prachuap khiri khan	ND	ND	185/185	AAA	ND	ND
C52	Bang saphan, Prachuap khiri khan	ND	ND	185/195	AAA	ND	ND
C53	Bang saphan, Prachuap khiri khan	ND	ND	185/185	AAA	ND	ND
C54	Bang saphan, Prachuap khiri khan	ND	ND	185/195	AAA	ND	ND
C55	Bang saphan, Prachuap khiri khan	ND	ND	185/195	ADA	ND	ND
C56	Bang saphan, Prachuap khiri khan	ND	ND	185/195	AAA	ND	ND
C57	Bang saphan, Prachuap khiri khan	ND	ND	185/195	ADA	ND	ND
C58	Bang saphan noi, Prachuap khiri khan	ND	ND	185/185	ADA	ND	ND
C59	Bang saphan noi, Prachuap khiri khan	ND	ND	195/195	CED	ND	ND
C60	Bang saphan noi, Prachuap khiri khan	ND	ND	185/195	ADA	ND	ND
C61	Thap sakae, Prachuap khiri khan	ND	ND	185/195	AAA	ND	ND
C62	Thap sakae, Prachuap khiri khan	ND	ND	185/195	AAA	ND	ND

Code of colonies	Sampling area	Size of alleles (bp)			Composite haplotype		
		Locus A28	Locus A107	**Locus A113**	I	II	III
C63	Thap sakae, Prachuap khiri khan	ND	ND	185/185	AAA	ND	ND
C64	Bang saphan, Prachuap khiri khan	ND	ND	185/185	AAA	ND	ND
C65	Bang saphan, Prachuap khiri khan	ND	ND	185/185	AAA	ND	ND
C66	Bang saphan, Prachuap khiri khan	ND	ND	195/195	AAA	ND	ND
C67	Bang saphan, Prachuap khiri khan	ND	ND	185/185	ADA	ND	ND
C68	Bang saphan, Prachuap khiri khan	ND	ND	185/185	BAB	ND	ND
C69	Bang saphan, Prachuap khiri khan	ND	ND	185/185	AAA	ND	ND
C70	Bang saphan, Prachuap khiri khan	ND	ND	185/185	AAA	ND	ND
C71	Bang saphan, Prachuap khiri khan	ND	ND	185/185	AAA	ND	ND
C72	Bang saphan, Prachuap khiri khan	ND	ND	185/185	AAA	ND	ND
C73	Bang saphan noi, Prachuap khiri khan	ND	ND	185/185	AAA	ND	ND
C74	Bang saphan noi, Prachuap khiri khan	ND	ND	185/195	AAA	ND	ND
C75	Bang saphan noi, Prachuap khiri khan	ND	ND	185/185	BBB	ND	ND
C76	Bang saphan noi, Prachuap khiri khan	ND	ND	185/185	ADA	ND	ND
C77	Bang saphan noi, Prachuap khiri khan	ND	ND	185/185	BBB	ND	ND
C78	Bang saphan noi, Prachuap khiri khan	ND	ND	185/185	BAB	ND	ND
C79	Bang saphan noi, Prachuap khiri khan	ND	ND	185/185	ADA	ND	ND
C80	Bang saphan noi, Prachuap khiri khan	ND	ND	185/195	ADA	ND	ND
C81	Bang saphan noi, Prachuap khiri khan	ND	ND	185/195	AAA	ND	ND
C82	Bang saphan noi, Prachuap khiri khan	ND	ND	185/195	AAA	ND	ND
C83	Bang saphan noi, Prachuap khiri khan	ND	ND	185/195	AAA	ND	ND

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

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

III = Composite haplotype of mtDNA control region digested with *Taq*I, *Rsa*I and *Hinf*I (Pootong, 1999)

ND = non determined

Collection of *A. cerana* from the North-East Thailand

Code of colonies	Sampling area	Size of alleles (bp)			Composite haplotype		
		Locus A28	Locus A107	Locus A113	I	II	III
NE1	Na haeo, Loai	112/119	167/167	186/186	ND	ND	ND
NE2	Na haeo, Loai	115/115	167/167	186/196	ND	AAA	ND
NE3	Na haeo, Loai	119/119	167/167	186/196	AAA	AAA	ND
NE14	Muang, Khonkaen	115/123	167/167	186/186	AAA	AAA	ND
NE15	Muang, Khonkaen	118/118	167/167	186/186	ADA	AAA	AAE
NE16	Chum phae, Khonkaen	119/119	167/167	186/186	AAA	AAA	ND
NE17	Chum phae, Khonkaen	120/126	156/167	186/186	AAA	AAA	ND
NE18	Chum phae, Khonkaen	108/112	167/167	186/196	AAH	AAD	ND
NE19	Chum phae, Khonkaen	114/119	167/167	196/196	AAH	ND	ND
NE20	Ubol ratana, Khonkaen	121/121	167/167	186/186	AAA	ACA	ND
NE21	Non sang, Nong bua lamphu	118/120	167/167	186/186	AAA	AAA	AAF
NE22	Non sang, Nong bua lamphu	115/120	167/169	186/196	AAA	AAA	ND
NE23	Non sang, Nong bua lamphu	120/127	167/169	182/186	AAA	AAA	AAB
NE24	Nong wua so, Udon thani	119/123	159/167	196/196	AAA	AAA	ND
NE25	Muang, Udon thani	119/119	156/167	196/196	AAA	AAA	ND
NE26	Muang, Udon thani	119/127	158/167	186/186	AAA	AAA	ND
NE27	Muang, Udon thani	116/116	167/167	186/186	AAA	AAA	ND
NE28	Muang, Nong khai	121/121	156/159	186/186	AAA	AAA	ND
NE29	Muang, Nong khai	120/123	165/165	186/196	AAA	AAA	ND
NE30	Nong han, Udon thani	122/127	157/167	186/186	AAA	AAA	ND
NE31	Phang khon, Sakon nakhon	119/119	167/167	ND	AAA	AAA	ND
NE32	Nong bun nak, Nakhon ratchasima	114/127	167/167	ND	AAA	AAA	ND
NE33	Nong bun nak, Nakhon ratchasima	119/119	167/167	186/186	AAA	AAA	ND
NE34	Nong bun nak, Nakhon ratchasima	127/127	167/167	186/186	AAA	ND	ND
NE35	Nong bun nak, Nakhon ratchasima	108/125	167/167	196/196	ND	ND	ND
NE36	Kranuan, Khonkean	121/121	159/166	186/196	ND	ND	ND
NE39	Kranuan, Khonkean	117/121	159/167	186/186	ND	ND	ND
NE40	Kranuan, Khonkean	115/117	167/167	186/186	ND	ND	ND

