จุฬาลงกรณ์มหาวิทยาลัย

ทุนวิจัย
กองทุนรัชดาภิเษกสมโภช

รายงานผลการวิจัย

การศึกษาการแพร่กระจายของประชากรผึ้งโพรง *Apis cerana* กลุ่มตอนเหนือและตอนใต้ในบริเวณพื้นที่รอยต่อโดยใช้ดีเอ็นเอเครื่องหมาย

โดย

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บทคัดย่อ

การตรวจสอบการกระจายของกลุ่มประชากรผึ้งโพรงกลุ่มเหนือและกลุ่มใต้ โดยใช้ PCR-RFLP ของไมโทคอนเดรียลดีเอ็นเอ 3 บริเวณ (ยีน srRNA, ยีน lrRNA และบริเวณระหว่างยีน COI – COII) ศึกษาผึ้ง 89 รังจากจังหวัดประจวบคีรีขันธ์และชุมพร พบว่าดีเอ็นเอที่เพิ่มปริมาณด้วยเทคนิค PCR ของยีน srRNA, ยีน lrRNA และบริเวณระหว่างยีน COI – COII มีขนาด 410, 755 และ 1710 คู่เบส ตามลำดับ หลังตัดด้วยเรสทริกชันเอนไซม์ *DraI* จะให้รูปแบบของแถบดีเอ็นเอเป็น 3, 4 และ 8 รูปแบบ ตามลำดับ เมื่อรวมรูปแบบของแถบดีเอ็นเอทั้ง 3 บริเวณของไมโทคอนเดรียลดังกล่าวเข้าด้วยกันจะให้รูปแบบรวม 11 รูปแบบ และจะได้รูปแบบรวม 12 รูปแบบ เมื่อรวมตัวอย่างผึ้งโพรงจากยูนนานและฮานอยเข้าไว้ด้วย เมื่อนำค่า genetic distance มาสร้างความสัมพันธ์เชิงวิวัฒนาการตามแบบ UPGMA จะสามารถแบ่งกลุ่มตัวอย่างในสองจังหวัดที่ศึกษาได้เป็น 2 กลุ่มคือ กลุ่มผึ้งโพรงตอนเหนือและกลุ่มตอนใต้ บริเวณที่มีการกระจายของผึ้งทั้ง 2 กลุ่มปนกันได้แก่ อำเภอบางสะพาน และบางสะพานน้อยของจังหวัดประจวบคีรีขันธ์ และอำเภอท่าแซะและประทิวของจังหวัดชุมพร จากการที่พบผึ้งซึ่งมีรูปแบบของไมโทคอนเดรียลเป็นรูปแบบผสมของผึ้งทั้ง 2 กลุ่ม เพียงรูปแบบเดียวคือ BAB ด้วยความถี่ที่ต่ำมาก แสดงให้เห็นว่ากลุ่มประชากรผึ้งตอนเหนือและตอนใต้น่าจะเกิดจากการเข้ามาตั้งรกรากจากผึ้งคนละกลุ่มกัน โดยผึ้งกลุ่มตอนเหนืออาจจะมาจากเวียดนาม

จากการพบรูปแบบรวมของไมโทคอนเดรียล 3 บริเวณในผึ้งโพรงหนึ่งตัวอย่างจากจังหวัดประจวบคีรีขันธ์เป็น CED ซึ่งมีความแตกต่างจากตัวอย่างอื่น ๆ อย่างมาก ทั้งที่มีสัณฐานคล้ายคลึงกัน น่าสงสัยว่าเป็นผึ้งต่างสปีชีย์ ดังนั้นจึงควรมีการศึกษาเพิ่มเติมเพื่อทราบสถานะทางอนุกรมวิธานที่แน่ชัดต่อไป

การวิเคราะห์ microsatellite DNA ในตัวอย่างผึ้งโพรง 4 บริเวณของประเทศไทย (1. ภาคกลาง 2. ภาคใต้ 3. ประจวบคีรีขันธ์ และ 4. ชุมพร) โดยใช้ microsatellite primer ของผึ้งพันธุ์ (*A. mellifera*) ที่ตำแหน่ง A28, A107 และ A113 พบว่าตำแหน่ง A28 และ A107 ให้ผลเป็นแถบดีเอ็นเอซ้อนทับกันมาก การอ่านขนาดดีเอ็นเออาจผิดพลาดได้ง่าย จึงวิเคราะห์ผลจาก microsatellite ที่ตำแหน่ง A113 เพียงตำแหน่งเดียว โดยได้ค่า heterozygosity เป็น 0.451 – 0.550 การวิเคราะห์ความแตกต่างของผึ้งโพรงทั้ง 4 บริเวณของประเทศไทย พบว่าไม่สามารถแบ่งกลุ่มประชากรออกจากกันได้ แสดงให้เห็นว่าน่าจะมีการผสมพันธุ์ข้ามบริเวณของผึ้งเพศผู้ในบริเวณซ้อนทับซึ่งอยู่ในจังหวัดประจวบคีรีขันธ์และชุมพร

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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ABSTRACT

PCR-RFLP of three mtDNA regions (srRNA gene, lrRNA gene and intergenic COI – COII region) were used to investigated the distribution of northern and southern *Apis cerana* populations of 89 colonies from Prachuap Khiri Khan and Chumphon provinces. Three, four and eight haplotypes were obtained from *Dra*I digestion of PCR- amplified 410 bp srRNA gene, 755 bp lrRNA gene and 1710 bp intergenic COI – COII region, respectively. These three mtDNA regions generated 11 composite haplotypes. Twelve composite haplotypes were generated when samples from Yunnan and Hanoi were included. A UPGMA phenogram based on genetic distance allocated *A. cerana* in these provinces into 2 distinct groups : northern and southern. Their distribution areas had overlapped in Amphur Bang Sapan (Prachuap Khiri Khan), Bang Saphan Noi (Prachuap Khiri Khan), Tha Sae (Chumphon) and Pa Thiu (Chumphon). Only one type of intermediate haplotype, BAB was found in this contact zone with low frequency indicated that northern and southern populations of bees in Thailand were colonized by separate population. The northern population of *A. cerana* in Thailand might be colonized by bee from Vietnam.

Notably, private haplotype of all amplified regions were found from one sample in Prachuap Khiri Khan. The composite haplotype, CED, was extremely different from all samples having morphological similarity. It was suspected to be the other species. Further study is needed to be carried out to clarify 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 nature. 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*. Therefore, crossed mating of male bees in the contact zone (Prachuap Khiri Khan and Chumphon) might occurred.

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สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CONTENTS

	Page
THAI ABSTRACT	ii
ENGLISH ABSTRACT	iii
ACKNOWLEDGEMENT	iv
CONTENTS	v
LIST OF TABLES	vi
LIST OF FIGURES	vii
ABBREVIATIONS	viii
INTRODUCTION	1
MATERIALS AND METHODS	2
RESULT	8
DISCUSSION	32
CONCLUSION	35
REFERENCES	36

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF TABLES

Table		Page
1	Sampling areas and sample sizes of *A. cerana* used in this study	2-3
2	Primer sequence, annealing temperatures, concentrations of primers and $MgCl_2$ used for PCR amplification of *A. cerana* mitochondria genes	4
3	Primer sequences and PCR conditions for the 3 microsatellite loci used to screen for polymorphic loci in *A. cerana*	5
4	Summary of restriction patterns of *Dra*I digested amplified DNA from three different regions in mitochondrial genome of *A. cerana*	14
5	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	16
6	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.	19
7	Haplotype and nucletide diversity within population for four geographic locations of *A. cerana* in Thailand	23
8	Nucleotide diversity and divergence between populations for four geographic locations of *A. cerana*	23
9	Geopraphic 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.	24
10	Genotypic disequilibrium of srRNA gene, lrRNA gene and intergenic COI –COII regions of *A. cerana* in Thailand	24
11	F-statistic among four *A. cerana* locations based on *Dra*I digestion of srRNA gene, lrRNA gene and intergenic COI – COII regions	25
12	Allele frequencies, number of allele, observed and expected heterozygosity of microsatellite A113 in four geographic samples of *A. cerana* in Thailand	30
13	Geographic heterogeneity analysis of four geographic samples of *A. cerana* in Thailand using microsatellite locus A113	30
14	F-statistics for microsatellite analysis loci A113 each pair of four geographic samples of *A. cerana* in Thailand	31

LIST OF FIGURES

Figure		Page
1.	PCR -amplified DNA from each individual was electrophoresed : a) srRNA gene (410 bp) b) lrRNA gene (755 bp) and C) intergenic COI – COII region (1710 bp)	10
2.	Three haplotypes were observed from *Dra*I digestion of amplified mitochondrial srRNA gene of *A. cerana* in Thailand	11
3.	Four haplotypes were observed from *Dra*I digested of amplified mitochondrial lrRNA gene of *A. cerana* in Thailand	12
4.	Eight haplotypes were observed from *Dra*I digested of amplified mitochondrial intergenic COI – COII regions of *A. cerana* in Thailand	13
5.	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	15
6.	Distribution of mtDNA composite haplotypes within each sampling locaiton of A. cerana in Thailand. Especially, Prachuap Khiri Khan and Chumphon provinces	17
7.	Phylogeographic pattern deduced from mtDNA composite haplotypes of *A. cerana* in borderline region between northern and southern populations in Thailand	18
8.	A UPGMA dendogram showing the relationships among twelve composite haplopyypes based on *Dra*I 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	20
9.	Distribution of distinct groups (based on UPGMA phonogram of genetic distance) of *A. cerana* in Prachuap Khiri Khan and Chumphon provinces	22
10.	Microsatellite pattern of *A.cerana* individuals at locus (a) A28 (b) A107	27
11.	Microsatellite pattern of *A.cerana* individuals at locus A113	28
12.	Allele frequency distributions at the microsatellite locus A113	29

