ความสัมพันธ์ทางพันธุกรรมระหว่างผึ้ง 2 ชนิด

(*Apis mellifera* LINNAEUS, 1758 และ *Apis cerana* FABRICIUS, 1753)

กับไรวาร์รัว ในประเทศไทย

นางสาวทิพย์วรรณ สรรพสัตย์

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาศาสตร์ชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2550
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

GENETIC RELATIONSHIPS BETWEEN TWO HONEY BEES (*Apis mellifera* LINNAEUS, 1758 AND *Apis cerana* FABRICIUS, 1753) AND VARROA MITES IN THAILAND

Miss Tipwan Suppasat

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biological Sciences
Faculty of Science
Chulalongkorn University
Academic Year 2007

Copyright of Chulalongkorn University

Thesis Title:	GENETIC RELATIONSHIPS BETWEEN TWO HONEY BEES (*Apis mellifera* LINNAEUS, 1758 AND *Apis cerana* FABRICIUS, 1753) AND VARROA MITES IN THAILAND
By	Miss Tipwan Suppasat
Field of Study	Biological Sciences
Thesis Advisor	Professor Siriwat Wongsiri, Ph.D.
Thesis Co-advisor	Associate Professor Deborah R. Smith, Ph.D.
Thesis Co-advisor	Assistant Professor Sureerat Deowanish, D.Agr.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

S. Hannongbua ..... Dean of the Faculty of Science

(Professor Supot Hannongbua, Ph.D.)

THESIS COMMITTEES

K. Thirakhupt ..... Chairman

(Associate Professor Kumthorn Thirakhupt, Ph.D.)

..... Thesis Advisor

(Professor Siriwat Wongsiri, Ph.D.)

..... Thesis Co-advisor

(Associate Professor Deborah R. Smith, Ph.D.)

Sureerat Deowanish ..... Thesis Co-advisor

(Assistant Professor Sureerat Deowanish, D.Agr.)

Chariya Lekprayoon ..... Member

(Associate Professor Chariya Lekprayoon, M.Sc)

Siriporn Sittipraneed ..... Member

(Associate Professor Siriporn Sittipraneed, Ph.D.)

Wandee ..... Member

(Assistant Professor Wandee Wattanachaiyingcharoen, Ph.D.)

ทิพย์วรรณ สรรพสัตย์: ความสัมพันธ์ทางพันธุกรรมระหว่างผึ้ง 2 ชนิด (*Apis mellifera* LINNAEUS, 1758 และ *Apis cerana* FABRICIUS, 1753) กับไรวาร์รัว ในประเทศไทย (GENETIC RELATIONSHIPS BETWEEN TWO HONEY BEES (*Apis mellifera* LINNAEUS, 1758 AND *Apis cerana* FABRICIUS, 1753) AND VARROA MITES IN THAILAND) อ. ที่ปรึกษา: ศ. ดร. สิริวัฒน์ วงษ์ศิริ, อ. ที่ปรึกษาร่วม: Assoc. Prof. Deborah R. Smith, Ph. D., ผศ. ดร. สุรีรัตน์ เตี่ยววาณิชย์. 158 หน้า.

การตรวจสอบต้นกำเนิดทางพันธุกรรมจากแม่โดยอาศัยรูปแบบทางพันธุกรรมในไมโทคอนเดรียลดีเอ็นเอของผึ้งพันธุ์จำนวน 476 รัง ในภาคเหนือ ภาคกลาง ภาคตะวันออกเฉียงเหนือและภาคใต้ของประเทศไทย ด้วยวิธีอาร์เอฟแอลพี และการหาลำดับนิวคลีโอไทด์ พบความแตกต่างของสามกลุ่มรูปแบบทางพันธุกรรมได้แก่ กลุ่ม ThaiA1 (22%) ตรงกับผึ้งพันธุ์สายพันธุ์ *A. m. ligustica* กลุ่ม ThaiA2 (60%) ตรงกับผึ้งพันธุ์สายพันธุ์ *A. m. carnica* ซึ่งจัดอยู่ในสายความสัมพันธ์ทางวิวัฒนาการแบบ C ทั้งสองกลุ่ม และกลุ่ม ThaiB (18%) จัดอยู่ในสายความสัมพันธ์ทางวิวัฒนาการแบบ O ซึ่งพบน้อยในประชากรผึ้งพันธุ์ของภาคเหนือและภาคกลาง

สองสายความสัมพันธ์ทางวิวัฒนาการของไมโทคอนเดรียลดีเอ็นเอในผึ้งโพรงไทยคือ เมนแลนด์เอเชีย และซุนดาแลนด์เชื่อมต่อกันที่บริเวณคอคอดกระ การศึกษาโครงสร้างประชากรของผึ้งโพรงไทย 184 ตัวอย่าง จาก 6 ประชากร (เหนือ กลาง ตะวันออกเฉียงเหนือ ประจวบคีรีขันธ์ ชุมพร และเพนนิซุลา) ด้วยวิธีเอเอฟแอลพี และทีอี เอเอฟแอลพี และการวิเคราะห์ข้อมูลทางพันธุกรรมพบความแตกต่างระหว่างประชากรเล็กน้อย

สามรูปแบบของสายความสัมพันธ์ทางวิวัฒนาการในไมโทคอนเดรียลดีเอ็นเอของไรวาร์รัวในรังผึ้งพันธุ์ ได้แก่ *Varroa destructor* สายพันธุ์เกาหลี 38 ตัวอย่าง สายพันธุ์ญี่ปุ่น 3 ตัวอย่าง และสายพันธุ์เวียดนาม 1 ตัวอย่าง ซึ่งไรวาร์รัวสายพันธุ์เวียดนามนี้พบในรังผึ้งโพรงไทยแถบเทือกเขาสูงที่ละติจูด 18 องศาเหนือขึ้นไป ไรวาร์รัวชนิด *V. jacobsoni* เข้าทำลายเฉพาะรังผึ้งโพรง โดยไรวาร์รัวสายพันธุ์มาเลเซียพบในรังผึ้งโพรงสายความสัมพันธ์ทางวิวัฒนาการแบบซุนดาแลนด์ และสายพันธุ์นอร์ทไทยพบในรังผึ้งโพรงสายความสัมพันธ์ทางวิวัฒนาการแบบเมนแลนด์เอเชีย

สาขาวิชา....วิทยาศาสตร์ชีวภาพ.... ลายมือชื่อนิสิต.....

ปีการศึกษา.....2550..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

467 38107 23: MAJOR BIOLOGICAL SCIENCES
KEY WORDS: *Apis mellifera*/ *Apis cerana*/ *Varroa jacobsoni*/ *Varroa destructor*/ RFLP/ AFLP/ TE-AFLP/ AMOVA/ DNA/ HOST/ PARASITE/ GENETIC/ POPULATION

TIPWAN SUPPASAT: GENETIC RELATIONSHIPS BETWEEN TWO HONEY BEES (*Apis mellifera* LINNAEUS, 1758 AND *Apis cerana* FABRICIUS, 1753) AND VARROA MITES IN THAILAND. THESIS ADVISOR: PROF. SIRIWAT WONGSIRI, Ph. D., THESIS CO-ADVISORS: ASSOC. PROF. DEBORAH R. SMITH, Ph. D., ASST. PROF. SUREERAT DEOWANISH, D. Agr., 158 pp.

To determine the matrilineal origin of Thai *Apis mellifera*, mtDNA haplotypes of 476 colonies in North, Central, Northeast and South Thailand were determined by RFLPs and DNA sequencing. Three haplotype groups were found: ThaiA1 (22%) matched *A. m. ligustica*, ThaiA2 (60%) matched *A. m. carnica* (both C lineage), ThaiB (18%) matched O lineage; O lineage was rare in North and Central populations.

Two mtDNA lineages of *A. cerana* occur in Thailand: Mainland Asia and Sundaland, meeting at Kra ecotone. Investigation of population structure of 184 samples from 6 populations (North, Central, Northeast, Prachuap Khiri Khan, Chumporn, Peninsular), using AFLPs, TE-AFLPs and AMOVA revealed little differentiation among populations.

Three mtDNA lineages of *Varrroa destructor* were found on *A. mellifera* colonies: Korea (n=38), Japan (n=3) and Vietnam (n=1); the Vietnam haplotype was found on *A. cerana* in mountainous regions north of 18 °N latitude. *V. jacobsoni* occurred only in *A. cerana* colonies: Malaysia haplotype on Sundaland *A. cerana* lineage, NorthThai haplotype on Mainland Asia lineage.

Field of study...Biological Sciences... Student's signature.....
Academic year.....2007..... Advisor's signature.....
Co-advisor's signature.....
Co-advisor's signature.....

ACKNOWLEDGEMENTS

I would like to express my deepest sense of gratitude to my advisor and co-advisors: Prof. Siriwat Wongsiri, Assoc. Prof. Deborah R. Smith and Asst. Prof. Sureerat Deowanish for their valuable guidance, kindliness suggestions, encouragement and supports throughout my study. Also, thanks to thesis committees: Assoc. Prof. Kumthorn Thirakhupt, Assoc. Prof. Chariya Lekprayoon, Assoc. Prof. Siriporn Sittipraneed and Asst. Prof. Wandee Watanachaiyingcharoen.

I have been fortunate to have in valuable contributions from many people both in Thailand and USA. I would like to thank 5 offices of Apicultural Extension: Chiangmai, Khonkhaen, Phitsanulok, Chanthaburi and Chumporn stations, every bee farms, beekeepers, local people and drivers for their kindliness to give honey bee and mite samples. Many thanks for valuable friendship, love, and helpful thought my friends: Sucheera Insuan, Thadsanee Chaiyawong, Piyamas Nanork, Kamolporn Boonthaworn, Orawan Phupisut, Chayanee Oddsup, Sasikan Juancharoen, Thunyaluk Takodee, Orawan Duangpakdee for their friendship, helpful and wonderful assistants. Extend to Sarawut Pungrussamee, Alongkot Pollawat, Marut Chinnawatchareonporn, Monruedee Chaiyapo, Prapaipit Srimawong and Sirikul Thammajitsakul. I wouldn't be here if wasn't Natapot Warrit, thanks for his attendance, guidance, encouragement and all everything that you gave. I had a very great time in Lawrence with all people in Deborah Smith's Laboratory: Dr. Robert Hagen, Molly Rightmyer, Lynn Veriffette, Dr. Maria Elfler, Victor Gonzales and Randy&Susan Su for their friendship, kindliness several accommodations for my life in USA. Sincerely, thanks extend to Prof. Charles Michner for his attendance for bee identification, David Macleod and Daniel J. Bennett for their friendship.

The financial was supported from the Commission on Higher Education Scholarship through the Naresuan University, the Thailand Research Fund: Royal Golden Jubilees Ph.D. Program (No. PHD/0169/2547) and research grant (RTA45008-Siriwat Wongsiri). Also, thank to Biological Sciences Ph. D. Program and Center of Excellence in Entomology, Faculty of Science, Chulalongkorn University. My research could be done completely by additional supported by the University of Kansas for giving an opportunity to do research.

I express my deepest appreciation to my parents and older sister for their love, kindness, encouragement and believing me. Thank you for valuable chance, knowledge and everything that you gave to my life. Finally, I have to thank every valuable bees and mites.

TABLE OF CONTENTS

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Page

LIST OF TABLES

Page

Page

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF FIGURES

Page

Page

Page

CHAPTER I

INTRODUCTION

Two species of multiple combs nesting cavity honey bees, *Apis mellifera* has been widely managed for commercial beekeeping in the world and *A. cerana* is an Asian honey bee which has distributed and maintained around Asia. In Thailand, *A. mellifera* is an exotic species which was introduced about 60 years ago for research and beekeeping propose while *A. cerana* is a naturally native species.

The parasitic honey bee mites belonging to genus *Vorroa* is a major pest of honey bees, *A. mellifera*. Asian honey bee, *A. cerana*, has probably co-evolved with *Varroa* mites. Firstly, *V. jacobsoni* was described from *A. cerana* in Java by Oudemans (1904). Insight of *V. jacobsoni* had begun parasitizing *A. mellifera* when honey bees introduced into Asia.

Anderson and Trueman (2000) first reported within complex species of *V. jacobsoni* and described newly species, named *V. destructor* based on morphological and molecular data. The results supported that the relationship between *V. jacobsoni* and native host *A. cerana* in Asia. Two well separated groups were from both mites from Mainland Asia and Malay-Peninsula and other three groups were unresolved data from the Philippines in *A. cerana*. On *A. mellifera* colonies were also investigated which found *V. jacobsoni* had not series infested in *A. mellifera* colonies. Thus, these results concluded that the *Varroa* mite infesting on *A. cerana* at Mainland Asia group on the other hand which can be found in *A. mellifera* that is newly *V. destructor*.

Additional, Warrit (2002) investigated biogeography of *Varroa* mites in a native host, *A. cerana*, in Thailand which found that *V. destructor* (Vietnam haplotype) also in *A. cerana* colonies resided at mountain region of northern of Thailand. Striking at *V. destructor* infesting on *A. cerana* of Thailand was not Japan/Thailand haplotype as same previous data but it was more similar to Vietnam haplotype which as same as most of *Varroa* mites infesting on *A. cerana* at Vietnam. So, the results suggested that the *V. destructor* in Thailand could be collected from *A. mellifera* colonies. While, there were four haplotypes of *V. jacobsoni* infesting on *A. cerana* (NorthThai1, NorthThai2, Malaysia and Samui1 haplotypes) depended on almost area in the Thai-Malay peninsula (excepted Northeastern area of Thailand). *V. jacobsoni* Malaysia and Samui1 haplotypes distributed lower longtitude than the Isthmus of Kra that related with Sundaland *A. cerana* but NorthThai1 and NorthThai2 haplotypes which mixed at the Isthmus of Kra and higher longitude not covered though mountainous region of Thailand.

Recently, genetic data are accurate to investigate in honey bees and their parasites. *A. mellifera* is most popular one model which has used molecular techniques that has initially studied since 1986 to resolve the intraspecific species, the evolutionary of origin, population differentiation and biogeography. Extended to *A. cerana* and *Varroa* mites which infected to honey bee colonies have investigated to resolve in many questions. This dissertation would like to verify genetic diversity, population differentiation, biogeography and distribution of *A. mellifera*, *A. cerana*, and their parasites, *Varroa* mite, infestation. Additional, the host-parasite relationship would be examined between honey bees and *Varroa* mites to give up information at northeastern and at Isthmus of Kra regions of Thailand.

Objectives are:

1.) To determine genetic variation, distribution and population differentiation of two honey bees, *A. mellifera* and *A. cerana*, also *Varroa* mites parasitized in their host colonies into Thailand.

2.) To examine the genetic relationship among two honey bees (*A. mellifera* and *A. cerana*) and their parasites (*Varroa* spp.) in Thailand.

Anticipated benefits are:

1) Information of subspecies and distribution of *A. mellifera* in Thailand will be useful for strain selection and development of commercial beekeeping program.

2) The results will provide new information of genetic variation, distribution and relationships among *A. mellifera*, *A. cerana* and *Varroa* mites in Thailand.

3) The results will provide biogeography and specificity of *Varroa* mites in their native hosts, *A. cerana*, and shifted hosts, *A. mellifera*, in Thailand.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

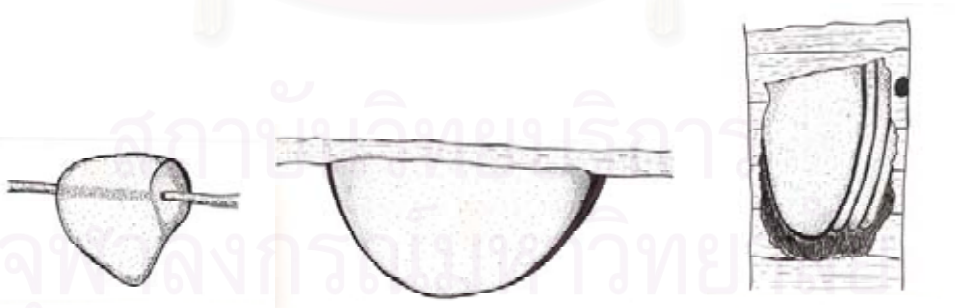
CHAPTER II

LITTERATURE REVIEWS

2.1 HONEY BEES

Honey bees are highly eusocial insect which belong to the genus *Apis*. This genus is an ancient lineages of bees that has evolved in tropical Eurasia and has migrated north and west global geographic (Grimaldi and Engle, 2005). Two types of nest construction provide for *Apis* classification. Firstly, an open-air nesting build a single-comb on branch of tree. There are two patterns of single comb nesting: dwarf honey bees (*A. florea, A. andreniformis*) (Figure 2.1A) and giant honey bees (*A. dorsata, A. laboriosa*) (Figure 2.1B). The secondly a multiple-combs cavity nest (Figure 2.1C) compose of *A. mellifera, A. cerana, A. nuluensis, A. koschevnikovi* (Oldroyd and Wongsiri, 2006). In Thailand, there are four native species: *A. dorsata, A. florea, A. andreniformis* and *A. cerana* and one introduced species, *A. mellifera* (Wongsiri, 1988).

Single comb **Multiple combs**



(A) (B) (C)

Figure 2.1 Drawing pictures of honey bees nesting: single comb open air nesting on branch of tree: *A. florea, A. andreniformis* (A); *A. dorsata* (B); multiple combs nesting cavity honey bees (*A. mellifera, A. cerana, A. koschevnikovi* and *A. nuluensis*) in natural darkness hole of tree or under roof (C).

Cavity nesting honey bees

Honey bees encounter a wide range of climate habitats, behaviors, physiology and morphology, which is reflected in numerous geographic races or subspecies (Ruttner, 1988). Evolution within the honey bees suggest that the present open-nesting species appeared in Southeast Asia 6-10 million years ago. The cavity nesting allow for precise thermoregulation with less energetic cost, so the new cavity-nesting species could colonize temperate as well as tropical regions few million years ago (Engel, 1999). Eventually, cavity nesting species spread into temperate regions from Europe or Asia by human (Figure 2.2A).

The European lineage has become isolated from Asian lineage by the expansion of desert areas of the Middle East. *A. mellifera* is now distributed most of Europe, Scandinavia and Africa (Figure 2.2A). Asian lineages commonly distributed around the Asia. Asian honey bees consisted of *A. cerana* and its related species: *A. koschevnikovi, A. nigrocincta* and *A. nuluensis* (Oldroyd and Wongsiri, 2006). Nest construction of *A. cerana* and *A. mellifera* are composed of multiple combs nesting cavity (Figure 2.1C). They normally build their multiple comb nests in concealed places. Cavity nesting honey bees may arise from the diversity of biotypes and climates which may have facilitated evolutionary change (Ruttner, 1988). Phylogeny of *Apis* agreed that cavity nesting is the derived condition and open-nesting honey bee is ancestor of the genus *Apis*. If the phylogeny at Figure 2.2B is correct, there are two branches ancestral of *Apis*, open-nesting honey bee i.e. *florea-andreniformis, dorsata-laboriosa* and cavity nesting branch i.e. *cerana-mellifera* (Oldroyd and Wongsiri, 2006). It is believed that *A. mellifera* split from its closest relative, *A. cerana* in western or central Asia and subsequently expanded into Europe and Africa (Ruttner, 1988; Garnery *et al*., 1991; Cameron, 1993; Shappard and Meixner, 2003; Whitfield *et al*., 2006). At present, there are four species of Asian cavity

nesting honey bee exists: *A. cerana*, *A. koshevnikovi*, *A. nigrocincta* and *A. nuluensis*. From the phylogenic tree, *A. nuluensis* is closely related to *A. cerana* (Tanaka *et al*., 2001) and this support that *A. koshevnikovi* is a sister group of *A. cerana*+*A. nuluensis* which distribute in Sundaland (Otis, 1996). *A. nigrocincta* has inferred it to be a sister of *A. cerana*+*A. koschevnikovi* (Engel, 1999).

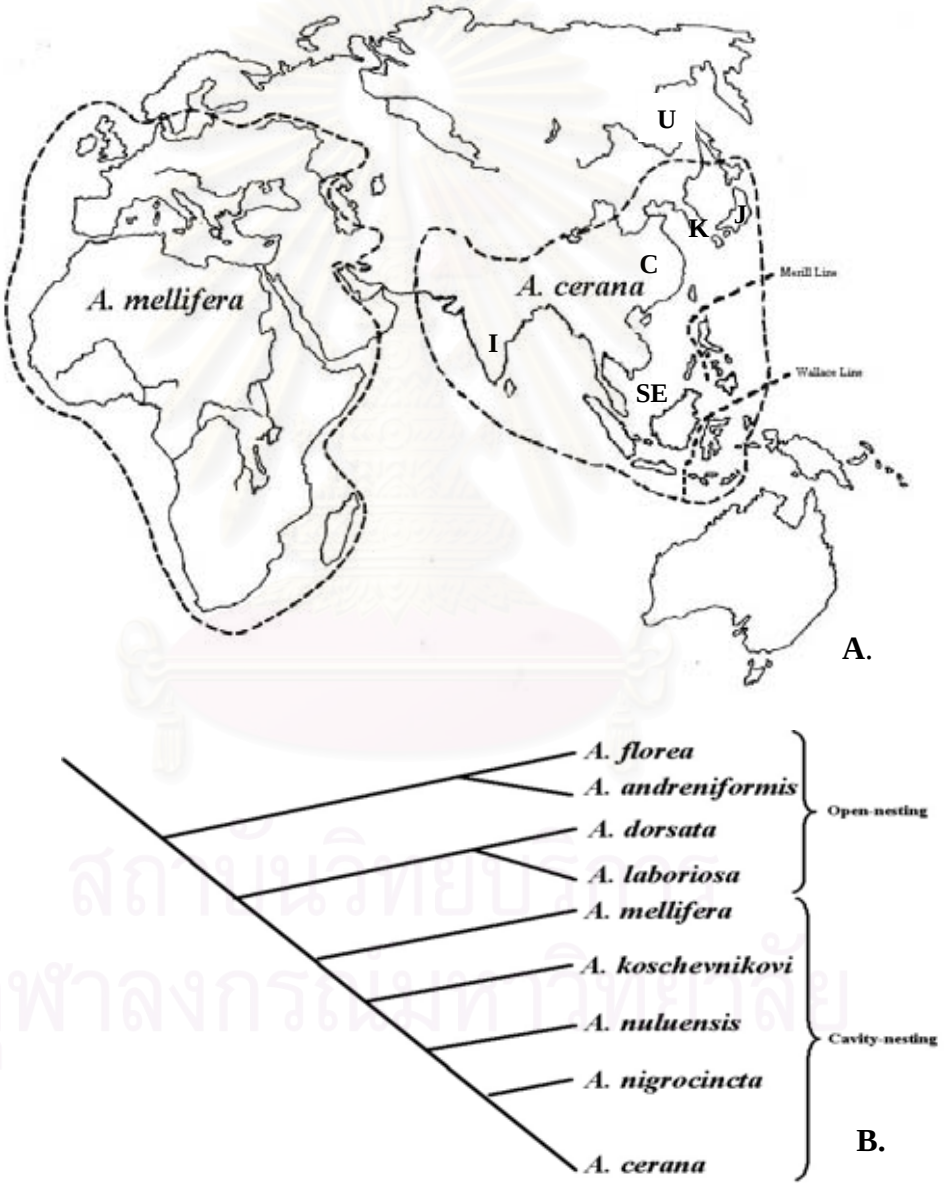


Figure 2.2 Distribution areas of multiple nesting cavity honey bees: *A. mellifera* and *A. cerana* (A) (adapted from Ruttner, 1988), phylogeny of honey bee genus *Apis* (B) (Oldroyd and Wongsiri, 2006).

Genetic variability in honey bees

Basically, molecular approaches have had a major impact on molecular ecological and evolutionary studies. Molecular genetics have numerous applications in population identification, population genetics and systematic (Avise, 2004). The principle of honey bee races or subspecies is based on the recognition of population within species. The genetic differences have achieved some measure in population. Any system of phylogenetic classification is the measurement of genetic variation. Example of genetic variation have been investigated in protein, enzyme, deoxy ribonucleic acid (DNA), ribonucleic acid (RNA) of honey bees. DNA analyses have valuable potential to increase the understanding in genetic information of honey bee populations (Smith, 1991a).

Figure 2.3 Complete mitochondrial genome of *A. mellifera* (Crozier and Crozier, 1993).

Implications of mitochondrial DNA are more suitable to study of the origin, population biology of hybrid population in honey bees (Hall and Muralidharan, 1989; Smith *et al.*, 1989; Smith, 1991a). The mitochondrial DNA of honey bees consist of the gene encoding 22 transfer RNAs, 2 ribosomal RNAs and 13 proteins. There are 6 genes for enzyme synthesis: cytochrome b (Cytb), Cytochrome Oxydase subunit I-III

and ATPase complex (ATPase 6 and ATPase 8). Seven protein encoding genes are subunits of the respiratory chain NADH dehydrogenase complex (ND 1-6 and ND 4L) (Wolstenholme, 1992). The complete sequence of honey bees, *A. mellifera*, mitochondrial genome is between 16,000-17,000 bp long (Figure 2.3), composes of 43.2 % A, 41.7% T, 5.5% G and 9.6% C. Normally, A-T rich region is at non-coding between $tRNA^{leu}$ and COII genes (Crozier and Crozier, 1993). The duplicate of intergenic gap between $tRNA^{leu}$ and COII genes represent essential function (Cornuet *et al*., 1991). This region may be prone to repeated duplicative events during evolution (Crozier and Crozier, 1993).

Recently, genome sequence of nuclear copies of mitochondrial sequences in honey bees, *A. mellifera*, now in hand. It is possible to use flanking sequences that be specific amplify the required. The sequences can be used as genetic markers from different individual or population. To understand the evolution of these sequences within and between closely related species or subspecies (Behura, 2007). Genomic DNA of honey bee is more A+T rich than other insect. Gene length of honey bee shows a striking relation to G+C content indicates that total intron length increase. In contrast to vertebrate which intron length decrease with G+C contents (The Honeybee Genome Sequencing Consortium, 2006).

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

2.1.1 Diversity of *Apis mellifera*

Classification and natural history

The western honey bee, *A. mellifera*, is an economic insect to produce high amount of honey, pollen and other products. Human can manage and carry its worldwide for beekeeping. More than 25 subspecies (Table 2.1) are currently recognized in morphology (Engel, 1999). The origin of *A. mellifera* has been believed that to be original in Asia,

the Middle East, Europe or Africa (Figure 2.2A) (Ruttner, 1988). In Shappard and Meixner (2003) noted about origin of *A. mellifera* populations occurred at Kazakhstan of Central Asia (Figure 2.2A and 2.4). Recently, The Honeybee Genome Sequencing Consortium (2006) suggested the origin of *A. mellifera* was derived from Africa lineage (Figure 2.5).

Figure 2.4 Distribution of naturally origin area of *A. mellifera* subspecies and mitochondrial lineages at Europe, Near East and Africa (adapted from Franck *et al.*, 1998).

Genetic variability of *A. mellifera*

Initially, whole genome of mitochondrial DNA has been proved to be a remarkable tool for study the genetic differentiation and natural range origin of *A. mellifera*. Restriction Fragment Length Polymorphism (RFLP) is a powerful technique to investigate honey bees subspecies (Moritz *et al.*, 1986). Whole of mitochondrial DNA has been used restriction enzyme cleavage to study *A. mellifera* polymorphism (Smith and Brown, 1988; 1990; Smith *et al*., 1989; Hall and Smith, 1991).

Study of *A. mellifera* mitochondrial DNA has revealed at least 4 major mitochondrial lineages: M or West European, C or Eastern Mediterranean, A or African, (Hall and Muralidharan, 1989; Smith *et al*., 1989; Cornuet and Garnery, 1991; Arias and Sheppard, 1996) and O or Middle Eastern lineage (Franck *et al*., 2000a; Palmer *et al*., 2000). The fifth newly lineages is Y in which found in Ethiopia (Franck *et al*., 2001).

Partial of mitochondrial genome sequencing of large subunit RNA (lsRNA) and cytochrome oxydase subunit I and II (COI-COII) have been completed (Crozier *et al.*, 1989). The COI-COII region including intergenic (between $tRNA^{leu}$-COII) contains an important length polymorphism. Restriction patterns of *Dra* I digestion show combination of two sequences, P and Q in the non-coding sequence. P element displays into two forms as P or Po. Q element can be more than one copy which shows different in *A. mellifera* lineages (Cornuet *et al*., 1991). Until, Crozier and Crozier (1993) has been sequenced complete mitochondrial genome of *A. mellifera* (Figure 2.3) which served to study in other regions

Most of portion of mitochondrial DNA amplify with Polymerase Chain Reaction (PCR). Several of 6-bases recognition sites of restriction enzymes are popular to check variation of *A. mellifera*. Initiation of PCR-RFLP between $tRNA^{leu}$-COII

restricts with *Dra* I (tRNA^{leu}-COII/ *Dra* I). *A. mellifera* showed eighteen different haplotypes which can be divided into three lineages (C, M, and A) as same as morphological data (Garnery *et al*., 1992; 1993). During 19th to 20th century, origin of *A. mellifera* lineages have investigated using the region of tRNA^{leu}-COII digested with *Dra* I restriction enzyme (Garnery *et al*., 1992; 1993; Meixner *et al*., 1993; Smith, 1991; Smith *et al*., 1997; Pinto *et al*., 2005; 2007; Kandemir *et al.*, 2006b; 2006c).

In addition to detection of *Bgl* II digest in cytochrome b (cytb) region of mitochondrial DNA can separate African lineage (A) from the European lineages (C, M, O) (Crozier *et al*., 1991; Smith *et al*., 1997). East Mediterranean (C) honey bee lineage can detect in large subunit ribosomal RNA (lsRNA) region digest with *EcoR* I. Within West European (M), Middle East (O) and African (A) lineages can not restrict with the same enzyme in this region (Smith and Brown, 1990; Hall and Smith, 1991; Smith *et al*., 1997). Only of West European (M) can digest with *Hinc* II in cytochrome oxydase I (COI) region but no restriction site in other lineages (Hall and Smith, 1991; Smith *et al*., 1997).

Furthermore, restriction enzyme, *Hin*f I which has a 4-base recognition site can discriminate whole mtDNA of *A. m. intermissa* from *A. m. scutellata* (Smith *et al*., 1991). *Hin*f I restriction patterns in mitochondrial DNA indicated differentiation in European and African honey bee subspecies. Especially, O lineage can be detected using *Hin*f I digestion of COI region which identified *A. m. lamarckii* subspecies (Shappard *et al*., 1996).

Genetic variations of *A. mellifera* have been studied in many countries to prove the origin of *A. mellifera* or the invasion of African lineage with the others, for examples, within south African populations (Moritz *et al.*, 1994; Franck *et al.*, 1998; 2000a; 2000b; 2001), Spanish and Moroccan populations (Garnery *et al.*, 1995), Turkish populations (Smith *et al*., 1997; Palmer *et al*., 2000; Kandemir *et al.*, 2006b),

Costa Rica populations (Segura, 2000), Yucatan peninsula populations (Clarke *et al*., 2001; 2002), Canary Islands populations (De la Rúa *et al*., 2001; 2002), Western Andalusia populations (De la Rúa *et al*., 2004), southern Brazil and Uruguay populations (Diniz *et al*., 2003), central Asia populations which separate new *A. m. pomonella* (Sheppard and Meixner, 2003), Slovenian populations (Sušnik *et al.*, 2004), Iberian Peninsula populations (Arias *et al*., 2005) and northern Cyprus populations (Kandemir *et al*., 2006c) (Table 2.1).

Mitochondrial DNA polymorphisms have been used broadly in population of Neotropical African and Africanized honey bees in America (Shappard, 1988; Shappard *et al*., 1999; Hall and Muralidharam, 1989; Hall and Smith, 1991; Smith and Brown, 1988; 1990, Smith, 1991b; Schneider *et al*., 2004; Pinto *et al*., 2003; 2004; 2005; 2007). In the America, both of mitochondrial DNA markers and genomic DNA microsatellite markers can be identified Africanized or neotropical African bees subspecies (Clarke *et al*., 2001; 2002; Coulson *et al*., 2005; Diniz *et al*., 2003).

The currently population genetic analysis of *A. mellifera* were studied using new genome-based single nucleotide polymorphisms (SNPs). The result suggested *A. mellifera* originated from A lineage in Africa. There were at least two subsequent expansions from A lineage: one expansion in Europe (M lineage). Another one or more expanded into Asia and the Middle East (O and C). The SNPs shows first evidence of extensive admixture between C, M, and O lineages. African alleles are dominant in population after Africanization. C lineages alleles are replaced by African alleles. M lineages are unclear replacement of African alleles. Because of may be close relationship between A and M lineages (Figure 2.5) (Whitfield *et al*., 2006; The Honeybee Genome Sequencing Consortium, 2006).

Table 2.1 Twenty-six subspecies of *A. mellifera* are divided into 3 branches by morphometric and molecular data.

A. mellifera subspecies	Morphometric lineages [a]	DNA lineages
A.m. adami[a]	Near East M	O
A.m. cypria[ac]		
A.m. syriaca[abce]		
A.m. armeniaca[a]		
A.m. anatoliaca[abce]		C
A.m. meda[abc]		
A.m. caucasica[abce]		
A.m. pomonella[*de]	Cental Asia	???
A.m. litorea[abeg]	Tropical Africa A	Y[b]
A.m. jemenitica[ab]		
A.m. lamarckii[abceh]		A
A.m. scutellata[abcegh]		
A.m. adansonii[ab]		
A.m. monticola[agh]		
A.m. capensis[abe]		
A.m. unicolor[abe]		
A.m. sahariensis[abh]	North Africa A	
A.m. intermissa[abceh]		
A.m. iberica[abceh]	West Mediterranean M	A[b] M
A.m. mellifera[abceh]		
A.m. sicula[ab]	Central Mediterranean C	C
A.m. ligustica[abceh]		
A.m. cecropia[ab]		
A.m. macedonica[af]		
A.m. carnica[abcefh]		
A.m. siciliana[*b]		

Remarks: Based on molecular data, *A. mellifera* divided into 5 branches (A, M, C, O and Y) additional to two new subspecies, *A. m. siciliana*[*b] and *A. m. pomonella*[*d] are reports. [(a)] Ruttner, 1988; [(b)] Garnery *et al.*, 1993; 1995; Moritz *et al.*, 1994; Arias and Shappard, 1996; Franck *et al.*, 1998; 2000a; 2000b; 2001; [(c)] Pinto *et al.*, 2003; Kandemir *et al.*, 2006a; 2006b; 2006c; [(d)] Shappard and Miexner, 2003; [(e)] Whitfield *et al.*, 2006; [(f)] Sušnik *et al.*, 2004; [(g)] Meixner *et al.*, 2000; [(h)] Shappard *et al.*, 1995.

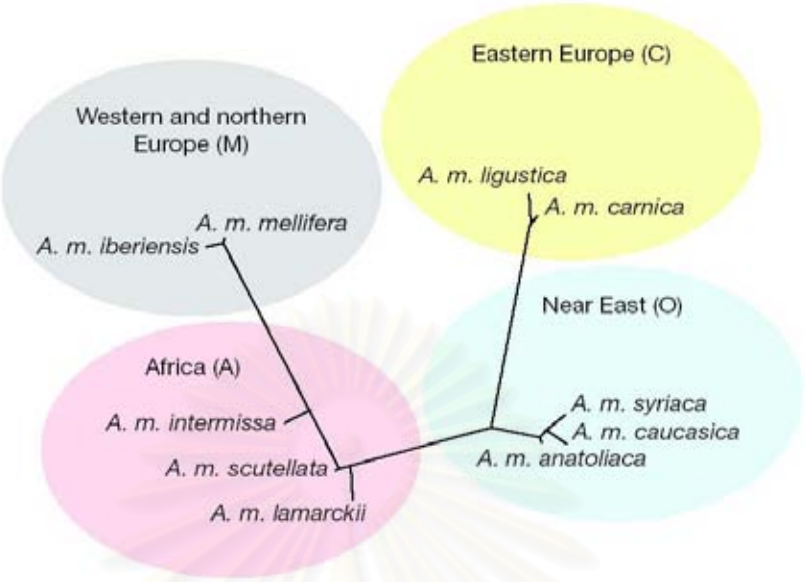


Figure 2.5 Neighbour-joining tree of ten different geographic *A. mellifera* subspecies which branches are supported by 100% bootstrap using SNPs data (The Honeybee Genome Sequencing Consortium, 2006)

A. mellifera in Thailand

A. mellifera (Figure 2.6) was introduced to Thailand in the early 1940s (Wongsiri, 1988) and 1950s (Akratanakul, 2000) for research at Chulalongkorn and Kasetsart Universities in Bangkok. But neither of these early introductions appears to have established lasting populations of *A. mellifera*. In the 1970s large numbers of *A. mellifera* are imported from Taiwan to Lampoon and Chiang Mai in northern Thailand for commercial purposes (Wongsiri *et al*., 1995). Additional importations may have come from Australia, Europe and Russia (Wongsiri *et al*., 2000; Kavinseksan *et al.*, 2004). *A. mellifera* beekeeping spread rapidly in northern Thailand, especially Chiang Mai province, and beekeeping with *A. mellifera* was later extended through the rest of Thailand (Thapa and Wongsiri, 1997; Chantawannakul *et al*., 2004). Today, there

are about 300,000 managed colonies of *A. mellifera* in Thailand, with more than half in the northern provinces (Wongsiri *et al*., 2000). There is little evidence of wild or feral populations. Currently, the survey of *A. mellifera* in northern Thailand indicated a potential problem for native beekeeping operation which be developed into a sustainable development activity in the region (Chantawannakul *et al*., 2004). The matrilineal origin of *A. mellifera* currently in Thailand is unknown but could include a mixture of many subspecies, the identity of Thai honey bee populations is potentially of interest for maintenance and improvement of Thai honey bee stocks.