Code of colonies	Sampling area	Size of alleles (bp)			Composite haplotype		
		Locus A28	Locus A107	Locus A113	I	II	III
NE41	Kranuan, Khonkean	118/122	167/167	186/186	ND	ND	ND
NE42	Prakhon chai, Buriram	120/120	167/167	186/186	ND	ND	ND
NE43	Prakhon chai, Buriram	114/118	167/167	186/196	ND	ND	AAC
NE44	Prakhon chai, Buriram	118/126	167/167	196/196	ND	ND	ND
NE45	Prakhon chai, Buriram	114/118	167/167	196/196	ND	ND	ND
NE46	Prakhon chai, Buriram	114/114	167/167	196/196	ND	ND	ND
NE47	Prakhon chai, Buriram	119/119	167/167	186/196	ND	ND	AAD
NE52	Rattanaburi, Surin	118/125	167/167	186/196	ND	ND	AAD
NE55	Prasat, Surin	119/120	167/167	186/186	ND	ND	AAB
NE56	Prasat, Surin	120/126	167/167	186/186	ND	ND	AAB
NE57	Prasat, Surin	126/126	167/167	186/186	ND	ND	ND
NE59	Prasat, Surin	126/126	167/167	186/196	ND	ND	ND
NE60	Prasat, Surin	126/126	167/167	186/186	ND	ND	ND
NE61	Nong bun nak, Nakhon ratchasima	118/120	159/167	186/186	ND	ND	AAB
NE62	Nong bun nak, Nakhon ratchasima	120/122	159/167	186/186	ND	ND	AAB
NE63	Nong bun nak, Nakhon ratchasima	116/120	159/167	186/186	ND	ND	AAD
NE64	Nong bun nak, Nakhon ratchasima	121/122	167/167	186/186	ND	ND	AAB
NE65	Nong bun nak, Nakhon ratchasima	117/122	167/167	186/196	ND	ND	ND
NE66	Nong bun nak, Nakhon ratchasima	117/118	167/167	186/186	ND	ND	AAD
NE70	Thawatchaburi, Roiet	126/126	167/167	186/196	ND	ND	AAB
NE71	Thawatchaburi, Roiet	122/126	167/167	186/186	ND	ND	ND
NE72	Warin chamrab, Ubon ratchathani	115/126	167/167	196/196	ND	ND	AAB
NE73	Muang, Si sa ket	118/120	167/167	186/196	ND	ND	ND
NE74	Muang, Kalasin	115/120	159/167	196/196	ND	ND	ND
NE75	Muang, Khonkaen	119/120	167/167	186/186	ND	ND	AAB

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

II = Composite haplotype of ATPase6-ATPase8 gene of mtDNA

digested with *Taq*I, *Ssp*I and *Vsp*I (Songram, 1997)

III = Composite haplotype of mtDNA control region digested with *Taq*I, *Rsa*I and *Hinf*I (Pootong, 1999)

ND = non determined

Collection of *A. cerana* from the South of Thailand

Code of colonies	Sampling area	Size of alleles (bp)			Composite haplotype		
		Locus A28	Locus A107	Locus A113	I	II	III
S1	Thalang, Phuket	116/120	167/167	186/196	BBB	ND	ND
S2	Muang, Chumphon	121/125	167/167	196/196	CED	AEF	ND
S3	Muang, Chumphon	117/123	167/167	186/196	BBB	ND	ND
S4	Tha chana, Suratthani	123/128	169/169	196/196	ND	ND	BBA
S5	Muang, Chumphon	120/120	167/167	186/186	ND	ND	ND
S6	Tha chana, Suratthani	114/122	167/169	186/196	BBB	ND	BBA
S8	Tha chana, Suratthani	118/124	167/167	196/196	ND	ND	BBA
S9	Tha chana, Suratthani	118/121	167/169	186/186	ND	ND	BBA
S10	Tha chana, Suratthani	ND	159/169	196/196	BBB	ND	BBA
S11	Muang, Phuket	121/121	167/167	ND	ND	BBB	ND
S13	Muang, Phuket	116/126	166/167	186/196	BBB	ND	BBA
S14	Thalang, Phuket	117/119	167/169	196/196	BBB	ND	ND
S15	Thalang, Phuket	ND	167/167	186/186	BBB	ND	ND
S16	Muang, Phuket	126/130	167/167	196/196	BBB	ND	BBA
S17	Sawi, Chumphon	115/117	167/167	186/186	BBB	ND	BBA
S18	Sawi, Chumphon	118/118	167/167	186/196	BBB	BBB	ND
S19	Sawi, Chumphon	119/119	167/167	186/186	ND	ND	ND
S20	Sawi, Chumphon	ND	167/169	186/186	BBB	ND	ND
S21	Muang, Chumphon	114/114	161/169	186/196	BBB	ND	ND
S22	Muang, Chumphon	114/119	167/167	186/186	BBB	ND	BBA
S23	Muang, Chumphon	114/118	155/167	186/196	BBB	BBB	ND
S24	Muang, Chumphon	124/128	167/169	186/196	ND	BBB	ND
S25	Kra buri, Ranong	116/126	167/167	196/196	BBB	ND	ND
S26	Kra buri, Ranong	117/123	167/169	186/196	ND	ND	ND
S27	Kapoe, Ranong	114/118	167/167	186/196	ND	ND	BBA
S28	Kapoe, Ranong	121/121	167/167	186/196	BBB	BBB	ND
S29	Kapoe, Ranong	121/125	169/169	196/196	BBB	ND	BBJ
S30	Kapoe, Ranong	119/124	167/167	186/196	BBB	BBB	ND