ABBREVIATIONS

A,T,C,G	=	nucleotide containing the base adenine, thymine, guanine and cytosine, respectively
bp	=	base pair
^{o}C	=	degree celeius
DNA	=	deoxyribonucleic acid
dNTPs	=	deoxyribonucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
ddNTPs	=	dideoxyribonucleotide triphosphates (ddATP, ddTTP, ddGTP, ddCTP)
EDTA	=	ethylenediamine tetraacetic acid
HCl	=	hydrochloric acid
kb	=	kilobase
KCl	=	potassiumchloride
$MgCl_2$	=	magnesiumchloride
ml	=	millilitre
mM	=	millimolar
mtDNA	=	mitochondial DNA
ng	=	nanogram
PCR	=	polymerase chain reaction
RFLP	=	restriction fragment length polymorphism
RNA	=	ribonucleic acid
rpm	=	revolution per minute
SDS	=	sodium dodecyl sulfate
TEMED	=	N, N, N', N' –tetramethylethy lenediamine
Tris	=	tris (hydroxy methyl) aminomethane
UPGMA	=	unweighted pair group method with arithmetic averages
UV	=	ultraviolet
V	=	volt
W	=	watt
µg	=	microgram
µl	=	microlitre
µM	=	micromolar

INTRODUCTION

The eastern honeybees, *Apis cerana* is widely distributed over vast areas of Asia. Rutter (1988) has distinguished four subspecies : 1) *A. cerana cerana* (samples from Afghanistan, Pakistan, north India, China, north Vietnam, 2) *A. cerana indica* (south India, Sri Lan Ka, Bangladesh, Berma, Malaysia, Thailand, Indonesia and the Philippines), 3) *A. cerana japonica* (Japan and Korea), 4) *A. cerana himalaya* (Napal and including the mountains in Thailand : Chiang Mai).

Geographic population differentiation of *A. cerana* in Thailand was studied using morphometric analysis (Sysvester, 1998), PCR-RFLP of three regions of mtDNA ; small subunit ribosomal RNA (srRNA) and large subunit ribosomal RNA (lrRNA) genes and intergenic COI-COII (Sihanuntavong *et al.*, 1999), microsatellite analysis (Sittipraneed *et al.*, 2001 a) and sequencing of lrRNA gene (Sittipraneed *et al.*, 2001 b). Results from morphometric, PCR-RFLP and sequencing analysis defined *A. cerana* in Thailand into three different groups (northern, southern and Samui Island). However, further differentiation of the north-east population from the northern latitude bees were found from microsatellite analysis.

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 200 meters rise in sea level in this area accured approximately 5000 years ago (Pinaka, 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 might cause the spitting 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 hypotheses is most likely, this research 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-RELP on 3 mitochondrial DNA (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. If the hypothesis of colonization is correct, the common haplotype found in the contact zone will be the same as common haplotype of the other areas. Also, gene flow of both male and female will be studied by using micosatellite analysis (locus A28, A107 and A113).

MATERIALS AND METHODS

Samples collection

Adult honeybees worker (*A. cerana*) from natural colonies or unmanage beekeeping's cases were individually collected by immediately preserved in 95% ethanol at ambient temperature for 1 – 5 days during transportation and then stored at 4 ^{0}C for later use. The sampling areas and sample sizes was shown in Table 1.

Table 1 Sampling areas and sample sizes of *A. cerana* used in this study

Sampling area	colonies (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
Central	
Nakorn pathom	2
Suphan buri	2
Samut songkhram	8
Samut phakan	1
Chunthaburi	3

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Sampling area	colonies (N)
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

DNA extraction

Total DNA was extracted from a thorax of each *A. cerana* individual using the modified method of Smith and Hagen (1997). The DNA concentration was then roughly estimated using a mini-gel method (Maniatis *et al.*, 1982).

Amplification of mitochondrial DNA

Three mitochondrial DNA regions (srRNA 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), lxPCR 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 predenatured 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. Aliquots of the amplified DNA fragments were electrophoretically analyzed in agarose gel. The remaining solution of the successed amplification was then subjected to restriction analysis.

RFLP analysis of amplified DNA fragment

Approximately 500 ng of PCR products from each individual was digested with 5 unit of *DraI* in 20 µl of reaction mixture using the conditions recommended by the manufacturer (Bochringer Mannheim). The digests were electrophoratically analyzed through 2.5 – 3.5% MetaPhor agarose gel (FMC) and visualized under a UV light after ethidium bromide staining (Maniatis *et al.*, 1982).

Table 2 Primer sequence, annealing temperatures, concentrations of primers and $MgCl_2$ used for PCR amplification of *A. cerana* mitochondria genes. Primer sequences and their positions were inferred from *A. mellifera* (Crozier and Crozier, 1993).

Gene	Sequence	Position	Annealing temp. (°C)	Primer conc. (µM)	MgCl conc. (mM)
lrRNA	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
srRNA	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
InterCOI-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

Microsatellite analysis

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, lxPCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl), 0.4 unit of 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 an optimal annealing temperature and extension step at 72 °C for 10 minutes (Table 3). After the amplification process was completed, the size of amplified microsatellite DNA was estimated using denaturating polyacrylamide gels electrophoresis.

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/M 13 forward primer (4.5 pmol), 3.4 μl of sterile water and 5 units of sequencing grade *Taq* DNA polymerase. The reaction mixture was completed by added 16 μl mixture in four microfuge tubes which each tube contained d/ddNTP mix. The reaction tube was 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 stop 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 the amplified microsatellite DNA from various samples.

Table 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')	$MgCl_2$ (mM)	Annealing Temp. (°C)
A28	$(CCT)_3GCT(CCT)_6(CT)_5TT(CT)_4$	GAAGAGCGTTGGTTGCGAGG GCCGTTCATGGTTACCACG	1.6	55
A107	$(GCTC)_7(GCT)_2(CT)_{23}$	CCGTGGGAGGTTTATTGTCG CCTTCGTAACGGATGACACC	1.2	55-57
A113	$(TC)_2C(TC)_2TT(TC)_2TT(TC)_8TT(TC)_5$	CTCGAATCGTGGCGTCC CCTGTATTTTGCAACCTCGC	1.6	58

Size estimation of amplified microsatellite DNA using denaturating polyacrylamide gels

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 of 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.

Sliver staining

Sequencing gel was fixed in 10% acetic acid for 20 minutes at room temperature. After washed 3 times for 2 minutes each with deionized water, then the gel was soaked by shaking for 30 minutes in a staining solution (0.6 mM of silver nitrate and 0.056% formaldehyde). After rinsed with ultrapure water for 5 – 10 seconds then placed the gel in cooled developing solution (0.03 M sodium thiosulfate) until microsatellite bands were clearly visualized. The gel was fixed in 10% acetic acid for 5 minutes and briefly rinsed with deionized water. The gel was air-dried and could be kept permanently at room temperature.

Data analysis

Restriction fragment data analysis

Restriction profiles from each region were alphabetically coded in order of appearance. Each bee was assigned a three-letter code arranged according to DNA region from that which gave the lowest number of restriction patterns (srRNA gene) to that which provided the highest number of restriction patterns (intergenic COI – COII region) to describe its composite haplotype. The genetic distance between mtDNA composite haplotypes, haplotype and nucleotide diversity within samples, and nucleotide divergence between samples (dxy) were calculated (Nei, 1987). All statistical parameters described above were calculated using REAP (McElroy *et al.*, 1992). Fst was computed after Weir and Cockerham (1984) using GENEPOP (Roymond and Rousset, 1995). A phenogram, based on percentage of sequence divergence between pairs of composite haplotypes, was constructed using UPGMA implemented in Phylip 3.56 C (Felsenstein, 1993). Genetic heterogeneity between geographic samples was estimated after Roff and Bentzen (1989) using the MONTE routine from REAP.

Microsatellite analysis

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.

The number of alleles, allele frequencies, observed and expected heterozygosity of each microsattelite locus were estimated in each population (Nei, 1987). Geographic heterogeneity in alleles distribution frequencies between compared geographic populations or regions were carried out with the Markov chain approach for X^2 analysis (Guo and Thompson, 1992). The Fst between pairs of population was tested to determine if Fst was statistically significant different from zero (Weir and Cockerham, 1984). These population genetic parameters were analyzed using GENEPOP (Raymond and Rousset, 1995).

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RESULT

DNA extraction

High molecular weight DNA larger than 23.1 kb was extracted from thorax of each *A. cerana* individual. The concentration of extracted DNA was 1.0 – 1.5 µg per individual.

PCR-RFLP of lrRNA gene, srRNA gene and intergenic COI –COII region

The amplified products of the srRNA and lrRNA genes and the intergenic COI –COII region obtained were 410,755 and 1710 bp, respectively (Figure 1). Length polymorphism were not observed in any individuals investigated. Digestion of PCR products of srRNA gene, lrRNA gene and the intergenic COI –COII with restriction endonuclease *Dra*I yielded three, four and eight digestion patterns (haplotypes), respectively (Figure 2 - 4). The DNA fragments size of all haplotypes were summarized in Table 4.

The interconnection between three haplotypes (A, B and C) obtained from *Dra*I digestion of srRNA gene was shown in Figure 5a. For example, interconnection of haplotype A (320, 42 and 38 bp) and haplotype B (160, 160, 42 and 38 bp) can be explained by a loss (or gain) of a single restriction site. Haplotype C (160, 160 and 80 bp) interconnected with haplotype B (160, 160 42 and 38 bp) by a single loss (or gain) of restriction site.

The network showing relationships among four haplotypes (A, B, C and D) of *Dra*I digestion of lrRNA genes and eight haplotypes (A, B, C, D, E, F, G and H) of *Dra*I digestion of the intergenic COI – COII were shown in Figure 5 b and Figure 5 c, respectively.