(A) (B) (C)

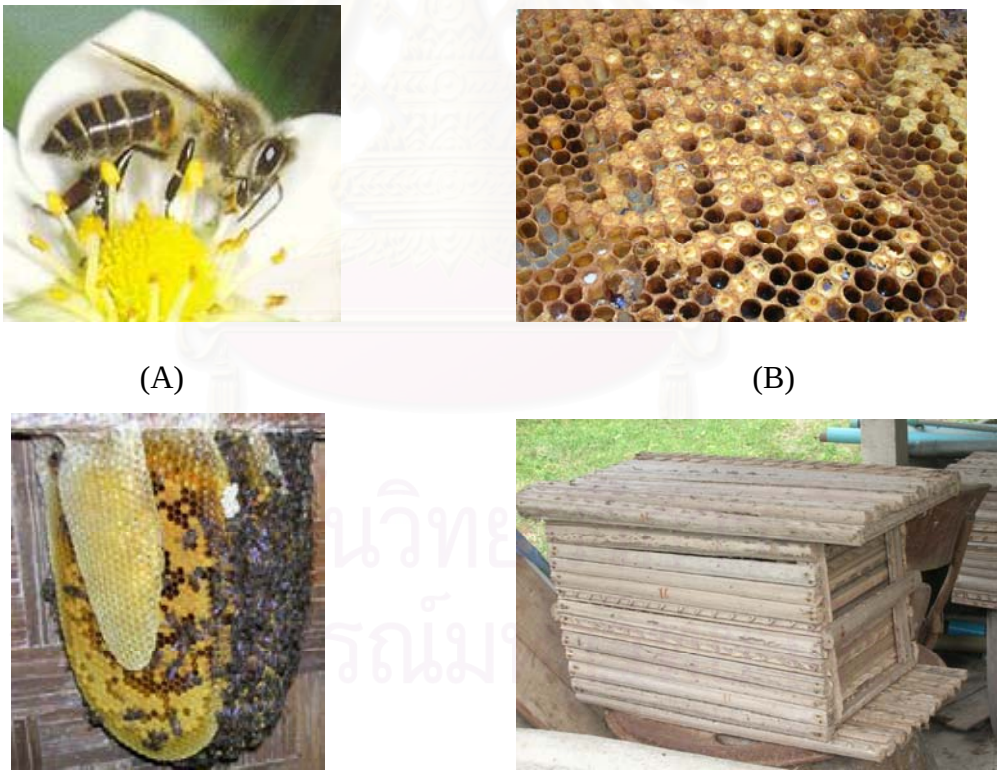
(D)

Figure 2.6 Adult worker of Thai *A. mellifera* on flower (A), yellow color (B), black color (C) of Thai *A. mellifera* queen and *A. mellifera* hives in an orchard in Thailand (D).

2.1.2 Diversity of *Apis cerana*

Classification and natural history

The Eastern honey bee or Asian honey bee, *Apis cerana*, (Figure 2.7) is widespread in temperate and tropical Asia. *A. cerana* distributed in all of Asia east of Iran, south of the great mountain ranges and the central deserts, Ussuria (U), Japan Island (J), Korea (K), China (C), and southeast Asia (SE) including to mainland, Malay-peninsula and west of the Wallace line (Figure 2.2A) (Ruttner, 1988; Oldroyd and Wongsiri, 2006). *A. cerana* subspecies are classified on basis of morphometrical and geographical distribution showed detail of subspecies distribution in figure 2.8 (Ruttner, 1988; Hepburn *et al*., 2001).



(A) (B)

(C) (D)

Figure 2.7 Adult workers of *A. cerana* collecting nectar and pollen from a flower (A). Drone broods of *A. cerana* (B) natural multiple combs nesting (C) and *A. cerana* hive for beekeeping (D).

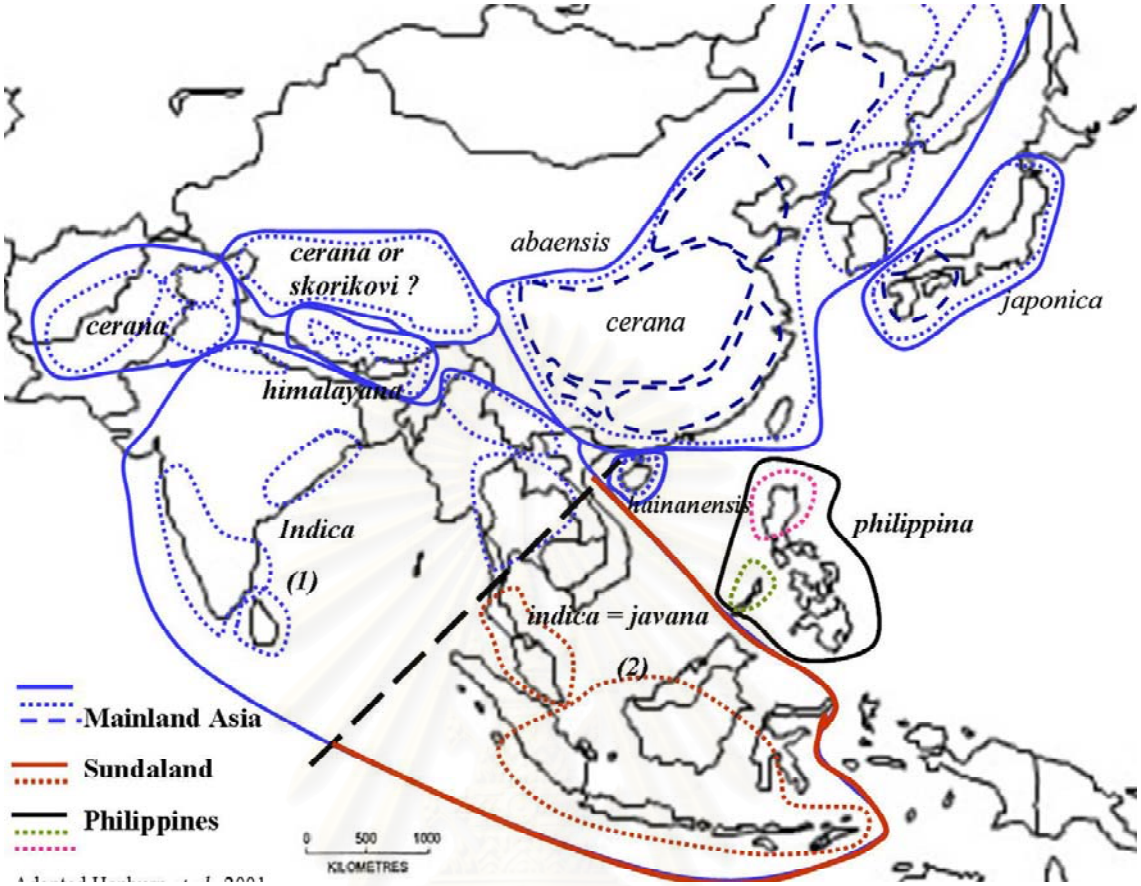


Figure 2.8 Distribution of distinct subspecies and ecotypes of *A. cerana*. ***A. c. cerana*** includes area of eastern Afghanistan, northern Pakistan, Kashmir, Himachal Pradesh, Korea, China and Ussuria sometimes, closely subspecies ***A. c. skorikovi*** covers at Tibet possibly as same as *A. c. cerana*. ***A. c. himalayana*** distribute at Nepal, Himalayas, Brahmaputra, Manipur, Mizoram and Nagaland and ***A. c. abaensis*** at Central China. ***A. c. indica*** is widespread ranges distributes at Southern India, Sri Lanka, Yunan, northern of Myanmar, Northern Thailand, Southern Thailand and continental Malaysia, Phuket, Samui, Sumatra, Java, Borneo, Lombok, Bali, Flores, most of Sulawesi, southern Sulawesi, Timor and Sabah. ***A. c. hainanensis*** is only at Haina Island, ***A. c. japonica*** is at Japan also, ***A. c. philippina*** spread at Mindanoa, Luzon and Palawan of the Philippines (adapted from Hepburn *et al*., 2001).

Asian cavity-nesting bees are more complex and even more speculative (Oldroyd and Wongsiri, 2006). *A. koschevnikovi* which distribute in Sabah and north Bornoa has reproductive isolation and different mating time with sympatric *A. cerana* species (Tingek *et al*., 1988; Ruttner *et al*., 1989; Rinderer *et al.*, 1989). The currently picture of Asian nesting honey bees, *A. koschevnikovi* is widely diverged and extant in Borneo and parts of peninsular Malaysia, southern Thailand, Java, Sumatra and Plain morph bee of India. *A. nigrocincta* has separately evolved in the island of Sulawesi. *A. nuluensis* is found in the mountains of Borneo *A. cerana* (Oldroyd and Wongsiri, 2006) is closely related to *A. cerana* (Tanaka *et al*., 2001).

Biogeography of *A. cerana* and other Asian honey bee species is undergoing changes as additional data become available (Smith *et al*., 2000). Recently, Hepburn *et al*. (2001) reviewed the infraspecific of *A. cerana* subspecies in Asia. *A. cerana* was grouped into three distinct groups, depending on the region of distribution i.e. 1) Western Asia (Afghanistan to the north and Pakistan to the south, at about longitude 60°, eastwards below the Himalayan mountain range and across the Indian sub-continent to Myanmar, at about longitude 94°), 2) Northeast Asia (China, the Manchurian plans of the former USSR, Korea and Japan) and 3) Southeast Asia (East of longitude 98° and southwards from about latitude 20°N to Timor below the equator at 10°S).

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Genetic variability in *A. cerana*

The molecular data on the evolutionary history of honey bees infer from mitochondrial DNA analysis indicates that *A. cerana* is the closest relative to *A. mellifera* (Garnery *et al*., 1991; 1992). Genetic variation in mitochondrial DNA of *A. cerana*, by using RFLP investigation, has been studied by Smith (1991a) who proposed that *A. cerana*

can be divided into three groups; mainland Asian group including Japan, Thailand, Malaysia, Borneo and south India, Luzon group , and Andaman Islands group.

The analysis of the genetic variation at a non-coding sequence between Cytochrome OxidaseI (COI) and Cytochrome Oxydase II (COII) in mtDNA is popular proven to the genus *Apis* (Cornuet *et al*., 1991). Subsequently, the portion of $tRNA^{leu}$-COII in mitochondrial DNA of *A. cerana* (*indica*, *cerana*, *japonica* and *himalayana*) in Asia also investigates with PCR-RFLP and separate *A. cerana* into six groups; 1) Japanese group, 2) Nepal, Vietnam and north-to-central Thailand group, 3) Korea-Tsushima group, 4) Taiwan group, 5) south Thailand group, and 6) Philippines group (Deowanish *et al*., 1996). The non-coding sequence between $tRNA^{leu}$-COII in mitochondrial DNA separated *A. nigrocincta* which related to *A. cerana* in Sulawesi. Geography of Sulawesi Island in Indonesia is explained that Island never connected to the mainland Asia (Smith *et al*., 2000; 2003).

The mitochondrial DNA data indicate four mitotypes i.e. mainland Asia group, Sundaland group, Palawan group and Luzon-Mindanao group (Figure 2.8) (Smith and Hagen, 1996; 1999; Smith *et al*., 2000; Hepburn *et al*., 2001; Smith *et al*., 2004). The mainland Asia group is extremely widespread and clearly separate from the other groups (Deowanish *et al*., 1996; Sihanuntavong *et al*., 1999; Sittipraneed *et al*., 2001a; Smith *et al*., 2000; Hepburn *et al*., 2001; Smith *et al*., 2004). Recently, the mitochondrial lineages of *A. cerana* in Burma indicate a clear break line between Sundaland and Mainland Asia at Isthmus of Kra (10˚ 34’ N latitude) (Smith *et al*., 2004).

Additional Cytochrome oxydase II sequence show that *A. nuluensis* is very close to *A. cerana*, especially to the geographic group of Thailand and Far Eastern Russia (Tanaka *et al*., 2001). Within Eastern mitochondrial haplotype of *A. cerana*, there are at

least five lineages: Mainland Asia (including the northern of Thailand), Sundaland (including the southern peninsular of Thailand), Sulawesi Indonesia, Palawan and Luzon-Mindanoa (Figure 2.8) (Smith *et a*l., 2000; Tanaka *et al*., 2001; Oldroyd *et al*., 2006; Oldroyd and Wongsiri, 2006).

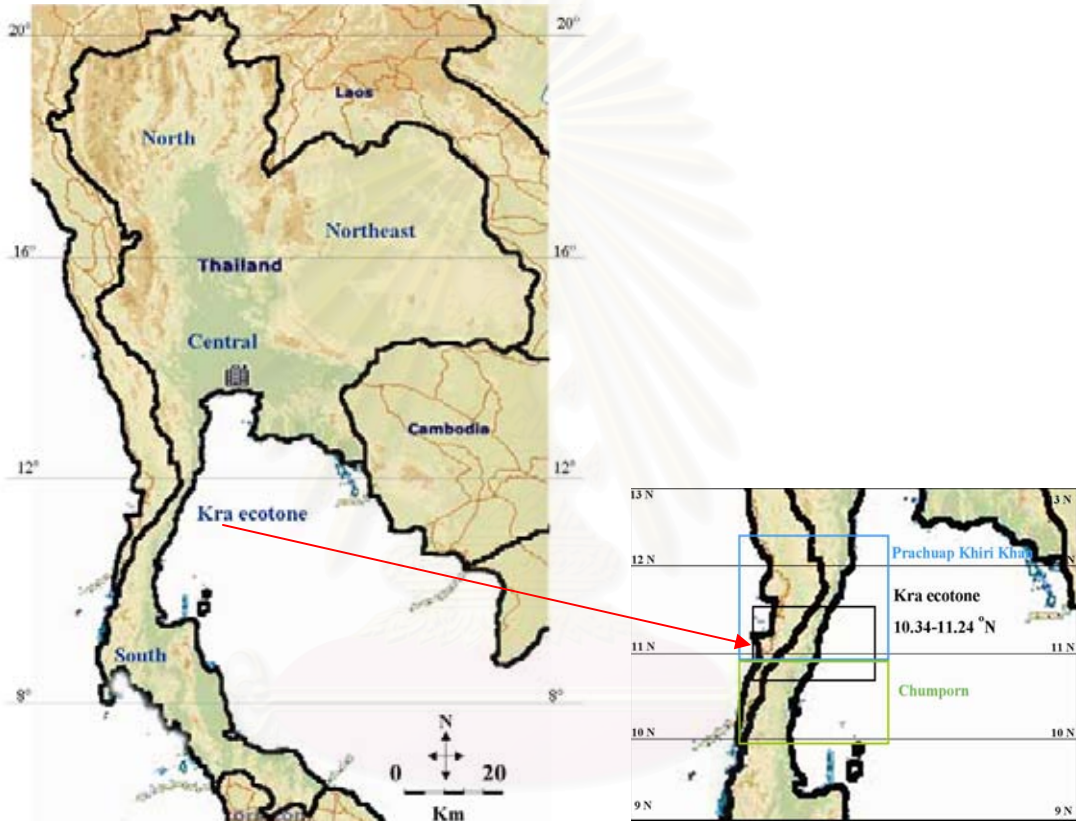


Figure 2.9 Geographic map of Thailand.

A. cerana in Thailand

In Thailand, *A. cerana* is a native species. It is interested for commercial beekeeping. Because it can be maintained in hive similar to *A. mellifera* (Wongsiri *et al*., 2000). The morphometric analysis of *A. cerana* from the northern to the southern of Thailand including Samui Island shows the separation of those populations into three groups: the northern latitude (Chiang Rai to Petchaburi), the southern latitude (Chumporn to

Songkhla) and Samui Island (Limbipichai, 1990; Sylvester *et al*., 1998). The Isthmus of Kra or Kra ecotone, where there is a transition from evergreen rainforest to more seasonal, semi-evergreen forest, between 10°34'N and 11°24'N (Figure 2.9) (Whitmore, 1984).

Genetic diversity and population differentiation of *A. cerana* in Thailand is one point to resolve biogeography of *A. cerana* in Asia (Smith *et al*., 2000; Hepburn *et al*., 2001). Scenario series of PCR-RFLP within Thai *A. cerana* examine within three regions of mitochondrial DNA: $tRNA^{leu}$-COII, srRNA, lsRNA (Sihanunthavong *et al*., 1999). Also, extend to the sequence of lrRNA gene (Sittipraneed *et al*., 2001a) and ATPase6-ATPase8 region of *A. cerana* (Songram *et al*., 2006). There are two distinct mitochondrial lineages of Thai *A. cerana*: Mainland Asia (north-central-northeast of Thailand) and Sundaland (south peninsular, Samui and Phuket Island of Thailand).

Combination of the mainland Asia and the Sundaland mitotypes of *A. cerana* display at the Isthmus of Kra. (Deowanish *et al*., 1996; Sihanunthavong *et al*., 1999; Smith and Hagen, 1996; 1999; Smith *et al*., 2000; Hepburn *et al*., 2001; Sittipraneed *et al*., 2001a; 2001b; Smith *et al*., 2004; Songram *et al*., 2006; Warrit *et al*., 2006).

The non-coding sequence between $tRNA^{leu}$ and COII mitochondrial DNA based phylogeograpic study of *A. cerana*. Recently, there are three new haplotypes of *A. cerana*, as Thai2, Thai3 group into mainland Asia lineages and ThaiSouth1 group into Sundaland lineages (Smith *et al*., 2000; Warrit, 2002; Warrit *et al*., 2006).

Thailand is overlap place of two mitotypes of *A. cerana* lineages. Mainland Asia *A. cerana* reaches its southern limit in the Thai-Malay Peninsula at Bang Sapan district, Prachuap Khiri Khan province (11°24’N and 99°31’E). While, Sundaland *A. cerana* reaches the farthest north at Tha Sae district, Chumporn province (10°34’N and 99°06’E) (Warrit, 2002). Two different of *A. cerana* lineages are also explained using

nuclear DNA markers. Microsatellite polymorphism analysis displayed intraspecific genetic differentiation of *A. cerana*. Heterogeneity of Thai *A. cerana* populations indicates the gene pool. The north population of *A. cerana* in Thailand suggested rate of gene flow may vary among male and female lineages (Sittipraneed *et al*., 2001b). Subsequently, restriction endonuclease analysis (REA) of genomic DNA with *Hae* III develop to examine *A. cerana* of Thailand indicate that comparable results with PCR-RFLP in mitochondrial DNA (Imjongjirak *et al*., 2004).

Amplified fragment length polymorphism-polymerase chain reaction (AFLP-PCR) which is a powerful method for assessing genetic differences among individuals, populations and independently evolving lineages such as species will be use to prove this hypothesis (Warrit, 2002). Recently, Amplified Fragment Length Polymorphisms (AFLP: Vos *et al*., 1995) and Three Enzyme Amplified Fragment Length Polymorphisms (TE-AFLP: van der Wurff *et al*., 2000) have been used to investigate population differentiation and gene flow of *A. cerana* population and to confirm status of *A. nigrocincta* which can be separated from *A. cerana* population (Smith *et al*., 2003).

2.2 PARASITIC BEE MITES

The parasitic mites (Acari) have become worldwide pest of honey bees. And, the mite has been a suppression role in beekeeping industry and crop pollinations. There are 3 parasitic mites that infest honey bees of the genus *Apis*: tracheal, varroa, and tropilaelaps (De Jong, 1997; Sammataro *et al*., 2000). Most of the parasitic bee mites of five native Asian honey bees are more specific to their own hosts than they are to the European honey bees (Lekprayoon *et al*., 1994).

2.2.1 Diversity of *Varroa* mites

Classification and natural history

The ectoparasitic mite in the genus *Varroa* (Acari: Varroidae) is cosmopolitans pest of honey bees. *Varroa* feed on the haemolymph of social cavity-nesting bees. *V. jacobsoni* firstly described from *A. cerana* in Java, Indonesia (Oudemans, 1904). Subsequently, *V. underwoodi* first discovered from *A. cerana* in Nepal (Delfinado-Baker and Aggarwal, 1987). *V. rindereri* is found to be a parasite of *A. koschevnikovi* (DeGuzman and Delfinado-Baker, 1996). These confine to the range of *A. koschevnikovi* in Borneo, Sumatra and south part of Malay Peninsular. *Varroa* may infest *A. nigrocincta* and *A. nuluensis* and often co-infests *A. cerana* colonies with *V. jacobsoni*, depending on locality (Oldroyd and Wongsiri, 2006). Since, Anderson and Trueman (2000) have described new *Varroa* species, *V. destructor*. The new species differ from *V. jacobsoni* which parasitized on *A. cerana* colonies. *V. jacobsoni* infests only within *A. cerana* recently distributes covering *A. cerana* Sundaland lineage: Java, Bali, Ambon, Sumatra, Lombok, Sumbawa, Flores of Indonesia, Borneo of Malaysia, Malay peninsula and Samui Island of Thailand. *V. destructor* infested mostly northern regions of *A. cerana* mainland Asia lineage. There are three unresolved genotypes of *Varroa* spp. infesting within *A. cerana* colonies in the Philippines (Anderson and Trueman, 2000; Anderson, 2000).

During 19th to 20th century, *V. jacobsoni* has been major pest to destroy *A. mellifera* in several countries. Until late of 19th found that the original *V. jacobsoni* can not reproduce in *A. mellifera* colonies (Anderson and Fuchs, 1998). There are least 2 complexes *V. jacobsoni* using morphological, behavioral and genetic identification. For example, mophological identification of female mites infesting on *A. cerana* (*V. jacobsoni*) are significantly spherical shape and smaller body size than those infesting

on *A. mellifera* (*V. destructor*). Even though there are some overlap in the body size of mites (Figure 2.10A and B) (Anderson and Trueman, 2000; Zhang *et al*., 2000). *Varroa* complexes species infested on five species of Asian honey bees (Anderson, 1994; Anderson and Sukarsih, 1996; Anderson and Fuchs, 1998; DeGuzman *et al*., 1997; DeGuzman *et al*., 1998; DeGuzman and Rinderer, 1999). Anderson and Trueman (2000) described newly *V. destructor* which shifted from *A. cerana* to *A. mellifera* host in Asia, it cause to become widespread pests in the world.

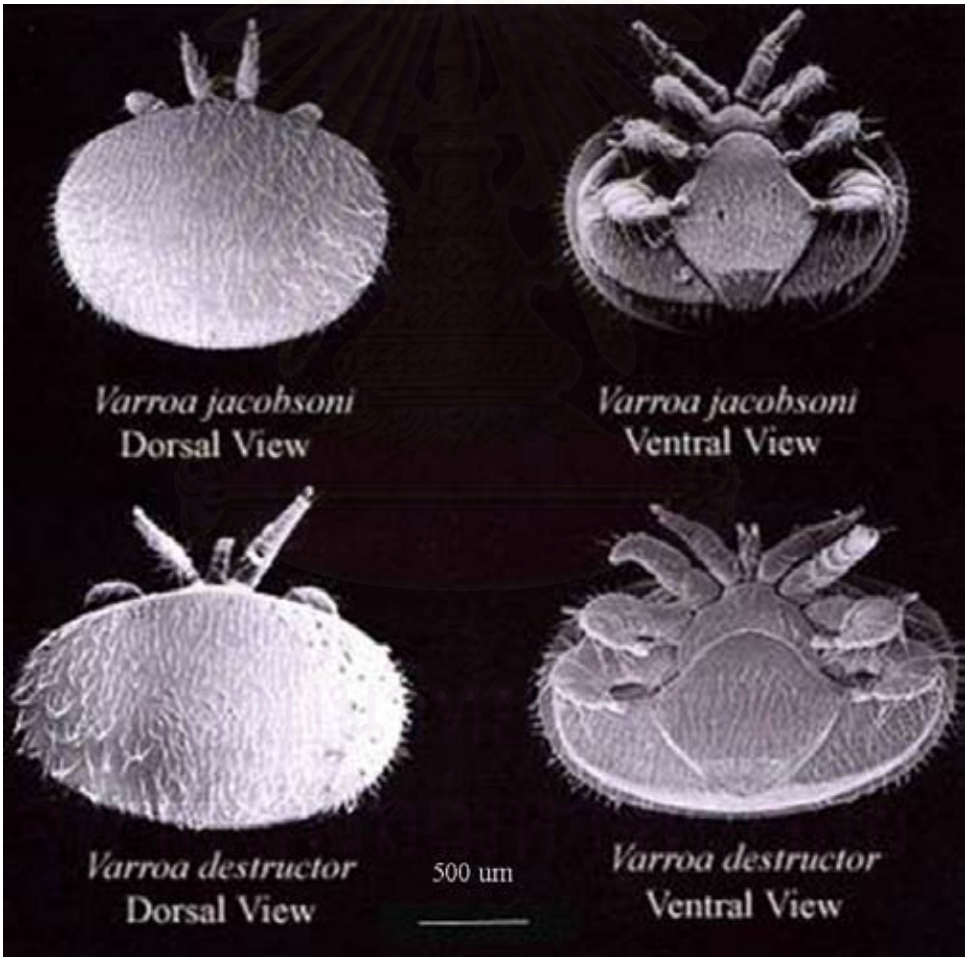


Figure 2.10 Dorsal and ventral views of *V. jacobsoni* (A.) and *V. destructor* (B.) (Anderson and Trueman, 2000).

Varroa mite shifted host from *A. cerana* to *A. mellifera* that is the most violence in new host. The switched evidences of *Varroa* mites have become in Japan at the 1950s, in China at the 1960s, in Europe at the late 1960s, in Israel at the 1970s (Sammataro *et al*., 2000). Next, the evidence of *Varroa* mites shifted in the 1960 have found on *A. mellifera* in the Philippines. In 1971, they have been present in *A. mellifera* colonies out side Asia. In 1975, they have spread to Europe, North Africa and South America. Exceptional Australia, New Zealand, and the state of Hawaii free from this pest (De Jong, 1997). But currently report has found *V. destructor* damage on *A. mellifera* colonies in New Zealand (Zhang, 2000).

Life cycle of *Varroa* mites

Varroa mites have a haplo-diploid or arrhenotokous sex determination system. Male is produced from unfertilized eggs (haploid), female develop from fertilized eggs (diploid) as same as honey bees. *Varroa* female, initially enter the honey bee brood cells of the last stage of worker or drone larvae, normally within 20 or 40 hours before the bee brood cells are sealed (Boot *et al*., 1992).

After that mother mite hide in the larval food at bottom of cell for 5 hours. In the time host develop to spin cocoon and mite fed on brood food. About 60 hours after the cell is completely capped then mother mite lay her first egg on the wall of the cell near the top. Up to five more female mite eggs are laid at approximately 30 hours intervals (Martin, 1995).

The mother mite maintained feeding site for her brood as the nymphs are unable to penetrate the skin of host. *Varroa* eggs hatch within 36 hours, develop rapidly to nymphs. Male is ready to mate within 190 hours of being laid. The mother mite prepares a rendezvous point for her faces place on the cell wall. And, it is here that the

male copulates with her sister. Until, the second matures, whereupon the male concentrates his attentions on hers. Adult mated female mites emerge from the cell along with the host. The mother mite can invaded a second cell. The first egg is generally male, and the rest female. Males develop from egg to adult in about 5-6 days and females in 7-8 days (Figure 2.11) (De Jong, 1997; Oldroyd, 1999; Sammataro *et al*., 2000)

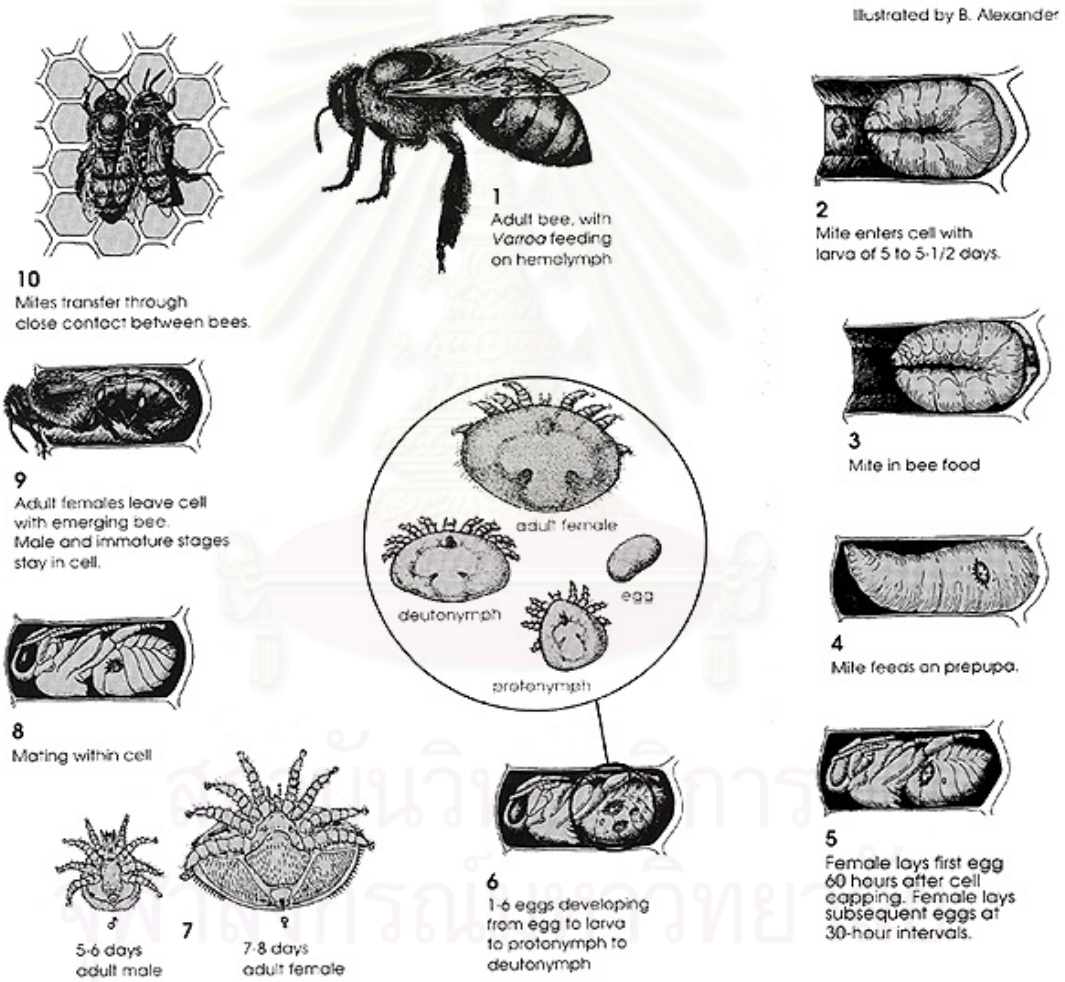


Figure 2.11 Life cycle of *Varroa* mite infesting honey bee, illustrated by B. Alexander (De Jong, 1997).

Varroa mites parasitized within honey bee colonies

Varroa is generally considered the most severe threat to the world beekeeping. In *A. cerana*, the original host of *Varroa*. They are likely to reproduce inside sealed drone brood cells (Figure 2.12A-C). Consequently, mite population within *A. cerana* colony is lower than *A. mellifera* (Webster and Delaplane, 2001). A tolerance to *Varroa* is exhibited in *A. cerana* consisted of several factors. A grooming behaviour followed by puncturing and subsequently killed the mites, is one of the important resistance factors (Peng *et al*., 1987). *A. cerana* remove mites more frequently and remove more mites which are found to be disorder or dead. Whereas those remove by *A. mellifera* workers are alive in hive (Wongsiri *et al*., 1987). Hygienic behavior is resistance mechanism of *A. cerana* to *Varroa* (Peng *et al*., 1987). *Varroa* reproduces only in the drone brood cells *A. cerana* (Koeniger *et al*., 1983). The developmental time of other castes from egg to emergence are insufficient for the mite development.

There are four characteristics of selective breeding of resistant bees to *V. destructor*: proportion of mites in brood, duration of the capped period, hygienic behaviour and suppression of mite reproduction (Harbo and Harris, 1999). The mite population growth is limited by the *A. cerana* behavioral and physiological adaptations which are lacked in *A. mellifera* (Boecking and Ritter, 1994). *V. destructor* reproduced in the much more numerous worker brood cells of *A. mellifera* (European honey bee = EHB) colonies. In *A. melllifera* (Africanized honey bee = AHB) have a unique tolerance to *V. destructor* that differed in the EHB colonies. Level of Korea haplotype reproduced in *A. m. scutellata* colonies similar to in EHB colonies while, be lacking of tolerance of AHB colonies (Figure 2.12 D-E) (Martin and Kryger, 2002; Medina *et al*., 2002; Martin and Medina, 2004).

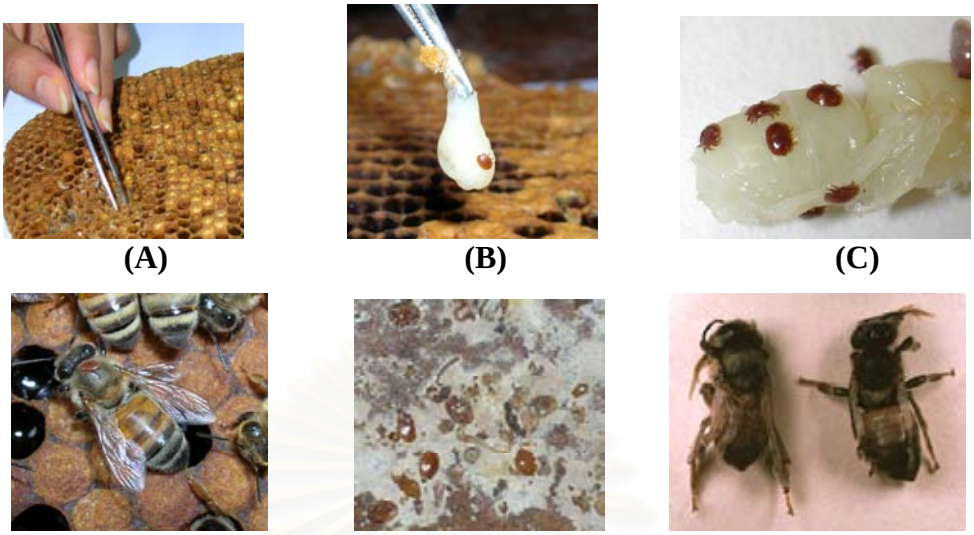


Figure 2.12 Collecting *Varroa* mites inside *A. cerana* drone brood cells (A-B). Adult of *Varroa* mites feed on pupa stage of *A. cerana* (C). Adult *Varroa* mites attach on adult *A. mellifera* in Thailand (D), *Varroa* mites are killed with chemical then they drop at bottom of *A. mellifera* hive (E) and adult of *A. mellifera* disorder (F).

Size of honey bee brood cells effects for mites developing. Comparison of the reproductive ability of *V. destructor* are reproduced successfully in *A. cerana* drone broods >> *A. m. scutellata* drone broods = EHB drone broods >> *A. m. scutellata* worker broods = EHB worker broods > AHB workerbroods (Martin and Kryger, 2002). *Varroa* mites prefer the largest brood cells such as in EHB colonies (Piccirillo and DeJong, 2003).

The infestation of *Varroa* mites in honey bee colony could be used as a model to study the complexities of the mite-bee relationship (Figure 2.13). This relationship depends on colony sizes, queen egg-laying rates and seasonal brood qualities which are easier than seasonal factor (Martin, 1998; Wilkinson and Smith, 2002). The reproductive biology of *V. jacobsoni* in *A. mellifera* (now is *V. destructor*) from tropical and sub-tropical regions of the

United States of America supported that some tolerance to that pest (Medina and Martin, 1999). *V. destructor* normally reproduce in brood cells of *A. mellifera* (Fuchs, 1994). In the tropical area, *A. m. scutellata* and hybrid of *A. m. carnica* in Brazil and Uruguay have shown female mites in worker brood cells (De Jong *et al*., 1984; Rosenkkranz and Engel, 1994). The climate type has an impact on *Varroa* population (Moretto *et al*., 1991). Population growth of *Varroa* mites is unexpectedly low under tropical conditions relative humidity values are only 9-25% within brood cells. But, tropical climate effect of environmental factors upon parasite virulent (Kraus and Velthuis, 1997).