Code of colonies	Sampling area	Size of alleles (bp)			Composite haplotype		
		Locus A28	Locus A107	Locus A113	I	II	III
S31	Muang, Ranong	118/121	155/167	186/186	BBB	BBB	BBA
S32	Muang, Ranong	117/125	167/169	186/196	ND	BBB	BBA
S33	Khuraburi, Phang nga	125/125	167/167	186/196	BBB	BBB	ND
S34	Khuraburi, Phang nga	117/128	167/169	186/196	BBB	BBB	BBA
S35	Phanom, Suratthani	114/117	167/169	186/186	BBB	BBB	BBA
S36	Ao luk, Krabi	109/123	167/167	186/186	BBB	BBB	BBA
S37	Ao luk, Krabi	116/119	ND	186/186	BBB	BBB	ND
S38	Ao luk, Krabi	114/122	167/167	186/186	BBE	BBB	BBA
S39	Ao luk, Krabi	114/118	167/167	186/186	ND	BBB	ND
S40	Ao luk, Krabi	121/124	167/167	ND	BBB	BBB	ND
S41	Muang, Krabi	125/125	167/167	186/186	ND	BBB	ND
S42	Muang, Krabi	114/119	167/167	186/196	BBB	BBB	BBA
S43	Muang, Krabi	118/123	167/167	186/186	BBB	BBB	ND
S44	Muang, Krabi	119/119	167/167	186/186	ND	BBB	BBA
S45	Muang, Krabi	119/124	167/167	186/196	ND	BBB	BBA
S46	Nua khlohg, Krabi	124/124	167/167	186/196	ND	BBB	ND
S47	Nua khlohg, Krabi	116/116	167/167	186/186	BBB	BBB	BBA
S48	Nua khlohg, Krabi	116/125	167/167	186/196	BBB	BBB	BBA
S49	Nua khlohg, Krabi	111/124	167/167	186/186	ND	BBB	BBA
S50	Khao phanom, Krabi	118/127	167/167	186/196	BBB	BBB	ND
S51	Thung yai, Nakhon sri thammarat	124/127	167/167	196/196	BBC	BBB	BBG
S52	Thung yai, Nakhon sri thammarat	118/118	ND	ND	BBB	BBB	ND
S53	Thung yai, Nakhon sri thammarat	125/125	167/169	186/186	BBC	BBB	BBG
S54	Sikao, Trang	120/127	167/167	186/186	BBB	BBB	BBA
S55	Huai yot, Trang	118/126	167/167	186/196	ND	BBB	ND
S56	Huai yot, Trang	124/127	167/167	186/186	BBB	BBB	ND
S57	Huai yot, Trang	121/125	167/169	196/196	BBB	BBB	BBA
S58	Huai yot, Trang	124/127	167/167	196/196	ND	BBB	ND

Code of colonies	Sampling area	Size of alleles (bp)			Composite haplotype		
		Locus A28	Locus A107	Locus A113	I	II	III
S59	Sri banphot, Phatthalung	118/118	167/167	186/196	BBF	BBB	ND
S60	Sri banphot, Phatthalung	109/126	ND	196/196	CED	AEE	ND
S61	Sri banphot, Phatthalung	127/127	167/169	186/186	ND	BBB	BBA
S62	Pa bon, Phatthalung	120/124	167/167	186/196	BBB	BBB	BBA
S63	Hat yai, Songkhla	113/121	167/169	186/186	BBB	BBB	BBA
S64	Muang, Songkhla	109/121	167/167	196/196	BBB	BBB	ND
S65	Muang, Songkhla	119/119	167/167	186/186	BBE	BBB	ND
S66	Muang, Songkhla	125/125	167/167	186/196	BBC	BBB	BBA
S67	Muang, Songkhla	121/121	167/167	196/196	ND	BBB	ND
S68	Muang, Songkhla	121/125	167/167	186/186	BBB	BBB	BBA
S69	Nakhon sri thammarat	117/121	167/167	186/196	BBB	BBB	BBA
S70	Nakhon sri thammarat	125/125	167/167	ND	BBB	BBB	BBA
S71	Tha sala, Nakhon sri thammarat	119/119	167/167	186/196	BBB	BBB	BBA
S72	Tha sala, Nakhon sri thammarat	117/117	167/169	186/196	ND	BBB	BBA
S73	Tha sala, Nakhon sri thammarat	118/118	167/167	186/196	ND	ND	BBA
S74	Tha sae, Chumphon	ND	ND	185/185	ADH	ND	ND
S75	Tha sae, Chumphon	ND	ND	185/195	BBB	ND	ND
S76	Tha sae, Chumphon	ND	ND	185/195	BBB	ND	ND
S77	Tha sae, Chumphon	ND	ND	185/195	BBB	ND	ND
S78	Tha sae, Chumphon	ND	ND	185/185	BBB	ND	ND
S79	Tha sae, Chumphon	ND	ND	185/195	BBB	ND	ND
S80	Tha sae, Chumphon	ND	ND	185/185	BBB	ND	ND
S81	Tha sae, Chumphon	ND	ND	185/195	BBB	ND	ND
S82	Pathiu, Chumphon	ND	ND	195/195	BBB	ND	ND
S83	Pathiu, Chumphon	ND	ND	185/195	ADA	ND	ND
S84	Pathiu, Chumphon	ND	ND	185/185	AAA	ND	ND
S85	Tha sae, Chumphon	ND	ND	185/195	AAA	ND	ND
S86	Tha sae, Chumphon	ND	ND	185/185	ADH	ND	ND