Geographic distributions of composite haplotypes in *A. cerana*

A restriction fragment pattern of each *A. cerana* individual from the single enzyme digestion was combined to generate composite haplotypes. The composite haplotypes for each individual (colony) were designed by 3 letter code representing the *Dra*I digestion of three regions, srRNA gene, lrRNA and the intergenic COI – COII region, respectively. The total 12 composite haplotypes were observed (Table 5). Composite haplotype, AAA, was the most common composite haplotype found in 39% of 95 individuals investigated. This composite haplotype, was distributed in Prachuap Khiri Khan (31 colonies), Chumphon (5 colonies) and Hanoi (1 colony). The composite haplotype, BBB, was found in 22 (23%) out of 95 individuals investigated. Twenty and two

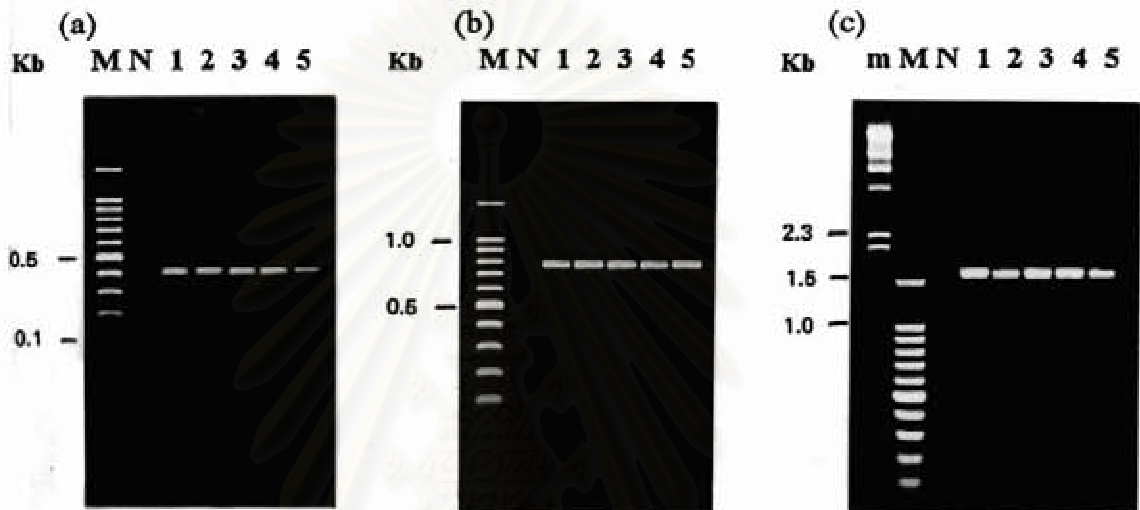

Figure 1 PCR –amplified DNA from each individual was electrophoresed : a) srRNA gene (410 bp) b) lrRNA gene (755 bp) and C) intergenic COI – COII region (1710 bp)

lane m = λ/*Hind* III standard DNA marker

lane M = 100 bp DNA ladder

lane N = Negative control

lane 1 – 5 = An example of PCR –amplified DNA patterns from 5 *A. cerana* individuals

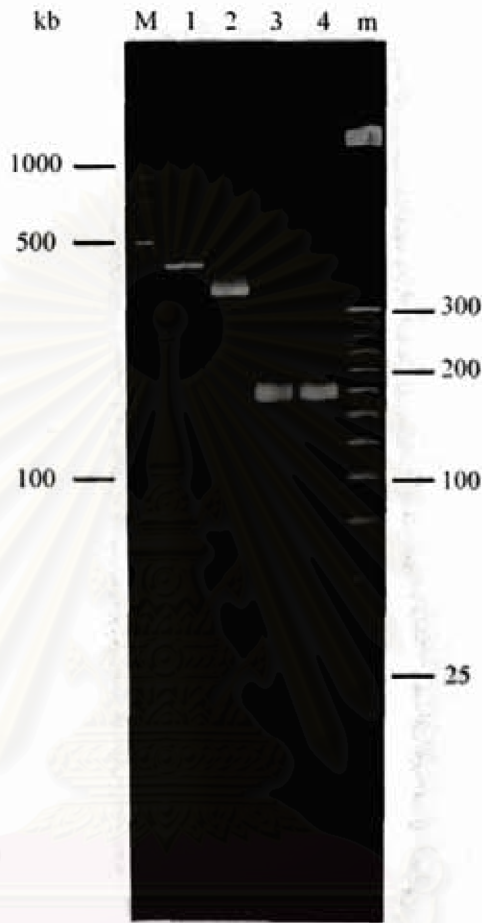

Figure 2 Three haplotypes were observed from *Dra*I digestion of amplified mitochondrial srRNA gene of *A. cerana* in Thailand.

lane M = 100 bp DNA ladder

lane m = 25 bp DNA ladder

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

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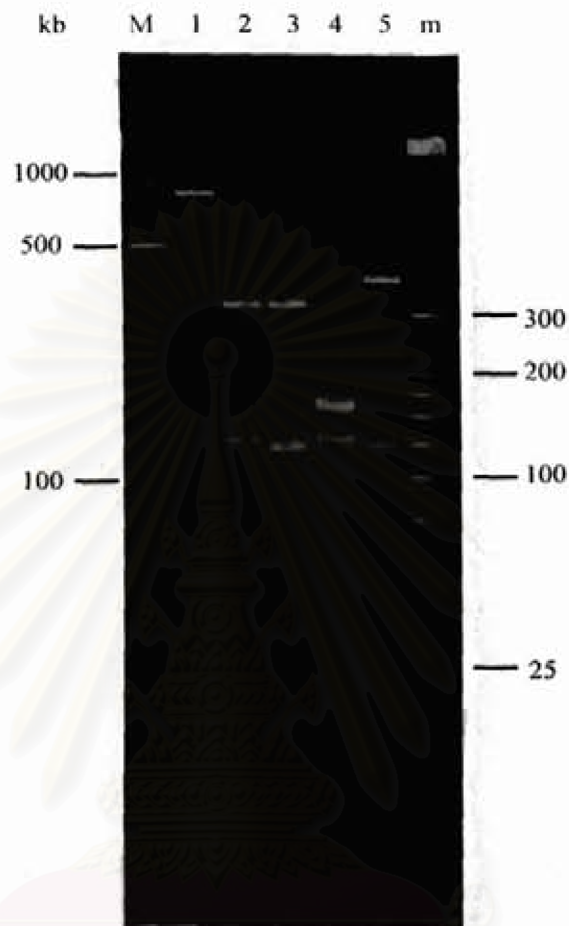

Figure 3 Four haplotypes were observed from *Dra*I digested of amplified mitochondrial lrRNA gene of *A. cerana* in Thailand.

lane M = 100 bp DNA ladder

lane m = 25 bp DNA ladder

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

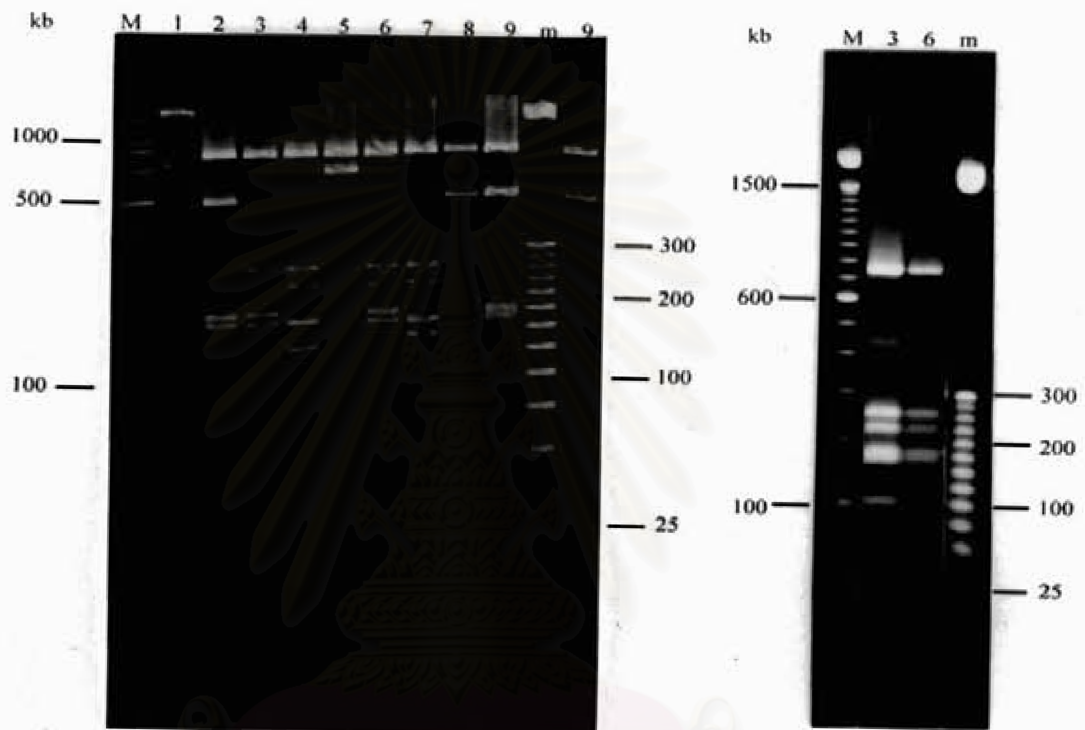

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Figure 4 Eight haplotypes were observed from *Dra*I digested of amplified mitochondrial intergenic COI – COII regions of *A. cerana* in Thailand