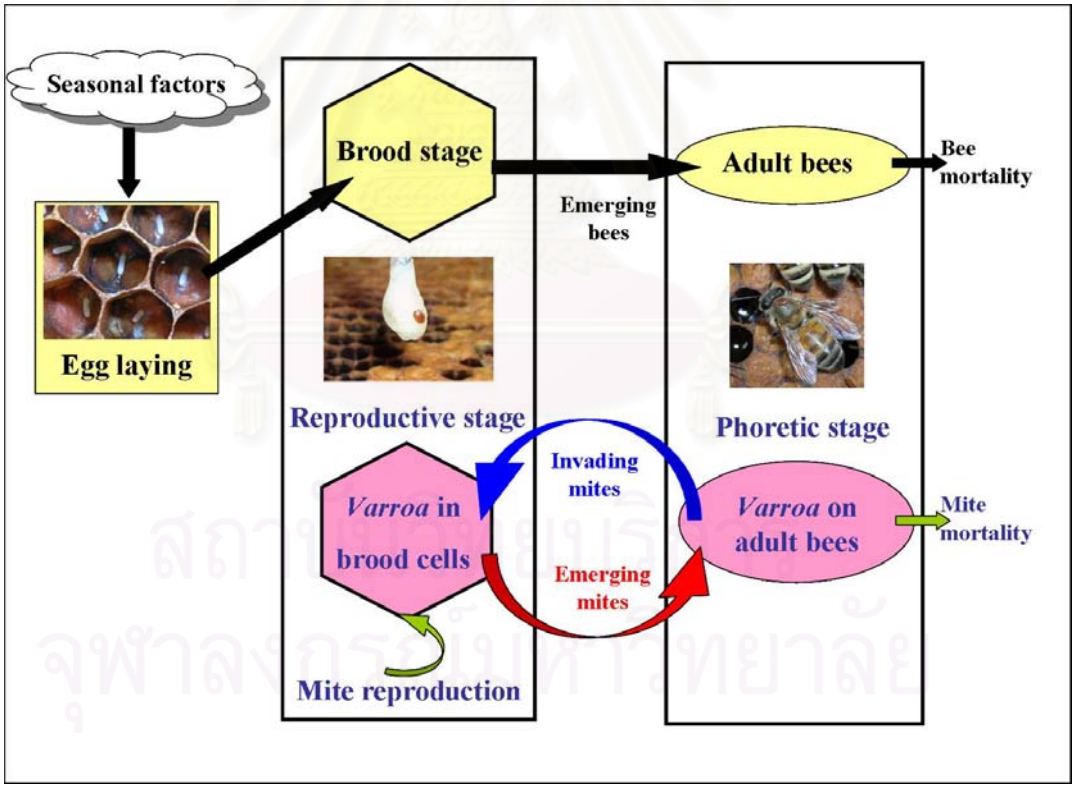


Figure 2.13 Basic model structure of *Varroa* mite parasitized on honey bee colony illustrated by Suppasat, T.

Table 2.2 *Varroa* mites (Korea, Japan/Thailand and Java haplotypes) infested on honey bees (Anderson, 2000).

Haplotypes	synonyms	Locations		Species
		Natural parasite of *A. cerana*	Infests&reproduces on *A. mellifera*	
Korea	Russian genotype R genotype GER genotype	Korea	Europe, Middle East, South Africa, Asia, North and South America	*V. destructor*
Japan/Thailand	Japan genotype J genotype	Japan and Thailand	Japan Thailand South America	*V. destructor*
Java	PNG genotype	Indonesia (Java, Sulawesi, Timor, Irian Jaya), Papua New Guinea	Not reproduce	*V. jacobsoni*

Genetic variability in *Varroa* mites

Molecular techniques are examined complexity of *V. jacobsoni* species. Initially, the Random Amplification of Polymorphic DNA technique (RAPD) indicated that *V. jacobsoni* infesting *A. cerana* in Malaysian Borneo were different from *V. jacobsoni* infesting *A. mellifera* in the USA and Germany (Kraus and Hunt, 1995). Two different genotypes of *V. jacobsoni* infests on *A. mellifera* in USA. There are R genotype which is found in Russia, Europe and five states of USA. Other, J genotype is found in Japan, Brazil and Puerto Rico (De Guzman and Rinderer, 1997; De Guzman *et al*., 1998).

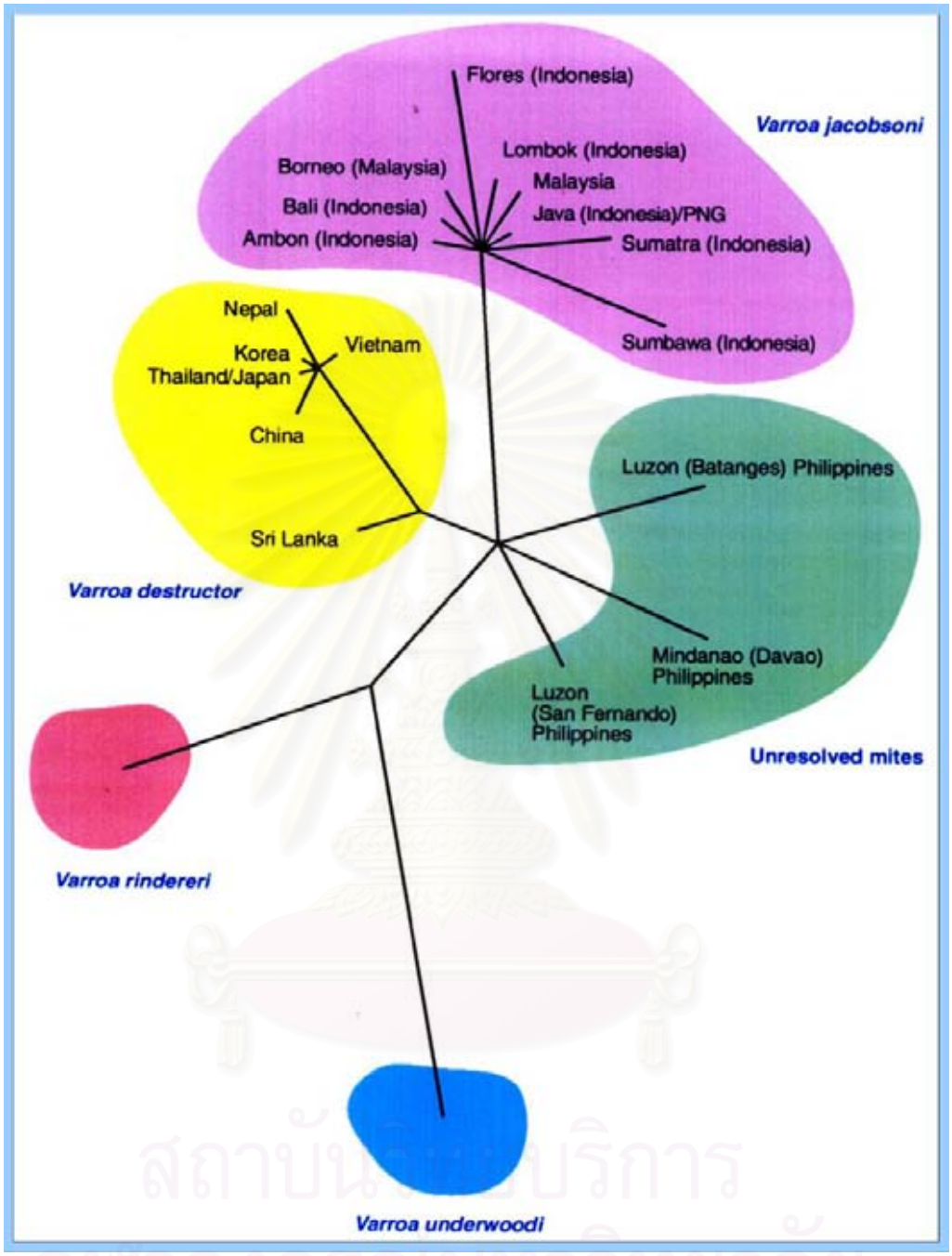


Figure 2.14 A bootstrap consensus tree of *Varroa* complex species, *Varroa* mites diverse into five distinct groups i.e. *V. underwoodi*, *V. rindereri*, *V. jacobsoni*, *V. destructor* and unresolved species at the Philippines (Anderson, 2000).

Subsequently, *V. jacobsoni* from *A. cerana* samples from Java Island differ from *A. mellifera* (Table 2.2). Cytochrome oxydase I gene in mitochondrial DNA of *V. jacobsoni* from Germany (GER genotype) digested by *Xho* I. *V. jacobsoni* from Papua New Guinea (PNG genotype) digested by *Sac* I restriction enzyme. Three distinct genotypes of *Varroa* consisted of R or GER genotype, J genotype and PNG genotype (DeGuzman *et al*., 1998; Anderson and Fuchs, 1998). Until 2000, *V. jacobsoni* infesting *A. cerana* in Asia using RFLP and sequencing at cytochrome oxydase I coding gene. Eighteen haplotypes of *Varroa* mites can be grouped into five different groups (Figure 2.14). *Varroa* haplotypes have assigned name from the country's or island's name. For example, Korea haplotype is *Varroa* mite parasitized on natural *A. cerana* colonies of Korea (Anderson, 2000). Nine mitochondrial COI haplotypes of *V. jacobsoni* which distributed in Malaysia-Indonesia region related to *A. cerana* Sundaland lineage. And, seven haplotypes of *V. destructor* can be infected on *A. cerana* in Mainland Asia lineage (Figure 2.15). There are Korea and Japan/Thailand haplotype of *V. destructor* could be also colonized on *A. mellifera* (Anderson and Trueman, 2000; Anderson, 2000).

The evidence of *Varroa* infesting in *A. cerana* and *A. mellifera* colonies in several countries in Asia has been investigation. In Vietnam found only *V. destructor*. Vietnam haplotype mostly colonize in *A. cerana* colonies and a few is also colonize in *A. mellifera*. While, Korea haplotype is the major pest infesting *A. mellifera* colonies which seems to be link between hosts (Fuchs *et al*., 2000). In China, three different haplotypes of *V. destructor* have been detected infesting *A. cerana* (China1, China2 and Vietnam haplotype). Major pests Korea haplotype can parasitize on *A. mellifera* colonies (Zhou *et al*., 2004). In Thailand, both of *V. destructor* (Vietnam haplotype)

and *V. jacobsoni* (North Thai1, North Thai2, Samui and Malaysia haplotypes) have been reported infesting *A. cerana* colonies in Thailand (Warrit, 2002; Warrit *et al*., 2006).

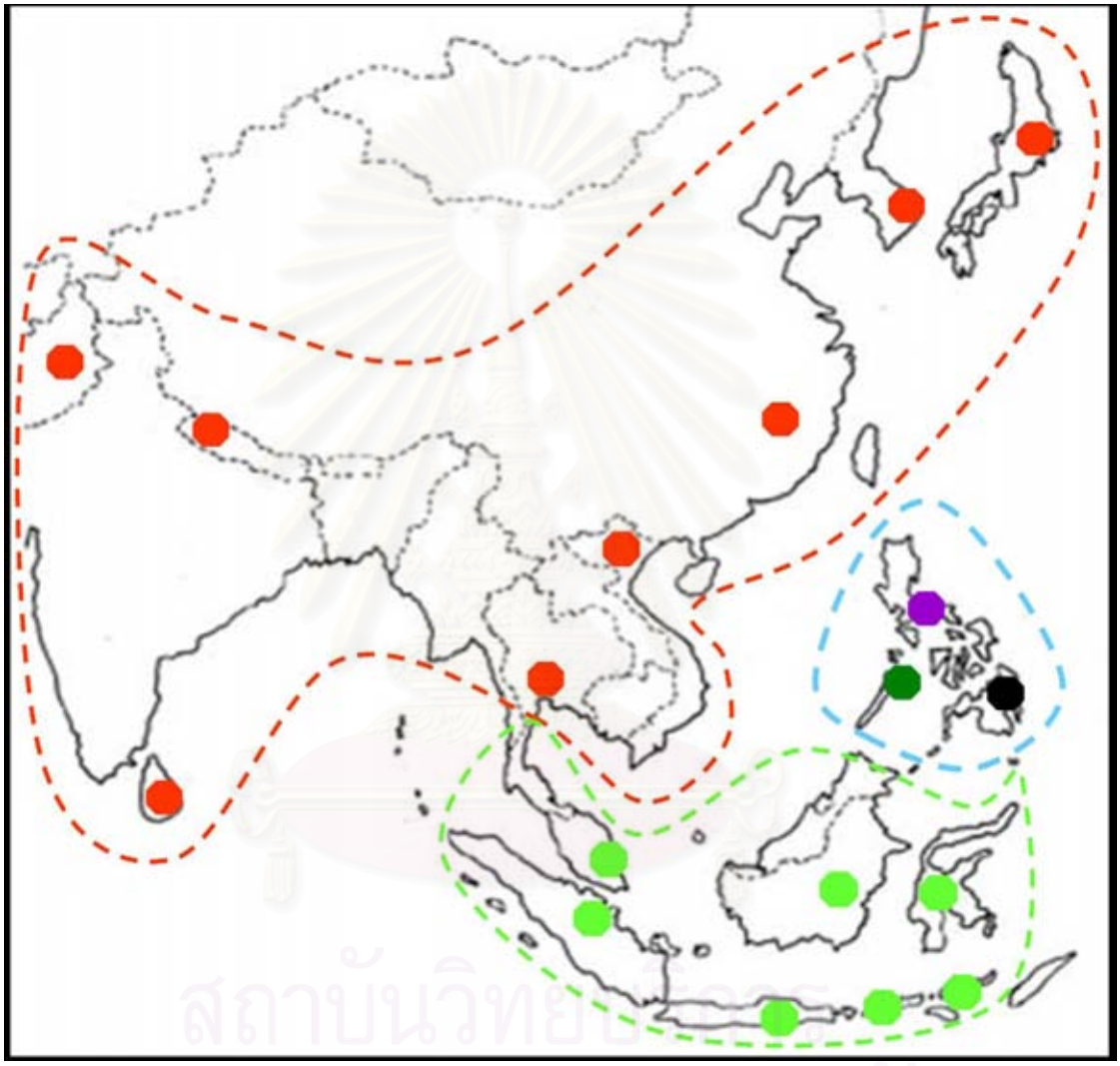


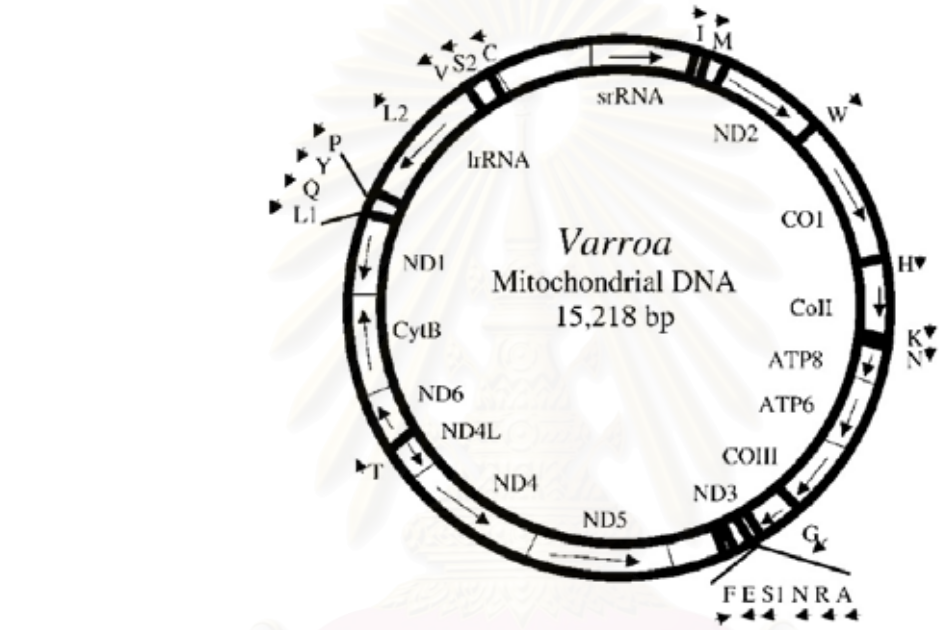
Figure 2.15 Host-parasite relationships of *Varroa* mites infested on natural host *A. cerana* in Asia show that *V. destructor* infested on *A. cerana* mainland Asia group (red line), *V. jacobsoni* infested on *A. cerana* Sundaland group (green line) and unresolved *Varroa* within *A. cerana* of the Philippines group (blue line) (B) (Warrit, 2002).

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

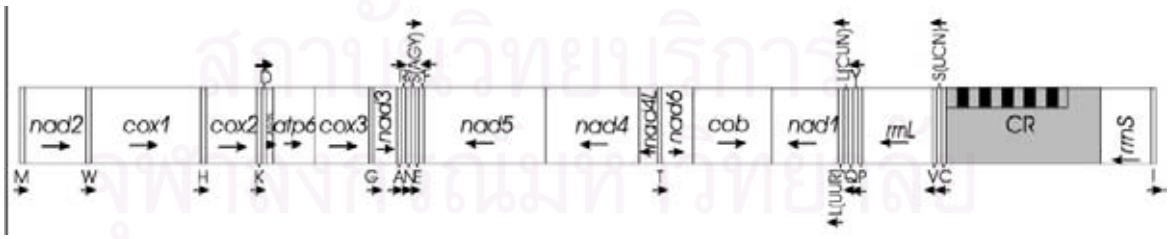
Presently, the majority western honey bee pest, *V. destructor* (Korea and Japan/ Thailand haplotypes) is current parasitized in *A. mellifera* colonies which resulted from the shifted from native host (Anderson 2000). Example of *Varroa* mites infesting on *A. mellifera* in North and South America, has two haplotypes become a pest in *A. mellifera* (De Guzman and Rinderer, 1997; Anderson, 2000). Only Japan haplotype widespread destroyed on *A. mellifera* in Brazil and Puerto Rico. But, the Japan showed low infestation rate in North America, Canada and Thailand. The most prevalent of Korea haplotype widely destroyed *A. mellifera* in Africa, Europe, the Middle East, Asia and the North America (Table 2.2) (Anderson, 2000). The newly *V. destructor* have been investigated in *A. mellifera* colonies to identify major haplotypes i.e. in New Zealand (Zhang, 2000), in United Kingdom (Martin, 2001), in *A. m. carnica* in the Mediteranean (Murilhas, 2002), in Brazil (Garrido *et al*., 2003), in Middle east of *A. mellifera* colonies in Turkey (Warrit *et al*., 2004), in co-mingled Russian and Italian honey bee stocks of *A. mellifera* in USA (Rinderer *et al*., 2004) and in *A. m. macedonica* in Greece (Kokkinis and Liakos, 2004).

The invasive patterns of Korea or K type and Japan or J type have been studied using microsatellite, PCR-RFLP at COI and sequencing in mitochondrial DNA. The K type is most abundant, far more virulent, majority population and displaced J type around the world. J type is now present in Japan,Taiwan, French, Guyama and Chile. Between two haplotypes of *V. destructor* display a nucleotide divergence for 0.40%. The divergence value of two haplotypes of *V. destructor* differed from *V. jacobsoni* for 4.15%. Several references have suggested that Korea and Japan" probably originated from different populations by founder events. The parasites infesting on *A. cerana* and *A. mellifera* share the same polymorphism. There is one received the parasite from the

other. For example, the parasites (J type) in *A. mellifera* are the same all *A. cerana* in Japan which appear to be the recipient species from the local *A. mellifera*. It is probable that after a first host transfer of *Varroa* from *A. cerana* to *A. mellifera*. Then, the parasite can shift back from *A. mellifera* to *A. cerana* (Solignac *et al*., 2005).



A



B

Figure 2.16 The *V. destructor* mitochondrial DNA: circular map of the genomic organization (A) details at Evans and Lopez, 2002 and mitochondrial gene arrangement (B) details at Navajas *et al*., 2002.

The recent evidence especially genetic variation of mitochondrial DNA has spurred an important revision of *Varroa* taxonomy (Anderson and Trueman, 2000). Size of *Varroa* mitochondrial firstly estimated approximately 16,477 bp (De Guzman *et al*., 1997). The mitochondrial genome size of *Vaorroa* is larger than of the Acari previously studied, approximately 15 kb (Boore, 1999). Complete mitochondrial DNA sequence of Korea haplotype of *V. destructor* is available under the accession number AJ493124 of EMBL database (Navajas *et al*., 2002). The mitochondrial genome size has a relatively compact mitochondrial genome about 15,218 bp (Figure 2.15B). Ten of twenty-two transfer RNAs are in different locations relative to hard ticks and the 12S ribosomal RNA subunit is inverted and separate from 16SrRNA by a non-coding region (Evans and Lopez, 2002) (Figure 2.16A and B). The bases composition of *V. destructor* showed 39% adenine, 41% thymine, 8% cytosine and 12% guanine. Usually, AT-rich at control region showed A+T content for 80% (Navajas *et al*., 2002). However, sequencing of some regions in mitochondrial DNA suggested useful for resolving population difference, challenge the origin view of *Varroa* taxonomy and informative for phylogenetic questions (Evans and Lopez, 2002).

Varroa mites in Thailand

Anderson and Trueman (2000) noted that *V. jacobsoni* parasitize *A. cerana* in southern of Thailand was Malaysia haplotype. While, *V. destructor* parasitize on natural host from Bangkok was Japan/Thailand haplotype. Subsequently, Warrit (2002) investigated biogeography of *Varroa* mites infests on *A. cerana* in the Thai-Malay Peninsula. The *Varroa* mites and *A. cerana* were collected at south (07° 35’ N and 100° 17’ E) past the Isthmus of Kra though out the north (18° 50’ N and 98° 53’ E) (Figure 2.9).

V. jacobsoni is found in the most area in Thai-Malay peninsula and can be separated into 4 hapltypes: NorthThai1, NorthThai2, Malaysia and Samui1 haplotypes. NorthThai1 and NorthThai2 haplotypes parasitize the mainland Asia *A. cerana*. Malaysia and Samui1 haplotypes colonizes the Sundaland *A. cerana* lineage. *V. destructor* is found on *A. cerana* at the mountainous region of Thailand. It is not similar to Japan/Thailand haplotype but resemble to Vietnam haplotype. The correlation of the *Varroa* haplotypes and *A. cerana* reveals a question about *V. jacobsoni* has colonized the Mainland Asia *A. cerana* or has been there for long time (Warrit *et al*., 2006).

2.3 PHYLOGEOGRAPHY CONCEPTS

2.3.1 Phylogenetic species concept

Nixon and Wheeler (1990) defined a phylogenetic species as the smallest aggregation of populations or lineages diagnosable by a unique combination of character states in comparable individuals. The concept identified diagnosability as a criterion for determining whether a particular grouping of organisms should be treated as a terminal for phylogenetic analysis. Many currently recognizes species may be more appropriately interpreted as racial variants or varieties, and levels of homoplasy often reports in analyses of closely related species may be attributable in part to the nonhierarchic descent relationships among these entities. Population aggregation analysis is a method for the identification of phylogenetic species. This method is designed to distinguish traits from characters on the basis of variation patterns observed within local population. All individuals of a local population are regarded as belonging to the same phylogenetic species (Davis and Nixon, 1992).

2.3.2 Population structure and phylogeography

In general, by revealing how genetic variation is partitioned within any plant or animal species, molecular methods can facilitate to characterize the intraspecific genetic resources that conservation biology seeks to preserve (Avise, 2004). The genetic diversity of such collections can be miximized through knowledge of how natural variation is partitioned within and among populations a task for which molecular genetic markers are well suited (Schoen and Brown, 1991).

The most intriguing connection between population demography and matrilineal structure occurs when female dispersal is extremely low and male dispersal high. Matrilines may exhibit little spatial structure, suggesting at face value that local population can re-colonize quickly, yet extirpation of critical source populations can doom an entire regional assemblage. In that case, gene flow estimates based solely on nuclear loci can, if taken at face value, provides a grossly misleading base for management decisions requiring a demographic perspective, such as how many distinct management units, or stocks, how they might couple otherwise separated populations. (Avise, 2004).

2.3.3 Biogeoraphy and Host-Parasite Co-evolution concept

Allele frequencies in populations can be compared to assess the extent to which populations differ from one another. Comparisons of populations are, sometimes, criticized because the allocation of individuals into groups imposes a pre-existing structure and might influence the outcome of a genetic study (Schuh, 2000). In several instances, molecular markers that distinguish genetic exotics from natives have been employed to monitor the success of these introductions and to assess whether hybridization and introgression with native species have taken place (Avise, 2004). A

common approach in studying individual genetic variation is to compute the genetic similarity between all possible pairs of individuals in a sample and then to search for clusters of individuals who are most similar to one another. At least two such studies have concluded that the observed clustering patterns do not correspond well to the subjects geographic origin. Hennig (1996) believed that the relationship of parasites to their hosts contains much information of great value to phylogenetic systematics, but laments the unsatisfactory state of its theories basis. The similarities between host-parasite relationships and biotic distributions may be listed as follow: some hosts may have no parasites, the biogeographic equivalent of certain taxa being absent from certain areas, some parasites may occur on more than one host, the biogeographic equivalent of widespread distributions and some hosts have more than one parasite, the biogeographic equivalent of redundant distributions (Schuh, 2000).

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER III

MATRILINEAL ORIGINS OF *Apis mellifera* IN THAILAND

(*Note: a version of this chapter accepted to publish in Apidologie at January 23, 2007*)

ABSTRACT

Apis mellifera was imported to Thailand approximately 60 years ago, but the subspecies that contributed to honey bee populations in this country are unknown. We collected 476 colonies from North, Central, Northeast and South Thailand and used PCR-RFLP and direct DNA sequencing to identify mitochondrial lineages and subspecies present. Three common and five rare composite haplotypes were found. Haplotype group ThaiA1 (22% of colonies) and group ThaiA2 (60%) match C or east European lineage *A. m. ligustica* and *A. m. carnica*. Haplotype group ThaiB (18%) belongs to the O or Middle Eastern lineage. Non-coding mitochondrial sequences of ThaiB are similar to those of *A. m. syriaca* and *A. m. lamarckii*, although no published sequence is an exact match. Analysis of Molecular Variation (AMOVA) showed most of the observed genetic variation occurred within individual apiaries, but significant differentiation between North+Central and Northeast+South regions was observed.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

INTRODUCTION

Apis mellifera was introduced to Thailand in the early 1940s (Wongsiri, 1988) and 1950s (Akratanakul, 2000) for research at Chulalongkorn and Kasetsart Universities in Bangkok, but neither of these early introductions appears to have established lasting populations of *A. mellifera*. In the 1970s large numbers of *A. mellifera* were imported from Taiwan to Lampoon and Chiang Mai in northern Thailand for commercial purposes (Wongsiri *et al*., 1995). Additional importations may have come from Australia, Europe and Russia (Wongsiri *et al*., 2000; Kavinseksan *et al*., 2004). *Apis mellifera* beekeeping spread rapidly in northern Thailand, especially Chiang Mai province, and beekeeping with *A. mellifera* was later extended through the rest of Thailand (Thapa and Wongsiri, 1997). Today there are about 300,000 managed colonies of *A. mellifera* in Thailand, with more than half in the northern provinces (Wongsiri *et al*., 2000); there is little evidence of a wild or feral population.

Thus, the ancestry of *A. mellifera* currently in Thailand is unknown but could include a mixture of many subspecies. Since subspecies are known to vary in disease resistance, defensive behavior and other economically important traits (Ruttner, 1988), the identity of Thai honey bee populations is potentially of interest for maintenance and improvement of Thai stocks.

At least 24 subspecies have been described from *A. mellifera*'s native range in Europe, Africa and the Middle East, based primarily on morphometric variation (Ruttner, 1988; Engel, 1999). Studies of *A. mellifera* mitochondrial DNA have revealed at least 4 major mitochondrial lineages: M or West European, C or Eastern Mediterranean, A or African, (Hall and Muralidharan, 1989; Smith *et al*., 1989; Cornuet and Garnery, 1991; Arias

and Sheppard, 1996) and O or Middle Eastern lineage (Franck *et al*., 2000; Palmer *et al*., 2000). A possible fifth lineage, Y, has been described from Ethiopia (Franck *et al*., 2001).

In recent years, several studies have used microsatellites and mitochondrial DNA restriction site polymorphisms to investigate which subspecies and mitochondrial lineages have contributed to mixed populations, especially Africanized or neotropical African bees in the Americas (e.g., Clarke *et al*., 2001; 2002; Coulson *et al*., 2005; Diniz *et al*., 2003; Pinto *et al*., 2004; 2005). A combination of mitochondrial restriction site polymorphisms can diagnose major mitochondrial lineages, as shown in Table 3.1 (Smith *et al*., 1989; Hall and Smith, 1991; Palmer *et al*., 2000). The major *A. mellifera* mitochondrial lineages also exhibit size variation in the mtDNA non-coding region due to the presence of repetitive elements, called "P", "P_0", "P_1", and "Q" that are characteristic of major mitochondrial lineages (Cornuet *et al*., 1991; Garnery *et al*., 1992; Palmer *et al*., 2000).

To clarify the maternal ancestry of *A. mellifera* in Thailand, we used PCR-RFLP analysis of four regions of mtDNA restricted with the 6- base restriction enzymes *Dra* I, *Bgl* II, *Eco*R I, *Hin*c II and a 4-base restriction enzyme, *Hin*f I, as well as DNA sequencing of the non-coding region between $tRNA^{leu}$ and cytochrome *c* oxidase II (COII) genes.

MATERIALS AND METHODS

Collections: *Apis mellifera* samples were collected between July 2003 and January 2006 from 476 colonies from 4 regions in Thailand (Figure 3.1, Table 3.2).

For comparison, 27 samples of honey bees from outside Thailand were also examined: *A. m. ligustica*-derived colonies from Australia (5 colonies), and New Zealand (1); *A. m. carnica* from Gratz (2 colonies), Klagenfurt (1), and Lunz-am-See (3), Austria, from the Institute fur Bienenkunde, Oberursel, Germany (3 colonies) and from Medvode, Slovenia (1); *A. m. mellifera* from Asker, Norway (2 colonies); *A.m. scutellata* from South

Africa (2 colonies); and *A. mellifera syriaca* (7 colonies) from Hatay, Turkey. European samples were collected by DRS in 1987 (Smith 1991 for details), African by Orley Taylor, Jr. in 1991, and Turkish samples by O. Kaftanoglu in 1997. All samples were kept in 95% ethanol and stored at -4 C°.

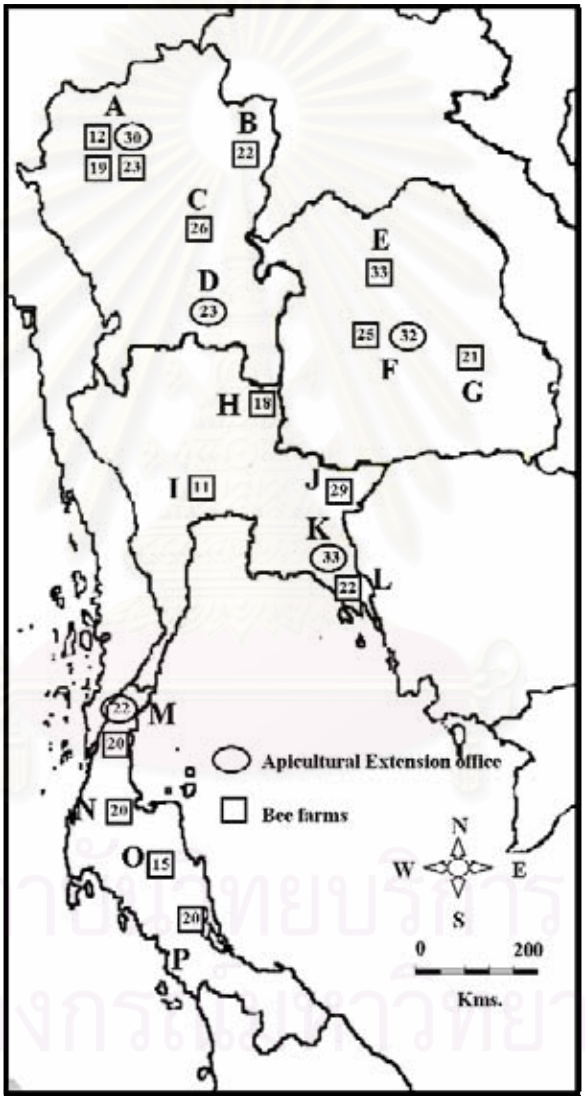


Figure 3.1 Collection locations for *Apis mellifera* in Thailand. Capital letters indicate collection sites. Circles represent samples collected from Apicultural Extension Offices, boxes represent collections from bee farms. Numbers in boxes and circles show number of honeybee colonies sampled at each site.

Mitochondrial DNA analysis: Total DNA was extracted from one bee thorax per colony by standard phenol-chloroform extraction methods. Four regions of mtDNA were PCR amplified: $tRNA^{leu}$ to the 5′ end of COII (including the non-coding region between $tRNA^{leu}$ and COII), a portion of cyt-b, a portion of the large ribosomal subunit and the 5′ end of COI. Primers, PCR thermal profiles and restriction enzymes are shown in Table 3.1.

The intergenic region between $tRNA^{leu}$ and COII was sequenced in one example of each major Thai mitotype detected by restriction fragment polymorphisms. PCR product was purified using a gel purification kit (QIAGEN) and sequenced in both directions on an ABI-3100 AVANT sequencer using primers E2 and H2 (Table 3.1). The resulting sequences were compared to published sequences and sequence available in Genbank.

Statistical analysis: Composite haplotypes were generated from the combined restriction patterns of the four mitochondrial regions (Figure 3.2, Table 3.2). Genetic distances (Nei and Li, 1979) among haplotypes and frequency distribution among populations were calculated using Restriction Enzyme Analysis Package, REAP (McElroy *et al*., 1992). A dendrogram showing similarity among haplotypes was constructed from the genetic distances using the neighbor-joining method in Mega3 (Kumar *et al*., 2004). Genetic differentiation among regions and among apiaries within regions was estimated using AMOVA (Analysis of Molecular Variance: Excoffier *et al*., 1992) implemented in the program AMOVA1.55 (Excoffier, 1995). Significance testing was carried out by comparison to 1000 random permuations of observed haplotypes into apiaries and regions of the same sizes. Sequence data were aligned by eye and by using Clustal W implemented in Mega 3.1 (Kumar *et al*., 2004).

RESULTS

RFLP haplotypes: Restriction enzyme digests of PCR products are summarized in Figure 3.2 and Table 3.2. All Thai samples possess a *Bgl* II restriction site in cytochrome b (pattern "b", Figure 3.2B), indicating that none belongs to the African or A mitochondrial lineage. Similarly, all Thai samples lack a *Hin*c II site in the 5′ end of COI (pattern "a", Figure 3.2D), indicating that none belongs to the West European or M lineage. The remaining restriction digests indicate the presence of both the Eastern Mediterranean (C) and the Middle East (O) lineages among Thai *A. mellifera*.

Amplification of $tRNA^{LEU}$-COII revealed fragments of two sizes: 573 bp and 837 bp. The smaller fragment, found in samples designated "ThaiA", is typical of the C lineage. Digestion of this fragment with *Dra* I produced a single pattern identical to that found in C lineage subspecies *A. m. ligustica* and *A. m. carnica* (ThaiA group, pattern "b", Figure 3.2A). Digestion of this fragment with *Hin*f I revealed two restriction patterns, "b," found in most of our *A. m. carnica* reference samples, and "c," found in *A. m. ligustica* and a small number of the *A. m. carnica* reference samples. Those with pattern "c" were designated ThaiA1, those with were pattern "b" designated ThaiA2 (Figure 3.2A).

Samples with the larger fragment were designated "ThaiB". Digestion of this fragment with *Dra* I produced a single pattern identical to that from Middle Eastern or O lineage (Frank *et al*., 2000 and our Turkish samples; ThaiB samples, pattern "a"). Digestion of this fragment with *Hin*f I produced only a single pattern ("a"), seen in all non-C lineage reference samples and all samples in the ThaiB group.

The results of the other amplification/digestion combinations *(Dra*I and *Hin*fI digests of cytochrome-b, *Eco*RI digests of the lsRNA region) are summarized in Table 3.2 and Figure 3.2. The patterns revealed by all seven restriction digests were combined into

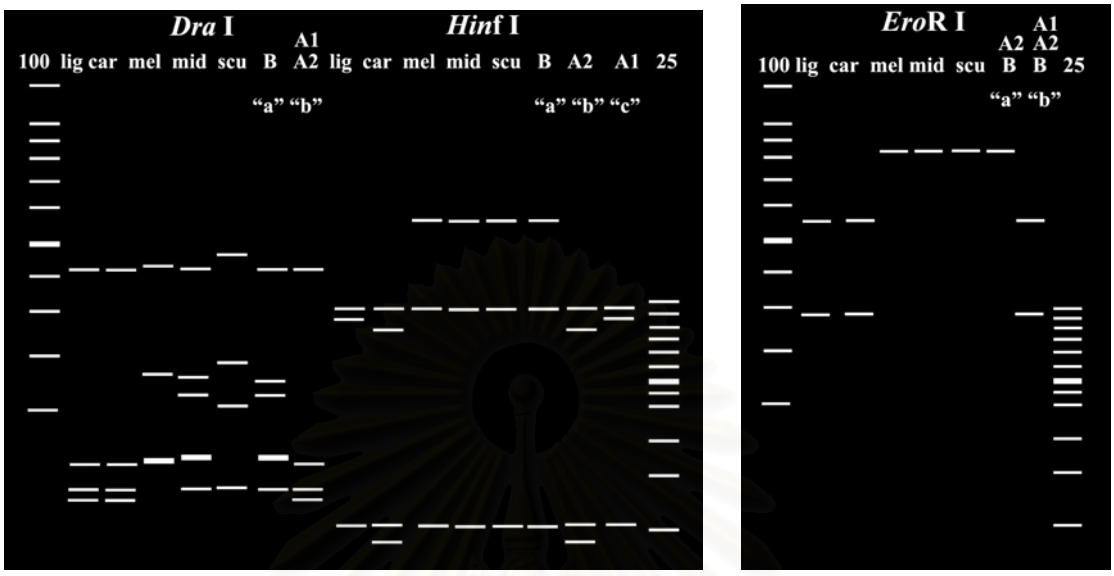
composite haplotypes, e.g., bbbbaba. The composite haplotypes indicate the restriction patterns generated by seven PCR product/restriction enzyme combinations: [tRNA^{LEU}–COII / *Dra* I] [tRNA^{LEU}-COII / *Hin*f I] [CytB / *Bgl* II] [CytB / *Dra* I] [CytB / *Hin*f I] [lsRNA / *Eco*R I] [COI / *Hin*c II].