Code of colonies	Sampling area	Size of alleles (bp)			Composite haplotype		
		Locus A28	Locus A107	Locus A113	I	II	III
S87	Pathiu, Chumphon	ND	ND	185/185	ADA	ND	ND
S88	Pathiu, Chumphon	ND	ND	185/185	ADA	ND	ND
S89	Pathiu, Chumphon	ND	ND	185/195	BBB	ND	ND
S90	Pathiu, Chumphon	ND	ND	185/195	AAA	ND	ND
S91	Pathiu, Chumphon	ND	ND	185/185	AAA	ND	ND
S92	Pathiu, Chumphon	ND	ND	185/195	AAA	ND	ND
S93	Sawi, Chumphon	ND	ND	185/185	BBB	ND	ND
S94	Muang, Chumphon	ND	ND	185/185	BBE	ND	ND
S95	Sawi, Chumphon	ND	ND	185/195	BAB	ND	ND
S96	Sawi, Chumphon	ND	ND	185/195	BAB	ND	ND
S97	Sawi, Chumphon	ND	ND	195/195	BAB	ND	ND
S98	Muang, Chumphon	ND	ND	185/195	BAB	ND	ND
S99	Muang, Chumphon	ND	ND	185/185	BAC	ND	ND
S100	Muang, Chumphon	ND	ND	185/185	BBB	ND	ND
S101	Muang, Chumphon	ND	ND	185/195	BBB	ND	ND
S102	Muang, Chumphon	ND	ND	185/195	BBB	ND	ND
S103	Muang, Chumphon	ND	ND	195/195	BBF	ND	ND
S104	Muang, Chumphon	ND	ND	185/185	BBB	ND	ND
S105	Muang, Chumphon	ND	ND	185/185	BBB	ND	ND
S106	Muang, Chumphon	ND	ND	185/185	BBB	ND	ND
S107	Muang, Chumphon	ND	ND	185/195	BBB	ND	ND
S108	Muang, Chumphon	ND	ND	185/185	BBC	ND	ND
S109	Muang, Chumphon	ND	ND	185/195	BBB	ND	ND
S110	Muang, Chumphon	ND	ND	185/185	BBB	ND	ND
S111	Muang, Chumphon	ND	ND	185/195	BBB	ND	ND

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

II = Composite haplotype of ATPase6-ATPase8 gene of mtDNA

digested with *Taq*I, *Ssp*I and *Vsp*I (Songram, 1997)

III = Composite haplotype of mtDNA control region digested with *Taq*I, *Rsa*I and *Hinf*I (Pootong, 1999)

ND = non determined

Collection of *A. cerana* from the Samui Island of Thailand

Code of colonies	Sampling area	Size of alleles (bp)			Composite haplotype		
		Locus A28	Locus A107	Locus A113	I	II	III
I4	Tham Bon Aungthong	118/124	167/167	196/196	ND	BBB	BBA
I5	Tham Bon Aungthong	124/124	167/167	196/196	ND	ND	ND
I6	Tham Bon Aungthong	118/124	167/167	196/196	ND	BBB	BCA
I9	Tham Bon Bophut	118/124	167/167	196/196	BBB	BBB	BCA
I10	Tham Bon Maret	118/118	167/167	196/196	BBB	BBB	BBA
I12	Tham Bon Maret	118/121	167/167	196/196	BBB	BBB	BBA
I14	Tham Bon Lipanoi	118/118	167/167	196/196	BBB	BBB	BBA
I16	Tham Bon Maenam	118/118	167/167	196/196	BCB	BBC	ND
I17	Tham Bon Maenam	124/124	167/167	196/196	BBB	BBB	BBA
I19	Tham Bon Maenam	118/126	167/167	196/196	BBB	BBB	BBA
I20	Tham Bon Maenam	119/119	167/167	196/196	BBB	BBB	BBA
I21	Tham Bon Maenam	118/124	167/167	196/196	BCB	BBC	BCH
I22	Tham Bon Maenam	116/124	167/167	196/196	BCB	BBC	BCI
I23	Tham Bon Maenam	124/124	167/167	196/196	BCC	BBC	ND
I24	Tham Bon Maenam	118/118	167/167	196/196	BCB	BBC	ND
I25	Tham Bon Bophut	124/124	167/167	196/196	BBB	BBB	BBA
I26	Tham Bon Bophut	118/124	167/167	196/196	BBB	BBB	BBA
I27	Tham Bon Bophut	114/124	167/167	196/196	BCC	BBC	BCA
I28	Tham Bon Bophut	124/124	167/167	196/196	BBB	BBB	BBA
I29	Tham Bon Maret	114/119	167/167	196/196	BCC	BBC	BCA
I30	Tham Bon Maret	124/124	167/167	196/196	BCC	BBC	BBA
I31	Tham Bon Maret	116/124	167/167	196/196	BCC	BBC	BBA
I32	Tham Bon Maret	118/126	167/167	196/196	BCC	BBC	BCH
I33	Tham Bon Maret	118/118	167/167	196/196	BCC	BBC	ND
I34	Tham Bon Maret	118/118	167/167	196/196	ND	BBB	BBA
I35	Tham Bon Maret	124/124	167/167	196/196	BBB	BBB	ND
I36	Tham Bon Maret	118/124	167/167	196/196	BCC	BBC	BBA
I37	Tham Bon Bophut	118/124	167/167	196/196	BBB	BBB	BBA