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 4 Summary of restriction patterns of *DraI* digested amplified DNA from three different regions in mitochondrial genome of *A. cerana*

a. srRNA

bp	A	B	C
320	—		
160		x2	x2
80			—
42	—	—	
38	—	—	
total(bp)	400	400	400

b. lrRNA

bp	A	B	D	E
350				—
300	—	—		
152			—	
148			—	
130	—		—	
120	—	x2	—	x2
90	—	—	—	—
80				
60	—	—	—	—
50	—	—	—	
40				
7*		—		
total(bp)	750	747	750	740

c. Intergenic COI-COII regions

bp	A	B	C	D	E	F	G	H
750	—	—	—	—	—	—	—	—
620				—				
450	—						—	—
240		—	—		—	—		
210		—	—		—	—		
170	—	—	—		—		—	—
160	—	—			—	—	—	—
140						—		
130			—					
100								—
90	x2			x3				
85		x2	x2		—	x2		
60				—				
50					x2			—
45							x4	
40			—					
30						—		
total(bp)	1710	1700	1700	1700	1715	1700	1710	1680

—— = one fragment, Xn = Repeated fragments

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

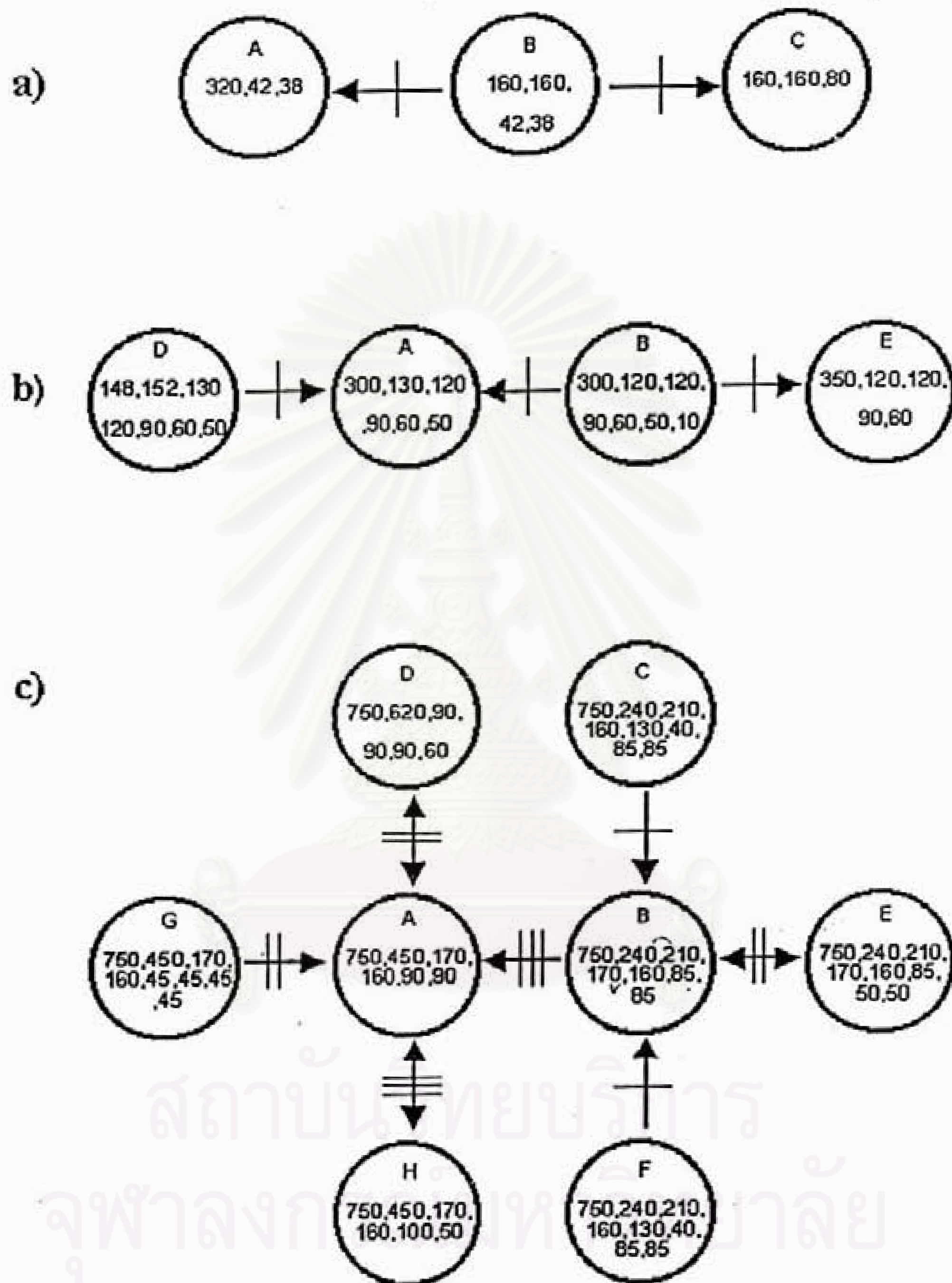

Figure 5 The most parsimonious networks based on *DraI* digestion of PCR-amplified DNA of a) srRNA gene, b) lrRNA gene and c) intergenic COI-COII region of *A. cerana* in Thailand. Arrows indicate restriction site losses and not necessarily indicate evolutionary direction. Cross bars indicate the number of point mutation.

Table 5 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)

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individuals were observed in Chumphon and Prachuap Khiri Khan, respectively. Composite haplotype ADA (18%) was found in 14 and 3 individuals from Prachuap Khiri Khan and Chumphon, respectively. Composite haplotype BAB (7%) was observed in 4 individuals from Chumphon and 2 individuals from Prachuap Khiri Khan. In addition, other 8 composite hapoltypes were found in low frequencies at particular location. All individuals from Yunnan (5 colonies) had AAB haplotype. Sample from Hanoi had AAA composite haploytpe. Distribution of composite haplotypes within each location is shown in Figure 6. The relationship among all composite haplotypes found in this study are illustrated in Figure 7. Five mutation steps were found between the most two common haplotypes (AAA and BBB). Composite haplotype AAB and BAB were the intermediate haplotypes between AAA and BBB. The genetic distances of these composite haplotypes are shown in Table 6. The value of genetic distances were ranged from 0.0047 to 0.0826. Distance values were used to reconstruct a dendogram using unweighted pair group method (UPGMA) (Figure 8). The phenogram indicated that the 12 composite haplotyes of *A. cerana* could be allocated into 3 groups (A, B and C). Group A composed of ADA, AAA, ADH and AAG, where as BBF, BBB, BBE, BBC, BAC, AAB and BAB were in Group B. C group 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 haplotypes (AAA, BBB, ADA and BAB) indicated the overlapping of these two groups in contact zone (Figure 9). 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 in Table 7. 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 might be changed because the sample size of both was only five and one colonies respectively. The average nucleotide diversity was 0.59%.

According to the result in Table 8, nucleotide diversity of all pairwise comparisons were low. The average nucleotide diversity between two populations was 1.58%. The total nucleotide divergence of all populations was low (1% in average).

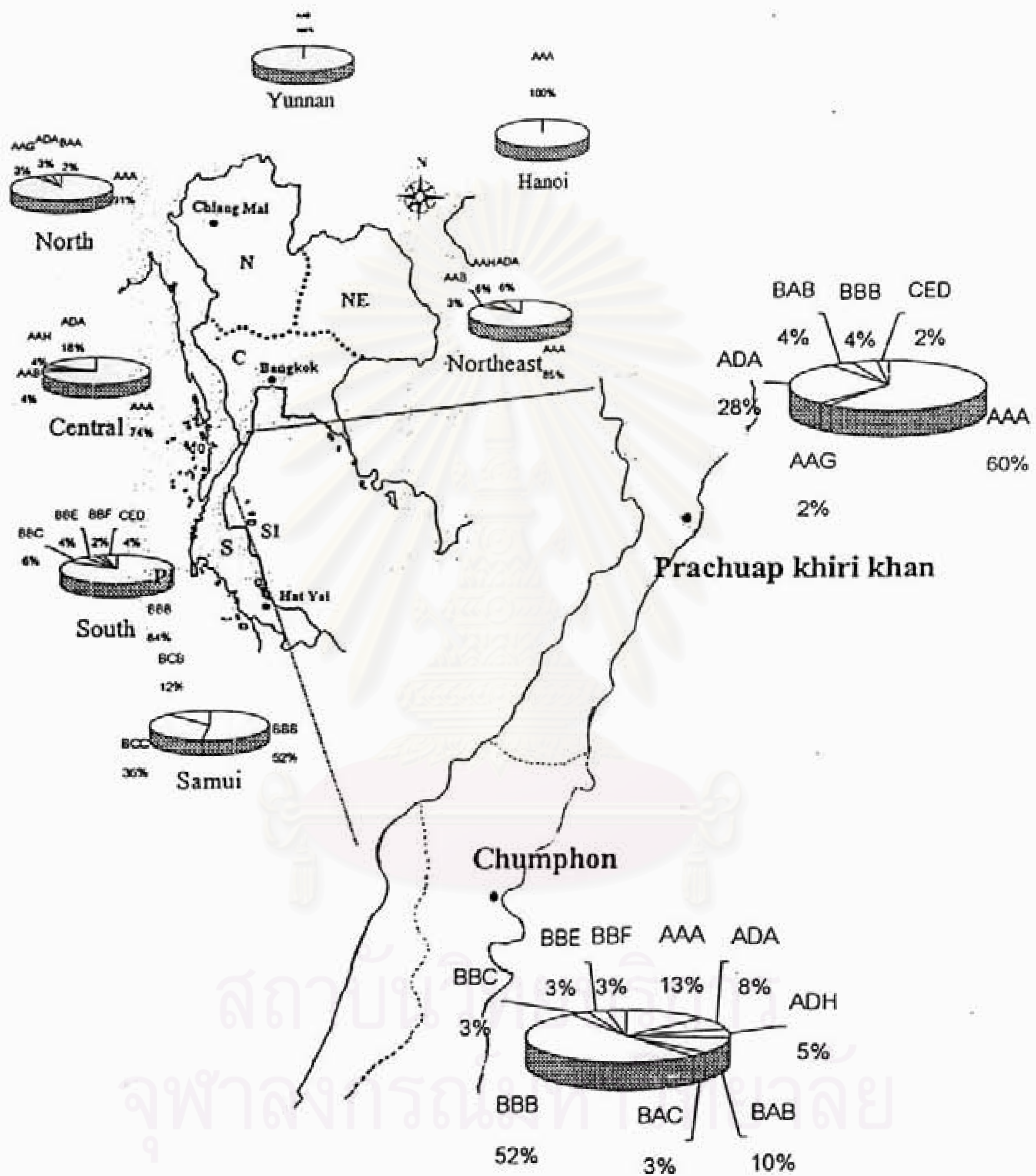

Figure 6 Distribution of mtDNA composite haplotypes within each sampling location of *A. cerana* in Thailand. Especially, Prachuap Khiri Khan and Chumphon provinces (North, North-east, Central, South and Samui datas referred to Sihanantavong, 1999)