Eight composite haplotypes were found among the 476 colonies of Thai *A. mellifera*. The ThaiA1 haplotype group includes haplotypes bcb-aba (dash indicates a or b), the ThaiA2 group includes bbb – – – a, and the ThaiB group includes aab – – – a (Tab. II). Pairwise distances among haplotypes as estimated from restriction site data (Nei and Li, 1979) ranged from 0.006 to 0.06. These distances were used to create a dendrogram by the neighbor-joining method (Figure 3.3), which clustered the eight haplotypes into two groups corresponding to the C or Eastern Mediterranean group and the Middle Eastern or O group.

ThaiA2.1 (bbbbaba) was the most common haplotype, found in 58.1% of samples. This was identical to 7 samples of *A. m. carnica* from Austria and Germany. A rarer haplotype, ThaiA1.2 (bcbbaba), matches three samples of *A. m. carnica* from Slovenia and Austria. The second most common haplotype, found in 19.7% of samples, was ThaiA1.1 (bcbaaba). This was identical to putative *A. m. ligustica* from Australia and New Zealand. The patterns of Thai A2.2 (bbbaaba) and ThaiA2.3 (bbbbbaa) were not detected in any of our reference samples, although they clearly are members of the C lineage (Figure 3.3) because they possess a *Bgl*II site in Cytb, lack a *Hinc*II site in 5′ portion of COI, the tRNA-COII fragment is small and lacking a P unit, and *Dra*I digests of this fragment produce a pattern seen in *A. m. carnica* and *A. m. ligustica*. Eighteen percent of the samples belong to the ThaiB group, which includes haplotypes ThaiB2.1 (aabbbaa, 17.0%), ThaiB2.2 (aababaa) and ThaiB2.3 (aabaaba). The ThaiB2.1 pattern was similar to reference samples of Middle East *A. m. syriaca* from southeastern Turkey.

(A) tRNA^{leu} -COII region

(C) lsRNA region



(B) Cytochrome b region

(D) COI region

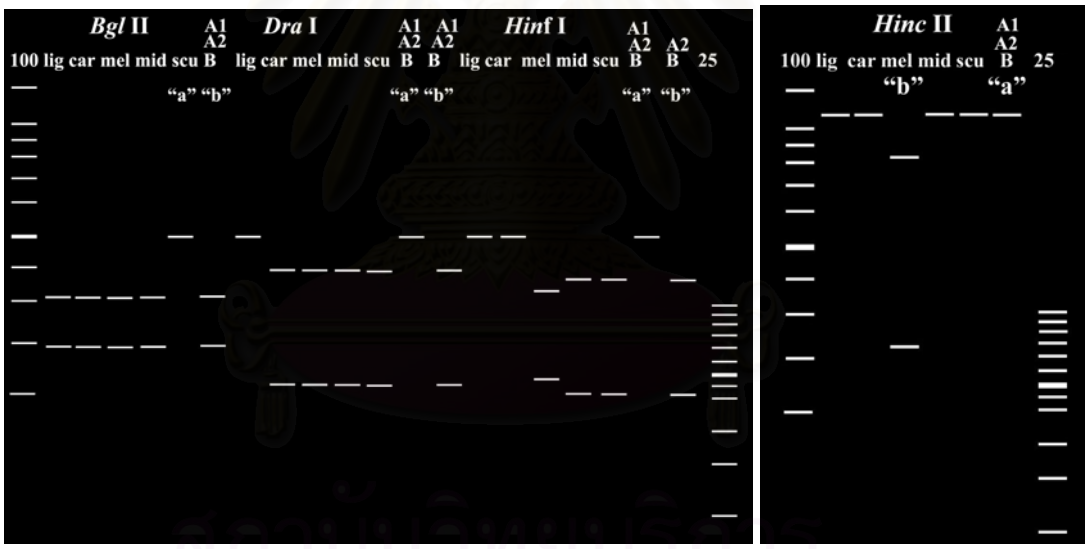


Figure 3.2 Schematic diagram of restriction patterns found in *A. m. ligustica* (lig), *A. m. carnica* (car), *A. m. mellifera* (mel), Turkish *A. m. syriaca* (mid) and *A. m. scutellata* (scu) reference samples and in Thai honeybees (A1 = ThaiA1 group, A2 = ThaiA2 group and B = ThaiB group). The symbol "a", "b" and "c" represent restriction patterns found in Thai honeybee haplotypes. (A) tRNA^{leu}-COII digested by *Dra* I and *Hinf*I; (B) cytochrome b digested by *Bgl* II, *Dra* I and *Hinf* I; (C) large subunit ribosomal RNA restricted by *EcoR* I; and (D) COI region digested by *Hinc* II. Sizes were determined with 100 bp and 25 bp ladder.

Table 3.1 Summary of procedures used to identify the mitochondrial DNA of *A. mellifera* in Thailand. M = West European lineage, C = East Mediterranean, A = African, O = Middle Eastern. *Dra*I digestions and size variation of $tRNA^{leu}$-COII are shown in Fig. 2.

Regions Amplified	Primers	PCR thermal profile	Restriction Enzyme	M	C	A	**O**
$tRNA^{leu}$-COII (Garnery et al., 1993)	E2: 5'GGCAGAATAAGTGCATTG 3' H2: 5'CAATATCATTGATGACC 3'	94°C-60s, (94°C -30 s, 55°C - 30s, 72°C - 60s)[30], 72°C -10 min	*Hinf* I	$+^{E}$	$+^{C}$, $+^{B}$	$+^{D}$	$+^{A}$
Cyt b (Crozier et al., 1991)	OLD1: 5'TATGTACTACCATGAGGACAAATATC3' OLD2: 5'ATTACACCTCCTAATTTATTAGGAAT 3'	94C°-60s, (92 C°- 60s, 50 C°- 60s, 72 C°- 90s)[35], 72 C°-5 min	*Bgl* II	+	+	-	+
			Dra I	+	-,+	+	-,+
			Hinf I	$+^{C}$	-	$+^{B}$	$+^{B}$
lsRNA (Hall & Smith, 1991)	Ls1: 5'TTTTGTACCTTTTGTATCAGGGTTG 3' Ls2: 5'CTATAGGGTCTTATCGTCCC 3'	94C°-60 s, (94 C°-30 s, 55 C°- 30 s and 72 C°- 60 s)[35], 72 C°-5 min	*EcoR* I	-	+	-	-
COI (Hall & Smith, 1991)	COI-1908: 5' TTAAGATCCCCAGGATCATG 3' COI-2932: 5' TGCAAATACTGCACCTATTG 3'	94C°-60s, (94 C°-60s, 50 C°- 60s, 72 C°-60 s)[35], 72 C°-5 min	*Hinc* II	+	-	-	-

+ indicates restriction site present, - indicates restriction pattern absent, $+^{A}$, $+^{B}$, $+^{C}$, $+^{D}$ and $+^{E}$ indicates different restriction pattern

Table 3.2 Geographic distribution of eight haplotypes from 476 Thai *Apis mellifera* colonies. The seven letters of the composite haplotype indicate restriction fragment patterns for seven gene/enzyme combinations ($tRNA^{leu}$ – COII/ *Dra* I, $tRNA^{leu}$ – COII / *Hinf* I, cyt-b/ *Bgl* II, cyt-b/ *Dra* I, Cytb/ *Hinf* I, lsRNA/ *EcoR* I and COI/ *Hinc* II, respectively). Localities A, B,…, P indicate collection sites (apiaries and agricultural offices) as shown in Figure 3.1.

Regions	Localities	Haplotpye								Total
		Eastern Medditerranean lineage					Middle Eastern lineage			
		ThaiA1			ThaiA2		ThaiB			
		ThaiA1.1 bcbaaba	ThaiA1.2 bcbbaba	ThaiA2.1 bbbbaba	ThaiA2.2 bbbaaba	ThaiA2.3 bbbbbaa	ThaiB1.1 aabbbaa	ThaiB1.2 aababaa	ThaiB1.3 aabaaba	
North	Chiangmai (A)	7		67		1	8	1		84
(155)	Nan (B)	20	1	1						22
	Utaradit (C)			25			1			26
	Phithsanulok (D)	16		7						23
Northeast	Udornthani (E)	12		16			5			33
(111)	Khon khan(F)	7	6	9			32	1	2	57
	Loi-Ed (G)			15		2	3	1		21
Central	Lopburi (H)			18						18
(113)	Bangkok (I)			11						11
	Srakeaw (J)	10		15	2		2			29
	Chantaburi (K)			33						33
	Trat (L)	20		2						22
South	Chumporn (M)			27		2	13			42
(97)	Surat thani (N)		3	11			6			20
	Nakornsrithammarat (O)		1	10		1	3			15
	Shong-Kla (P)		1	10		1	8			20
Total		92	12	277	2	7	81	3	2	476

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Sequence data: We detected no variation among Thai samples in $tRNA^{leu}$ and little in COII (not shown). Sequence of the non-coding intergenic region confirms that ThaiA1 and ThaiA2 haplotype groups belong to the C or East Mediterranean lineage as they lack a P sequence, and show high sequence similarity to each other and to the reference sequences of *A. m. carnica* and *A. m. ligustica*. ThaiB haplotypes are excluded from the C lineage because, unlike C lineage, they possess a P sequence, and from the M lineage because their P sequence does not show the 13 bp deletion characteristic of M lineage. Several base substitutions in both the P and Q units also distinguish the ThaiB haplotypes from the A lineage. On the other hand, the ThaiB sequences share features with *A. m. lamarckii* and *A. m. syriaca*, including 3 substitutions in the P element, a 3 bp insertion in Q2, and two substitutions in the Q3 sequence. Our ThaiB sequences show two repeats of the Q element (Q' and Q") while published sequences from *A. m. lamarckii* and *A. m. syriaca* have shown only a single copy of the Q element.

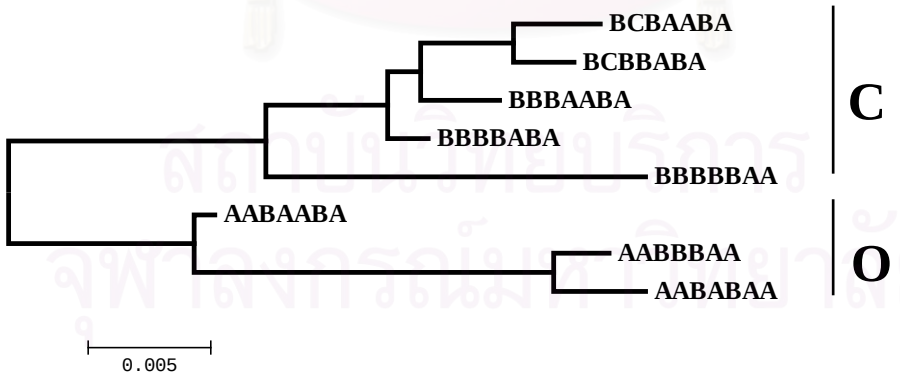


Figure 3.3 Neighbor-joining dendrogram showing similarity among composite mitochondrial DNA restriction site haplotypes. Haplotypes are as shown in Table 3.2; C and O refer to the East European and Middle Eastern mitochondrial lineages, respectively.

P or Po

```
intermissa (A-8)    TTAATAAATTAATATAAAATAAAACAAAATATAACAAAATATATTTATTAAAATTTAATTTATTAAAA-
mellifera  (M-6)    ..................................T...T.............................-
lamarckii (O-1c)    ........................T...T.......G................C..............-
syriaca    (O-3)    ........................T----------G................C..............-
ThaiB               ........................T..........G....-..........C..............-
carnica    (C)      ------------------------------------------------------------------
ThaiA2              ------------------------------------------------------------------
ligustica  (C)      ------------------------------------------------------------------
ThaiA1              ------------------------------------------------------------------
```

Q1

```
intermissa          -TTCCCCACTTAATTCATATTAATTTAAGAATAAATTAA-TAACAA-
mellifera           T..-..............T.........A..........A......T
lamarckii           T..-.........................A..........A......T
syriaca             T..-.........................A..........A......T
ThaiBQ1'            T..-.........................A..........A......T
ThaiBQ1''           -..-.........................A..........A......T
carnica             T.....-.......................A..........-......T
ThaiA2              T.....-.......................A..........-......T
ligustica           T.............................A..........-......T
ThaiA1              T.............................A..........-......T
```

Q2

```
intermissa          TTTTAATAAAATAAATAA---TTAATTTTATTTTTATATTGAATTTTTAATTCAATCTTAAAGATTTAATCTTTTTATTAAA
mellifera           ...................---.............................A................................
lamarckii           ..................TAA......-.......................A................................
syriaca             ..................TAA......-.......................A................................
ThaiBQ2             ..................TAA......-.......................A................................
ThaiBQ2''           ..................TAA......-.......................A................................
carnica             ...................---.............................A................................
ThaiA2              ...................---.............................A................................
ligustica           ...................---.............................A................................
ThaiA1              ...................---.............................A................................
```

Q3

```
intermissa          ATTAATAAATTAATATAAAA--TAAAACAAAATATAACAGAATATATTTATTAAAATTTAATTTATTAAAA-
mellifera           ......................AA..................A.....................................-
lamarckii           ......................--.....T.....................................C.................-
syriaca             ......................--.....T.................A.....................C.................-
ThaiBQ3'            ......................--.....T.................A.....................C.................T
ThaiBQ3''           ......................--.....T.................A.....................C.................-
carnica             ......................--.....................................................................-
ThaiA2              ......................--.....................A.....................................-
ligustica           ......................--.....................................................................-
ThaiA1              ......................--.....................................................................-
```

Figure 3.4 Sequence of the non-coding region between tRNA^{leu} and cytochrome oxidase II in *A. mellifera* mtDNA, including the 3' end of tRNA^{leu}, P or Po, and Q sequences. Q1, Q2, and Q3 refer to the 5', middle, and 3' portions of the Q sequence. The entire Q sequence may be present in 1, 2, 3 or more tandemly repeated copies. Primes (e.g., Q1', Q1") are used to indicate the sequence of the first, second, etc. copy of the Q sequence. Thai honeybees (ThaiA1, ThaiA2 and ThaiB) are compared with sequences from other *A. mellifera* subspecies: *A.m. intermissa*, *A. m. mellifera*, *A. m. lamarckii*, *A. m. syriaca* (Franck *et al*., 2000), *A. m. ligustica* (GenBank accession # L-06178), and *A. m. carnica* (sequence produced in this study). Capital letters in parentheses indicate the mitochondrial lineage of the reference sequences (C, East Mediterranean; M, West European; A, African; O, Middle Eastern). Numbers and small letters indicate which sequence from Franck *et al*. (2000) is displayed (e.g., O-1c = Middle East sequence 1c). Dots indicate the same base as in the first sequence, dashes indicate a gap in the sequence. East Mediterranean or C lineage haplotypes lack a P unit in the non-coding sequence.

Table 3.3 Results of Molecular Analysis of Variance (AMOVA: Excoffier *et al*., 1992; Excoffier, 1995). (A) Apiaries are grouped into four regions: North, Central, Northeast, and South. (B) Apiaries are grouped into two regions, North+Central, Northeast+South. Significance tested with 1000 random permutations; all Φ-statistics significant at $P < 0.001$.

AMOVA analysis	Variance components	(A) Partition of observed variance			(B) Partition of observed variance		
		Variance	%Total	Φ-statistics	Variance	%Total	Φ-statistics
Nested analysis	Among regions	0.002	19.20	Φct = 0.192	0.003	29.92	Φct = 0.299
	Among apiaries/ within regions	0.001	16.81	Φsc = 0.208	0.001	12.93	Φsc = 0.184
	Within apiaries	0.006	63.99	Φst = 0.360	0.006	57.15	Φst = 0.428
Analysis among apiaries	Among apiaries	0.003	33.14		0.003	33.14	
	Within apiaries	0.006	66.86		0.006	66.86	
Analysis among regions	Among regions	0.002	22.38		0.003	31.15	
	Within regions	0.008	77.62		0.007	68.85	

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Distribution and abundance of haplotypes: The three most common haplotypes made up 450 (95%) of the 476 samples. The most common, ThaiA2.1 (bbbbaba), was found at every collection site in all four regions. The second most common haplotype, ThaiA1.1 (bcbaaba), was absent in the South region, but present in the North, Northeast and Central regions. The third most common haplotype, ThaiB1.1 (aabbbaa), was almost absent from the North and Central regions but more common in the South and Northeast; (Figure 3.5A). A similar pattern is observed when all eight haplotypes are considered (Figure 3.5B).

AMOVA was used to examine differentiation among geographic regions and among apiaries (Table 3.3). Two groupings of apiaries into regions were particularly notable. When apiaries were grouped into the North, Central, Northeast and South regions, variance among regions accounted for 19.2% of the total (probability of a more extreme value from random permutations <0.001). Based on the observed distribution of haplotypes shown in Figure 3.5, apiaries were regrouped into two larger regions, corresponding to North+Central and Northeast+South; in this configuration variance among regions accounted for 29.9% of the total ($p<0.001$). In both cases, however, the greatest portion of variance occurred within apiaries (64% and 57%, respectively).

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

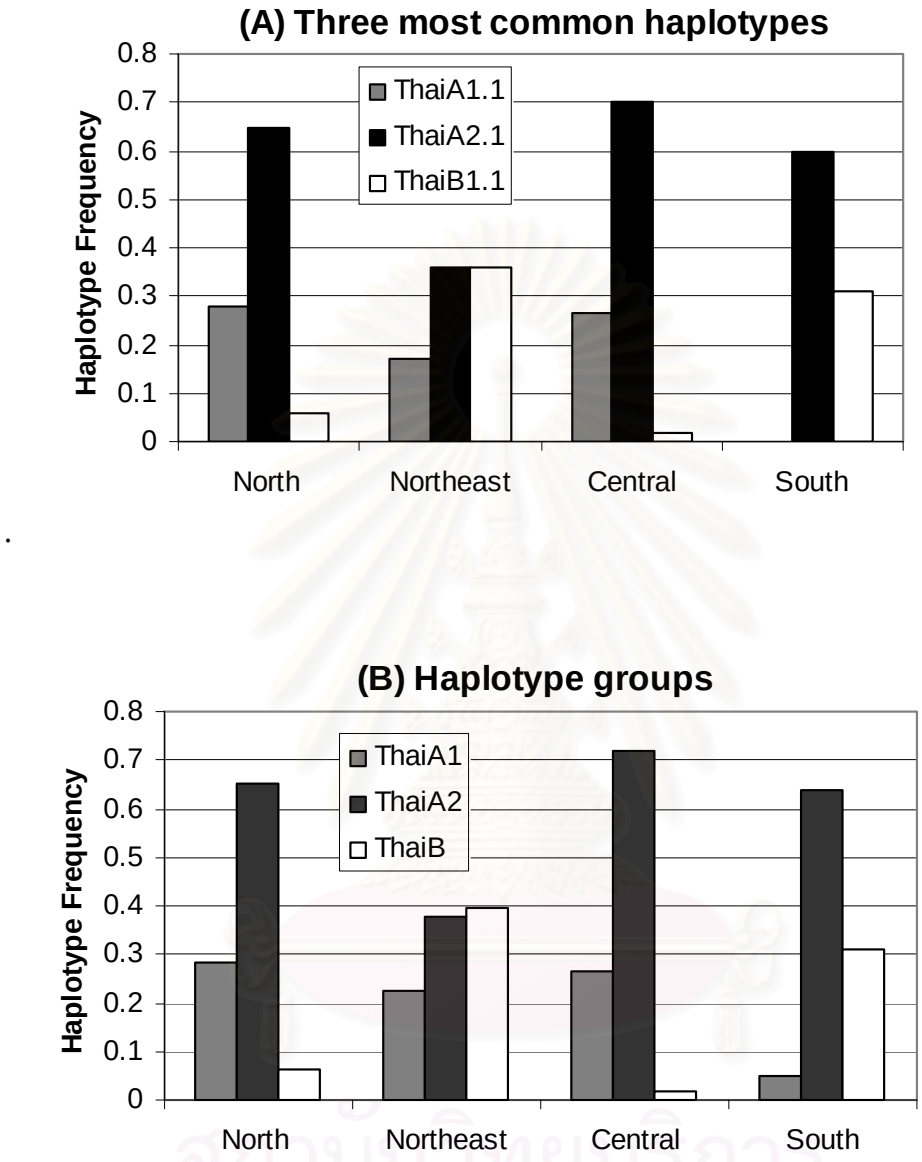


Figure 3.5 Distribution of *Apis mellifera* mitochondrial haplotypes in 4 areas of Thailand. Bars indicate frequency of colonies of each haplotype. (A) Frequencies of three most common haplotypes. (B) Frequencies of three haplotype groups. ThaiA1 group corresponds to *A. m. ligustica* mtDNA haplotypes, ThaiA2 group corresponds to *A. m. carnica* haplotypes, and ThaiB group corresponds to Middle Eastern mtDNA haplotypes (see text).

DISCUSSION

Thai *A. mellifera* trace their maternal ancestry to the east Mediterranean (C) and Middle Eastern (O) mitochondrial lineages. We infer that the maternal ancestry of Thai *A. mellifera* populations is approximately 60% *A. m. carnica* (ThaiA2.1 haplotype), 22% *A. m. ligustica* (ThaiA1.1) and 18% Middle Eastern or O lineage (the ThaiB haplotype group). We did not find any exact match to our ThaiB sequences, but this may not be surprising since the O or Middle Eastern mitochondrial lineage was identified relatively recently and is still poorly sampled. There was no evidence of either west European (M) or African (A) lineage mtDNAs in Thailand.

Compared to native honey bee species, there is relatively little geographic structure to the distribution of *A. mellifera* matrilines. *Apis cerana* shows clear genetic differentiation among regions of Thailand, particularly between populations north and south of a biogeographic transition in the Isthmus of Kra (e.g., Smith and Hagen, 1996, 1999; Warrit *et al*., 2006). In contrast, most variation of *A. mellifera* mtDNA haplotypes in Thailand occurs within individual apiaries. This suggests that these introduced bees have been transported widely, and that beekeepers acquire bees of several genetic backgrounds. Despite the variation observed within individual apiaries, a geographic pattern in haplotype distribution does exist in Thailand: the Middle East haplotypes are rare in the North and Northeast, and the *A. m. ligustica*-like haplotypes are rare in the south. These patterns may reflect importation history, patterns of migratory beekeeping, or other factors.

It is rather surprising that up to 20% of the Thai *A. mellifera* population appears to be matrilineal descendants of Middle Eastern honey bees. How and when bees with these mitotypes were introduced remains a mystery. Interviews with Apicultural Extension officers in Chiang Mai, Phisthsanulok, Khonkan, Chantaburi and Chumporn, and with Thai

beekeepers indicate researchers and beekeepers in those regions have introduced honeybee queens from the USA, Europe, Australia and Russia, which may have included hybrids with Middle Eastern ancestry. A co-operative project on *A. mellifera* between the Agriculture Department of Thailand and Israel 1990-1995 also may have introduced Middle Eastern queens or colonies from Israel to Thailand (Wongsiri, unpublished).

PCR-RFLP polymorphisms in the mitochondrial genome provide a fast method for determining matrilines present in honeybee populations, while sequencing provides finer discrimination of mtDNA genomes characteristic of particular subspecies. In this study we employed both approaches to investigate the maternal ancestry of Thai *A. mellifera*, an exotic species of great economic importance

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER IV

GENETIC DIFFERENTIATION OF *Apis cerana* POPULATIONS IN THAILAND BASED ON TE-AFLP AND AFLP ANALYSIS

ABSTRACT

The range of *Apis cerana* covers every region of Thailand. Two different mitochodrial lineages of *A. cerana* occur within Thailand, separated at Isthmus of Kra: the Mainland Asian lineage, north of the so-called Kra ecotone, and the Sundaland lineage to the south. Genomic DNA was extracted from 184 Thai *A. cerana* from six populations (North, Central, Northeast, Prachuap Khiri Khan, Chumporn and Peninsular) and analyzed by amplified fragment length polymorphisms (AFLP) and three-enzyme amplified fragment length polymorphisms (TE-AFLP). These DNA fingerprinting techniques revealed abundant genetic diversity within Thai *A. cerana* populations. An analysis of molecular variance (AMOVA) indicated no genetic differentiation among populations, and extensive overlap among subpopulations, indicative of gene flow that could be caused by movement of drones. North, Central, and Northeast populations were found to belong to the Mainland Asian mitochondrial lineage, as did most of the samples of the Prachuap Khiri Khan population, which occurs around Isthmus of Kra (10-11°N). The Chumporn and Peninsular populations, which occur south of 10 °N latitude, both belong to the Sundaland mitochondrial lineage.

INTRODUCTION

Apis cerana Fabricius, the Asian honey bee, is closely related to *A. mellifera* (the Western honey bee), and builds multiple-comb nests in dark cavities. The geographic range of the Asian honey bee reaches from Afghanistan to Ussuria in Russia and from Indonesia to Japan (Ruttner, 1988; Smith and Hagen, 1996). Morphological and geographical investigations indicate the Asian honey bee is subdivided into three regions: Western Asia, Northeast Asia and Southeast Asia (Hepburn *et al*., 2001).

Genetic variation at a mitochondrial non-coding sequence between Cytochrome OxidaseI (COI) and Cytochrome Oxydase II (COII) is popular and useful tool for study of *Apis* (Cornuet *et al*., 1991). A pioneer work on genetic variation in *A. cerana* in Asia used restriction fragment length polymorphisms (RFLPs) to survey mitochondrial DNA (mtDNA) (Smith, 1991a).

Mitochondrial DNA diversity has also been used to examine relationships among species of the Asian honey bees. Two major haplotype families have been proposed: 'Western' and 'Eastern." The Western haplotype group appears to be widely distributed, and includes A. cerana in southern Thailand (Smith and Hagen, 1996; Oldroyd *et al*., 2006). The Eastern haplotype group includes all other *A. cerana*, *A. nigrocincta* and *A. nuluensis*. Within the 'Eastern' mitochondrial haplotype there are at least five lineages of *A. cerana*: Mainland Asia (including *A. cerana* in northern Thailand), Sundaland (including the southern peninsular of Thailand), Sulawesi Indonesia, Palawan, and Luzon-Mindanao (Smith *et al*., 2000; Tanaka *et al*., 2001).

The Isthmus of Kra, where there is a transition from evergreen rainforest to more seasonal, semi-evergreen forest, between 10°34'N and 11°24'N (Whitmore, 1984), is also the site of two independent species boundaries: northern limit *A. koschevnikovi*

and southern limit *A. florea* (Oldroyd and Wongsiri, 2006). Morphometric analysis of *A. cerana* from the northern to the southern of Thailand, including Samui Island, is separated three groups: the northern latitude, the southern latitude and Samui Island (Sylvester *et al*., 1998). Mitochondrial DNA variation which is transmitted only along maternal lines of *A. cerana*, indicates clearly genetic differences between geographical origins. The diversity of *A. cerana* in Thailand separates into two mtDNA lineages: Mainland Asia (north-central-northeast of Thailand) and Sundaland (south peninsular, Samui and Phuket Island of Thailand) (Deowanish *et al*., 1996; Sihanunthavong *et al*., 1999; Smith and Hagen, 1996; 1999; Smith *et al*., 2000; Hepburn *et al*., 2001; Smith *et al*., 2004; Songram *et al*., 2006; Warrit *et al*., 2006). The Isthmus of Kra is a boundary zone between both of northern and southern *A. cerana* mitochondrial lineages.

Investigation of microsatellite variation in Thai *A. cerana* populations can separate northern populations [north-central and northeastern populations], and suggests that rate of gene flow due to male and female dispersal may differ. The geographic heterogeneity indicates the gene pool of this bee is not panmictic population (Sittipraneed *et al*., 2001b). Recently, Amplified Fragment Length Polymorphisms (AFLP: Vos *et al*., 1995) and Three Enzyme Amplified Fragment Length Polymorphisms (TE-AFLP: van der Wurff *et al*., 2000) have been used to investigate gene flow in *A. cerana* populations and to confirm status of *A. nigrocincta*. Analysis of Molecular Variance (AMOVA: Excoffier *et al*., 1992) of AFLP data indicates little or no gene flow between the two species (Smith *et al*., 2003).

Here we use AFLP and TE-AFLP DNA fingerprinting, to clarify genetic diversity and population differentiation of Thai *A. cerana* populations in Mainland Asia and Sundaland regions, with special attention to the Isthmus of Kra, where Mainland

Asian and Sundaland mitochondrial lineages of Thai *A. cerana* come into contact. Differentiation of Thai *A. cerana* populations in lowland and the mountainous areas within Mainland Asia region will be also examined.

MATERIALS AND METHODS

Collections: A total of 184 samples of *A. cerana* collected in Thailand were selected for these analyses; this includes fifty samples collected by Dr. Siriporn Sittipraneed, Chulalongkorn University, from 1996-1999. Eighty three samples were collected in a line transect from North to South Thailand by Natapot Warrit in 2001 and kept at the laboratory of DRS at the University of Kansas, and fifty-three samples were collected in 2004-2006 by Tipwan Suppasat from mountainous regions of north and northeast Thailand and from the Isthmus of Kra area. Samples were separated into 6 subpopulations: North (32), Northeast (24), Central (33), Prachuap Khiri Khan (27), Chumporn (30) and Peninsular (38), including Samui and Phuket Island (Figure 4.1).

Genomic DNA analysis: DNA was extracted from the thorax of one *A. cerana* per nest, using standard phenol-chloroform extraction methods (Smith *et al*., 1997) as detailed in Appendix IA. Two techniques of DNA fingerprinting (standard AFLP and TE-AFLP) as detailed in Appendix IC, were used to examine genomic DNA variation of 184 *A. cerana* individuals. Both AFLP and TE-AFLP primers were screened in thirty samples of Thai *A. cerana* selected from the six populations. Four different *Mse* I- XXX selective primers were screened for use in AFLP amplifications (XXX: CGA, CTA, CGC, CTG) and eight different *Xba* I-XX primers were screened for use in TE-AFLP amplifications (XX: CC, CT, AC, AG, GC, GG, TG, TC). The *Mse* I-CTA and *Xba* I-AC were selected for use in this study because the produced easily scored bands and showed some variation among individuals and populations.

The standard AFLP technique was carried out following the procedure described by Vos *et al*. (1995). High quality genomic DNA was digested with a pair of restriction enzymes (*Eco*R I and *Mse* I) then ligated to double-stranded *Eco*R I and *Mse* I linkers. The digest-ligation mixture was pre-amplified with non-selective primers that matched the sequence of the adaptors plus one arbitrary base (*Eco*R I-A and *Mse* I-C). Selective amplification was performed by using selective primers with arbitrary 3 base extensions (*Eco*R I-AAG* and *Mse* I-CTA). One selective primer (*Eco*R I-AAG*) was labeled with radioactive material (P-32) to detect amplification products. Labeled amplification products were separated by electrophoresis on 8% TBE polyacrylamide gels run for five hours; the gel was then dried and used to expose x-ray film. In the newer TE-AFLP technique genomic DNA was digested with three restriction enzymes, two with six base recognition sites (*Xba* I and *Bam*H I) and one with a four base recognition site (*Rsa* I). The mixture was ligated with ligase enzyme at the same time following the procedure described by Van der Wurff *et al*. (2000). The digest-ligation mixture was directly amplified with primers that matched the adaptor plus one or two arbitrary bases: Bam HI-C*, which was labeled with P-32, and *Xba* I-AC. Amplification products were detected as described for the AFLP technique.

Statistical analysis: Bands of standard AFLP and TE-AFLP were scored by eye and recorded as present (1) or absent (0), to create [individual X band] data matrices. AFLP and TE-AFLP data matrices were analyzed separately, and jointly in a combined matrix. Analyses were performed using the Excel add-in Genetic Analysis in Excel, (GenAlEx6: Peakall and Smouse, 2006). The percentage of polymorphic (%P) bands and mean of expected heterozygosity (*He*) were calculated for each of the six populations. A band was considered polymorphic if it was present in at least one

individual and absent in at least one individual. Allele frequency at each band locus was estimated using the approach of Lynch and Milligan (1994), which assumes populations are either in Hardy-Weinberg equilibrium or completely inbreeding. The frequency of individuals in which a band is absent is taken as the frequency of recessive homozygotes, q^2, and allele frequencies are estimated as ($\sqrt{q^2}$) and ($1 - \sqrt{q^2}$). Expected heterozygosity was estimated from inferred allele frequencies. .

Analysis of Molecular Variance Analysis (AMOVA: Excoffier *et al*., 1992) was used to investigate genetic differentiation among the six *A. cerana* populations. AMOVA, as implemented in GenAlEx (Peakall and Smouse, 2006), calculates three Phi (Φ) statistics (Φ_{RT}, Φ_{PR}, Φ_{PT}) from the presence/absence matrix of bands for each individual. These statistics are analogous to Wright's F-statistics (Wright, 1951; Wright, 1965) for co-dominant data, but are appropriate for use with dominant markers or binary data, such as RAPDs, AFLPs and TE-AFLPs. $\Phi_{PT,}$ which can range from 0 to 1, measures the similarity of pairs of individuals drawn at random from the same population, relative to pairs of individuals drawn from the entire sample. Φ_{RT} measures the similarity between pairs of individuals drawn at random from the same region (group of populations), compared to individuals drawn at random from the entire sample. Φ_{PR} is the correlation among individuals drawn at random from the same population, compared to individuals drawn at random from any population in the same region.

Pairwise genetic distance among individuals was estimated using the method of Huff et al. (1993). This pairwise distance was used to estimate mean pairwise genetic distances among populations. The genetic distance matrices were used to construct phenograms illustrating similarity among populations. Both Unweighted Pair-Group Method (UPGMA) and Neighbor-Joining methods (NJ) phenograms were generated

using Mega 3.0 program (Kumar *et al*., 2004) Later, Principle Coordinate Analysis (PCA) was used to plot the major distribution patterns between two coordinates of six subpopulations, using GenAlEx6 program.

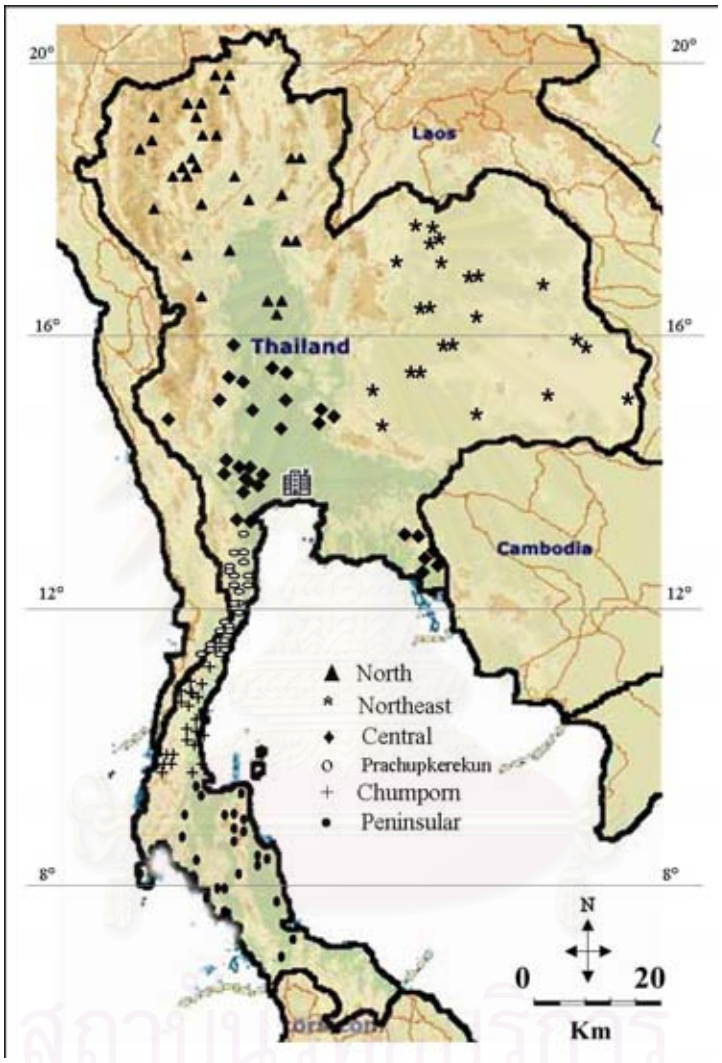


Figure 4.1 Collection sites for 184 *A. cerana* samples separated into 6 subpopulations in 2 regions. The Mainland Asia region, consisted of 4 subpopulations: North, 32 samples (20 ° N to 16° N), Central, 33 samples (16°N to 12° N), Northeast, 24 samples (18° N to 14° N) and Prachuap Khiri Khan, 27 samples (12° N to11° N). The Sundaland region consisted of 2 subpopulations: Chumporn, 30 samples (11° N to 9° N) and a Peninsular, 38 samples (9° N to 5° N).

RESULTS

Apis cerana genomic DNA was amplified o TE-AFLP primers *Xba* I-AC and *Bam*H I-C*, and the presence or absence of 27 bands were scored for each individual. Twenty-four bands were scored from standard AFLP reaction which used primers *Eco*R I-AAG* and *Mse* I-CTA.