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

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

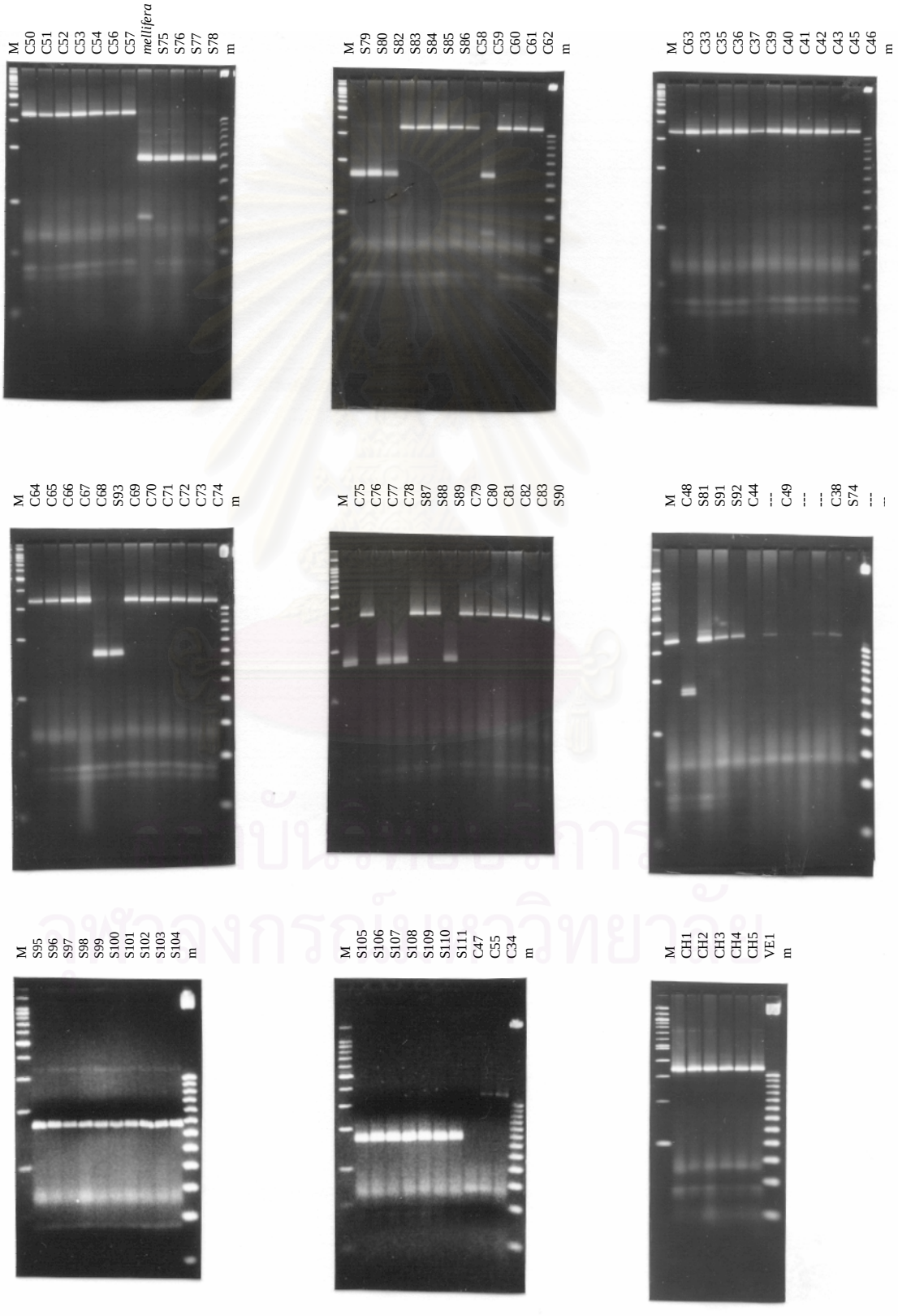
III = Composite haplotype of mtDNA control region digested with *Taq*I, *Rsa*I and *Hinf*I (Pootong, 1999)