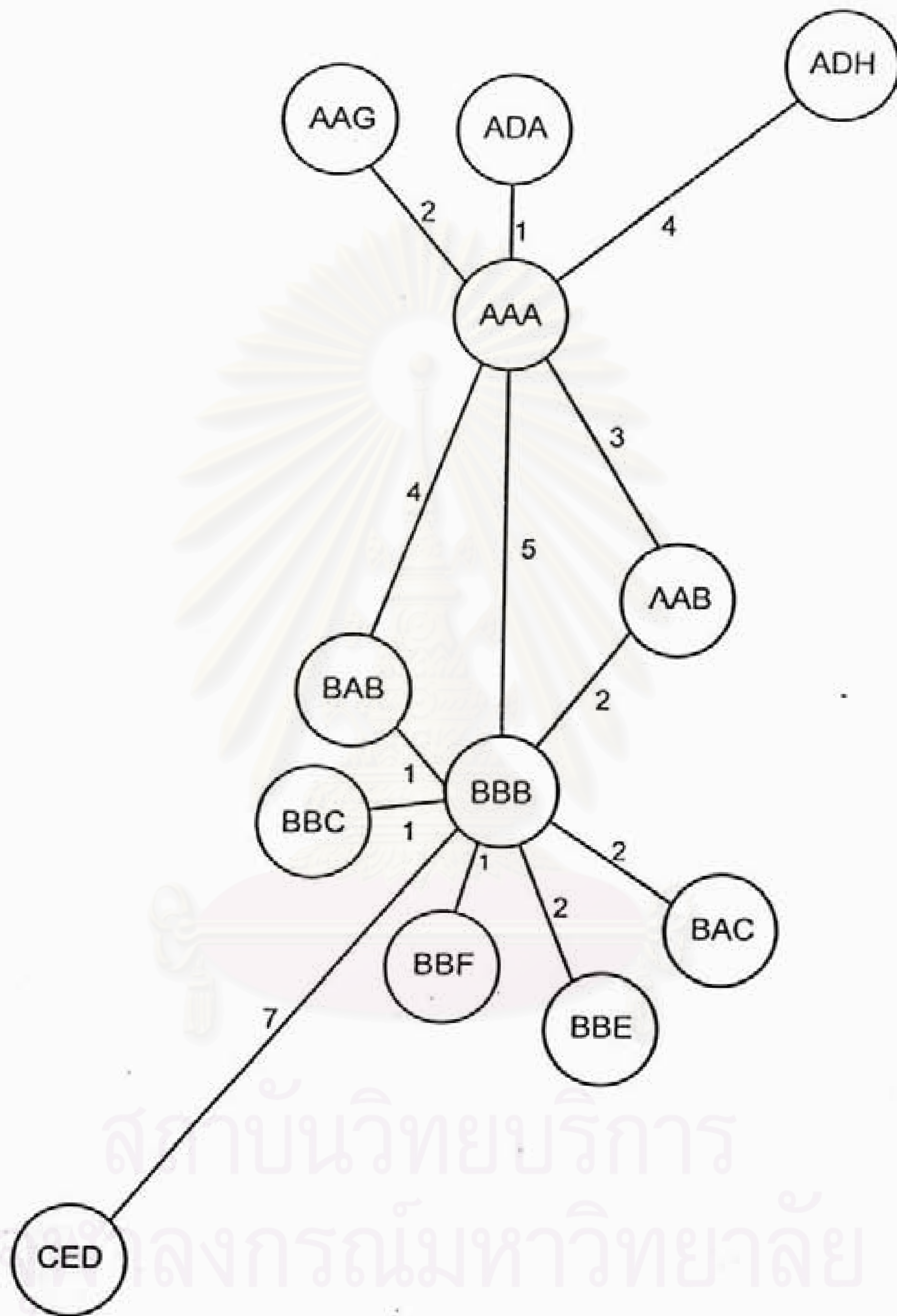

Figure 7 Phylogeographic pattern deduced from mtDNA composite haplotypes of *A. cerana* in borderline region between northern and southern populations in Thailand. Number along connected line indicated inferred mutation steps.

Table 6 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	-

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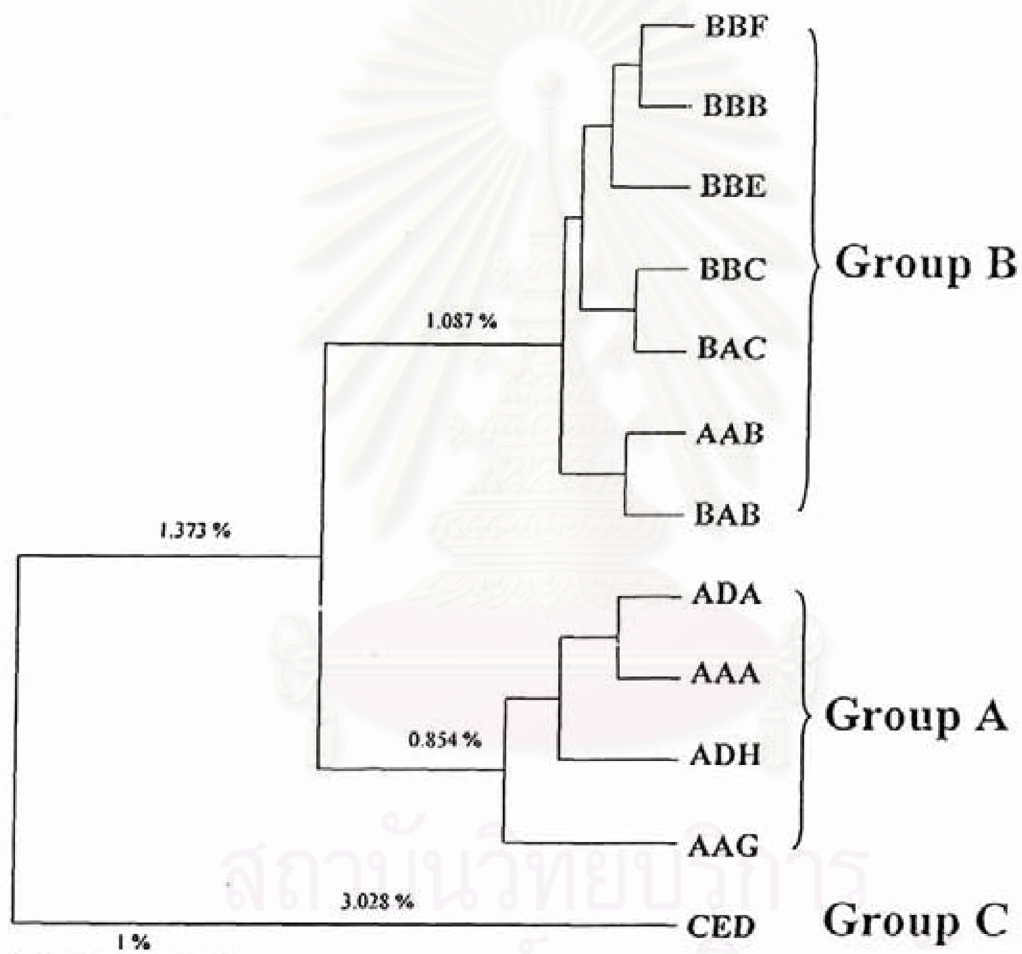

Figure 8 A UPGMA dendogram showing the relationships among twelve composite haplopypes based on *Dra*I digestion of PCR amplified DNA of srRNA gene, lrRNA gene and intergenic COI – COII regions of *A. cerana* in borderline region between northern and southern populations.