Genetic diversity: The frequency of polymorphic bands (%P) and expected heterozygostiy (H_e) in both AFLP and TE-AFLP reactions is shown in Table 4.1. The frequency of polymorphic TE-AFLP bands was generally lower (66-89% in the six populations) than for AFLP bands (79-96%). The Peninsular subpopulation showed the highest level of polymorphism while, the Northeast subpopulation displayed the lowest of value. Mean of expected heterozygosity (*He*) indicated same trend with percentage of polymorphism. The highest mean of heterozygosity also found in Peninsular subpopulation (*He* = 0.341 ± 0.022), the lowest (*He* = 0.282 ± 0.028) in Northeast subpopulation. Not only the standard AFLP may be higher polymorphic bands of *A. cerana* than TE-AFLP but also, datasets from both methods may be better to detect for resolving genetic diversity of Thai *A. cerana* populations. Most *He* values were between 0.2 and 0.4 (Table 4.1), suggesting high levels of genetic diversity in Thai *A. cerana* populations.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 4.1 Genetic diversity into 6 subpopulations of Thai *A. cerana* calculated from TE-AFLP, AFLP and TE-AFLP&AFLP data. Heterozygosity (*He*) value of genetic diversity for binary data was measured by dominant markers is necessarily bound between 0 and 0.5.

Subpopulation	**TE-AFLP**		**AFLP**		**TE-AFLP & AFLP**	
(sample size)	**%P**	**Mean *He*±SE**	**%P**	**Mean *He*±SE**	**%P**	**Mean *He*±SE**
North (32)	66.67	0.254±0.040	95.83	0.356±0.024	80.39	0.302±0.025
Northeast (24)	66.67	0.237±0.040	79.17	0.333±0.039	72.55	0.282±0.028
Central (33)	70.37	0.266±0.040	95.83	0.377±0.027	82.35	0.318±0.025
Prachuap Khiri Khan (27)	81.48	0.296±0.035	91.67	0.366±0.031	86.27	0.329±0.024
Chumporn (30)	74.07	0.247±0.035	79.17	0.343±0.038	76.47	0.292±0.026
Peninsular (38)	88.89	0.290±0.032	95.83	0.399±0.027	92.16	0.341±0.022
Mean±SE	74.692±8.118	0.265±0.022	89.583±7.510	0.362±0.022	81.698±6.371	0.311±0.021

Table 4.2 Comparison results of pairwise of genetic distance among populations from TE-AFLP, AFLP and both data sets combined were calculated from GenAlEx 6.0 program.

	Pairwise genetic distance among populations					
	North	**Northeast**	**Central**	**Prachuap Khiri Khan**	**Chumporn**	**Peninsula**
TE-AFLP	0.000					
	0.012	0.000				
	0.008	0.008	0.000			
	0.032	0.024	0.024	0.000		
	0.030	0.038	0.026	0.036	0.000	
	0.044	0.041	0.032	0.043	0.017	0.000
AFLP	0.000					
	0.066	0.000				
	0.026	0.026	0.000			
	0.039	0.039	0.018	0.000		
	0.067	0.061	0.035	0.017	0.000	
	0.066	0.041	0.028	0.016	0.016	0.000
TE-AFLP &AFLP	0.000					
	0.036	0.000				
	0.016	0.016	0.000			
	0.035	0.031	0.021	0.000		
	0.047	0.049	0.031	0.028	0.000	
	0.054	0.041	0.030	0.031	0.016	0.000

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Population differentiation: Pairwise genetic distances among the six populations are shown in Table 4.2. All values were lower than 0.05, indicating little differentiation among *A. cerana* subpopulations. Neighbor-joining (NJ) and Unweighted pair-group method with arithmetic mean (UPGMA) were used to cluster populations using genetic distance data generated from AFLP, TE-AFLP and combined data sets, as shown in Figure 4.2. All data sets, displayed by both UPGMA and NJ, grouped together the Chumporn and Peninsular populations (located between 11°N and upper 5°N latitude), which correspond to the Sundaland mtDNA lineage. All but the AFLP data set grouped together North, Northeast and Central populations (between 20°N and upper 11°N latitude), corresponding to the Mainland Asian mtDNA lineage. Prachuap Khiri Khan, in the Kra ecotone between the northern and southern population groups, was grouped with either the Mainland Asian or with the Sundaland populations.

Pairwise genetic distance data were also used in principle coordinate analysis (PCA). Three PCA diagrams, generated from pairwise genetic distances (NEI from GenAlEx) among subpopulations in each datasets, supported with cluster analysis (Figure 4.3). Also, pairwise Φ_{PT} values (PW from GenAlEx6) of subpopulation were imported into PCA analysis. Interestingly, coordinate 1 separated two regions, Mainland Asia and Sundaland. The Mainland Asia region is composed of North, Northeast and Central subpopulations. As was the case with the NJ and UPGMA dedrograms, the placement of the Prachuap Khiri Khan population as ambiguous. The PCA diagram of TE-AFLP data indicated that Prachuap Khiri Khan subpopulation was clustered with other subpopulations in the Mainland Asia region (Figure 4.3A). But using only the AFLP data, the PCA plot placed the Prachuap Khiri Khan subpopulation with Sundaland populations (Figure 4.3B). The PCA diagram from both TE-AFLP and

AFLP datasets is shown in Figure 4.3C. PCA plot of all 184 *A. cerana* individuals using only genetic distance (GD from GenAlEx) found that mostly individuals were distributed around the intersection of coordinate 1 and coordinate 2. Independent distribution of *A. cerana* individuals from different subpopulations seems to indicate mixing of genetic information (Figure 4.3).

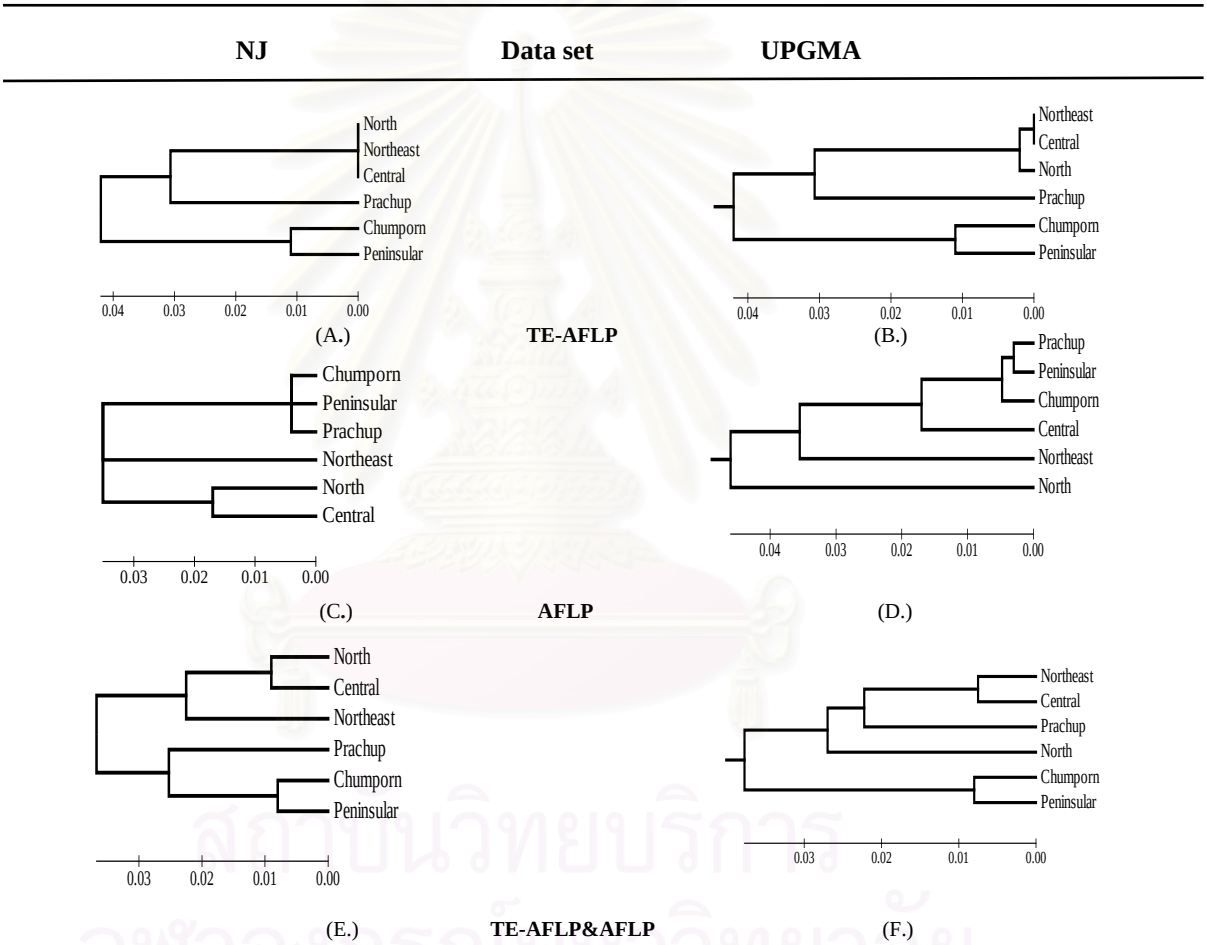


Figure 4.2 Cluster analyses were grouped from pairwise Nei's genetic distance by using NJ and UPGMA tests in Mega 3.0 program.

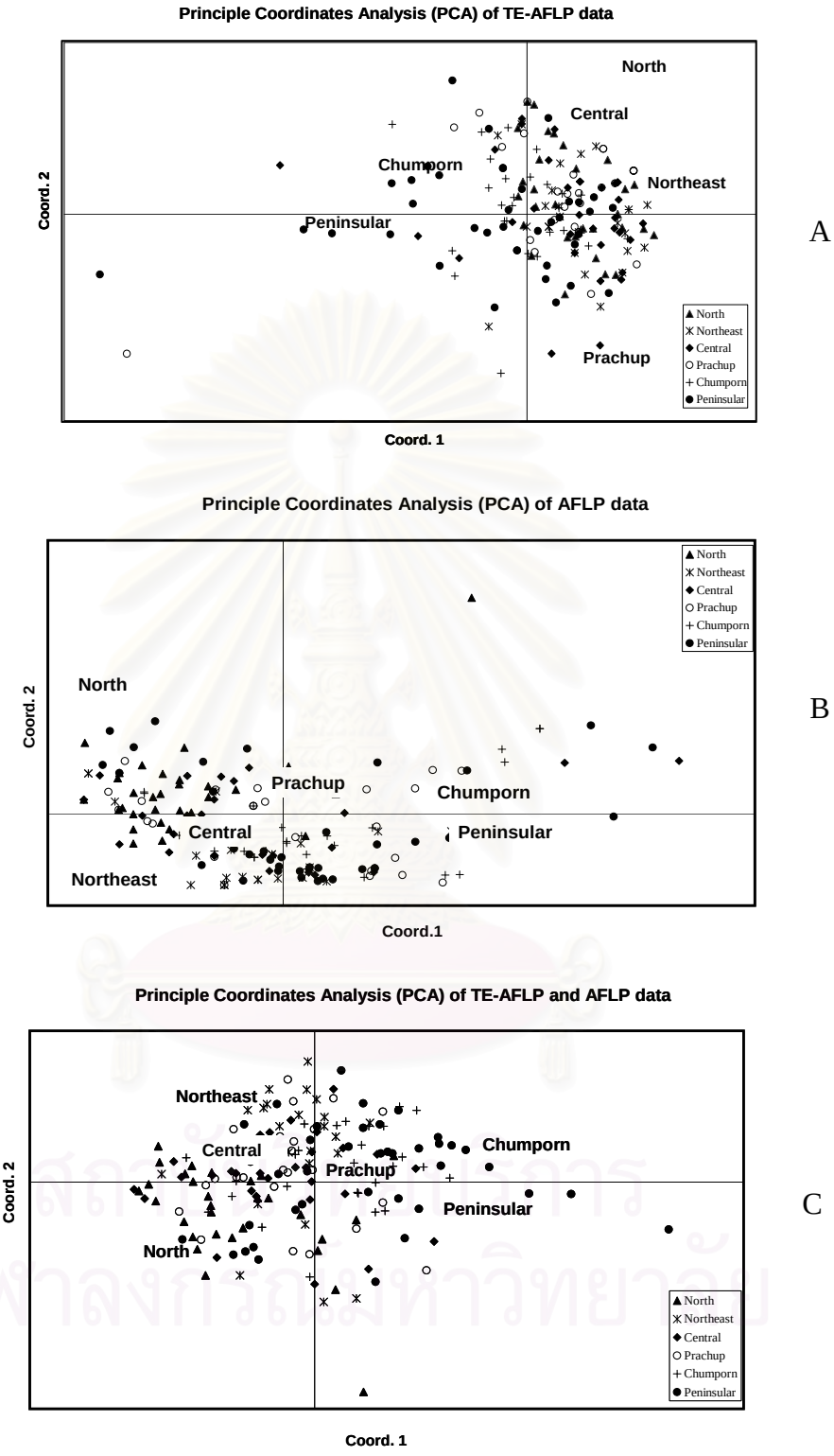


Figure 4.3 Principle Coordinates Analysis (PCA) diagram were plotted with two coordinates 1 and 2 using pairwise of Nei's genetic distance values (GD) of Thai *A. cerana* samples.

The AMOVA analyses using AFLP, TE-AFLP and combined data sets showed that more than 90% of observed molecular variance is within populations. Differentiation among population/ regions and among regions was low but statistically significant. Pairwise population Φ_{PT} values are shown in Table 4.4. Values of Φ_{PT} between the two Sundaland populations, Peninsula and Chumporn, were not significant, and values of Φ_{PT} among the Mainland populations. The Significant values of pairwise Φ_{PT} were observed between the Peninsular population and all others except Chumporn values among the North, Central, and Northeast populations were low. In contrast to the Chumporn and the Peninsular populations were classified into Sundaland region. TE-AFLPs did not detect differentiation between the North and the Northeast but as separated differentiation of the Chumporn+Prachuap Khiri Khan, the Central+Prachuap Khiri Khan and the Central +Peninsular. Anyway, pairwise Φ_{PT} could be detected differentiation into population although, Pairwise Nei's genetic distance could not clear in this investigation.

DISCUSSION

This is a part of additional data about genetic diversity and biogeography of *A. cerana* into Thailand which is analyzed at nuclear DNA by using standard AFLP and TE-AFLP fingerprinting. Two pairs of selective primers from standard AFLP (*Eco*R I-AAG* and *Mse* I-CTA) and TE-AFLP (*Bam*H I-C* and *Xba* I-AC) are effectively ones to detect polymorphism of Thai *A. cerana* that different from the previously investigation between *A. nigrocincta* and *A. cerana* at Sulawesi, Indonesia (Smith *et al*., 2003). Totally, fifty one bands from standard AFLP and TE-AFLP are dominant markers, are usually much more numerous and may expect that the loss of information encountered with these tools. Anyway, at least of forty AFLP markers

seem to be enough to correctly assign individuals to their population (Gaudeul *et al*., 2004). All of genetic diversity and population differentiation were analyzed based on three data of standard AFLP, TE-AFLP and both of data. The standard AFLP may be efficiency to detect higher genetic diversity of *A. cerana* than the TE-ALFP. But, TE-AFLP procedure has many advantages for selecting such as decrease unnecessary bands, immediately to amplification and low cost, etc. (van der Wruff *et al*., 2000). Further, this procedure will be popular to detect genetic differentiation population into other creatures. The mean of heterozygosity (*He*) indicate that *A. cerana* in Thailand are abundance genetic diversity. The TE-AFLP can be displayed lower *He* values than the standard AFLP. The *He* of standard AFLP support that it is still recommended procedure to investigate genetic differentiation into population which, it is too much complicate for amplification.

Table 4.3 Summary of AMOVA analysis using binary data matrix of TE-AFLP, AFLP and both. A total of 184 samples was separated into 2 regions: Mainland, with 4 populations (North, Northeast, Central, Prachuap Khiri Khan); and Peninsular, with 2 populations (Chumporn and Peninsular). Statistic value (Φ_{PT}) was repeated with 9999 permutations at $P < 0.001$ in calculation.

Summary of		**TE-AFLP**			**AFLP**			**TE-AFLP&AFLP**		
AMOVA	**df**	**Var.**	**%**	**Φ-statistics**	**Var.**	**%**	**Φ-statistics**	**Var.**	**%**	**Φ-statistics**
Among region	1	0.201	6	Φ_{RT} =0.061	0.115	3	Φ_{RT}=0.032	0.316	5	Φ_{RT} =0.046
Among Pops/Regions	4	0.084	3	Φ_{PR} =0.027	0.142	4	Φ_{PR}=0.040	0.226	3	Φ_{PR} =0.034
Within Pops	178	2.992	91	Φ_{PT} =0.087	3.376	93	Φ_{PT}=0.071	6.368	92	Φ_{PT} =0.078
Total	183	3.277			3.633			6.909		

Table 4.4 Comparison of pairwise population Φ_{PT} values among subpopulations.

Pair of Populations		Pairwise population Φ_{PT} values (PWT)		
Pop1	**Pop2**	**TE-AFLP**	**AFLP**	**TE-AFLP&AFLP**
North	Northeast	0.008	0.126*	0.075*
North	Central	0.000	0.034	0.018
Northeast	Central	0.000	0.034	0.015
North	Prachuap Khiri Khan	0.078*	0.062*	0.069*
Northeast	Prachuap Khiri Khan	0.053*	0.064*	0.059*
Central	Prachuap Khiri Khan	0.053*	0.010	0.030
North	Chumporn	0.067*	0.124*	0.098*
Northeast	Chumporn	0.093*	0.119*	0.107*
Central	Chumporn	0.057*	0.056*	0.057*
Prachuap Khiri Khan	Chumporn	0.088*	0.010	0.048
North	Peninsular	0.102*	0.114*	0.108*
Northeast	Peninsular	0.095*	0.067*	0.081*
Central	Peninsular	0.069*	0.036	0.052*
Prachuap Khiri Khan	Peninsular	0.101*	0.006*	0.053*
Chumporn	Peninsular	0.022	0.009	0.016

* showed the values > 0.05 which were significantly different between Pop1 and Pop2**.**

Consideration the genetic diversity and population differentiation into six subpopulations of *A. cerana* in Thailand, the Peninsular showed the highest diversity (Table 4.1) at all data that will be effect from *A. cerana* at island population which included into the same subpopulation. In addition to Nei's genetic distance values, NJ and UPGMA dendograms (Table 4.2, Figure 4.2) indicate that the Chumporn seem to be nearby genetically with the Peninsular more than the Prachuap Khiri Khan. Likewise of pairwise population Φ_{PT} values and PCA diagram support that Peninsular and Chumporn are in same group (Table 4.4, Figure 4.3). Subsequently, the Prachuap Khiri Khan subpopulation distributed around Isthmus of Kra area between longtitude 12° N to 11° N of Thailand will be explained that area is intermediate population between two groups (Mainland Asia and Sundaland) of Thai *A. cerana* and will effect from genetically inherit of drones. This result agree in this reason because of *He* values of the Prachuap Khiri Khan is higher than the Chumporn but nearby with the Central subpopulation. Pairewise of genetic distance and population Φ_{PT} values (Table 4.4) can

be supported the reason and confirm that is intermediate population between Mainland and Sundaland groups. Moreover, cluster dendograms (NJ and UPGMA) and PCA plot may indicate differentiation among subpopulations (Figure 4.2, Figure 4.3). In contrast to Mainland Asia group consist of three subpopulations as North, Northeast and Central. Suspect of the Northeast subpopulation differ significantly to the North, Prachuap Khiri Khan, Chumporn and Peninsular but classify into Mainland Asia group as well as the North and Central. Consequently, the Central subpopulation displays similarity into genetically of the North, Northeast and Prachuap Khiri Khan but to clearly differ from Chumporn and Peninsular (Table 4.4). Supporting about differentiation between North and Northeast subpopulations with Sittipraneed *et al*. (2001b), reported five *A. cerana* populations in their study could be divided into four difference groups including (A) northern and central, (B) peninsular Thailand, (C) Samui island and (D) north-east population. They pointed to further study of differentiation in *A. cerana* at northern, central and northeast population on Mainland Asia region. Another aim of this investigation examined differentiation of *A. cerana* population at mountainous area of Thailand which covered with the North subpopulation. All of statistic analysis from genetically data indicated that *A. cerana* at the mountainous region is similar to be with ground region (the Central and Prachuap Khiri Khan subpopulations) of Thailand. Reasonably, the mountain in Thailand may be not major geographic barriers of gene flow in *A. cerana* population but suspiciously at the Northeast subpopulation. However, the nuclear DNA is investigated by using standard AFLP and TE-AFLP indicate, population differentiation does exist in Thai *A. cerana*, as confirm the result from mtDNA and microsatellite variation. The idea about the gene pool of Thai *A. cerana* is not panmictic but reproductively isolated into several groups (Sittipraneed *et al*., 2001b), will be examine about drone flight mating between

Mainland Asia and Sundaland of *A. cerana* to confirm in the further. Together, the AMOVA analysis distinguish of Thai *A. cerana* subpopulation can not detect population differentiation among region (Φ_{RT} = 0.046) by using standard AFLP and TE-AFLP but is almost significant in this statistic value. Also, the Φ_{PR} value suggests that is not different among population in regions. Differentiation within population show significant into Φ_{PT} statistic indicate that *A. cerana* is exist high genetic variation within subpopulation (Table 4.4). However, Φ statistic may indicate about gene flow of *A. cerana*, which may inherit from drone bee. So, drone flight mating investigation of Thai *A. cerana* will be necessary to improve about reproductive isolation between these two groups example for the study of two color morphs of cavity nesting honey bee (*Apis*) in south India (Oldroyd *et al*., 2006).

The genetic relationship of Thai *A. cerana* into six subpopulations is generated by Nei's genetic distance by using cluster analysis (NJ and UPGMA test) and PCA analysis should be support them. The TE-AFLP data showed resemble dendograms from NJ and UPGMA. But, standard AFLP data displayed strongly genetic differentiation in the Northeast, it split from the North and Central subpopulation. The Prachuap Khiri Khan subpopulation showed nearby genetic distance with Sundaland group. While, both of AFLP datasets may be successful to explain in Thai *A. cerana* population which indicate that the Prachuap Khiri Khan is intermediate subpopulation. NJ test can be classified the Prachuap Khiri Khan at Sundaland while, UPGMA test group it at Mainland Asia. Furthermore, to also confirm the result of AFLP distribution within population, performed by PCA analysis which generated from genetic distance. The planes of coordinate reflect differences among *A. cerana* individuals within subpopulations. The three PCA axes explain the major part of *A. cerana* population

genetic diversity, the first and the second axes show at diagrams but the third axes is not shown in diagram (Figure 4.3). The statistics of Φ_{PT} show significant among subpopulations of two regions but the values are not high. The most of subpopulations are nearby 0.050 to 0.130, so will be found genetically overlap of *A. cerana* individuals into six subpopulations (Table 4.4). To clear up about genetic overlap among subpopulations suggest that with PCA plots of *A. cerana* individuals. Nuclear DNA as AFLP markers can be additional approved genetic differentiation of *A. cerana* population in Thailand that will be combined in its. *A. cerana* subpopulations are related with geographic pattern of Thailand. Absolutely, Prachuap Khiri Khan subpopulation may be intermediate population between Mainland Asia and Sundaland lineages while, Chumporn subpopulation belong to be Sundaland lineage. As same way, Central subpopulation may be intermediate among subpopulations into Mainland Asia region of Thailand. Extremely, drone bee can importantly effect to widespread genetic variation of Thai *A. cerana* also gene flow can occur among populations.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER V

GENETIC RELATIONSHIPS BETWEEN TWO HONEY BEES (*Apis mellifera* Linnaeus, 1758 AND *A. cerana* Fabricius, 1753) AND *Varroa* MITES IN THAILAND

ABSTRACT

The cytochrome oxydase I (COI) gene of mitochondrial DNA from all mite samples (n=73) were amplified and further analyzed using RFLP and sequencing. *V. destructor*, Korea haplotype (n=38), importantly invaded into *A. mellifera*. Others, Japan (n=3) and Vietnam (n=1) haplotypes were found only infesting major ThaiA2 *A. mellifera*. The percentage ratio of mites infested on each Thai *A. mellifera* haplotypes (ThaiA1, ThaiA2, ThaiB) was 19: 64: 17. This ratio was related to the ratio of Thai *A. mellifera*. The COI gene sequence of *Varroa* mites from their naturally colonies supported that *V. jacobsoni* have specific distribution related to *A. cerana* lineages. Malaysia haplotype of *V. jacobsoni* reproduce exactly on Sundaland *A. cerana* lineage. While, NorthThai haplotype colonize on Mainland Asia lineage. Isthmus of Kra is intermediate region of two haplotypes of *V. jacobsoni*. Vietnam haplotype of *V. destructor* could reproduce on host in mountainous region 18 °N latitude. Insight to lowland area could be contract area between *V. jacobsoni* and *V. destructor* in Thailand. Genomic DNA analysis, TE-AFLP, indicated that *V. destructor* could colonize in *A. mellifera* and *V. jacobsoni* could colonize in natural *A. cerana*. The genetic distribution of *V. jacobsoni* haplotypes related to *A. cerana* lineage in Thailand. These results suggested co-evolution between two *Apis* and their parasites. The Korea haplotype have been rapidly distributed around the world and not found parasitized on *A. cerana* meanwhile the Vietnam haplotypes could be detected inside two honey bee species.

INTRODUCTION

The major ectoparasitic honey bee mite was classified in genus *Varroa* (Mesostigmata: Varroidae). The native pest of *A. cerana* colonies which first described from Java by Oudemans (1904) was named *Varroa jacobsoni*. This natural parasite of *A. cerana* switched to the occidental *A. mellifera* when the later was introduced into Asia for apiculture reasons (Anderson, 2000; Solignac *et al*., 2005). The host transfer seems to have occurred on two occasions (De Guzman *et al*., 1997; Oldroyd, 1999). At the first, *A. mellifera* colonies were introduced into Japan in 1877 but there was no report about *Varroa* inside colonies until 1957. From Japan, *Varroa* invaded Paraguay in 1971, Brazil in 1972 and later North America (De Jong *et al*., 1982). A second pathway, *A. mellifera* colonies from Ukraine were introduced to the far east of the Soviet Union, where allowing the shift of *Varroa* infesting local colonies of *A. cerana* onto *A. mellifera*. Thus, infested colonies were later found in European USSR in 1975 (Crane, 1978) and there the parasite invaded Europe, following rapidly and almost worldwide expansion (Solignac *et al*., 2005).

A lot of genetic investigations were used to resolve complexity in *V. jacobsoni*. The random amplification of polymorphic DNA technique (RAPD) indicated *V. jacobsoni* infesting *A. cerana* at Malaysian Borneo differed from *V. jacobsoni* infesting *A. mellifera* at USA and Germany (Kraus and Hunt, 1995). Cytochrome oxydase I (CO-I) of mitochondrial DNA were investigated with PCR-RFLP and DNA sequencing showed that *V. jacobsoni* from Javanese origin differed from *V. jacobsoni* infesting *A. mellifera* colonies in Germany (Anderson and Fuchs, 1998). On the two host species, the parasite was first described as belonging to *V. jacobsoni* until Anderson and Trueman (2000) had revealed the existence of at least two species: *V. jacobsoni* and

newly named *V. destructor*. The mtDNA examination of *Varroa* mites infested on naturally *A. cerana* colonies in Asia showed eighteen genetic distinct types. They were separated into two groups on continental Asia (as same as *A. cerana* mtDNA lineages: mainland Asia and Sundaland) and three genetic distinct types on the Philippines (Anderson, 2000; Anderson and Trueman, 2000). *Varroa* mite infested on *A. mellifera* was same a one group of genetically distinct of *Varroa* infesting *A. cerana* Mainland Asia linegae which was *V. destructor* (Anderson, 2000). Two haplotypes of *V. destructor*, Korea haplotype or K type is present on *A. mellifera* almost worldwide, and Japan/Thailand haplotype or J type is present only in Japan, Thailand and the Americas (De Guzman *et al*., 1999; Anderson, 2000). K type was more virulent, displaced the J type in many countries that they were admixture between types and shared the same polymorphism (Solignac *et al*., 2005).

Initially, *Varroa* mites infesting on their native host, *A. cerana* in Asia was investigated to understand host and parasite relationship by Anderson and Trueman (2000). They found nine mitochondrial COI haplotypes of *V. jacobsoni*, which distributed in Malaysia-Indonesia region (Sundaland) and seven haplotypes of *V. destructor* distributed at Mainland Asia region. Exceptional Korea and Japan/Thailand haplotype of *V. destructor* can be also colonized on *A. mellifera* (Anderson, 2000). The evidence of *Varroa* infesting on *A. cerana* and *A. mellifera* colonies in Vietnam showed that almost Vietnam haplotype of *V. destructor* colonized on *A. cerana* colonies. And, a few was also colonized on *A. mellifera* while, Korea haplotype almost infested on *A. mellifera* colonies (Fuchs *et al*., 2000). In China, three different *V. destructor* haplotypes were detected on *A. cerana*: China1, China2 and Vietnam haplotypes and only Korea haplotype was a major pest infested on *A. mellifera* colonies

(Zhou *et al*., 2004). In Thailand, *Varroa* infested only on their native host (*A. cerana*) was recently reported four different haplotypes of *V. jacobsoni*: NorthThai1, NorthThai2, Samui and Malaysia haplotypes and one of *V. destructor*: Vietnam haplotype which, distributed at the mountainous region of Thailand (Warrit *et al*., 2006). Mentioning to Vietnam haplotype could infest both of *A. cerana* and *A. mellifera* colonies which seemed to be linked between hosts (Fuchs *et al*., 2000).

The mtDNA data of *Varroa* were related with geographical distribution of mitochondrial DNA lineages of *A. cerana* in Asia: Mainland Asia and Sundaland. Almost of results seemed to be clearly about geographical distribution of *Varroa* species and *A. cerana* in Asia. *V. jacobsoni* could colonize on *A. cerana* at Malaysia-Indonesia region while *V. destructor* could colonize on *A. cerana* at Mainland region (Anderson, 2000; Anderson and Trueman, 2000; Fuchs *et al*., 2000; Hepburn *et al*., 2001; Smith *et al*., 2000). Suspect to *Varroa* on natural *A. cerana* colonies in Thailand was not found *V. destructor*, Japan/Thailand haplotype, which different from previously examination but the Vietnam haplotype was found at the mountainous region in the north. Moreover, newly two haplotypes of *V. jacobsoni* (NorthThai1 and NorthThai2 haplotypes) were found along to apart of Isthmus of Kra and lowland region which distributed upper Isthmus of Kra. Two haplotypes of *V. jacobsoni* (Malaysia and Samui haplotypes) were still belonging to Sundaland mtDNA *A. cerana* lineage (Warrit *et al*., 2006). However, within Isthmus of Kra, highland mountainous and northeastern regions are still interesting places where contract region among *Varroa* haplotypes on their natural native hosts in Thailand. Additional, to completely explain about *Varroa* mites and their hosts into Thailand, *Varroa* mites infesting on introduced species (*A. mellifera*)

should be also investigated about their genetic diversity, population differentiation and host-parasite relationship.

MATERIALS AND METHODS

Collections

Varroa mites and their honey bee hosts (*A. mellifera* and *A. cerana*) were sampled in same colonies during October 2004 to January 2006. Totally, forty-two *Varroa* mites parasitized on *A. mellifera* colonies were collected from bee farms and apicultural extension offices around Thailand (Table 5.1). *Varroa* collections were sampled at different attachment on their honey bee hosts such as thirteen collections attached on adult stage of honey bee, nineteen collections were found inside brood cells and ten collections were collected at bottom of bee hive. Thirty-one *Varroa* samples were found from natural *A. cerana* colonies. Twenty-two *Varroa* samples were collected from drone brood cells and nine samples from worker brood cells (Table 5.1). Moreover, nineteen collections of *Varroa* infested on natural Thai *A. cerana* colonies from DRS laboratory were also selected to investigate the genomic DNA with TE-AFLP analysis.

Molecular and Statistical analysis

DNA from *Varroa* collections (n=73) were extracted using GeneElute Mamalian Genomic DNA procedure. Also, standard phenol/chloroform procedure was used to extract the forty-two *A. mellifera* and thirty-one *A. cerana* samples (Smith *et al*., 1997). *Varroa* mites and their *A. cerana* hosts 19 DNA were extracted using QIAGEN miniDNA Extraction Kit procedure (Appendix A).

Varroa mites

Each of DNA extracts (n=73) were amplified at Cytochrome oxidase I (COI) gene of mitochondrial DNA. PCR amplification was followed pair of primers as same as

Warrit *et al*. (2006) but was different in profile program (pre-amplification step at 94°C for 1 min; 35 cycles, denaturing at 94°C for 1 min, annealing at 50 °C for 1 min, extending at 72°C for 1 min; and post-extension at 72°C for 5 min). The products were digested with *Xho* I and *Sac* I restriction enzymes. The COI gene portion of *Varroa* was sequenced with V51 primer using automatic sequencer: 3730xl DNA analyzer of Macrogen, USA (detail in Appendix IB). Comparison of the Thai *Varroa* sequences and reference *Varroa* sequences from GenBank database [*V. jacobsoni* consisted of Indo-Java (AF106910), Malaysia (AF106906), NorthThai1 (DQ061189), NorthThai2 (DQ061190), Samui1 (DQ061188), *V. destructor* consisted of German (AF010478), Japan (AF106897), Nepal (AF106898), South Korea (AF106899), China (AF106900), Vietnam (AF106901), *V. rindereri* and *V. underwoodi*] were estimated the pairwise genetic distance using Kimura's 2-parameter model (Kimura, 1980). The COI gene sequences were aligned with ClustalW and constructed phylogenetic tree with neighbor-joining (NJ) and maximum parsimony (MP) methods, additional tested with 1,000 replications bootstrap in Mega version 3.0 (Kumar *et al*., 2004).

Genomic DNA of *Varroa* samples infested on *A. cerana* (n=48) and on *A. mellifera* (n=10) were determined using Three Restriction Enzyme Amplified Fragment Length Polymorphism (TE-AFLP), followed procedure of van der Wuff *et al*. (2000) (Appendix A). TE-AFLP bands were scored as present (1) and absent (0) by hands. Then, the matrices binary data were analyzed genetic diversity, percentages of polymorphism, heterogeneity values and genetic differentiation within Thai *Varroa* populations using AMOVA in the GenAlEx 6.0 (Peakall and Smouse, 2006). The matrices binary data were also exported to Winclada version 1.00.08: NONA (Nixon, 2002) to analyze phylogenetic tree tested with bootstrap 1,000 replications.

Table 5.1 Amount of *Varroa* samples (n=73) were collected within *A. mellifera* (n=42) and *A. cerana* (n=31) colonies. The mites were collected from several attachments: phoretic on thorax or abdomen of adult honey bee = (^{p}), inside worker brood cells = (^{w}), inside drone brood cells = (^{d}) and non-living at bottom of bee hive = (^{b}). All of *Varroa* samples infested on *A. cerana* were sampled from drone brood cells (n=22) and worker brood cells (n=9).

Latitude N° (populations)	**Symbol ***	**Locallity (No. of samples)**	***Varroa* haplotype on their host**	
			A. cerana	***A. mellifera***
Upper 18 N° (North)	N1	Nan (2)	ThaiV2 (1^{d})	ThaiV3 (1^{b})
	N2	Chiang Mai (6)	ThaiV2(1^{d}) NorthThai** (1^{d})	ThaiV3 (2^{w}), (3^{d})
	N3	Chiang Rai (1)	ThaiV2(1^{w})	
18 N° - 11 N° (North+Northeast+Central)	N4	Utaradit (14)	NorthThai** (1^{w})	ThaiV1 (1^{w}), (2^{b}) ThaiV2 (1^{w}) ThaiV3 (3^{w}), (3^{b}), (3^{p})
	NE1	Udonthani (4)	NorthThai** (1^{d})	ThaiV3 (2^{w}), (1^{d})
	NE2	Sakhonnakorn (1)	NorthThai** (1^{w})	
	NE3	Khonkhan (3)		ThaiV3 (2^{p}), (1^{w})
	NE4	Loi Et (4)	NorthThai** (3^{d})	ThaiV3 (1^{p})
	NE5	Yasothorn (1)	NorthThai** (1^{w})	
	C1	Phithsanulok (9)		ThaiV3 (8^{w}), (1^{b})
	C2	Srakeaw (1)		ThaiV3 (1^{w})
	C3	Samurtsongkarm (6)	NorthThai (4^{d}), (2^{w})	
	C4	Petchburi (1)	NorthThai (1^{d})	
11 N° - 10 N° (Central+South)	C5	Prachuap Khiri Khan (9)	NorthThai (7^{d}), (2^{w})	
	S1	Chumporn (4)	Malaysia (4^{d})	
Lower 10 N° (South)	S2	Surat Thani (1)		ThaiV3 (1^{p})
	S3	Songkla (5)		ThaiV3 (5^{p})

*referred the symbols for population's names into same locality, **referred haplotype of *V. jacobsoni* distributed at north and northeastern areas of Thailand were showed differences of sequences alignment (Figure 5.1) and phylogenetic tree (Figure 5.2)

Honey bees

Genetic variation of *A. mellifera* samples (n=42) were determined using PCR-RFLP into four regions of mitochondrial DNA following Suppasat *et al*. (2007). As same technique, *A. cerana* samples (n=31) examined some regions of mitochondrial DNA were examined following procedure of Deowanish *et al*. (1996) and Sittipraneed *et al*. (2001a). The biogeography of *Varroa* mites and their honey bee hosts in Thailand were discussed about host–parasite relationship.