ND = non determined

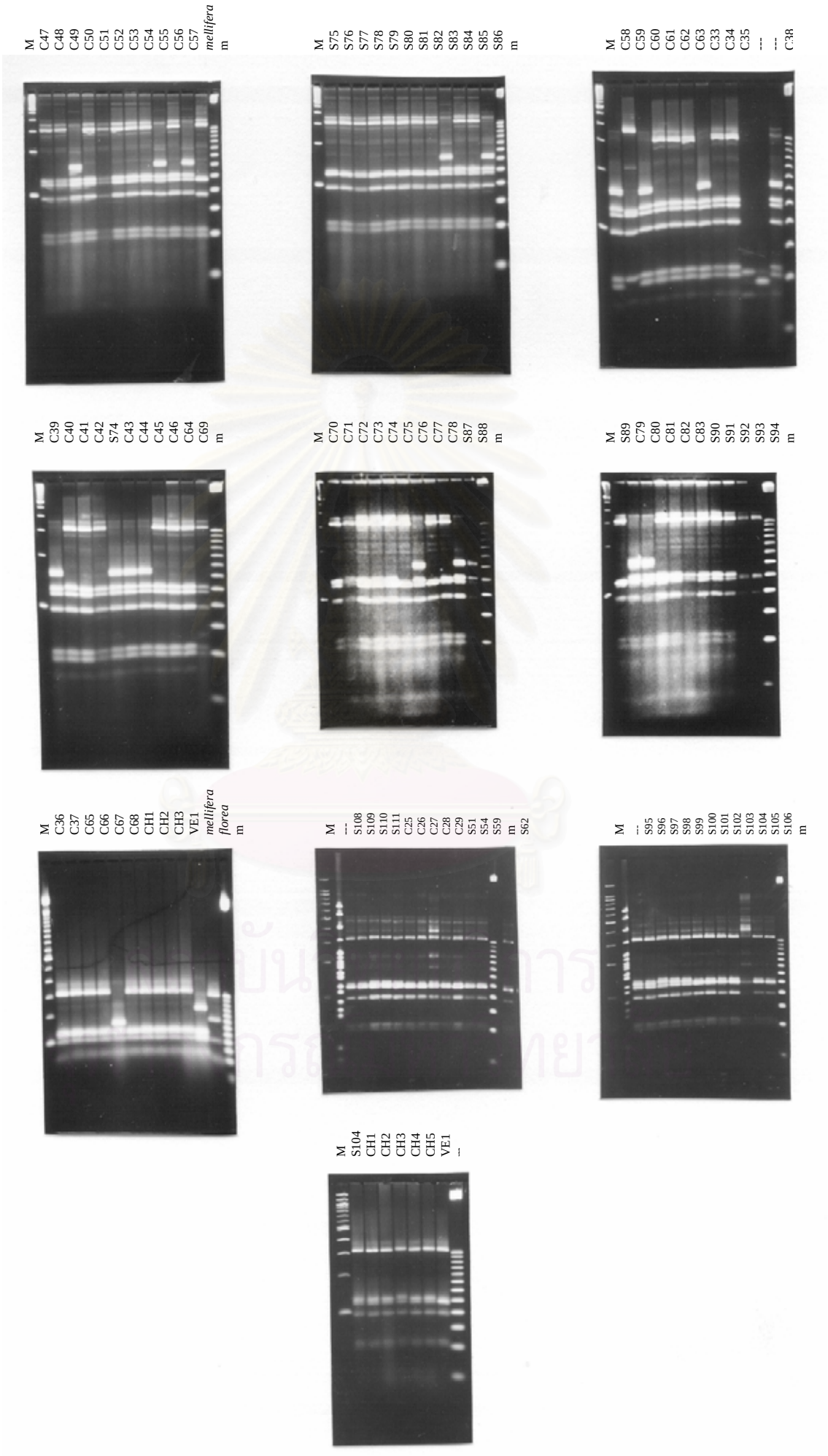
APPENDIX B

The *Dra*I restriction patterns of the amplified products of *A. cerana* samples in four geographic areas