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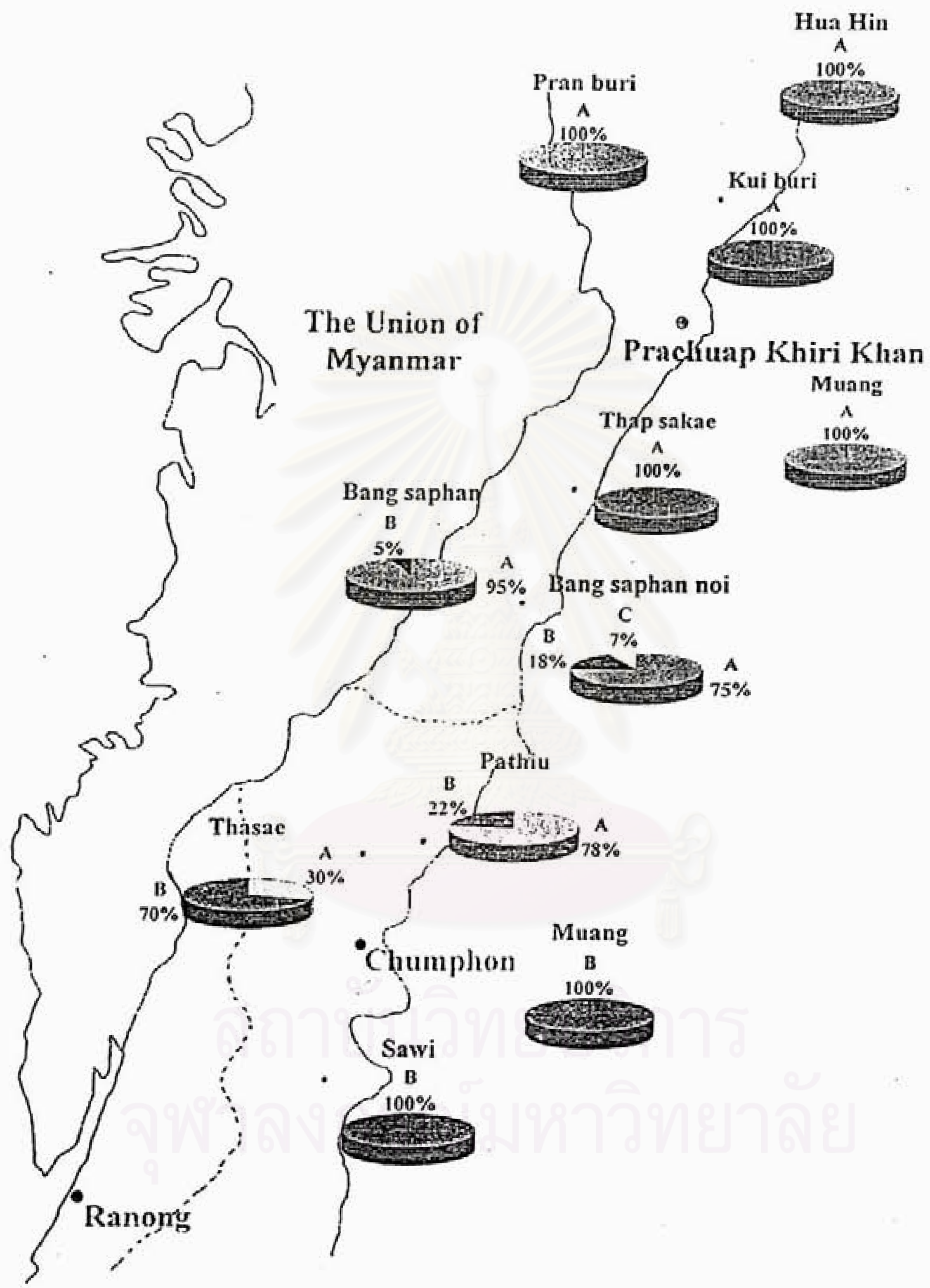

Figure 9 Distribution of distinct groups (based on UPGMA phonogram of genetic distance) of *A. cerana* in Prachuap Khiri Khan and Chumphon provinces.

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Table 7 Haplotype and nucleotide 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 8 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	-

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Monte Carlo simulation was used for geographic heterogeniety analysis. The significant difference in composite haplotype distribution frequencies among 4 geopgraphic locations of *A. cerana* were shown in Table 9. 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 10). Testing in Prachuap Khiri Khan and Chumphon populations, there was no linkage disequilibrium detected. High significance between two populations were shown (P = 0.0000 – 0.0071). For all populations, genotypic disequilibrium of mtDNA among 3 regions was also highly significant (P< 0.0001). Geographic heterogeneity analysis indicated highly significant differences in distribution of mtDNA haplotypes of Prachuap Khiri Khan and Cnumphon pairwise comparisons (P< 0.0001, Fst = 0.3061 – 0.5946) (Table 11). The overall Fst was 0.45871.

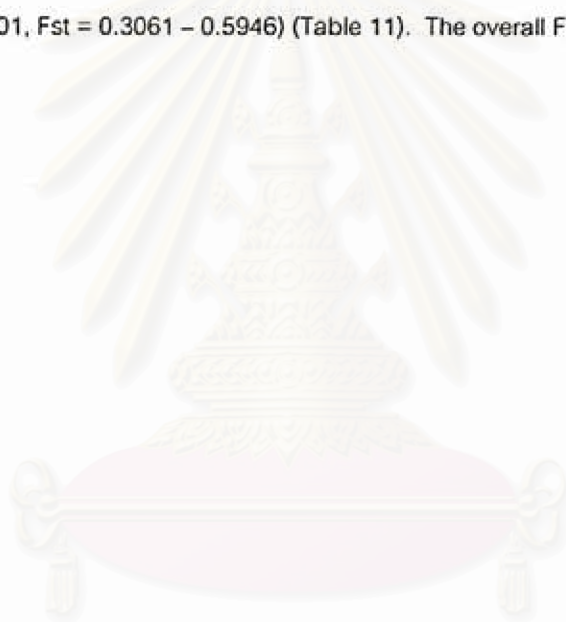

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Table 9 Geopraphic heterogeneity analysis in distribution frequency of composite haplotype among four *A. cerana* locations based on *Dra*I digestion of srRNA gene, lrRNA gene and intergenic COI –COII region.

	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	-

Table 10 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	-

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Table 11 F-statistic among four *A. cerana* locations based on *Dra*I digestion of srRNA gene, lrRNA gene and intergenic COI – COII regions

Sample	srRNA		lrRNA		Intergenic COI-CIOII	
	Fst	P-value	Fst	P-value	Fst	P-value
Pruchuap-Chumphon	0.5946	<0.0001	0.3061	<0.0001	0.4979	<0.0001
Pruchuap-Yunnan	-0.06643	1.0000	0.0899	0.2531	0.7963	<0.0001
Pruchuap-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

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Microsatellite analysis

Genetic variation of three microsatellites DNA

Microsatellite analysis using three microsatellite loci (A28, A107 and 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 10 a and 10b), 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 A113 locus was used for analysis (Figure 11).

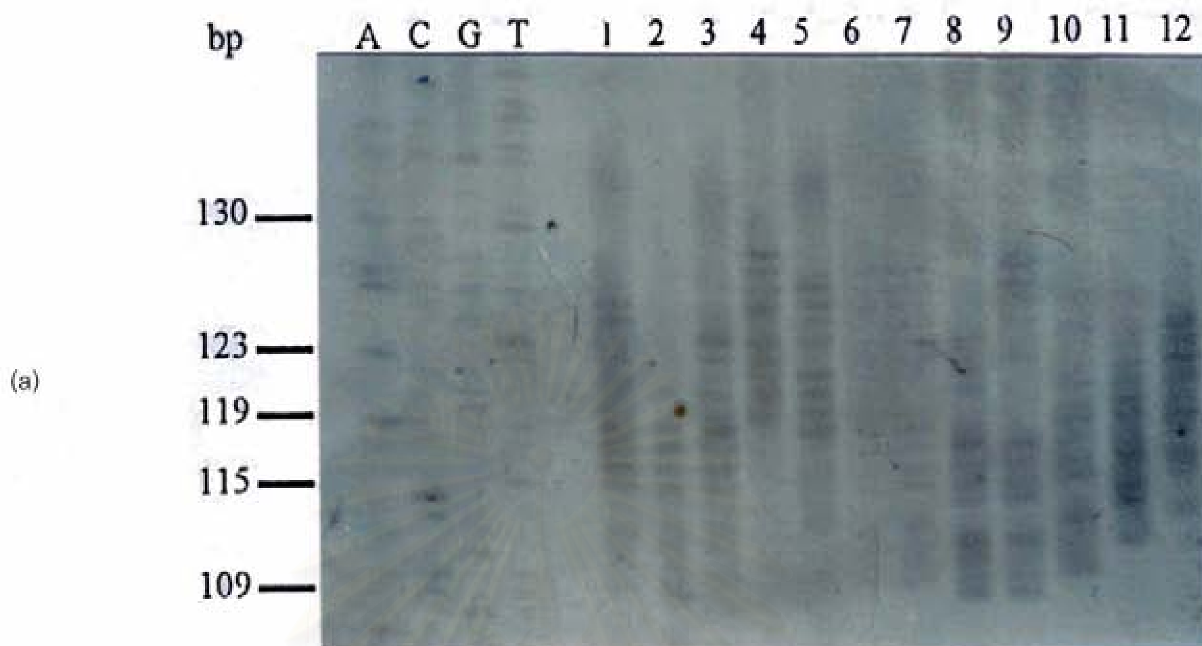
Central and southern populations of *A. cerana* from flanking region of Prachuap Khiri Khan and Chumphon were also used in this analysis. Therefore, 4 geographic samples included 129 individual colonies in total (central : 20, southern : 20, Prachuap Khiri Khan : 51 and Chumphon : 38), were analyzed. The results showed that locus A113 had four alleles (175, 180, 185 and 195 bp). Only a 175 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, 185 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 195 bp allele was 0.350, 0.325, 0.284 and 0.316 for central, southern, Prachuap Khiri Khan and Chumphon bee samples, respectively. All allele frequency distributions of microsatellite locus A113 were shown in Figure 12 and Table 12

The observed heterozygosity (Ho) and expected heterozygosity (He) of microsatellite loci A113 of four geographies were shown in Table 12. The average observed heterozygosities ranged from 0.451 (Prachuap Khiri Khan samples) to 0.550 (central samples). These observed heterozygosities were equal to the expected heterozygosities.

Geographic heterogeneity of allele frequency of four geographic samples was shown in Table 13. No significance in distribution of alleles frequency was observed in all populations ($p = 0.6017$).

Intraspecifically geographic differentiation of four geographies of *A. cerana* were supported by F-statistics (F_{ST}). The average F_{ST} value was -0.01035. Genetic differentiation do not exist in these populations (Table 14).