RESULTS

Varroa mite haplotypes infested on *A. mellifera* colonies

Varroa destructor was specifically pest of *A. mellifera* colonies in Thailand as same as in other countries. Restriction enzyme digestion at cytochrome oxydase I with *Xho* I and *Sac* I was basically method to identify *Varroa* complex species (Table 5.2). The completely partial sequences in COI region were 289 base pairs. There were shorter than those reported by Warrit *et al*. (2006) investigation because of they were not included V51 oligo-primer about 21 bases plus consequent base about 18 bases. The partial sequences alignment of Thai *Varroa* mites showed 100% identity and 99.7% similarity with partial of reference sequences: Japan, Vietnam and Korea haplotypes, respectively (Figure 5.1). There were three different haplotypes such as ThaiV1 = Japan, ThaiV2 = Vietnam and ThaiV3 = Korea (Table 5.1). Amount of samples (n=38) were identified into ThaiV3 for 90.5%, ThaiV1 (n=3) for 7.1% and ThaiV2 (n=1) for 2.4% of totally *Varroa* mites infested on Thai *A. mellifera* (Table 5.2).

The consensus trees calculated bootstrap 1,000 replications of Maximum Parsimony (MP) and Neighbor-Joining (NJ) methods showed the condense trees with

bootstrap values which supported that Thai *Varroa* infested on *A. mellifera* in Thailand were similar to three *V. destructor* haplotypes (Figure 5.2A and B).

In part of Thai *A. mellifera* (n=476), which were collected during July 2003 and January2006, were found *Varroa* mites (n=42) approximately 8.8% infestation. *Varroa* mites were abundantly found at the period of July to October and December to February. From the results of matrilineal of *A. mellifera* in Thailand found three major haplotypes (ThaiA1: *A. m. ligustica*, ThaiA2: *A. m. carnica* and ThaiB: (Middle East honeybees) *A. m. syriaca* or *A. m. larmarkii*. The percentage ratio of Thai *A. mellifera* haplotypes for ThaiA1: ThaiA2: ThaiB were 22%: 60%: 18% (Suppasat *et al*., 2007). Within the amount of percentages ratio of *Varroa* mites infested on three haplotypes of Thai *A. mellifera* showed as 19%: 64%: 17%. ThaiA2 of *A. mellifera* haplotype was major parasitized by three haplotypes of *V. destructor* (ThaiV1, ThaiV2 and ThaiV3). While, ThaiA1 and ThaiB haplotypes were parasitized by only one haplotype (ThaiV3) of *V. destructor* (Table 5.2). The distribution of ThaiV3 was commonly found in every apiaries of Thailand but was not related with Thai geography. There was only one locality at Utaradit (N4) detected three different *Varroa* haplotypes (Table 5.1).

Varroa mite haplotypes of infested on _A. cerana_ colonies

The RFLP patterns of COI region in mitochondrial DNA supported that almost of *Varroa* mites infested natural of Thai *A. cerana* colonies were *V. jacobsoni*. While, the *Varroa* samples at highland mountainous region of Thailand where was distributed higher than 18 N°latitude (Chiang Mai, Chiang Rai and Nan Province) were identified into *V. destructor* as same as a previous investigation (Table 5.2). The phylogram from NJ and MP analysis (Figure 5.2) indicated that two clusters of *V. jacobsoni* in Thailand also related with mitochondrial DNA lineages of *A. cerana*. This study found that

Malaysia haplotype of *V. jacobsoni* infested natural colonies of *A. cerana* colonies from Chumporn province (S1) where located below 10.34 N° of latitude was Sundaland lineage. Secondary cluster were NorthThai haplotypes which included NorthThai1 and NorthThai2 haplotypes (Warrit *et al*., 2006), infested on mainland Asia *A. cerana* lineage which distributed upper than 10.34 N° latitude of Kra ecotone (Table 5.1). The sequences alignments of these NorthThai haplotypes suspected of two substitution nucleotide bases (G to C, C to T) were differently among NorthThai, NorthThai1 and NorthThai2 haplotypes (Figure 5.1). Also, *Varroa* mites from northeast of Thailand (NE1-NE5 subpopulations) were identified into *V. jacobsoni* NorthThai haplotype which clustered closely to clad of *V. jacobsoni* from mountainous region at north population, N2.2 (Figure 5.2). The *V. destructor* Vietnam haplotype was found inside *A. cerana* from mountainous region of Thailand (Chiangmai, Chiangrai and Nan).

Genetic differentiation of *Varroa* mites in Thailand

Population differentiation of *Varroa* mites infested on *A. cerana* was firstly performed with AMOVA analysis using the sequence data. The populations were grouped into two partitions of observed. When populations were grouped into partition of observed A, variance among groups for 64.00% of the total while partition of observed B showed value for 49.36%. In part of variance among populations, the values were 24.60% and 25.38%, respectively (Table 5.3).

Later, within genomic DNA of *Varroa* infested on natural *A. cerana* analyzed by TE-AFLP analysis using one pair of primers: Bam-C* and Xba-AC for detection. The binary data of 0/1 matrices were exported to Winclada ver. 1.00.08 program (Nixon, 2002) with bootstrap 1,000 replications. This tree showed that *Varroa* mites infested on *A. cerana* clustered in same clad. Almost *V. destructor*, Vietnam haplotype, found

infested on mountainous *A. cerana* colonies. *V. jacobsoni*, NorthThai haplotype was major infesting to Mainland *A. cerana* mitochondrial lineage and Malaysia haplotype found in Sundaland *A. cerana* mitochondrial lineage. Suspect to *V. destructor*, Vietnam haplotype, was grouped into same clad with *V. jacobsoni*, NorthThai and Malaysia haplotypes (Figure 5.3A). In contrast to some of NorthThai haplotype infested on *A. cerana* of Northeast population and some of Malaysia haplotype which infested on *A. cerana* of Chumporn population were showed closely related of genetically information at their clad (Figure 5.3A). When determining, the 0/1 matrices were converted to A/T matrices before imported to Mega 3.0 program for phylogenetic tree construction. The maximum parsimony tree showed mixable of *Varroa* haplotypes that infested on *A. cerana* especially between NorthThai and Vietnam haplotypes. The Malaysia haplotype was still in one group. Anyway, the tree supported that NorthThai haplotype from Northeast population and two samples of Malaysia haplotype from Chumporn population were in same clad (Figure 5.3B). The AMOVA analysis of TE-AFLP generated with three different partitions of observed populations using GenAlEx 6.0 program. The greatest variances among populations of three portions (A, B, C) were about 65.48%, 65.86% and 61.85%, respectively indicated different within population. While, the extremely variances among regions were about 19.61%, 12.55% and 20.32%, respectively (Table 5.4II).

Distribution and abundance of *Varroa* mites in Thailand

In Thailand, two species of *Varroa* mites: *V. jacobsoni* found only in *A. cerana* while, *V. destructor* found in *A. mellifera*. Abundance of *V. destructor*, Korea haplotype, distributed all regions in Thailand and infested only inside *A. mellifera* colonies. While *V. jacobsoni*, NorthThai haplotype, was major in *A. cerana*. All of sequences (n=73) were performed population differentiation using AMOVA analysis which

grouped populations into two partitions of observed (probability of a more extreme value from 9999 random permutations). The nested analysis showed percentages of variance as almost equal into two portions as about 24.60% (A) and 25.38% (B) within populations. But, the percentages of variance among regions were different between portion A and B that were about 3.74% and 64.52%, respectively (Table 5.3I). Moreover, summary of AMOVA from TE-AFLP data supported that the percentages of variance among region portion A (17.19%) was higher than portion B (11.41%) but the greatest variance showed at portion C (10.77%). Although, the variance within population of portion C was the lowest (55.96%) while, others were also almost equal as same sequences, which were about 60.54% and 60.38%, respectively (Table 5.4I).

Table 5.2 The restriction sites of COI genes digested with *Xho* I and *Sac* I restriction enzymes and amount of Thai *Varroa* haplotypes which infested on different Thai honey bees, Thai *A. mellifera* and natural *A. cerana*. Symbols + showed restriction site of *Xho* I and *Sac* I, (*V.d.*) was *V. destructor* and (*V.j.*) was *V. jacobsoni*.

Haplotypes	**Restriction site**		***A.mellifera* haplotypes**			***A.cerana* haplotypes**		**Total**
(*Varroa* sp.)	***Xho* I**	***Sac* I**	**ThaiA1**	**ThaiA2**	**ThaiB**	**Mainland**	**Sundaland**	**(n)**
Japan (*V.d.*) ThaiV1	+	+		3				3
Vietnam (*V.d.*) ThaiV2	+	+		1		3		4
Korea (*V.d.*) ThaiV3	+	+	8	23	7			38
Malaysia (*V.j.*)		+					4	4
NorthThai 1(*V.j.*) NorthThai 2 NorthThai		+				24		24
Total			**8 (19%)**	**27 (64%)**	**7 (17%)**	**27 (87%)**	**4 (13%)**	**73**

```
#Indo-Java  TTT AGG GAA T TTA TGC TA TAA TAA GTA TTG GTA TTT TAG GTT TTA TTG TAT GAG CTC ATC ATA TAT TTA CAG
#Malaysia   ... ... ... . ... ... .. ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#S1         ... ... ... . ... ... .. ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Samui      ... ... ... . ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#NorthThai1 ... ... ... . ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#NorthThai2 ... ... ... . ... ... .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C5.1       ... ... ... . ... ... .. ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C5.2       ... ... ... . ... ... .. ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C4         ... ... ... . ... ... .. ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C3         ... ... ... . ... ... .. ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#NE4        ... ... ... . ... ... .. ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C
#N2.1       ... ... ... . ... ... .. ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C
#N2.2       ... ... ... . ... C.. .. ... ... C.. .C. ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#N3         ... ... ... . ... C.. .. ... ... C.. .C. ..G ... ... ... ... ... ... ... ... ... ... ... ... ...
#Vietnam    ... ... ... . ... C.. .. ... ... C.. .C. ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#ThaiV2     ... ... ... . ... C.. .. ... ... C.. .C. ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Japan      ... ... ... . ... C.. .. ... ... C.. .C. ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#ThaiV1     ... ... ... . ... C.. .. ... ... C.. .C. ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Korea      ... ... ... . ... C.. .. ... ... C.. .C. ... ... ... ... ... ... ... ... .G. ... ... ... ... ...
#ThaiV3     ... ... ... . ... C.. .. ... ... C.. .C. ... ... ... ... ... ... ... ... .G. ... ... ... ... ...

#Indo-Java  TAG GGA TAG ATA TTG ATA CTC GGG CTT ATT TTA CTG CGG CTA CAA TGA TTA TTG CGG TTC CCA CTG GTA TTA
#Malaysia   ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#S1         ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Samui      ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#NorthThai1 ... .T. ... ... ... ... ... ... ... ... .C. ... ... ... ... ... ... ... ... ... ... ... ... ...
#NorthThai2 ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C5.1       ... .T. ... ... ... ... ... ... ... ... .C. ... ... ... ... ... ... ... ... ... ... ... ... ...
#C5.2       ... .T. ... ... ... ... ... ... ... ... .C. ... ... ... ... ... ... ... ... ... ... ... ... ...
#C4         ... .T. ... ... ... ... ... ... ... ... .C. ... ... ... ... ... ... ... ... ... ... ... ... ...
#C3         ... .T. ... ... ... ... ... ... ... ... .C. ... ... ... ... ... ... ... ... ... ... ... ... ...
#NE4        ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#N2.1       ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#N2.2       ... .A. ... ... ... ... ... .A. .A. ... ... ... .A. ... ... .A. ... ... ... ... .T. ... ... ...
#N3         ... .A. ... ... ... ... ... .A. .A. ... ... ... .A. ... ... .A. ... ... ... ... .T. ... ... ...
#Vietnam    ... .A. ... ... ... ... ... .A. .A. ... ... ... .A. ... ... .A. ... ... ... ... .T. ... ... ...
#ThaiV2     ... .A. ... ... ... ... ... .A. .A. ... ... ... .A. ... ... .A. ... ... ... ... .T. ... ... ...
#Japan      ... .A. ... ... ... ... ... .A. .A. ... ... ... .A. ... ... .A. ... ... ... ... .T. ... ... ...
#ThaiV1     ... .A. ... ... ... ... ... .A. .A. ... ... ... .A. ... ... .A. ... ... ... ... .T. ... ... ...
#Korea      ... .A. ... ... ... ... ... .A. .A. ... ... ... .A. ... ... .A. ... ... ... ... .T. ... ... ...
#ThaiV3     ... .A. ... ... ... ... ... .A. .A. ... ... ... .A. ... ... .A. ... ... ... ... .T. ... ... ...

#Indo-Java  AAA TTT TTT CTT GAT TAG CGA CAA TTC ATG GTT CTA TAG TAA AAT TAG ATG TTC CAA TAA TCT GAT CCT TGG
#Malaysia   ... ... ... ... ... ... .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#S1         ... ... ... ... ... ... .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#Samui      ... ... ... ... .G. ... .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#NorthThai1 ... ... ... ... ..C ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#NorthThai2 ... ... ... ... ..C ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
#C5.1       ... ... ... ... ..C ... .T. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ...
#C5.2       ... ... ... ... ..C ... .T. ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ... .T. ...
#C4         ... ... ... ... ..C ... .T. ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ... .T. ...
#C3         ... ... ... ... ..C ... .T. ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ... .T. ...
#NE4        ... ... ... ... ..C ... .T. ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ... .T. ...
#N2.1       ... ... ... ... ..C ... .T. ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ... .T. ...
#N2.2       ... ... ... ... ... ... .A. ... ... ... ... ... ... ... .T. ... ... ... .C. .G. ... .T. ... .T. .A.
#N3         ... ... ... ... ... ... .A. ... ... ... ... ... ... ... .T. ... ... ... .C. .G. ... .T. ... .T. .A.
#Vietnam    ... ... ... ... ... ... .A. ... ... ... ... ... ... ... .T. ... ... ... .C. .G. ... .T. ... .T. .A.
#ThaiV2     ... ... ... ... ... ... .A. ... ... ... ... ... ... ... .T. ... ... ... .C. .G. ... .T. ... .T. .A.
#Japan      ... ... ... ... ... ... .A. ... ... ... ... ... ... ... .T. ... ... ... ... .G. ... .T. ... .T. .A.
#ThaiV1     ... ... ... ... ... ... .A. ... ... ... ... ... ... ... .T. ... ... ... ... .G. ... .T. ... .T. .A.
#Korea      ... ... ... ... ... ... .A. ... ... ... ... ... ... ... .T. ... ... ... .C. .G. ... .T. ... .T. .A.
#ThaiV3     ... ... ... ... ... ... .A. ... ... ... ... ... ... ... .T. ... ... ... .C. .G. ... .T. ... .T. .A.
```

```
#Indo-Java  GTT TTA TTT TTT TAT TTA CTT TGG GGG GAA TTA CTG GTG TGA TTT TAG CTA ATT CTT CTA TTG ATA TTG A  289
#Malaysia   ... ... ... ... ... ... ... ... .A. ... ... ... ... ... ... ... ... ... ... ... ... ... . .  289
#S1         ... ... ... ... ... ... ... ... .A. ... ... ... ... ... ... ... ... ... ... ... ... ... . .  289
#Samui      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... . .  289
#NorthThai1 .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... . .  289
#NorthThai2 .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... . .  289
#C5.1       .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... . .  289
#C5.2       .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... . .  289
#C4         .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... . .  289
#C3         .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... . .  289
#NE4        .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... . .  289
#N2.1       .A. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... . .  289
#N2.2       ... ... ... ... ... ... ... .A. ... .T. ... ... ... .A. ... ... ... ... ... ... ... ... . .  289
#N3         ... ... ... ... ... ... ... .A. ... .T. ... ... ... .A. ... ... ... ... ... ... ... ... . .  289
#Vietnam    ... ... ... ... ... ... ... .A. ... .T. ... ... ... .A. ... ... ... ... ... ... ... ... . .  289
#ThaiV2     ... ... ... ... ... ... ... .A. ... .T. ... ... ... .A. ... ... ... ... ... ... ... ... . .  289
#Japan      ... ... ... ... ... ... ... .A. ... .T. ... ... ... .A. ... ... ... ... ... ... ... ... . .  289
#ThaiV1     ... ... ... ... ... ... ... .A. ... .T. ... ... ... .A. ... ... ... ... ... ... ... ... . .  289
#Korea      ... ... ... ... ... ... ... .A. ... .T. ... ... ... .A. ... ... ... ... ... ... ... ... . .  289
#ThaiV3     ... ... ... ... ... ... ... .A. ... .T. ... ... ... .A. ... ... ... ... ... ... ... ... . .  289
```

Figure 5.1 Partial sequence alignments of COI gene (289 base pairs) were from references of *V. jacobsoni* (Indo-Java, Malaysia, Samui, NorthThai1 and NothThai2 haplotypes), *V. destructor* (Japan, Vietnam and Korea haplotypes) (Anderson and Trueman, 2000; Warrit *et al.*, 2006). And, the partial sequences of Thai *Varroa* mites: S1, C5.1, C5.2, C4, C3, NE4, N2.1, N2.2 and N3 infested natural *A. cerana* colonies also, ThaiV1, ThaiV2 and ThaiV3 infested Thai *A. mellifera*. The nucleotides similarity with Indo-Java (*V. jacobsoni*) sequence showed with dot (.) but the alphabets (A, T, C and G) showed different nucleotides. Highlight alphabets indicated nucleotide different among sequences of NorthThai1, NorthThai2 and these Thai *Varroa* sequences. Underline alphabets showed base substitution sites within North and Northeast of *Varroa* populations which were different with NothThai1 and NorthThai2 sequences.

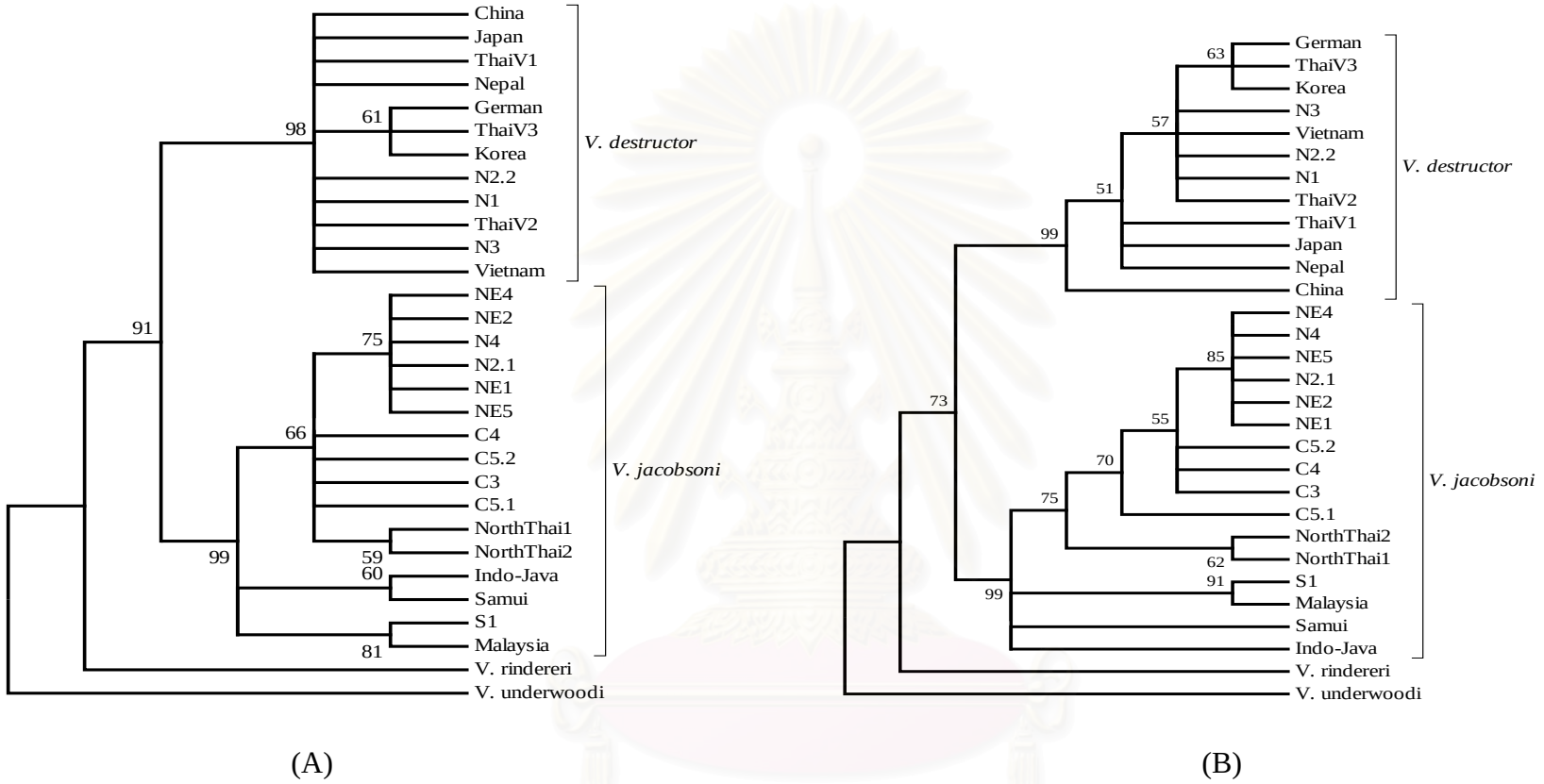


Figure 5.2 The bootstrap consensus phylogenetic tree of Maximmum Parsimony (A) and Neighbor-Joining (B) methods showed statistic values at branches of references and Thai *Varroa* sequences. The reference sequences of *V. underwoodi*, *V. rindereri*, *V. jacobsoni* (Indo-Javva, Malaysia, Samui, NorthThai1 and NorthThai2 haplotypes) and *V. destructor* (Japan, Vietnam, Korea and German haplotypes) showed in the trees.

Table 5.3 The nested analysis of *Varroa* mites used COI sequences data were generated into AMOVA 155 (Excoffier *et al*., 1992; Excoffier, 1995). (I) Summary table of *Varroa* mites infected on *A. mellifera* and *A. cerana* colonies, (II) Summary table of *Varroa* infested only on natural *A. cerana* colonies. There were two partition of observed variance. Partition (A) consisted of North, Northeast, Central and South while, partition (B) consisted of North (upper 18°N), North+Northeast+Central (between 17-12°N), Central+South (between 12-10°N) and South (lower 10°N) or (Mountain, Lowland and Sundaland). Significance tested with 1,000 random permutations; all ϕ-statistics significant at $P < 0.001$.

(I) AMOVA analysis	**Variance components**	***Varroa* infected on *A. mellifera* & natural *A. cerana* colonies** A Partition of observed variance Variance (%)	ϕ-statistics	B Partition of observed variance Variance (%)	ϕ-statistics
Nested analysis	Among groups	0.008 (3.74%)	ϕct = 0.037	0.018 (64.52%)	ϕct = 0.645
	Among populations/within groups	0.015 (71.60%)	ϕsc = 0.744	0.002 (10.11%)	ϕsc = 0.285
	Among populations	0.005 (24.60%)	ϕst = 0.753	0.004 (25.38%)	ϕst = 0.746
Analysis among populations	Among populations	0.016 (75.15%)		0.008 (65.14%)	
	Within populations	0.005 (24.85%)		0.004 (34.86%)	
Analysis among groups	Among groups	0.005 (25.56%)		0.012 (69.85%)	
	Within groups	0.016 (74.44%)		0.005 (30.15%)	
(II) AMOVA analysis	Variance components	*Varroa* infected on natural *A. cerana* colonies A Partition of observed variance Variance (%)	ϕ-statistics	B Partition of observed variance Variance (%)	ϕ-statistics
Nested analysis	Among groups	0.009 (64.00%)	ϕct = 0.640	0.007 (49.36%)	ϕct =0.494
	Among populations/within groups	0.002 (15.91%)	ϕsc = 0.442	0.004 (30.48%)	ϕsc = 0.602
	Among populations	0.003 (20.09%)	ϕst = 0.799	0.003 (20.16%)	ϕst = 0.798
Analysis among populations	Among populations	0.009 (76.57%)		0.009 (76.57%)	
	Within populations	0.003 (23.43%)		0.003 (23.43%)	
Analysis among groups	Among groups	0.010 (70.34%)		0.008 (59.67%)	
	Within groups	0.004 (29.66%)		0.006 (40.33%)	

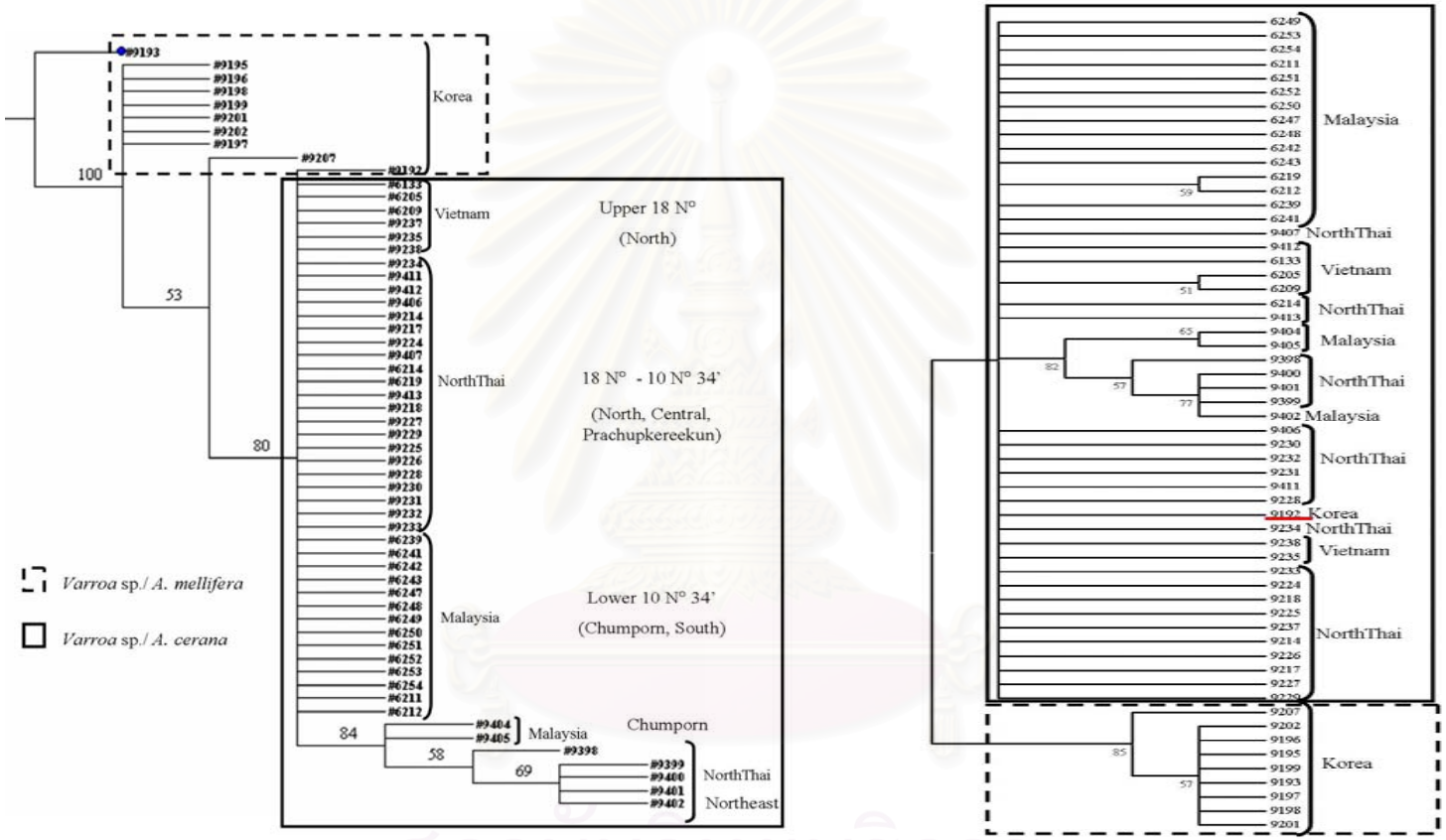


Figure 5.3 The bootstrap phylogenetic trees were generated using TE-AFLP binary data with Winclada program (A) and maximum parsimony methods in Mega program (B). The bootstrap values showed at branches.

Table 5.4 Summary AMOVA table were calculated using TE-AFLP binary data. The table I showed results of Thai *Varroa* infested on *A. mellifera* and *A. cerana* while, table II showed results of Thai *Varroa* infested *A. cerana*. Three partitions of observed variances: (A) consisted of four regions (North, Northeast, Central and South), (B) consisted of three regions (Mountain, Lowland and Sundaland) and (C) consisted of two regions (Mainland and Sundaland). Significance tested with 9999 random permutations.

(I)	***Varroa* mites infected on *A. mellifera* & natural *A. cerana* colonies**		
Partition of observed variances	Summary AMOVA	Variances (%)	ϕ-statistics
A	Among regions	1.551 (17.19%)	ϕ_{RT} = 0.172
	Among population /within region	2.009 (22.27%)	ϕ_{PR} = 0.269
	Among populations	5.462 (60.54%)	ϕ_{PT} = 0.395
B	Among regions	1.039 (11.41%)	ϕ_{RT} = 0.114
	Among population /within region	2.569 (28.21%)	ϕ_{PR} = 0.318
	Among populations	5.498 (60.38%)	ϕ_{PT} = 0.396
C	Among regions	1.929 (19.77%)	ϕ_{RT} = 0.198
	Among population /within region	2.369 (24.28%)	ϕ_{PR} = 0.303
	Among populations	5.462 (55.96)	ϕ_{PT} = 0.440
(II)	***Varroa* mites infected on natural *A. cerana* colonies**		
Partition of observed variances	Summary AMOVA	Variances (%)	ϕ-statistics
A	Among regions	1.804 (19.61%)	ϕ_{RT} = 0.196
	Among population /within region	1.367 (14.88%)	ϕ_{PR} = 0.185
	Among populations	6.015 (65.48%)	ϕ_{PT} = 0.345
B	Among regions	1.155 (12.55%)	ϕ_{RT} = 0.126
	Among population /within region	1.986 (21.59%)	ϕ_{PR} = 0.247
	Among populations	6.060 (65.86%)	ϕ_{PT} = 0.341
C	Among regions	1.991 (20.32%)	ϕ_{RT} = 0.203
	Among population /within region	1.747 (17.83%)	ϕ_{PR} = 0.224
	Among populations	6.060 (61.85%)	ϕ_{PT} = 0.382

DISCUSSION

These results were clarified the taxonomic status of *Varroa* mites parasitized to *A. mellifera* and *A. cerana* in Thailand. These were three haplotypes of *V. destructor* infested on Thai *A. mellifera* colonies called as ThaiV1, ThaiV2 and ThaiV3 resembled to Japan/Thailand, Vietnam and Korea (Anderson and Trueman, 2000), respectively. Firstly, Thai *Varroa* mites infested on *A. cerana* was recorded into Japan/Thailand haplotype (Anderson and Trueman, 2000) but no evidence to support that haplotype. Then, Warrit *et al*. (2006) suggested that study could be collected the sample from *A. mellifera* colonies. Presently, the *V. destructor*, Japan/Thailand (ThaiV1) haplotype, appeared into Japan haplotype because there was no evidence of this haplotype in Thai natural *A. cerana* colonies. Only Vietnam haplotype invaded to natural *A. cerana* at the mountainous region (Warrit *et al*., 2006). Commonly, *V. destructor* (Korea haplotype or ThaiV3) of, which has been detected in worldwide *A. mellifera* colonies but never detected in *A. cerana* colonies (Anderson, 2000). Mostly, Thai *A. mellifera* was also parasitized in colonies. Recently, two haplotypes of *V. destructor* have been found to be capable of reproducing on *A. mellifera* (Korea = K and Japan = J types) showed distribution of two haplotypes were more complex supported Korea haplotype, far more virulent, is displacing the Japan haplotype in many countries (Solignac *et al.*, 2005). According to mostly *V. destructor* invaded Thai *A. mellifera*, was identified into Korea haplotype, only three samples were identified into Japan haplotype and other one sample was Vietnam haplotype.

The genetic information of Thai *A. mellifera* haplotypes (Suppasat *et al*., 2007) have not directly related with their parasites at all of three *Varroa* haplotypes. In ThaiA2 *A. mellifera* colonies which were broadly distributed into Thailand could be parasitized

with three haplotypes of *V. destructor*. The mostly of *V. destructor*, Korea haplotype, could parasitize on every Thai *A. mellifera* haplotypes at all apiaries. The reasons of the physical changes in the bees, mediated by the climate, have some effects on *Varroa* reproduction and survival (De Jong, 1981). *Varroa* infestation rate in different *A. mellifera* lineages in three different climatic regions in Brazil indicated that was slightly lower than effect of the climate (Moretto *et al*., 1991). However, the reason of high humidity condition would be one factor affecting for *V. destructor* population growth in Africanized honey bee colonies in Texas (Kraus and Velthuis, 1997; Villa *et al*., 2002). The suitable periods to collecting *Varroa* mite infested on Thai *A. mellifera*, could be during August to October that be high humidity. The others, could collect after honey flow period during December to February that be low temperature period. The climate and high humidity factors could be effect to the distribution of *V. destructor* infested on Thai *A. mellifera* colonies but, these hypotheses were also difficult to apply to the distribution of *V. destructor* infested on *A. cerana* in Thailand (Warrit, 2002).

Suspiciously, the *V. destructor*, Vietnam haplotype, detected in drone and worker brood cells of Thai natural *A. cerana* colonies at upper 18°N latitude of mountainous regions (Chiang Mai, Chiang Rai and Nan) of Thailand and one parasitized in worker brood cell of Thai *A. mellifera* at Utaradit where located at 17.36°N latitude. When consideration using COI sequence at Utaradit locality,13 samples of *Varroa* from *A. mellifera* were identified into three haplotypes of *V. destructo*r and one sample from Thai *A. cerana* was identified into *V. jacobsoni*, NorthThai haplotype. But, the phylogram of TE-AFLP suggested that *V. jacobsoni* at Utaradit was closely to Vietnam haplotype at mountainous region of *A. cerana*. In addition to all of *Varroa* samples parasitized on *A. mellifera* at Chiang Mai locality where located at 18.47°N were

identified into Korea haplotype. While, two haplotyps of *Varroa* parasitized in *A. cerana* inside drone brood cell were found. One is *V. destructor* (Vietnam haplotype) and another is *V. jacobsoni* (NorthThai haplotype). Striking to *V. destructor*, Vietnam haplotype, could reproduce in drone brood cell of *A. cerana* at mountainous region, clearly supported the hypothesis of *V. destructor* distributed at mountainous region (Warrit *et al*., 2006) especially at upper 18°N latitude localities. Mention to *Varroa* parasitized in natural *A. cerana* colonies identified into *V. jacobsoni* NorthThai1 or NorthThai2 haplotypes (Warrit *et al*., 2006). The results of COI gene sequences suggested that two haplotypes of *V. jacobsoni* closely related in genetic information so that they should be classified as NorthThai haplotype. The NorthThai haplotype distributed at 18°N latitudes covering to 11.24°N latitudes at the Isthmus of Kra where located in part of lowland (including North, Central and Prachuap Khiri Khan populations) and northeastern of Korat plateau (Northeast population) in geographical region of Thailand. The result suggested that major NorthThai haplotype of *V. jacobsoni* which colonize in Thai *A. cerana* Mainland Asia lineage, was not included at the mountainous region of Thailand.

However, the *Varroa* infested *A. cerana* populations at the lowland region of Thailand were more closely related in genetically information among populations within region. The results of COI gene and TE-AFLP indicated that *Varroa* mites from the northeastern Thailand were not exactly similar to NorthThai1 or NorthThai2 but they resembled in their population and would distinct into NorthThai group. These also supported the previous investigation of genomic DNA of *A. cerana* in Thailand using microsatllite which indicated that *A. cerana* Northeast population differed from other populations (Sittipraneed *et al*., 2001).

Thailand is an overlap place where large populations of the two major mitochondrial lineages of *A. cerana* Mainland Asia and Sundaland lineages. Determining to the COI gene in mitochondrial DNA suggested that *V. jacobasoni* (NorthThai haplotype) was major parasitized on natural Thai *A.cerana* Mainland Asia lineage except to mountainous region was invaded by *V. destructor* (Vietnam haplotype). Thai *A. cerana* Sundaland lineage was infested with *V. jacobasoni* (Malaysia haplotype) as same as the previous report (Warrit *et al*., 2006). This conclusion showed that genomic DNA clearly indicated that clearly host-parasite relationship at species level. Because of the *Varroa* parasitized on Thai *A. mellifera* could be in *V. destructor* clad while, all of *Varroa* parasitized natural *A. cerana* could be in *V. jacobsoni* clad (Figure 5.3). Exactly, the Malaysia haplotype of *V. jacobsoni* infested the natural *A. cerana* Sundaland lineage has distributed below 10.34°N. This suggested that populations of *Varroa* from different host populations are genetically differences, and may be adapted to specific characteristics of their local host populations (Warrit *et al*., 2006). The genomic DNA of all *Varroa* infested on natural drone brood of *A. cerana* of northeast area of Thailand resembled to *Varroa* infested on drone brood cells of two colonies of *A. cerana* which identified into Malaysia haplotype. The *Varroa* parasitized in natural *A. cerana* colonies suggested that Thailand is not only the contact place between two *A. cerana* lineages but also the contact place of *V. jacobsoni* and *V. destructor*.