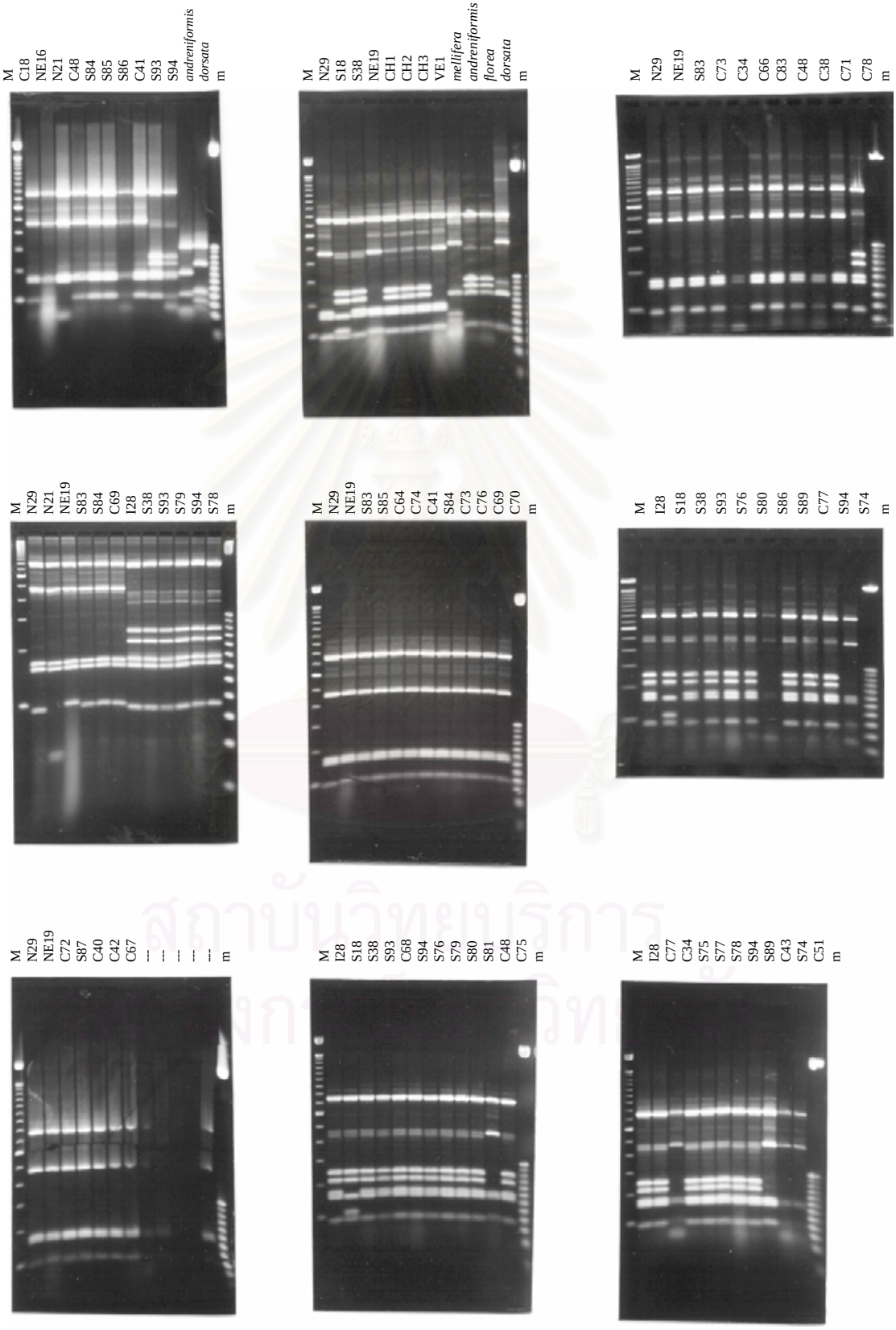
- srRNA gene / *Dra*I

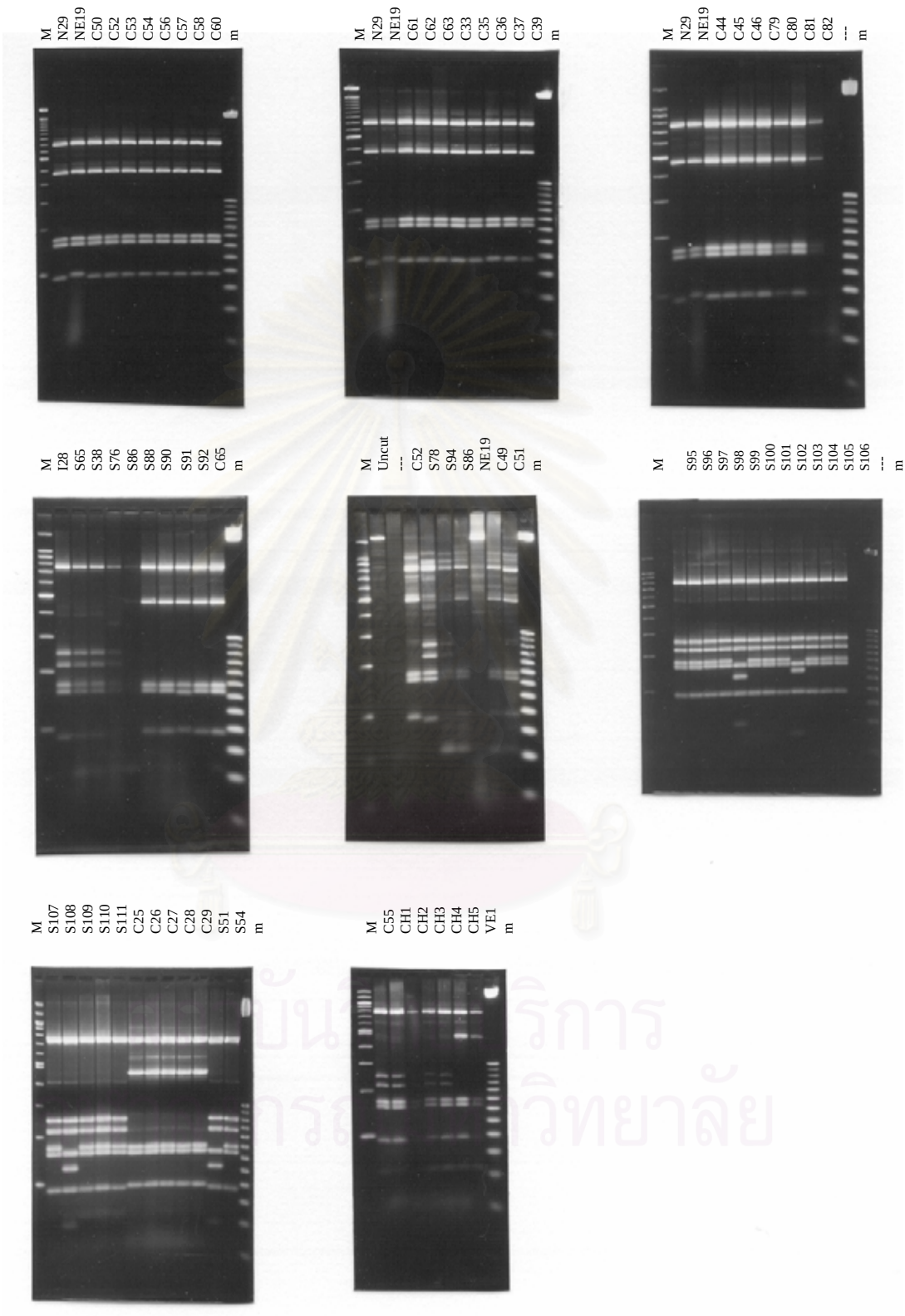


• lrRNA gene / *Dra*I



• intergenic COI-COII regions / *Dra*I



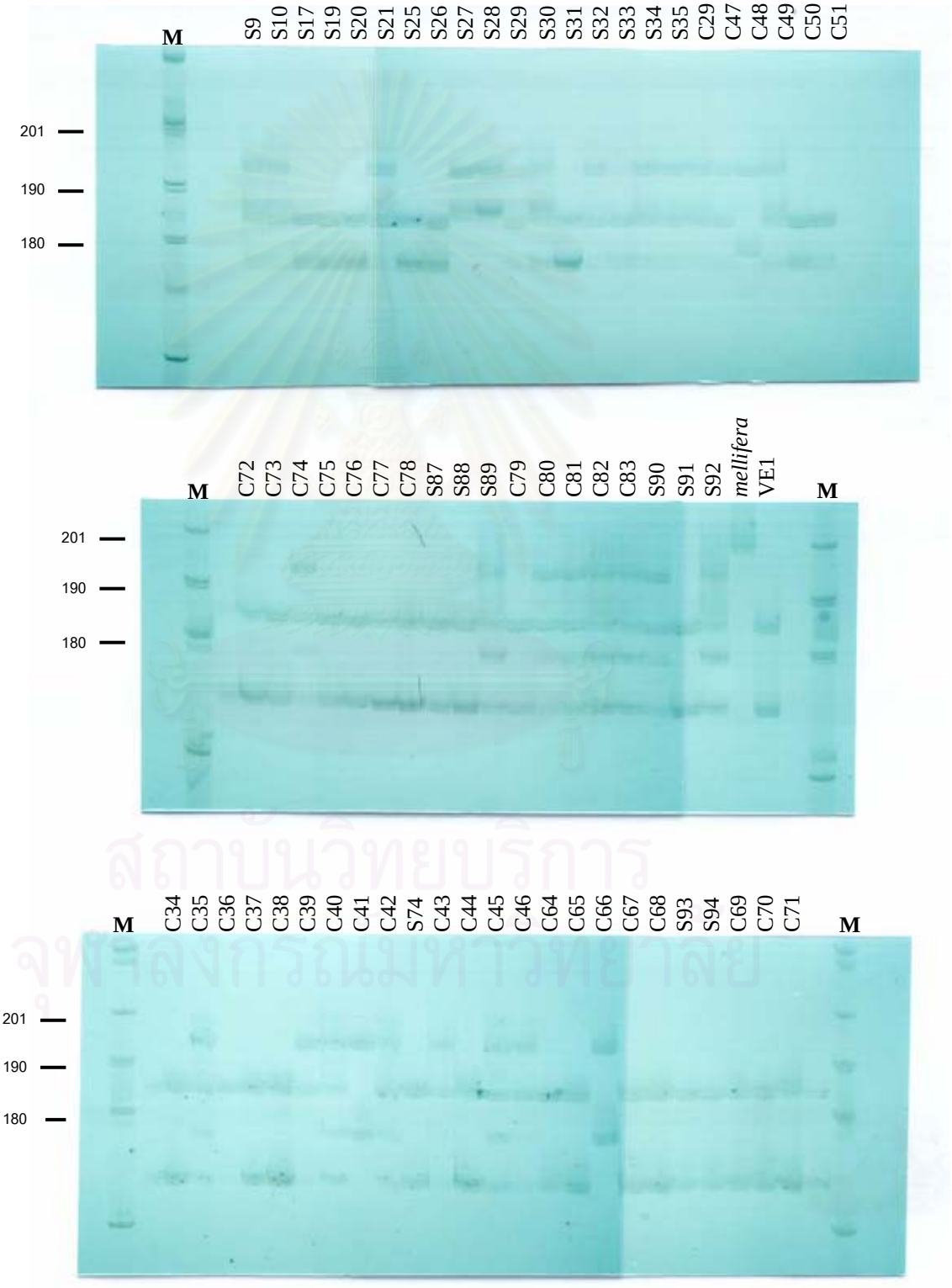


M : 100 bp DNA ladder

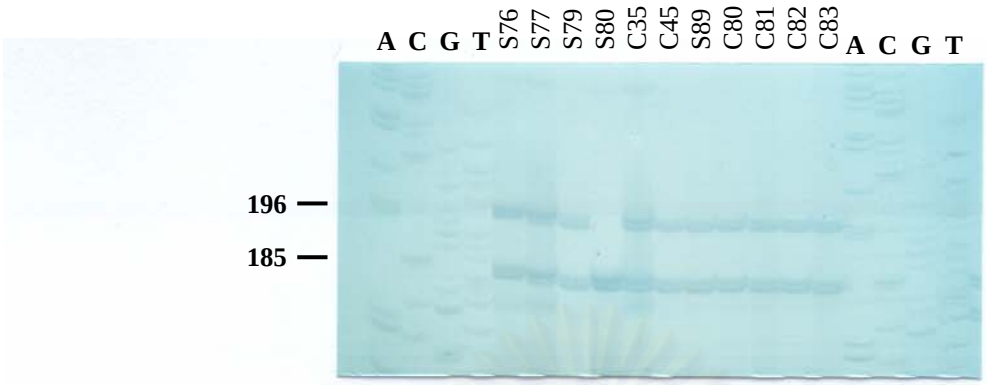
m : 25 bp DNA ladder

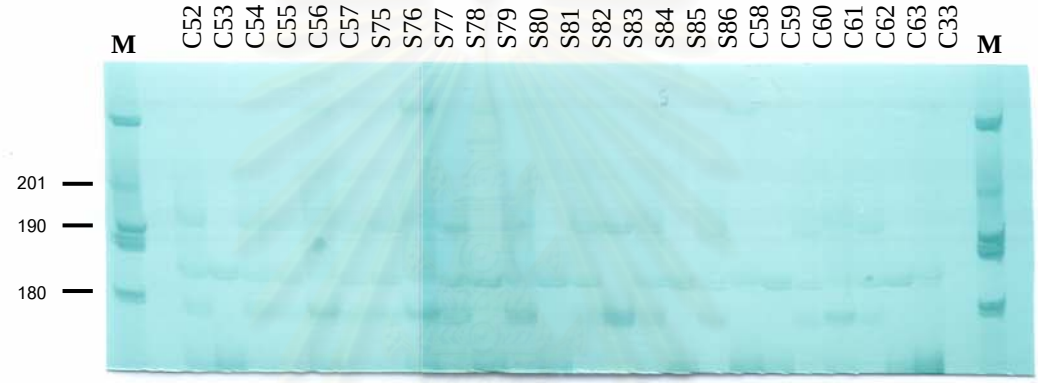