(a)



(b)

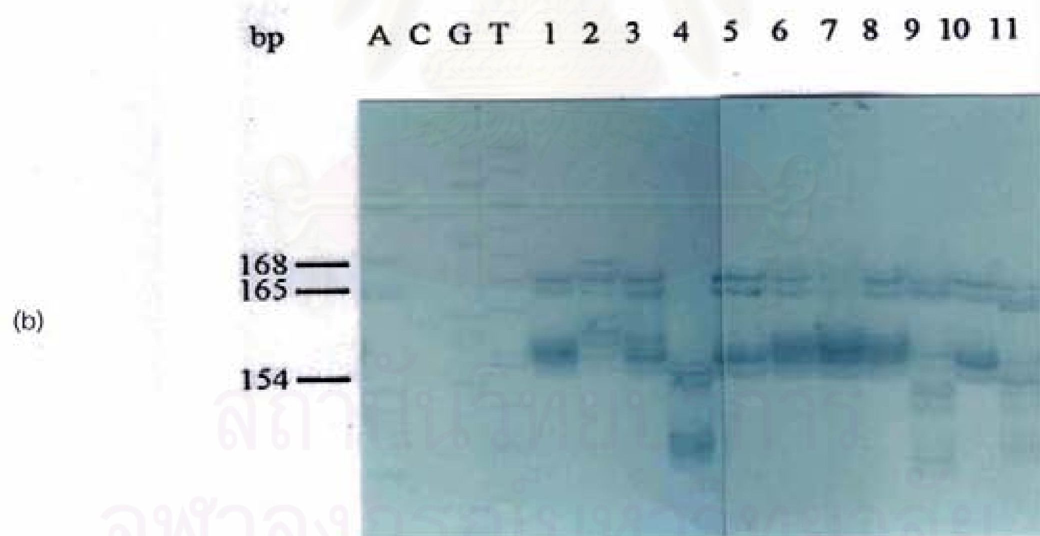
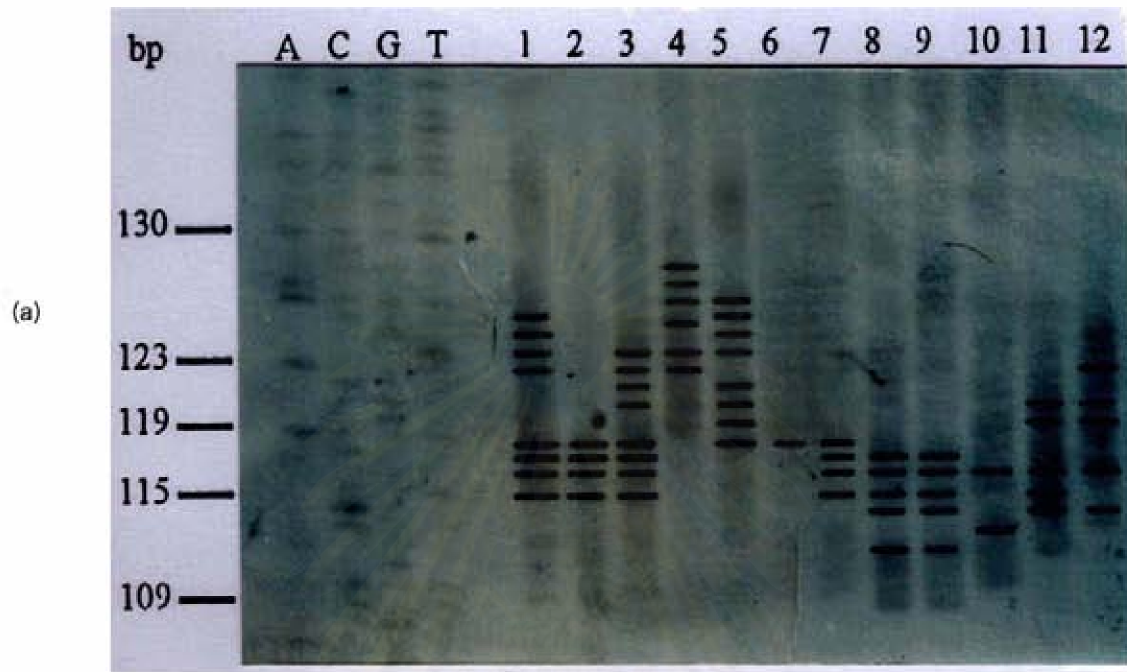


Figure 10 Microsatellite pattern of *A.cerana* individuals at locus (a) A28 (lane 1-12)

(b) A107 (lane 1-11)

pGEM sequencing marker was used as a standard

(a)



(b)

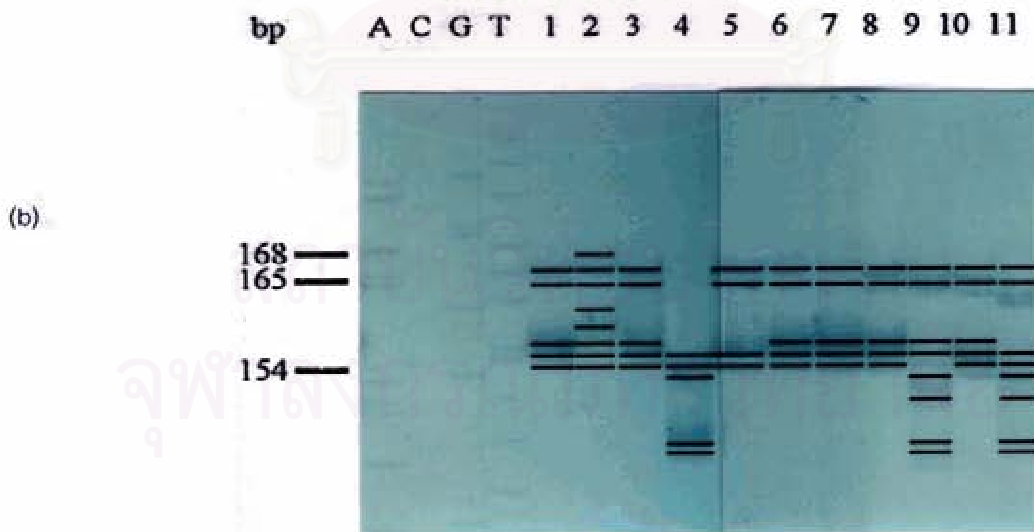
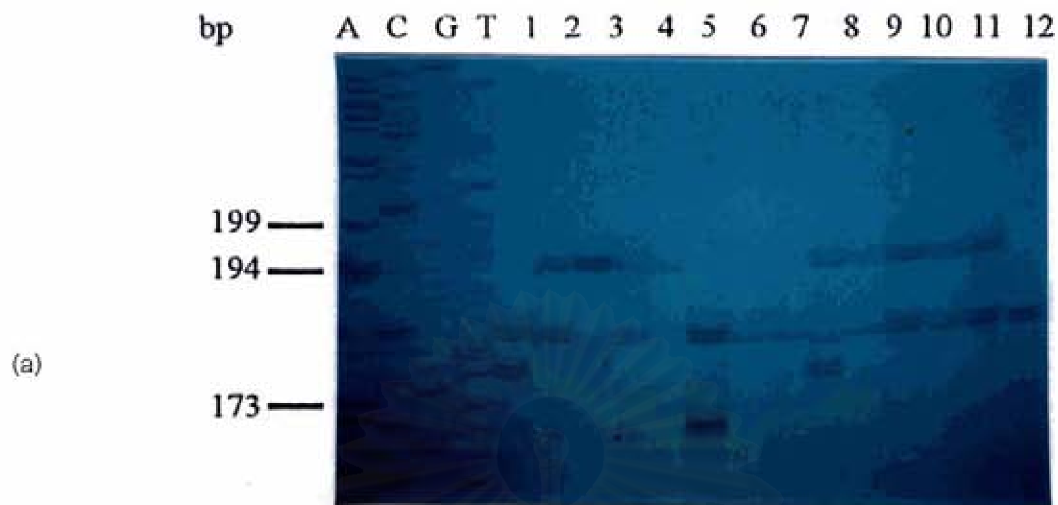


Diagram of Figure 10a and 10b

(a)



(b)

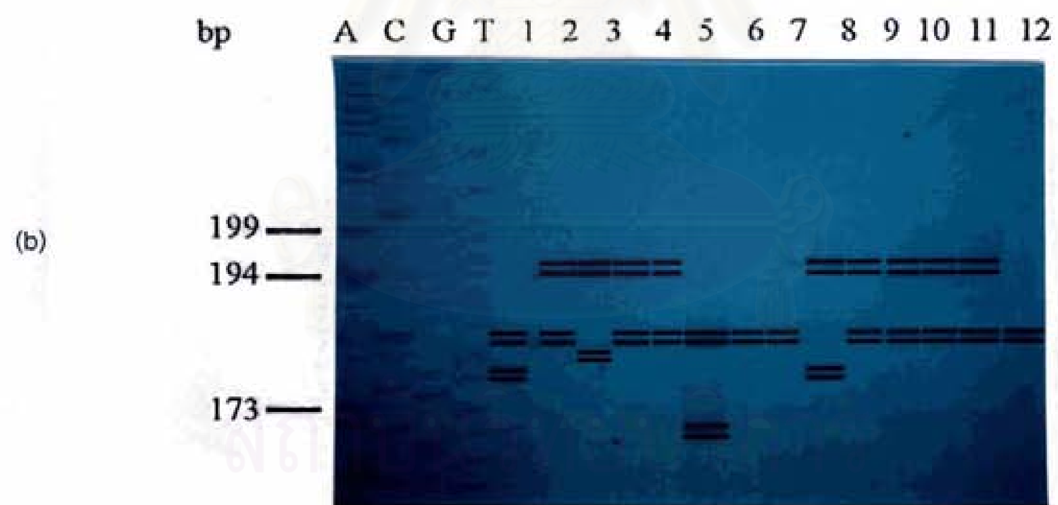


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Figure 11 a) Microsatellite pattern of *A.cerana* individuals at locus A113 (lane 1-11) pGEM sequencing marker was used as a standard

b) diagram of a)