CHAPTER VI

CONCLUSION

Honey bees

Two species of multiple nesting cavity honey bees in genus *Apis* compose of one an imported species, *A. mellifera*, and the natural native species, *A. cerana*. Both of honey bees are importantly for beekeeping into Asia.

A. mellifera was introduced to Thailand approximately 60 years ago. Today, there are over 300,000 colonies in Thailand, but its ancestry and genetic composition is unknown. Since different subspecies of *A. mellifera* differ in disease resistance and other economically important traits. These information should be useful for maintenance and improvement of Thai stocks. PCR-RFLP analysis and sequencing of the mitochondrial genome were used to clarify the maternal ancestry of *A. mellifera* in Thailand. Total samples of 476 colonies which collected from 4 regions of Thailand: Central (113), North (156), South (97) and East/North (111), were compared with 27 reference samples representing *A. m. carnica, A. m. ligustica, A. m. mellifera, A. m. scutellata* and Turkish *A. m. syriaca* and with published RFLP data.

Four regions of mtDNA were PCR amplified and digested with restriction enzymes: a fragment from $tRNA^{leu}$ to the 5′ end of COII was digested with *Dra* I and *Hinf* I; a portion of cyt-b digested with *Bgl* II, *Dra* I and *Hinf* I; a portion of lsRNA digested with *EcoR* I; and the 5′ end of COI digested with *Hinc* II. We sequenced the non-coding region between $tRNA^{leu}$ and COII and compared them to sequences in the literature and Genbank.

Three common and five rare composite RFLP haplotypes were showed in Table 3.2. Five haplotypes (ThaiA group) belong to the east Mediterranean (C) mitochondrial lineage

and three (ThaiB group) belong to the Middle Eastern (O) lineage. As expected, the C lineage was the most abundant, found in 82% of studied colonies. Unexpectedly, 18% of studied colonies had O lineage mtDNA. ThaiA2.1, ThaiA1.1 and ThaiB1.1 which are the most common hapotypes made up 95% of the samples. There was no evidence of either west European (M) or African (A) lineage mtDNAs in Thailand. The sequence of the non-coding region of Thai B samples resembles to the published sequences of both *A. m. syriaca* and *A.m. lamarkckii* but it is not an exact match to either.

Analysis of Molecular Variance (AMOVA) revealed significant differentiation among regions. The O lineage haplotypes are rare in the North and Northeast, and *A. m. ligustica*-like haplotypes are rare in the south, which may reflect importation history or migratory patterns of beekeeping. However, most observed variation occurred within apiaries.

Apis cerana had been naturally native of multiple nesting cavity honeybees of Thailand. Morphometric and mitochondrial DNA analysis could divide two groups of *A. cerana* as Mainland Asia and Sundaland lineages. The Isthmus of Kra where there is a transition from evergreen rainforest to more seasonal, semi-evergreen forest, between 10°34'N and 11°24'N (Whitmore 1984). *A. cerana* Mainland Asia and the Sundaland lineages contracted at the Isthmus of Kra area.

The Amplified Fragment Length Polymorphism (AFLP) of DNA fingerprinting is a powerful technique to identify genetic variation of organism within genomic DNA. Total of *A. cerana* (n=184) were separated into 6 populations: North (32), Northeast (24), Central (33), Prachupkerekun (27), Chumporn (30) and Peninsular including Phuket and Samui Islands (38). Two procedures of AFLP were selected to investigate genetic diversity and population differentiation which were standard AFLP and TE-AFLP. AFLP bands were recorded present (1)/ absent (0) bands. The dominant binary data analyzed

genetic diversity displayed with percentage of polymorphism (%P), Heterozygosity (*He*) values (Table 4.1). Genetic differentiation among populations analyzed with several methods. Such as Nei's genetic distances (Table 4.2), Clustering (Figure 4.2), AMOVA (Table 4.3, 4.4) and PCA (Figure 4.3) analysis using GenAlEx 6.0 program.

Thai *A. cerana* showed abundance genetic diversity. The AMOVA distinguished of Thai *A. cerana* populations could not detect population differentiation among regions but displayed genetic different within population. The genetic information of Thai *A. cerana* exist overlap among populations that could cause of gene flow among populations and would be effect of drones. AFLP markers showed different in Mainland Asia mtDNA lineage population which separated North, Central and Northeast subpopulations. In addition to the North and the Central could be concluded that mountainous area should be not a geographic barrier of gene flow of *A. cerana*. PCA plots of *A. cerana* individuals (Figure 4.3) suggested overlap of genetic information among subpopulations.

The AFLP markers could support genetic differentiation within genomic DNA of *A. cerana* population. Thai *A. cerana* subpopulations were related with Thailand geographic patterns. Below the Isthmus of Kra at 10°N latitude consists of Chumporn and Peninsula populations could cluster in same group, which distributed south of Thailand that could belong to Sundaland lineage. Prachuap Khiri Khan subpopulation could be intermediate between Mainland Asia and Sundaland lineages. Anyway, gene flow occurred among subpopulations. Drone of honey bees could be importance for gene flow to widespread genetic information of *A. cerana* lineages.

***Varroa* mites**

Parasitic bee mite genus *Varroa* is widely infestation in two species of multiple nesting honey bees. *A. cerana* was firstly discovered host of parasites named *V. jacobsoni* Oudemans, 1904. The *V. jacobsoni* was worldwide distributed in the world, which was a major destroy in *A. mellifera*. Since 2000, *V. jacobsoni* complex species had been parasitized within honey bee colonies. Newly, distinct one could be common widespread infestation into *A. mellifera* was named *V. destructor*.

Mitochondrial DNA at COI gene of *Varrroa* mites invaded on Thai *A. mellifera* colonies (n=42). Three different haplotypes of *V. destructor* infested on Thai *A. mellifera* colonies were identified into three haplotypes: ThaiV1 (n=3), ThaiV2 (n=1) and one numerous ThaiV3 (n=38) which resembled to Japan/Thailand, Vietnam and Korea, respectively. Commonly, the Korea haplotype of *V. destructor* was found mostly parasite in *A. mellifera*, which has been detected in worldwide *A. mellifera* colonies but never detected in *A. cerana* colonies (Anderson, 2000). The genetically haplotypes of Thai *A. mellifera* (Suppasat *et al*., 2007) have not directly related with their parasites at all of three *Varroa* haplotypes. The ratios of Thai *A. mellifera* haplotypes: ThaiA1 (19%), ThaiA2 (64%) and ThaiB (17%) were parasitized by *V. destructor*. Distribution of *A. mellifera*, ThaiA2 haplotype could be parasitized with three haplotypes of *V. destructor*. The mostly of Korea haplotype could parasitize on every Thai *A. mellifera* haplotypes at all apiaries. Basically, climate and high humidity factors could be effect for the distribution *V. destructor* infested on Thai *A. mellifera* colonies also colonies moved by beekeepers.

Also, *Varroa* invaded natural *A. cerana* colonies (n=31) were mostly collected in drone brood cells from the mountainous region: North (4), the Korat plateau region: Northeast (6), the lowland region: central (8) and contract zone of Kra ecotone regions:

Prachuap Khiri Khan (9) and Chumporn (4). Mitochondrial DNA of CO I gene supported that Vietnam haplotype of *V. destructor* could exactly reproduce on natural *A. cerana* at mountainous region upper than 18 °N latitudes. Together *V. jacobsoni*, Malaysia haplotype, exactly parasitized on *A. cerana* Sundaland lineage while, NorthThai haplotype colonized on Mainland Asia lineage. Insight to lowland area could be contract area between *V. jacobsoni*, NorthThai haplotype and Malaysia haplotype in Thailand. The *Varroa* populations at the lowland region of Thailand were more closely related in genetically information among populations within region. The Kra ecotone was intermediate region of two haplotypes of *V. jacobsoni*. The result of COI gene indicated that *Varroa* mites from the northeastern Thailand were not similar to with NorthThai1 or NorthThai2. But they resembled in their populations. And they may be identified to NorthThai group. Also they could be different when detected with TE-AFLP. Genomic DNA analyzed with TE-AFLP into *Varroa* mites infested on *A. mellifera* (n=10) could be parasitized by *V. destructor*. And, natural *A. cerana* (n=48) could be almost infested by *V. jacobsoni*. However, the TE-AFLP (Figure 5.3 and Table 5.4) result suggested clearly the specificity of species level between honey bee hosts and *Varroa* parasites in Thailand. The results indicated that *A. mellifera* was colonized by *V. destructor* while, *A. cerana* was colonized by *V. jacobsoni*. In same species of *Varroa* mites indicated variation among populations or regions when using genomic DNA. While, mitochondrial DNA at COI gene is still powerful to check *Varroa* complex species.

REFERENCES

Akratanakul, P. 2000. Apiculture development in Thailand. in: Anonymous (Ed.). *Proceedings of the Seventh International Conference on Tropical Bees*: Management and diversity, and Fifth Asian Apicultural Association Conference, Chiang Mai, Thailand, IBRA. 395-398

Anderson, D. L. 1994. Non-reproduction of *Varroa jacobsoni* in *Apis mellifera* colonies in Papua New Guinea and Indonesia. *Apidologie*. 24:375-382.

Anderson, D. L. and Sukarsih. 1996. Changed *Varroa jacobsoni* reproduction in *Apis mellifera* colonies in Java. *Apidologie*. 27: 461-466.

Anderson, D. L. and Fuchs, S. 1998. Two genetically distinct populations of *Varroa jacobsoni* with contrasting reproductive abilities on *Apis mellifera*. *Journal of Apicultural Research*. 37: 69-78.

Anderson, D. L. 2000. Variation in the parasitic bee mite *Varroa jacobsoni* Oud. *Apidologie*. 31: 281-292.

Anderson, D. L. and Trueman, J. W. H. 2000. *Varroa jacobsoni* (Acari: Varroidae) is more than one species. *Experimental and Applied Acarology*. 24: 165-189.

Arias, M. C. and Sheppard, W. S. 1996. Molecular phylogenetics of honey bee subspecies (*Apis mellifera* L.) inferred from mitochondrial DNA sequence. *Molecular Phylogenetics and Evolution*. 5: 557-566.

Arias, M. C., Rinderer, T. E. and Sheppard, W. S. 2005. Further characterization of honey bees from the Iberian Peninsula by allozyme, morphometric and mitochondrial DNA haplotype analyses. *Journal of Apicultural Research*. 45(4): 188-196.

Avise, J. C. 2004. *Molecular Markers, Natural History, and Evolution*, (2nd). Sinauer Associates, Inc. Publisher, Sunderland, Massachusetts, USA.

Behura, S. K. 2007. Analysis of nuclear copies of mitochondrial sequences in honeybee (*Apis mellifera*) genome. *Molecular Biology and Evolution*. 24(7): 1492-1505.

Boecking, O. and Ritter, W. 1994. Current status of behavioral tolerance of the honey bee *Apis mellifera* to the mite *Varroa jacobsoni*. *American Bee Journal*. 134: 689-694.

Boore, J. L. 1999. Animal mitochondrial genomes. *Nucleic Acids Research*. 27: 1767-1780.

Boot, W. J., Calis, N. M. and Beetsma, J. 1992. Differential periods of *Varroa* mite invasion into worker and drone brood cells of honey bees. *Experimental and Applied Acarology*. 165:295-301.

Cameron, S. A. 1993. Multiple origins of advanced eusociality in bees inferred from mitochondrial DNA sequences. *Proceedings of the National Academy of Sciences*. 90: 8687-8691.

Chantawannakul, P., Peterson, S. and Wongsiri, S. 2004. Conservation of honey bee species in South East Asia: *Apis mellifera* or native bees?. *Biodiversity*. 5(2): 25-28.

Clarke, K. E., Oldroyd, B. P., Javier, J., Quezada-Euan, G. and Rinderer, T. E. 2001. Origin of honeybees (*Apis mellifera* L.) from the Yucatan peninsula inferred from mitochondrial DNA analysis. *Molecular Ecology*. 10: 1347-1355.

Clarke, K. E., Rinderer, T. E., Franck, P., Quezada-Euán, J. G. and Oldroyd, B. P. 2002. The Africanization of honey bees (*Apis mellifera* L.) of the Yucatan: A study of a massive hybridization event across time. *Evolution*. 56: 1462-1474.

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

Cornuet, J.-M. and Garnery, L. 1991. Mitochondrial DNA variability in honeybees and its phylogeographic implications. *Apidologie*. 22: 627-642.

Coulson, R. N., Pinto, M. A., Tchakerian, M. D., Baum, K. A., Rubink, W. L. and Johnston, J. S. 2005. Feral honey bees in pine forest landscapes of east Texas. *Forest Ecology and Management*. 215: 91-102.

Crane, E. 1978. The *Varroa* mite. *Bee World*. 59: 164.

Crozier, R. H., Crozier, Y. C. and Mackinlay, A. G. 1989. The *COI* and *COII* region of honeybee mitochondrial DNA: evidence for variation in insect mitochondrial evolutionary rates. *Molecular Biology and Evolution*. 6: 399-411.

Crozier, Y. C., Koulianos, S. and Crozier, R. H. 1991. An improved test for Africanized honey bee mitochondrial DNA. *Experientia*. 47: 968-969.

Crozier, R. H. and Crozier, Y. C. 1993. The mitochondrial genome of the honeybee *Apis mellifera*: Complete sequence and genome organization. *Genetics*. 133: 97-117.

Davis, J. I. and Nixon, K. C. 1992. Populations, genetic variation, and the delimitation of phylogenetic species. *Systematic Biology*. 41(4): 421-435.

De Guzman, L. I. and Delfinado-Baker, M. 1996. A new species of *Varroa* (Acari: Varroidae) associated with *Apis koschevnikovi* (Hymenoptera: Apidae) in Borneo. *International Journal of Acarology*. 22: 23-27.

De Guzman, L. I., Rinderer, T. E. and Stelzer, J. A. 1997. DNA evidence of the origin of *Varroa jacobsoni* Oudemans in the Americas. *Biochemical Genitics*. 35: 327-335.

De Guzman, L. I., Rinderer, T. E., Stelzer, J. A. and Anderson, D. L. 1998. Congruence of RAPD and mitochondrial DNA markers in assessing *Varroa jacobsoni* genotypes. *Journal of Apicultural Research*. 37: 49-51.

De Guzman, L. I. and Rinderer, T. E. 1999. Identification and comparison of *Varroa* species infesting honey bees. *Apidologie*. 30: 85-95.

De Jong, D., Morse, R. A. and Eickworth, G. C. 1982. Mite pests of honey bees. *Annual Review of Entomology*. 27: 229-252.

De Jong, D., Gonçalves, L. S. and Morse, R. A. 1984. Dependence on climate of the virulence of *Varroa jacobsoni*. *Bee World*. 65: 117-121.

De Jong, D. 1997. Mites: *Varroa* and other parasites of brood, in: *Honey bee pests, Predators and diseases* (3rd ed). Edited by Morse, R. A. and Flottum, K. The A. I. Root Company, Medina, Ohio, U.S.A. 278-322.

De la Rúa, P., Galián, J., Serrano, J. and Moritz, R. F. A. 2001. Genetic structure and distinctness of *Apis mellifera* L. populations from the Canary Islands. *Molecular Ecology*. 10: 1733-1742.

De la Rúa, P., Serrano, J. and Galián, J. 2002. Biodiversity of *Apis mellifera* populations from Tenerife (Canary Islands and hybridization with East European races. *Biodiversity and conservation.* 11: 59-67.

De la Rúa, P., Hernández-Garcia, R., Pedersen, B. V. Galián, J. and Serrano, J. 2004. Molecular diversity of honeybee *Apis mellifera iberica* L. (Hymenoptera: Apidae) from Western Andalusia. *Archivos de zootecnia*. 53: 195-203.

Delfinado-Baker, M. and Aggarwal, K. 1987. A new *Varroa* (Acari: Varroidae) from the nest of *Apis cerana* (Apidae). *International Journal of Acarology*. 13: 233-237.

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

Diniz, N. M., Soares, A. E. E., Sheppard, W. S. and Del Lama, M. A. 2003. Genetic structure of honeybee populations from southern Brazil and Uruguay. *Genetics and Molecular Biology*. 26(1): 47-52.

Engel, M. S. 1999. The taxonomy of recent and fossil honeybees (Hymenoptera: Apidae: *Apis*). *Journal of Hymenopteran Research*. 9: 165-196.

Evans, J. D. and Lopez, D. L. 2002. Complete mitochondrial DNA sequence of the important honey bee pest, *Varroa destructor* (Acari: Varroidae). *Experimental and Applied Acarology*. 27: 69-78.

Excoffier, L., Smouse,P. E. and Quattro, J.-M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes application to human mitochondrial DNA restriction data. *Genetics*. 131: 479-491.

Excoffier, L. 1995. *AMOVA 1.55* (Analysis of Molecular Variance). University of Geneva, Geneva.

Franck, P., Garnery, L., Solignac, M. and Cornuet, J.-M. 1998. The origin of west European subspecpies of honeybees (*Apis mellifera*): New insights from microsatellite and mitochondrial data. *Evolution*. 52(4): 1119-1134.

Franck, P., Garnery, L., Solignac, M. and Cornuet, J.-M. 2000a. Molecular confirmation of a fourth lineage in honeybees from Near East. *Apidologie*. 31: 167-180.

Franck, P., Garnery, L., Celebrano, G., Solignac, M. and Cornuet, J.-M. 2000b. Hybrid origins of honeybees from Italy (*Apis mellifera ligustica*) and Sicily (*A. m. sicula*). *Molecular Ecology*. 9: 907-921.

Franck, P., Garnery, L., Loiseau, A., Oldroyd, B. P., Hepburn, H. R., Solignac, M. and Cornuet, J.-M. 2001. Genetic diversity of the honeybee in Africa: Microsatellite and mitochondrial data. *Heredity*. 86: 420-430.

Fuchs, S. 1994. Nonreproducing *Varroa jacobsoni* Oud. in honey bee worker cells-status of mites or effect of brood cell?. *Experimental Applied Acarology*. 18: 309-317.

Fuchs, S., Long, L. T. and Anderson, D. L. 2000. A scientific note on the genetic distinctness of *Varroa* mites on *Apis mellifera* and *Apis cerana* in North Vietnam. *Apidologie*. 31: 459-460.

Garnery, L., Vautrin, D., Cornuet, J.-M. and Solignac, M. 1991. Phylogenetic relationships in the genus *Apis* inferred from mitochondrial DNA sequence data. *Apidologie*. 22: 87-92.

Garnery, L., Cornuet, J.-M. and Solignac, M. 1992. Evolutionary history of the honey bee *Apis mellifera* inferred from mitochondrial DNA analysis. *Molecular Ecology*. 1:145-154.

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

Garnery, L., Mosshine, E. H., Oldroyd, B. P. and Cornuet, J.-M. 1995. Mitochondrial DNA variation in Moroccan and Spanish honey bee populations. *Molecular Ecology*. 4: 465-471.

Garrido, C., Rosenkranz, P., Paxton, R. J. and Goncalves, L. S. 2003. Temporal changes in *Varroa destructor* fertility and haplotype in Brazil. *Apidologie*. 34: 535-541.

Gaudeul, M., Till-Bottraud, I., Barjon, F. and Manel, S. 2004. Genetic diversity and differentiation in *Eryngium alpinum* L. (Apiaceae): comparison of AFLP and microsatellite markers. *Heredity*. 92: 508-518.

Grimaldi, D. and Engle, M. S. 2005. *Evolution of the insect*. Cambridge University Press, Cambridge.

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

Hall, H. G. and Smith, D. R. 1991. Distinguishing African and European honeybee matrilines using amplified mitochondrial DNA. *Proceedings of the National Academy of Science USA*. 88: 4548-4552.

Harbo, J. R. and Harris, J. W. 1999. Selecting honey bees for resistance to *Varroa jacobsoni*. *Apidologie*. 30: 183-196.

Hennig, W. 1966. *Phylogenetic systematics*. University of Illinois Press, Urbana, Ill.

Hepburn, H. R., Smith, D. R., Radloff, S. E. and Otis, G. W. 2001. Infraspecific categories of *Apis cerana*: morphometric, allozymal and mtDNA diversity. *Apidologie*. 32: 3-23.

Huff, D. R., Peakall, R., Smouse, P. E. 1993. RAPD variation within and among natural populations of outcrossing buffalograss *Buchloe dactyloides* (Nutt) Engelm. *Theoretical and Applied Genetics*. 86: 927-934.

Imjongjirak, C., Ngewsra, L., Pramual, C., Pala-or, K., Klinbunga, S. and Sittipraneed, S. 2004. Development of a simple and rapid method for typing population origins of the honey bee (*Apis cerana*) in Thailand. *Journal of Apicultural Research* . 43(1): 17-20.

Kavinseksan, B., Wongsiri, S., Rinderer, T. E. and De Guzman, L. I. 2004. Comparison of the hygenic behavior of ARS Russian commercial honey bees in Thailand. *American Bee Journal*. 144: 870-872.

Kandemir, I., Pinto, M. A., Meixner, M. D. and Sheppard, W. S. 2006a. *Hinf*-I digestion of *cytochrome oxidase I* region is not a diagnostic test for *A. m. lamarckii*. *Genetics and Molecular Biology*. 29(4), 747-749.

Kandemir, I., Kence, M., Sheppard, W. S. and Kence, A. 2006b. Mitochondrial DNA variation in honeybee (*Apis mellifera* L.) populations from Turkey. *Journal of Apicultural Research*. 45(1): 33-38.

Kandemir, I., Meixner, M. D., Ozkan, A. and Sheppard, W. S. 2006c. Genetic characterization of honey bee (*Apis mellifera cypria*) populations in northern Cyprus. *Apidologie*. 37: 547-555.

Koeniger, N., Koeniger, G. and Delfinado-Baker, M. 1983. Observations on mites of the Asian honey bee species (*Apis cerana*, *Apis dorsata*, *Apis florea*). *Apidologie*. 14: 197-204.

Kokkinis, M. and Liakos, V. 2004. Population dynamics of *Varroa destructor* in colonies of *Apis mellifera macedonica* in Greece. *Journal of Apicultural Research*. 43(4): 150-154.

Kraus, B. and Hunt, G. 1995. Differentiation of *Varroa jacobsoni* Oud populations by random amplification of polymorphic DNA (RAPD). *Apidologie*. 26: 283-290.

Kraus, B. and Velthuis, H. H. W. 1997. High humidity in the honey bee (*Apis mellifera* L.) brood nest limits reproduction of the parasitic mite *Varroa jacobsoni* Oud. *Naturwissenschaften*. 84: 217-218.

Kumar, S., Tamura, K. and Nei, M. 2004. *MEGA3*: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Briefings in Bioinformatics*. 5: 150-163.

Lekprayoon, C., Wongsiri, S., Rinderer, T. E. and Sylvester, H. A. 1994. Biodiversity of parasitic mites of honey bees in southeast Asia. *Acarology IX: Symposia, section8: Parasitic mites of honey bees: A crisis in world agriculture*. 325-329.

Limbipichai, K. 1990. *Morphometric studies on the eastern honey bee (Apis cerana Fabricius) in Thailand and Malaysian peninsula*. Thesis, Chulalongkorn University, Bangkok.

Lynch, M. and Milligan, B. G. 1994. Analysis of population genetic structure with RAPD markers. *Molecular Ecology*. 3: 91-99.

Martin, S. J. 1995. Reproduction of *Varroa jacobsoni* in cells of *Apis mellifera* containing one or more mother mites and the distribution of these cells. *Journal of Apicultural Research*. 34: 187-196.

Martin, S. J. 1998. A population model for the ectoparasitic mite *Varroa jacobsoni* in honey bee (*Apis mellifera*) colonies. *Ecological Modelling*. 109: 267-281.

Martin, S. J. 2001. *Varroa destructor* reproduction during the winter in *Apis mellifera* colonies in UK. *Experimental and Applied Acarology*. 25:321-325.

Martin, S. J. and Kryger, P. 2002. Reproduction of *Varroa destructor* in South African honey bees: does cell space influence *Varroa* male survivorship?. *Apidologie*. 33: 51-61.

Martin, S. J. and Medina, L. M. 2004. Africanized honeybees have unique tolerance to *Varroa* mites. *Trends in Parasitology*. 20(3): 112-114.

McElroy, D., Moran P., Bermingham, E. and Kornfield, I. 1992. The Restriction Enzyme Analysis Package (REAP) Version 4.0. *Journal of Heredity*. 83: 157-158.

Medina, L. M. and Martin, S. J. 1999. A comparative study of *Varroa jacobsoni* reproduction in worker cells of honeybees (*Apis mellifera*) in England and Africanized bees in Yucatan. *Mexico Experimental and Applied Acarology*. 23: 659-667.

Medina, L. M., Martin, S. J., Espinosa-Montanõ, L. and Ratnieks, F. L. W. 2002. Reproduction of *Varroa destructor* in worker brood of Africanized honey bees (*Apis mellifera*). *Experimental and Applied Acarology*. 27: 79-88.

Meixner, M. D., Sheppard, W. S. and Poklukar, J. 1993. Asymmetrical distribution of a mitochondrial DNA polymorphism between two introgressing honey bee subspecies. *Apidologie*. 24: 147-153.

Meixner, M. D., Arias, M. C. and Sheppard, W. S. 2000. Mitochondrial DNA polymorphisms in honey bee subspecies from Kenya. *Apidologie*. 31: 181-190.

Moretto, G., Gonçalvas, L. S., De Jong, D. and Michuette, M. Z. 1991. The effects of climate and bee race on *Varroa jacobsoni* Oud. infestation in Brazil. *Apidologie*. 22: 197-203.

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

Moritz, R. F. A., Cornuet, J.-M., Kryger, P., Garnery, L. and Hepburn, H. R. 1994. Mitochondrial DNA variability in South African honeybees (*Apis mellifera* L.). *Apidologie*. 25: 169-178.

Murilhas, A. M. 2002. *Varroa destructor* infestation impact on *Apis mellifera carnica* worker brood production, bee population and honey storage in a Mediterranean climate. *Apidoologie*. 33: 271-281.

Navajas, M., Le Conte, Y., Solignac, M., Cros-Arteil, S. and Cornuet, J.-M. 2002. The complete sequence of the mitochondrial genome pf the honeybee ectoparasite mite *Varroa destructor* (Acari : Varroidae). *Molecular Biology and Evolution.* 19(2): 2313-2317.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from small number of individuals. *Genetics*. 89: 583-590.

Nei, M. and Li, W.-S. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceeding of Natural Academic Science*. 50: 5269-5273.

Nei, M. 1987. *Molecular Evolutionary Genetics.* Columbia University Press, New York.

Nixon, K. C. and Wheeler, Q. D. 1990. An amplification of the phylogenetic species concept. *Cladistics*. 6: 211-223.

Nixon, K. C. 2002. *NONA: Winclada ver 1.00.08 program*. Ithaca, New York, USA.

Oldroyd, B. P. 1999. Coevolution while you wait: *Varroa jacobsoni*, a new parasite of western honey bees. *Trends in Ecology and Evolution*. 14: 312-315.

Oldroyd, B. P. and Wongsiri, S. 2006. *Asian Honey Bees*: *Biology, Conservation and Human interactions*. Harvard University Press, Cambridge.

Oldroyd, B. P., Reddy, M. S., Chapman, N. C., Thompson, G. J. and Beekman, M. 2006. Evidence for reproductive isolation between two color morphs of cavity nesting honey bees (*Apis*) in south India. *Insectes Sociaux*. 53: 428-434.

Otis, G. W. 1996. Distributionns of recently recognized species of honey bee (Hymenoptera: Apidae; *Apis*) in Asia. *Journal of the Kansas Entomological Society*. 69:311-333.

Oudemans, A. C. 1904. Note VIII. On a new genus and species of parasitic Acari. *Notes Leyden Museum*. 24: 216-222.

Palmer, M. R., Smith, D. R. and Kaftanoglu, O. 2000. Turkish honey bees: genetic variation and evidence for a fourth lineage of *Apis mellifera* mtDNA. *Journal of Heredity*. 91: 42-46.

Peng, Y. S., Fang, Y., Xu, S. and Ge, L. 1987. The resistance machanism of the Asian honey bee *Apis cerana* Fabr. To an ectoparasitic mite *Varroa jacobsoni* Oudemans. *Journal of Invertebrate Pathology*. 49: 54-60.

Peakall, R. and Smouse, P. E. 2006. *GenAlEx 6*: genetic analysis in Excel, population genetic software for teaching and research. *Molecular Ecology Notes*. 6: 288-295.

Piccirillo, G. A. and De Jong, D. 2003. The influence of brood comb cell size on the reproductive behavior of the ectoparasitic mite *Varroa destructor* in Africanized honey bee colonies. *Genetics and Molecular Research*. 2(1): 36-42.

Pinto, M. A., Johnston, J. S., Rubink, W. L., Coulson, R. N., Patton, J. C. and Sheppard, W. S. 2003. Identification of Africanized honey bee (Hymenoptera: Apidae) mitochondrial DNA: Validation of a Rapid Polymerase Chain Reaction based-assay. *Annals of the Entomological of America*. 96(5): 679-684.

Pinto, M. A., Rubink, W. L., Coulson, R. N., Patton, J. C. and Johnston, J. S. 2004. Temporal pattern of Africanization in a feral honey bee population from Texas inferred from mitochondrial DNA. *Evolution*. 58: 1047-1055.

Pinto, M. A., Rubink, W. L., Patton, J. C., Coulson, R. N. and Johnston, J. S. 2005. Africanization in the United States: Replacement of feral European honeybees (*Apis mellifera* L.) by an African hybrid swarm. *Genetics*. 170: 1653-1665.

Pinto, M. A., Sheppard, W. S., Johnston, J. S., Rubink, W. L., Coulson, B. N., Schiff, N. M., Kandiermir, I. and Patton, J. C. 2007. Honey bee (Hymenoptera: Apidae) of Africa origin exist in non-Africaniized areas of the southern United States: Evidence from mitochondrial DNA. *Annals of the Entomological Society of America*. 100(2): 289-295.

Rinderer, T. E., Koeniger, N., Tingek, S., Mardan, M. and Koeniger, G. 1989. A morphological comparison of the cavity dwelling honeybees of Borneo *Apis koschevnikovi* (Buttel-Reepen, 1906) and *Apis cerana* (Fabricius, 1793). *Apidologie*. 20: 405-411.

Rinderer, T. E., De Guzman, L. I. and Narper, C. 2004. The effects of co-mingled Russian and Italian honey bee stocks and sunny or shaded apiaries on *Varroa* mites infestation level, worker bee population and honey bee production. *American Bee Journa*l. June: 481-485.

Rosendahl, S. and Taylor, J. W. 1997. Development of multiple genetic markers for studies of genetic variation in arbuscular mycorrhizal fungi using AFLPTM. *Molecular Ecology*. 6: 821-829.

Rosenkkranz, P. and Engel, W. 1994. Infertility of *Varroa jacobsoni* females after invasion into *Apis mellifera* worker brood as a tolerance factor against varroatosis. *Apidologie*. 25: 402-411.

Ruttner, F. 1988. *Biogeography and Taxonomy of Honeybees*. Springer-Verlag, Berlin.

Ruttner, F., Kauhausen, D. and Koeniger, N. 1989. Position of the red honey bee, *Apis koschevnikovi* (Buttel-Reepen 1906), within the Genus *Apis*. *Apidologie*. 20: 395-404.

Sammataro, D., Gerson, U. and Needham, G. 2000. Parasitic mites of honey bees: life history, implications and impact. *Annual Reviews of Entomology*. 45: 519-548.

Schneider, S. S., De Grandi-Hoffman, G. and Smith, D. R. 2004. The African honey bee: Factors contributing to a successful biological invasion. *Annual Review of Entomology*. 49: 351-376.

Schoen, D. J. and Brown, A. H. D. 1991. Intraspecific variation in population gene diversity and effective population size correlates with the mating system in plants. *Proceedings of the National Academy of Sciences of the United States of America*. 88(10): 4494-4497.

Schuh, R. T. 2000. *Biological Systematics: Principles and Applications (1st Ed)*. Cornell University Press, New York, USA.

Segura, A. A. L. 2000. Highly polymorphic DNA markers in and Africanized honey bee population in Costa Rica. *Genetics and Molecular Biology*. 23(2): 317-322.

Sheppard, W. S. 1988. Comparative study of enzyme polymorphism in United States and European honey bee (Hymenoptera: Apidae) populations. *Annals of the Entomological Society of America*. 81(6): 886-889.

Sheppard, W. S., Rinderer, T. E., Meixner, M. D., Yoo, H. R., Stelzer, J. A., Schiff, N. M., Kamel, S. M. and Krell, R. 1996. *Hin*f I variation in mitochondrial DNA of Old World honey bee subspecies. *Journal of Heredity*. 87(1): 35-40.

Sheppard, W. S., Rinderer, T. E., Garnery, L. and Shimanuki, H. 1999. Analysis of Africanized honey bee mitochondrial DNA reveals further diversity of origin. *Genetics and Molecular Biology*. 22(1).

Sheppard, W. S. and Smith, D. R. 2000. Identification of African-derived bees in the Americas: A survey of methods. *Annals of the Entomological Society of America*. 93: 159-176.

Sheppard, W. S. and Meixner, M. 2003. *Apis mellifera pomonella*, a new honey bee subspecies from Central Asia. *Apidologie*. 34: 367-375.

Sihanunthavong, D., Sittipraneed, S. and Klinbunga, S. 1999. Mitochondrial DNA diversity and population structure of the honey bee (*Apis cerana*) in Thailand. *Journal of Apicultural Research.* 38: 211-219.

Sittipraneed, S., Sihanunthavong, D. and Klinbunga, S. 2001a. Genetic differentiation of the honey bee (*Apis cerana*) in Thailand revealed by polymorphism of a large subunit of mitochondrial ribosomal DNA. *Insectes Sociaux*. 48: 266-272.

Sittipraneed, S., Laoaroon, S., Klinbunga, S. and Wongsiri, S. 2001b. Genetic differentiation of the honey bee (*Apis cerana*) in Thailand: evidence from microsatellite polymorphism. *Journal of Apicultural Research*. 40(1): 9-16.

Smith, D. R. and Brown, W. M. 1988. Polymorphisms in mitochondrial DNA of European and Africanized honeybees (*Apis mellifera*). *Experientia*. 44: 257-260.

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

Smith, D. R. and Brown, W. M. 1990. Restriction endonuclease cleavage site and length polymorphisms in mitochondrial DNA of *Apis mellifera mellifera* and *A. m. carnica* (Hymenoptera: Apidae). *Annals of the Entomological Society of America*. 83(1): 81-88.

Smith, D. R. 1991a. Mitochondrial DNA and honey bee biogeography, in: Smith D. R. (Ed.). *Diversity in the Genus Apis*. Westview, Boulder, CO.

Smith, D. R. 1991b. Honey bee biogeography and the African bees in The Americas: insights from biogeography and genetics. *Trends in Ecology and Evolutions*. 6: 17-21.

Smith, D. R. and Hagen, R. H. 1996. The biogeography of *Apis cerana* as revealed by mitochondrial DNA sequence data, *Journal of the Kansas Entomological Society* 69, 294-310.

Smith, D. R., Slaymaker, A., Palmer, M. and Kaftanoglu, O. 1997. Turkish honey bees belong to the east Mediterranean mitochondrial lineage. *Apidologie*. 28: 269-274.

Smith, D. R. and Hagen, R. H. 1999. Phylogeny and Biogeography of *Apis cerana* subspecies: testing alternative hypotheses, in: Hoopingarner R., Connor L. (Ed.), Apiculture for the 21st Century, Wicwas Press, Cheshire, CT.

Smith, D. R., Villafuerte, L., Otis, G. W. and Palmer, M. R. 2000. Biogeography of *Apis cerana* F. and *A. nigrocincta* Smith: insights from mtDNA studies. *Apidologie*. 31: 265-279.

Smith, D. R., Palmer, M. R., Otis, G. and Damus, M. 2003. Mitochondrial DNA and AFLP markers support species status of *Apis nigrocincta*. *Insectes Sociaux*. 50: 185-190.

Smith, D. R., Warrit, N. and Hepburn, H. R. 2004. *Apis cerana* from Myanmar (Burma): unusual distribution of mitochondrial lineages. *Apidologie*. 35: 637-644.

Songram, O., Sittipraneed, S. and Klinbunga, S. 2006. Mitochondrial DNA diversity and genetic differentiation of the honeybee (*Apis cerana*) in Thailand. *Biochemical Genetics*. 44: 256-269.

Solignac, M., Cornuet, J-M., Vautrin, D., Le Conte, Y., Anderson, D. L., Evans, J., Cros-Arteil, S. and Navajas, M. 2005. The invasive Korea and Japan types of *Varroa destructor*, ectoparasitic mites of the Western honeybees (*Apis mellifera*), are two partly isolated colonies. *Proceedings of the Royal B Society*. 272: 411-419.

Sušnik, S., Kozmus, P., Poklukar, J. and Meglič, V. 2004. Molecular characterisation of indigenous *Apis mellifera carnica* in Slovania. *Apidoloie*. 35: 623-636.

Suppasat, T., Smith, D. R., Deowanish, S. and Wongsiri, S. 2007. Matrilineal origins of *Apis mellifera* in Thailand. *Apidologie*. 38: 323-334.