APPENDIX C

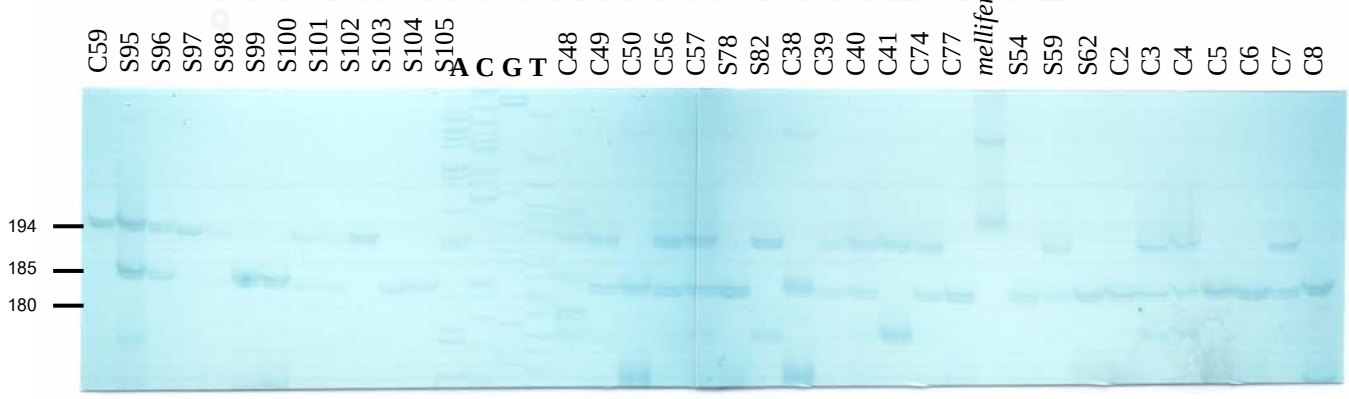
PCR amplification patterns of microsatellite locus A113 of *A. cerana* in four geographic areas



M = *pBR*322 / *Msp*I







BIOGRAPHY

Mr. Wachira Suktawonjarearnpon was born on August 3, 1977. He graduated with the Bachelor's degree of Science and Technology from Mahidol University in 1998.