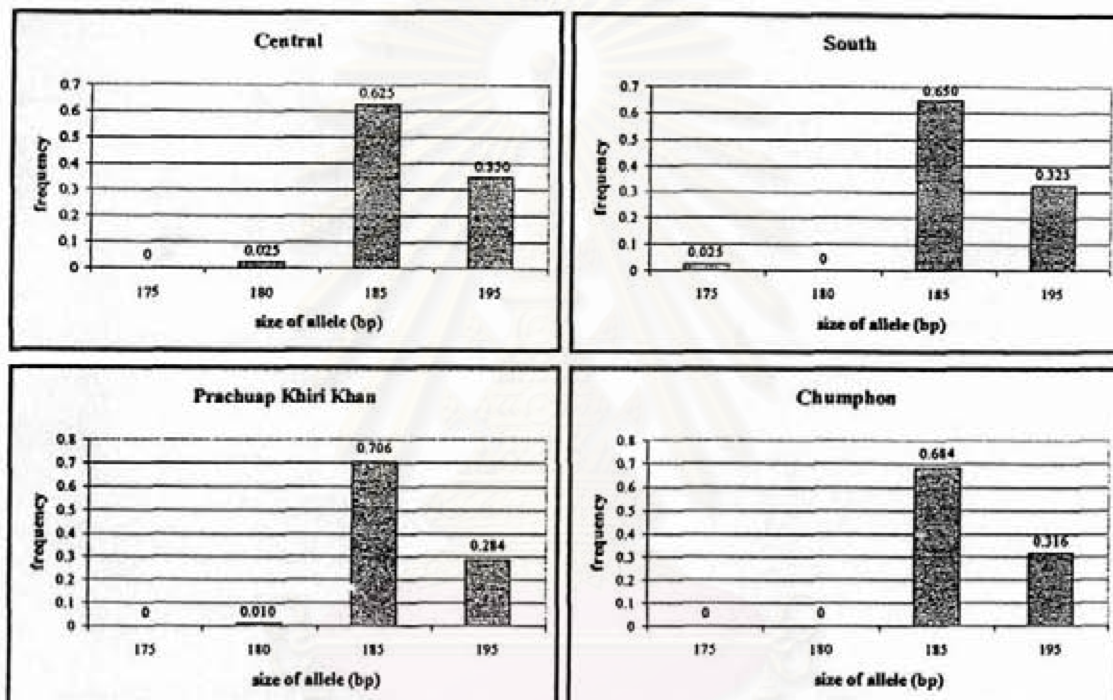

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Figure 12 Allele frequency distributions at the microsatellite locus A113 from Central (n = 20), South (n = 20), Prachuap Khiri Khan (n = 51) and Chumphon (n = 38)

Table 12 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
190	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 13 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

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 14 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 F_{ST} value across overall geographic samples was -0.0104 (P = 08287).

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

DISCUSSION

The PCR products of 410, 755 and 1710 bp were obtained from amplification of srRNA genes, lrRNA gene and intergenic COI – COII regions, respectively. *Dra*I digestion of amplified products of srRNA genes, lrRNA gene and intergenic COI – COII regions showed three, four and eight haplotypes, respectively. Eleven composite haplotypes (AAA, ADA, ADH, AAG, BBB, BBF, BBE, BBC, BAC, BAB, CED) were found in 89 investigated colonies from Prachuap Khiri Khan and Chumphon provinces. Twelve composite haplotypes (AAA, ADA, ADH, AAG, BBB, BBF, BBE, BBC, BAC, BAB, CED, AAB) were obtained when samples from Yunnan (5 colonies) and Hanoi (1 colony) were include. Among these twelve composite haplotypes, 9 composite haplotypes had already reported (Sihanuntavong, 1999) and 3 new composite haplotypes (ADH, BAB, BAC) was 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 composed of composite haplotypes, ADA, AAA, ADH and AAG. Group B composed of BBF, BBB, BBE, BBC, AAB and BAB and group C 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 2 and 3 mutation steps a 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 5 colonies from Yunnan (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.

From our previous study (Sihanuntavong, 1999), the distribution of *A. cerana* group A was completely different from group B. Group A was northern population, whereas group B was southern population. In this study, 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 A and seventy-four percent were group B. The result from this study showed that amphur Bangsaphan (Prachuap Khiri Khan province), amphur Tasae (Chumphon province) and amphur Prathe (Chumphon province) were the contact zone of the northern and southern *A. cerana* because overlapping between group A and B of *A. cerana* was found in these area.

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 the values of 5 geographic areas (north, north-east, central, south and Samui Island) which ranged from 0.1686 to 0.5967 and 0.0014 to 0.0048, investigated by Sihanuntavong (1999). This clearly showed that genetic diversity was maintained in the contact area. Haplotype diversity and nucleotide diversity of *A. cerana* in Yunnan and Hanoi were zero. The geographic heterogeneities between Prachuap Khiri Khan, Chumphon and Yunnan were significant different ($P < 0.0001$). In the opposite, each pairwise comparison with Hanoi was not significant different (P = 0.02849 – 0.6732) (Table 9). However, it should be not that haplotype and nucleotide diversity of *A. cerana* in Yunnan and Hanoi might be under estimated because the number of sample was too small (5 colonies of Yunnan and one colony of Hanoi).

The result from mtDNA analysis supported that *A. cerana* in borderline region or contact zone had high genetic diversity. The highest diversity was in Chumphon where presented highest haplotype diversity and nucleotide diversity.

The intermediat composite haplotypes, which could define by mutation steps between the haplotype AAA and BBB, was found with low frequency in Prachuap Khiri Khan and Chumphon provinces. Only one type of intermediate haplotype, BAB was found in six samples out of 89 investigated samples. The other intermediate haplotype, AAB which was found in one sample each from Kanchanaburi and Ubonratchatani provinces by Sihanunthavong (1999) was not found in the contact zone. However, the intermediat haplotype (AAB) was observed in all five samples from Yunna (China). Since the intermediate haplotype was found least in contact zone, therefore it is unlikely that these two separate mitochondrial lineages are evolved independently from the same ancient *A. cerana*. Alternatively, the northern (group A) and southern (group B) is most likely colonized by separate populations. *A. cerana* group A might be colonized by Hanoi honeybees. As only one sample from Hanoi (Vieatnam) was analyzed in this study, an increase in sample size investigated may be required. In addition Bugharuang (1996) studied genetic variation of *A. cerana* in Thailand using the sequences of mtDNA intergenic COI – COII region. The comparison of these sequences with those from other countries. Bugharuang found that southern population of Thai *A. cerana* was in the same group of Malaysian *A. cerana*. Therefore, group B of Thai *A. cerana* might be continuously colonized by Malaysian honeybees.

It should be emphasized that composite haplotype CED (group C) which was carried by one individual in Prachuap Khiri Khan was allocated into different clone. Its genetic distance from A or B was 3.03% sequence divergence, which was extremely different from all samples. It was suspected to be the other species. This kind of composite haplotype was previously reported in 2 colonies from southern part of Thailand (Sihanuntavong, 1997).

Microsatellite marker was additional chosen for this study because it previously showed genetic differentiation within different geographic samples of *A. cerana* in Thailand (Sittipraneed, 2001 a). In addition, gene flow mediated from male can be investigated. Three microsatellite loci (A28, A107 and A113) which had been previously used (Sittipraneed, 2001 a) were selected for this studied. In order to reduce cost of the analysis, detection of microsatellite alleles were performed by using silver stain instead of autoradiography of (^{32}P) dATP incorporated microsatellite DNA. As the result, many stutter bands were obtained when microsatellite loci A28 and A107 were analyzed. The mistake of scoring the molecular size of microsatellite alleles can effect the data analysis. Therefore, only the result from microsatellite locus A113 was used for the data analysis. Moreover, the result from our previous study (Sittipraneed, 2001 a) showed that geographic heterageneity from microsatellite locus A113 could indicate the different between northern and southern *A. cerana* population.

Microsatellite A113 analysis of 135 samples from four geographic regions (central, south, Prachuap Khiri Khan and Chumphon) (Table 1) showed that 185 bp was the most common alleles. All populations carried this 185 bp alleles with high frequencies. The 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 that genetic variations among four *A. cerana* populations and subpopulations did not existed. Eventhough, genetic difference between central and southern populations was not detected in this study but clear genetic difference between these population had been reported (Sittipraneed, 2001). The different result may cause by the error of sampling area in this study which was near the contact zone.

The result from mtDNA study showed clear genetic difference between Prachup Khiri Khan and Chumphon *A. cerana* whereas microsatellite analysis (nuclear DNA) showed that genetic difference between samples from these provinces did not exist. The possible explanation was the crossed mating of male honey bees between these areas. This explanation also supported the absent of genetic difference in central and southern bees near the contact zone (Prachuap Khiri Khan and Chumphon provinces) as detected by microsatellite analysis.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CONCLUTIONS

1. Restriction of amplified srRNA gene lrRNA gene and intergenic COI –COII region of Phrachup Khiri Khan and Chumphon *A. cerana* mtDNA with *Dra*I revealed 3, 4 and 8 haplotypes, respectively. A total of 11 composite haplotypes was found in this area.
2. The UPGMA dendogram of populations derived from PCR-RFLP data can devide *A. cerana* in Prachuap Khiri Khan and Chumphon into 2 destinct groups : the northern and southern bees.
3. Contact zone of northern and southern *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.
4. Northern and southern populations of *A. cerana* in Thailand is colonized by different bee populations. The northern population might be colonized by bee from Vietnam, whereas the southern population might be from Malaysian's bee.
5. Microsatellite analysis of specimens from Prachuap Khiri Khan and Chumphon using microsatellite locus A113 showed that genetic population differentiation did not exist in the contact zone. Therefore, crossed mating of male bees might occurred in this area.

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สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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