Sylvester, H. A., Limbipichai, K., Wongsiri, S., Rinderer, T. E. and Mardan M. 1998. Morphometric studies of *Apis cerana* in Thailand and the Malaysian peninsula. *Journal of Apicultural Research*. 37: 137-145.

Tanaka, H., Roubik, D. W., Kato, M., Liew, F. and Gunsalam, G. 2001. Phylogenetic position of *Apis nuluensis* of northern Borneo and phylogeography of *A. cerana* as inferred from mitochondrial DNA sequences. *Insectes Sociaux*. 48: 44-51.

Thapa, R. and Wongsiri, S. 1997. *Eupatorium odoratum*: a honey bee plant for beekeepers in Thailand. *Bee World*. 78: 175-178.

The Honeybee Genome Sequencing Consortium. 2006. Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature*. 443, 931-949.

Tingek, S., Mardan, M., Rinderer, T. E., Koeniger, N. and Koeniger, G. 1988. Rediscovery of *Apis vechti* (Maa, 1953): The Sabah honey bee. *Apidologie*. 19(1): 97-102.

van der Wurff, A. W. G., Chan, Y. L., van Stralen, N. M. and Schouten, J. 2000. TE-AFLP: combining rapidity and robustness in DNA fingerprinting. *Nucleic Acids Research*. 28(24): e105.

Villa, J. D., Rinderer, T. E. and Stelzer, J. A. 2002. Answer to the puzzling distribution of Africanized bees in the United States. *American Bee Journal*. 142: 480-483.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van de Lee, T., Homes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*. 23: 4407-4414.

Warrit, N. 2002. *Biogeography of the Varroa mites on their native host, Apis cerana F., in the Thai-Malay-Peninsula*. M. A. Thesis. University of Kansas, Kansas, USA.

Warrit, N., Hagen, T. A. R., Smith, D. R. and Çakmak, I. 2004. A survey of *Varroa destructor* strains of *Apis mellifera* in Turkey. *Journal of Apicultural research*. 43(4): 190-191.

Warrit, N., Smith, D. R. and Lekprayoon, C. 2006. Genetic subpopulations of *Varroa* mites and their *Apis cerana* hosts in Thailand. *Apidologie*. 37: 19-30.

Webster, T. C. and Delaplane, K. S. 2001. *Mites of the Honey bee*. Dadant publication. Illinois, USA.

Whitfield, C. W., Behura, S. K., Berlocher, S. H., Clark, A. G., Johnston, J. S., Sheppard, W. S., Smith, D. R., Suarez, A. V., Weaver, D. and Tsutsui, N. D. 2006. Thrice out of Africa: Ancient and recent expansions of the honey bee, *Apis mellifera*. *Sciences*. 314: 642-645.

Whitmore, T. C. 1984. *Tropical Rain Forests of the Far East*. 2nd edition, Oxford Scienctific Publications, Clarendon Press, Oxford.

Wilkinson, D. and Smith, G. C. 2002. Modeling the efficiency of sampling and trapping *Varroa destructor* in the Drone Brood of Honey bees (*Apis mellifera*). *American Bee Journal*. 209-211.

Wolstenholme, D. R. 1992. Animal mitochondrial DNA: Structure and evolution. *International Review of Cytology*. 141: 173-216.

Wongsiri, S., Tangkanasing, P. and Sylvester, H. A. 1987. Mites, pest and beekeeping with *Apis cerana* and *Apis mellifera* in Thailand. *American Bee Journal*. 127:500-503.

Wongsiri, S. 1988. The effect of import of *Apis mellifera* L. to Thailand. *Proceedings of Fourth International Conference on Apiculture in Tropical Climates*. Cairo, Egypt. 162-167

Wongsiri, S., Chanchao, C., Deowanish, S., Aemprapa, S., Chaiyawong, T., Peterson, S. and Leepitakrat, S. 1995. Honeybee diversity and beekeeping in Thailand. *Bee World*. 81: 20-29.

Wongsiri, S., Chanchao, C., Lekprayoon, C., Wattanasermkit, K., Deowanish, S. and Leepitakrat, S. 2000. Honeybee diversity and management in the new millennium in Thailand. in: Anonymous (Ed.), *Proceeding of the Seventh international conference on tropical bees: management and diversity*. Chiang Mai, Thailand. 9-14

Wright, S. 1951. The genetical structure of populations. *Annals of Eugenics*. 15: 323-354.

Wright, S. 1965. The interpretation of population structure by F-Statistics with special regard to systems of mating. *Evolution*s. 19: 395-420.

Zhang, Z. Q. 2000. Note on *Varroa destructor* (Acari: Varroidae) parasitic on honeybees in New Zealand. *Systematic and Applied special publications*. 5: 9-14.

Zhou, T., Anderson, D. L., Huang, Z. Y., Huang, S., Yao, J., Ken, T. and Zhang, Q. 2004. Identification of *Varroa* mites (Acari: Varroidae) infesting *Apis cerana* and *Apis mellifera* in China. *Apidologie*. 35: 645-654.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDICES

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX A

MOLECULAR METHODS

I.) DNA PREPARATIONS

1. Standard phenol chloroform Extraction (Smith *et al*., 1997)

Procedure: Honey bee samples

Total DNA was extracted from single thorax of 95 % ethanol-preserved. Each thorax was cut in 500 μl of sterile STE buffer (0.1 M NaCl, 0.05 M Tris pH 7.5, 0.001 M EDTA), 25 μl of 20% sodium lauryl sulfate (SDS) and 25 μl of 10 mg/ml proteinase K in STE. The mixture was incubated from 2 h to overnight in the 55-56 °C degree water bath. After digestion the mixture was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/ isoamyl (24:1). DNA was precipitated with 1:10 volume of 3 M sodium acetate and two volumes of ice-cold absolute ethanol. DNA was pelleted by centrifuging for 10 min at 12,000 rpm. The pellet was rinsed once in 70% ethanol, air dried and re-suspended in 100 μl of 1/10 TE (TE= 10mM Tris, 1mM EDTA pH 7.8)

2. GENELUTE™ Mammalian Genomic DNA Kit (Sigma, USA)

Procedure: *Varroa* mite samples

Total DNA was extracted from one *Varroa* mite of 95% ethanol-preserved. Each mite was transferred into 1.5 microcenrifuge tube, added 180 μl of lysis solution T and 20 μl of 10 mg/ml proteinase K. Then, the mite was minced on ice. The mixture was shaken on vortex for 30 s and incubated at 55 °C degree for 2-4 h shaking water bath. Then, 200 μl of lysis solution C was added. The mixture was incubated at 70 °C degree for 10 min. The GenElute Miniprep Binding Column was prepared with 500 μl

of column preparation solution then, was centrifuged at 12,000 rpm for 1 min and discard flow though liquid. Next, the mixture was added 200 μl of 95% ethanol and mixed on vortexing until monogeneous solution. The lysate was transferred to the column that already to preparation approximately 700 μl then was centrifuged at 6,500 rpm for 1 min and discarded the collection tube. At washing step, the column was placed inside new collection tube, added 500 μl of wash solution, then centrifuged at 6,500 rpm for 1 min and discarded the collection tube. Repeating the washing step centrifuged at 12,000 rpm for 3 min. The binding column must be free of ethanol before eluting DNA so that additional centrifuged at 12,000 rpm for 1 min. The binding column was placed inside 1.5-2.0 microcentrifuge tube, added Elute Solution for 50 μl in center of the column, incubated at room temperature for 5-10 min and centrifuged at 6,500 rpm for 1 min. The DNA was kept in 1.5 microcentrifuge tube at -20 °C degree.

II.) PCR-RFLP AND DNA SEQUENCING

PCR preparation

The PCR reaction contained 2 μl of 10X PCR buffer (Promega, USA), 1.2 μl of 25 mM $MgCl_2$, 0.8 μl of 10 mM deoxynucleotide phosphates (dNTPs mixed: dATP, dTTP, dGTP and dCTP), 0.8 μl of 10 μM forward primer, 0.8 μl of 10 μM reverse primer, 0.2 μl of 5 unit/μl *Taq* polymerase, 1 μl of honey bee DNA extract and sterile distilled water adjust the volume at total 20 μl but in *Varroa* DNA extract was added for 4 μl in the mixture. The pairs of oligo-nucleotide primer sequences were selected to investigate genetic variation in mitochondrial DNA of honey bees into 4 regions and 1 region of *Varroa* mites see detail at the table1.

Table A.1 Honey bee and *Varroa* mite primers were selected for PCR-RFLP study

Honey bee primers		
MtDNA region	Oligonucleotide Sequences	References
$tRNA^{leu}$-COII	E2: 5'GGCAGAATAAGTGCATTG 3' H2: 5'CAATATCATTGATGACC 3'	(Garnery *et al*., 1993)
Cyt b	OLD1: 5'TATGTACTACCATGAGGACAAATATC3' OLD2: 5'ATTACACCTCCTAATTTATTAGGAAT 3'	(Crozier *et al*., 1991)
lsRNA	Ls1: 5'TTTTGTACCTTTTGTATCAGGGTTG 3' Ls2: 5'CTATAGGGTCTTATCGTCCC 3'	(Hall & Smith, 1991)
COI	COI-1908: 5' TTAAGATCCCCAGGATCATG 3' COI-2932: 5' TGCAAATACTGCACCTATTG 3'	(Hall & Smith, 1991)
***Varroa* mite primer**		
MtDNA region	Oligonucleotide Sequences	References
COI	V51: 5'GTAATTTGTATACAAAGAGGG 3' V400: 5'CAATATCAATAGAAGAATTAGC 3'	(Anderson & Trueman, 2000)

RFLP preparation

Five microliter aliquots of each PCR amplification product was digested with appropriate restriction enzymes following the New England Biolabs inc. recommended temperature and buffer conditions showed in the table 2.

Table A.2 Restriction enzymes information

Restriction enzyme	Recognition sites	Buffer and temperature
Bgl II	5’ A / GATAT 3’	NE buffer no. 3
	3’ TCTAG / A 5’	37 °C
Dra I	5’ TTT / AAA 3’	NE buffer no. 4
	3’ AAA / TTT 5’	37°C
*Eco*R I	5’ G / AATTC 3’	NE buffer no. 1-4
	3’ CTTAA / G 5’	37°C
*Hin*C II	5’ GTY / RAC 3’	NE buffer no. 3 + BSA
	3’CAR / YTG 5’	37°C
Hinf I	5’ G / ANTC 3’	NE buffer no. 2
	3’ CTNA / G 5’	37°C
*Sac*I	5’ GAGCT / C 3’	NE buffer no. 1 + BSA
	3’ C / TCGAG 5’	37°C
Xho I	5’ C / TCGAG 3’	NE buffer no. 2 +BSA
	3’ GAGCT / C 5’	37°C

Electrophoresis

The PCR products were checked with 1.5% agarose/ TBE gel (110 volts room temperature for 30 min). The resulting restriction fragments were separated with 2.5% agarose/ TBE gel (110 volts room temperature for 60-90 min). The gel were stained with ethidium bromide and viewed and photographed under ultraviolet illumination.

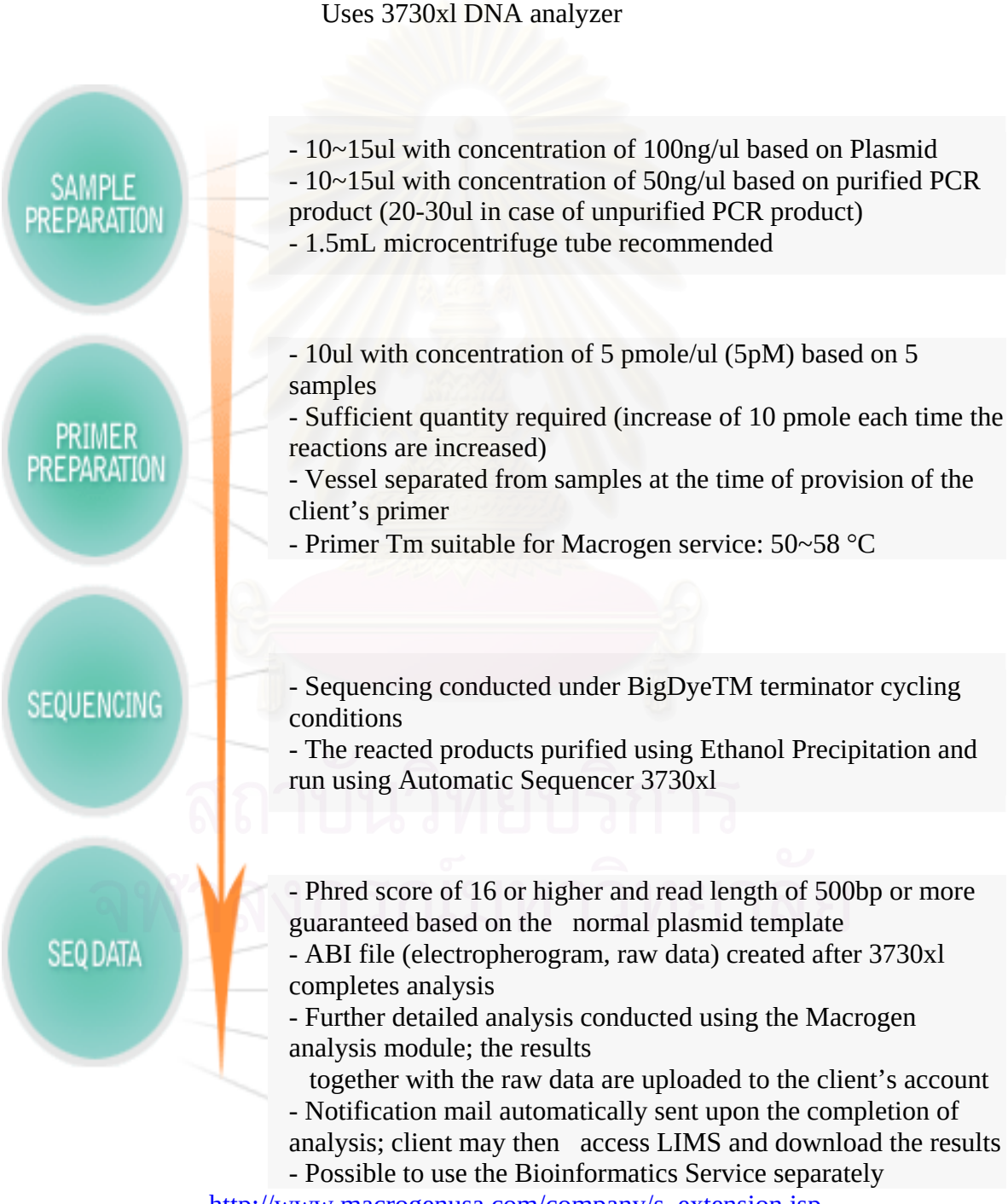
PCR purification for sequencing

QIAquick Gel Extraction Kit procedure: using a microcentrifuge

The QIAquick Gel Extraction Kit procedure (QIAGEN, USA) was designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose could be processed per spin column. The PCR product was run on 1.0% agarose/TBE (110 volts room temperature for 30 min). The PCR band was cut from the agarose gel with a clean sharp scalpel and removed the gel slice into newly.5 microcentrifue tube. The gel slice was weighed, added 3 volume of buffer QG to 1 volume of gel slice (gel 100 mg ~ 300 ml buffer QG) and incubated at 50 °C for 10 min or until the gel slice had completely dissolved. The gel was mixed by vortexing the tube for 2-3 times during incubation. The color of the mixture was checked after completely gel dissolved as same as the yellow of buffer QG. The isopropanol was added for 1 volume of gel weight (gel 100 mg ~ isopropanol 100 µl). The spin column was placed in a provided 2 ml collection tube and added the mixture of sample to QIAquick column and centrifuged 10,000 rpm for 1 min then, discarded flow-through and placed QIAquick column back in the same tube. Optional, buffer QG for 500 µl was added and centrifuged 10,000 rpm for 1 min. The washing step, the QIAquick column with DNA binding was cleaned with buffer PE for 750 µl and centrifuged 10,000 rpm for 1 min, then discarded flow-though and additional centrifuged at high speed 13,000 rpm for 1 min. The QIAquick column was moved to newly clean 1.5 microcentrifuge tube and eluted with sterile distilled water for 50 µl at center of column for 5 to 10 min and centrifuged at 13,000 rpm for 1 min.

DNA sequencing

The PCR purification products were sent to the Macrogen USA for sequence using primer extension which showed in figure.



http://www.macrogenusa.com/company/s_extension.jsp

III.) STANDARD AFLP AND TE-AFLP ANALYSIS

1. Standard AFLP procedure (Vos *et al*., 1995; Smith *et al*., 2003).

The amplified fragment length polymorphism (AFLP) techniques was one of a number of DNA fingerprinting procedures that used advantage of the PCR to amplify a limited set of DNA fragments from a specific DNA samples. The AFLP was quickly becoming the tool of choice for many applications and organisms. Potential applications include screening DNA markers linked to genetic traits, parentage analysis, population genetics, etc.

Recognition sites of restriction enzyme for AFLP

Restriction enzymes	Recognition sites
*Eco*R I	5’ G / AATTC 3’
	3’ CTTAA / G 5’
Mse I	5’ T / TAA 3’
	3’ AAT / T 5’

AFLP linkers

*Eco*R I-linkerL1: 5’CTCGTAGACTGCGTACC 3’

*Eco*R I-linkerL2: 5’AATTGGTACGCAGTCTAC 3’

‘Linker *Eco*R I’: 5’AATTGGTACGCAGTCTAC 3’
3’CCATGCGTCAGATGCTC 5’

Mse I-linkerL1: 5’GACGATGAGTCCTGAG 3’

Mse I-linkerL2: 5’TACTCAGGACTCAT 3’

’Linker- *Mse* I’: 5’TACTCAGGACTCAT 3’
3’GAGTCCTGAGTAGCAG 5’

AFLP primers

Selective primers

*Eco*R I-A: 5'CTCGTAGACTGCGTACCAATTCA 3'

Mse I-C: 5'GACGATGAGTCCTGAGTAAC 3'

Amplification primers

*Eco*R I-AAG: 5'CTCGTAGACTGCGTACCAATTCAAG 3'

Mse I-CTA: 5'GACGATGAGTCCTGAGTAACTA 3'

Procedure

DNA digestion and ligation

Three microliters of each total DNA extract were digested for 4 h with 1 unit each of 2 restriction enzymes, *Eco*R I and *Mse* I, in 1X universal buffer and 10X BSA (Promega, USA). The sticky ends of restriction fragments were ligated to DNA linkers having complementary sticky ends by addition to 1 μl of 'linker *Eco*R I' (10 mM stock solution), 2 μl of 'linker *Mse* I' (10 mM stock solution), 1X ligase buffer and 0.2 μl (0.6 units) of *T4* DNA ligase (Promega, USA) incubated overnight at room temperature.

Selective amplification reaction

The digestion and ligation of genomic DNA mixture was selectively amplified using primer that exactly matched the linkers plus one base at the ends of: primers, '*Eco*R I-A' and '*Mse* I-C'. Amplification was performed by adding 2.0 μl of digested and ligated DNA mixture to 23 μl of amplification mixture containing 2.5 μl of 10x PCR buffer (Promega, USA), 0.75 μl of 25 mM $MgCl_2$, 0.25 μl of primer stock 10 μM of '*Eco*R I-A', 1.0 μl of primer stock 10 μM of '*Mse* I-C', 0.5 μl of 10 mM dNTPs mix, 0.125 μl of *Taq* DNA polymerase 5 unit/μl (Promega, USA) and sterile distilled water.

The reaction was amplified using the profile 94 °C for 1 min, 20 cycles of 94 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min and extension at 72 °C for 1 min.

Second amplification reaction

A second amplification was performed using primers matching the linker plus three bases at the end of primers, '*Eco*R I-AAG' and '*Mse* I-CTA'. The reaction mixture included 1.0 μl of the selective amplification product, 0.375 μl of a 10 μM stock of primer *Eco*R I-AAG*, end-labeled with ^{32}P, 1.0 μl of 10 μM stock primer *Mse* I-CTA, 0.25 μl of 10 mMdNTPs stock, 0.125 μl of 5 unit/μl stock *Taq* polymerase (Promega, USA) and sterile distilled water to make 25 μl totally. Annealing temperatures were slowly decreased over the first 13 amplification cycles: beginning with a cycle of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min, each step reduced the annealing temperature by 0.7 °C for 1 min (Rosendahl and Taylor, 1997). The amplification products were then electrophoresed through an 8% denaturing polyacrylamide/TBE gel for 5 h at 1,500 volts. Gel was dried on filter paper and used to expose Fuji X-ray film 18 h to overnight. After autoradiogram was developed, the bands were read and recorded by hand.

2. TE-AFLP procedure (van der Wurff *et al*., 2000; Smith *et al*., 2003)

A new type of fingerprinting technique based on standard AFLP, the method was called three endonucleases amplified fragment length polymorphism (TE-AFLP) which differed from traditional AFLP by reducing the number of amplified fragments not only by primer extension, but also by selective ligation. Three endonucleases together with only two sets of adapters were added to a single reaction. The third

endonuclease provided discrimination at extra sites between genomes compared to typical AFLP, thereby increasing discriminatory power.

Recognition sites of restriction enzyme for TE-AFLP

Restriction enzymes	Recognition sites
*Bam*H I	5’ G / GATCC 3’
	3’ CCTAC / G 5’
Xba I	5’ T / CTAGA 3’
	3’ AGATC / T 5’
Rsa I	5’ GT / AC 3’
	3’ CA / TG 5’

TE-AFLP adaptors

*Bam*H I-adaptor1: 5’ ACGAAGTCCCGCGCCAGCAA 3’

*Bam*H I-adaptor2: 5’ GATCTTGCTGGCGCGGG 3’

*Bam*H I-adaptor: 5’ ACGAAGTCCCGCGCCAGCAA 3’
3’GGGCGCGGTCGTTCTAG 5’

Xba I-adaptor1: 5’ ACGTTGTGGCGGCGTCGAGA 3’

Xba I-adaptor2: 5’ CTAGTCTCGACGCCGCC 3’

Xba I-adaptor: 5’ ACGTTGTGGCGGCGTCGAGA 3’
3’CCGCCGCAGCTCTGATC 5’

TE-AFLP primers

Amplification primers

*Bam*H I-C: 5’ GTTTCGCGCCAGCAAGATCCC 3’

*Xba*I-AC: 5’ GGCGTCGAGACTAGAAC 3’

Procedure

DNA digestion and ligation

Each of DNA extract was digested with 6-base restriction enzymes, *Xba* I and *Bam*H I, and the 4-base enzyme *Rsa* I. Digestion-ligation mixture in final volume 20 μl

contained the following ingredients: 2 µl of 10X ligation buffer, 2 µl 500 mM of NaCl, 4 µl of 1 picomol/µl *Bam*H I adaptor, 4 µl of 1 picomol/µl *Xba* I adaptor, 0.6 µl of 10 unit/µl *Xba* I , 0.125 µl of 10 unit/µl *Bam*H I, 0.1 µlof 10 unit/µl *Rsa* I, 0.6 µl of 3 unit/µl *T4* DNA ligase, 2 µl of DNA extract (buffers and enzymes supplied by Fisher, USA) and 4.5 µl sterile distilled water. Reactions were incubated at 30 °C for 1.5 h, stored at -20 °C.

Amplification reaction

Each 12.5 µl PCR reaction contained the following ingredients: 1.25 µl of 10X PCR buffer, 0.75 µl of 25 mM $MgCl_2$, 0.25 µl of 2.5 picomol *Bam*H I-C primer, 0.25 µl of 2.5 picomol labeled ^{32}P *Xba* I-AC* primer, 0.125 µl of 5 unit/µl *Taq* polymerase, 0.25 µl of 10 mM dNTPs mix, 0.75 µl digestion-ligation DNA and 8.875 µl sterile distilled water (buffers enzymes and dNTPs supplied by Promega, USA). The thermal profile for amplification followed denaturation at 95 °C for 3 min, 10 cycles of 95 °C for 30 s, 70 °C for 30 s, 72 °C for 60 s, then 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s, finished at 72°C for 20 min. The products were then electrophoresed through an 8% denaturing polyacrylamide/TBE gel for 5 h at 1,500 volts. Gel was dried on filter paper and used to expose Fuji X-ray film 18 h to overnight. After auto-radiogram was developed, the bands were read and recorded by hands.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX B

SAMPLE COLLECTIONS

I. Total of 476 *A. mellifera* collections were selected to investigate genetic variation in mitochondrial DNA using PCR-RFLP procedure.

#DNA	Province	Location	Date(mm/dd/yy)	Haplotypes
8520	Bangkok	Chulalongkorn University	07/20/2003	BBBBABA
8521	Bangkok	Chulalongkorn University	07/20/2003	BBBBABA
8522	Bangkok	Chulalongkorn University	07/20/2003	BBBBABA
8523	Bangkok	Chulalongkorn University	07/20/2003	BBBBABA
8524	Bangkok	Chulalongkorn University	07/20/2003	BBBBABA
8525	Bangkok	Chulalongkorn University	07/20/2003	BBBBABA
8526	Bangkok	Chulalongkorn University	07/20/2003	BBBBABA
8527	Bangkok	Chulalongkorn University	07/20/2003	BBBBABA
8528	Bangkok	Chulalongkorn University	07/20/2003	BBBBABA
8529	Bangkok	Chulalongkorn University	07/20/2003	BBBBABA
8530	Bangkok	Chulalongkorn University	07/20/2003	BBBBABA
8531	Chiangmai	Chiangmai University	08/11/2003	BBBBABA
8532	Chiangmai	Chiangmai University	08/11/2003	BBBBABA
8533	Chiangmai	Chiangmai University	08/11/2003	BBBBABA
8534	Chiangmai	Chiangmai University	08/11/2003	BBBBABA
8535	Chiangmai	Chiangmai University	08/11/2003	BBBBABA
8536	Chiangmai	Chiangmai University	08/11/2003	BCBAABA
8537	Chiangmai	Chiangmai University	08/11/2003	BBBBABA
8538	Chiangmai	Chiangmai University	08/11/2003	BCBAABA
8539	Chiangmai	Chiangmai University	08/11/2003	BCBAABA
8540	Chiangmai	Chiangmai University	08/11/2003	BCBAABA
8541	Chiangmai	Chiangmai University	08/11/2003	BBBBABA
8542	Chiangmai	Chiangmai University	08/11/2003	BBBBABA
8543	Chiangmai	Rim Tai, Mae Rim District	08/11/2003	BBBBABA
8544	Chiangmai	Rim Tai, Mae Rim District	08/11/2003	BCBAABA

#DNA	Province	Location	Date(mm/dd/yy)	Haplotypes
8545	Chiangmai	Rim Tai, Mae Rim District	08/11/2003	BBBBABA
8546	Chiangmai	Rim Tai, Mae Rim District	08/11/2003	BBBBABA
8547	Chiangmai	Rim Tai, Mae Rim District	08/11/2003	BBBBABA
8548	Chiangmai	Rim Tai, Mae Rim District	08/11/2003	BBBBABA
8549	Chiangmai	Rim Tai, Mae Rim District	08/11/2003	BBBBABA
8550	Chiangmai	Rim Tai, Mae Rim District	08/11/2003	BBBBABA
8551	Chiangmai	Rim Tai, Mae Rim District	08/11/2003	BBBBABA
8552	Chiangmai	Rim Tai, Mae Rim District	08/11/2003	BBBBABA
8553	Chiangmai	Rim Tai, Mae Rim District	08/11/2003	BBBBABA
8554	Chiangmai	Rim Tai, Mae Rim District	08/11/2003	BBBBABA
8555	Chiangmai	Rim Tai, Mae Rim District	08/11/2003	BBBBABA
8556	Chiangmai	Rim Tai, Mae Rim District	08/11/2003	AABBBAA
8557	Chiangmai	Rim Tai, Mae Rim District	08/11/2003	BBBBABA
8558	Chiangmai	Rim Tai, Mae Rim District	08/11/2003	AABBBAA
8559	Chiangmai	Rim Tai, Mae Rim District	08/11/2003	BBBBABA
8560	Chiangmai	Rim Tai, Mae Rim District	08/11/2003	BBBBABA
8561	Chiangmai	Rim Tai, Mae Rim District	08/11/2003	BBBBABA
8562	Chiangmai	Sun Sai District	08/11/2003	BBBBABA
8563	Chiangmai	Sun Sai District	08/11/2003	BBBBABA
8564	Chiangmai	Sun Sai District	08/11/2003	BBBBABA
8565	Chiangmai	Sun Sai District	08/11/2003	BBBBABA
8566	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8567	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8568	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8569	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8570	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8571	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8572	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8573	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8574	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8575	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8576	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8577	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8578	Chantaburi	Tha Mai District	08/30/2003	BBBBABA

#DNA	Province	Location	Date(mm/dd/yy)	Haplotypes
8579	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8580	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8581	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8582	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8583	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8584	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8585	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8586	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8587	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8588	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8589	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8590	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8591	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8592	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8593	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8594	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8595	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8596	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8597	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8598	Chantaburi	Tha Mai District	08/30/2003	BBBBABA
8599	Chiangmai	Sun Khum Pang District	09/14/2003	BBBBABA
8600	Chiangmai	Sun Khum Pang District	09/14/2003	BBBBABA
8601	Chiangmai	Sun Khum Pang District	09/14/2003	BBBBABA
8602	Chiangmai	Sun Khum Pang District	09/14/2003	BBBBABA
8603	Chiangmai	Sun Khum Pang District	09/14/2003	BBBBABA
8604	Chiangmai	Sun Khum Pang District	09/14/2003	AABBBAA
8605	Chiangmai	Sun Khum Pang District	09/14/2003	BBBBABA
8606	Chiangmai	Sun Khum Pang District	09/14/2003	BBBBABA
8607	Chiangmai	Sun Khum Pang District	09/14/2003	BBBBABA
8608	Chiangmai	Sun Khum Pang District	09/14/2003	BBBBABA
8609	Chiangmai	Sun Khum Pang District	09/14/2003	BBBBABA
8610	Chiangmai	Sun Khum Pang District	09/14/2003	BBBBABA
8611	Chiangmai	Sun Khum Pang District	09/14/2003	BBBBABA
8612	Chiangmai	Sun Khum Pang District	09/14/2003	AABBBAA

#DNA	Province	Location	Date(mm/dd/yy)	Haplotypes
8613	Chiangmai	Sun Khum Pang District	09/14/2003	BBBBABA
8614	Chiangmai	Sun Khum Pang District	09/14/2003	BBBBBAA
8615	Chiangmai	Sun Khum Pang District	09/14/2003	BBBBABA
8616	Chiangmai	Sun Khum Pang District	09/14/2003	BBBBABA
8617	Chiangmai	Sun Khum Pang District	09/14/2003	BBBBABA
8618	Lopburi	Meaung District	09/26/2003	BBBBABA
8619	Lopburi	Meaung District	09/26/2003	BBBBABA
8620	Lopburi	Meaung District	09/26/2003	BBBBABA
8621	Lopburi	Meaung District	09/26/2003	BBBBABA
8622	Lopburi	Meaung District	09/26/2003	BBBBABA
8623	Lopburi	Meaung District	09/26/2003	BBBBABA
8624	Lopburi	Meaung District	09/26/2003	BBBBABA
8625	Lopburi	Meaung District	09/26/2003	BBBBABA
8626	Lopburi	Meaung District	09/26/2003	BBBBABA
8627	Lopburi	Meaung District	09/26/2003	BBBBABA
8628	Lopburi	Meaung District	09/26/2003	BBBBABA
8629	Lopburi	Meaung District	09/26/2003	BBBBABA
8630	Lopburi	Phattananikhom District	09/26/2003	BBBBABA
8631	Lopburi	Phattananikhom District	09/26/2003	BBBBABA
8632	Lopburi	Phattananikhom District	09/26/2003	BBBBABA
8633	Lopburi	Phattananikhom District	09/26/2003	BBBBABA
8634	Lopburi	Phattananikhom District	09/26/2003	BBBBABA
8635	Lopburi	Phattananikhom District	09/26/2003	BBBBABA
8636	Trat	Bori District	10/10/2003	BCBAABA
8637	Trat	Bori District	10/10/2003	BCBAABA
8638	Trat	Bori District	10/10/2003	BCBAABA
8639	Trat	Bori District	10/10/2003	BCBAABA
8640	Trat	Bori District	10/10/2003	BCBAABA
8641	Trat	Bori District	10/10/2003	BCBAABA
8642	Trat	Bori District	10/10/2003	BCBAABA
8643	Trat	Bori District	10/10/2003	BCBAABA
8644	Trat	Bori District	10/10/2003	BCBAABA
8645	Trat	Bori District	10/10/2003	BCBAABA
8646	Trat	Bori District	10/10/2003	BCBAABA

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

#DNA	Province	Location	Date(mm/dd/yy)	Haplotypes
8647	Trat	Bori District	10/10/2003	BCBAABA
8648	Trat	Bori District	10/10/2003	BCBAABA
8649	Trat	Bori District	10/10/2003	BCBAABA
8650	Trat	Bori District	10/10/2003	BCBAABA
8651	Trat	Bori District	10/10/2003	BCBAABA
8652	Trat	Bori District	10/10/2003	BCBAABA
8653	Trat	Bori District	10/10/2003	BBBBABA
8654	Trat	Bori District	10/10/2003	BCBAABA
8655	Trat	Bori District	10/10/2003	BBBBABA
8656	Trat	Bori District	10/10/2003	BCBAABA
8657	Trat	Bori District	10/10/2003	BCBAABA
8658	Chumporn	Thung Tha Go District	10/18/2003	BBBBABA
8659	Chumporn	Thung Tha Go District	10/18/2003	BBBBABA
8660	Chumporn	Thung Tha Go District	10/18/2003	AABBBAA
8661	Chumporn	Thung Tha Go District	10/18/2003	AABBBAA
8662	Chumporn	Thung Tha Go District	10/18/2003	BBBBABA
8663	Chumporn	Thung Tha Go District	10/18/2003	AABBBAA
8664	Chumporn	Thung Tha Go District	10/18/2003	AABBBAA
8665	Chumporn	Thung Tha Go District	10/18/2003	AABBBAA
8666	Chumporn	Thung Tha Go District	10/18/2003	BBBBABA
8667	Chumporn	Thung Tha Go District	10/18/2003	BBBBABA
8668	Chumporn	Thung Tha Go District	10/18/2003	AABBBAA
8669	Chumporn	Thung Tha Go District	10/18/2003	BBBBABA
8670	Chumporn	Thung Tha Go District	10/18/2003	AABBBAA
8671	Chumporn	Thung Tha Go District	10/18/2003	AABBBAA
8672	Chumporn	Thung Tha Go District	10/18/2003	BBBBBAA
8673	Chumporn	Thung Tha Go District	10/18/2003	BBBBBAA
8674	Chumporn	Thung Tha Go District	10/18/2003	AABBBAA
8675	Chumporn	Thung Tha Go District	10/18/2003	BBBBABA
8676	Chumporn	Thung Tha Go District	10/18/2003	AABBBAA
8677	Chumporn	Thung Tha Go District	10/18/2003	AABBBAA
8678	Chumporn	AEF, Meuang District	10/19/2003	AABBBAA
8679	Chumporn	AEF, Meuang District	10/19/2003	BBBBABA
8680	Chumporn	AEF, Meuang District	10/19/2003	BBBBABA

#DNA	Province	Location	Date(mm/dd/yy)	Haplotypes
8681	Chumporn	AEF, Meuang District	10/19/2003	BBBBABA
8682	Chumporn	AEF, Meuang District	10/19/2003	BBBBABA
8683	Chumporn	AEF, Meuang District	10/19/2003	BBBBABA
8684	Chumporn	AEF, Meuang District	10/19/2003	BBBBABA
8685	Chumporn	AEF, Meuang District	10/19/2003	BBBBABA
8686	Chumporn	AEF, Meuang District	10/19/2003	BBBBABA
8687	Chumporn	AEF, Meuang District	10/19/2003	BBBBABA
8688	Chumporn	AEF, Meuang District	10/19/2003	BBBBABA
8689	Chumporn	AEF, Meuang District	10/19/2003	BBBBABA
8690	Chumporn	AEF, Meuang District	10/19/2003	BBBBABA
8691	Chumporn	AEF, Meuang District	10/19/2003	BBBBABA
8692	Chumporn	AEF, Meuang District	10/19/2003	BBBBABA
8693	Chumporn	AEF, Meuang District	10/19/2003	BBBBABA
8694	Chumporn	AEF, Meuang District	10/19/2003	BBBBABA
8695	Chumporn	AEF, Meuang District	10/19/2003	BBBBABA
8696	Chumporn	AEF, Meuang District	10/19/2003	BBBBABA
8697	Chumporn	AEF, Meuang District	10/19/2003	BBBBABA
8698	Chumporn	Swee District	10/19/2003	BBBBABA
8699	Chumporn	Swee District	10/19/2003	AABBBAA
8700	Surat Thani	Karnjanadit District (Site 1)	10/21/2003	AABBBAA
8701	Surat Thani	Karnjanadit District (Site 1)	10/21/2003	AABBBAA
8702	Surat Thani	Karnjanadit District (Site 1)	10/21/2003	BBBBABA
8703	Surat Thani	Karnjanadit District (Site 1)	10/21/2003	AABBBAA
8704	Surat Thani	Karnjanadit District (Site 1)	10/21/2003	BBBBABA
8705	Surat Thani	Karnjanadit District (Site 1)	10/21/2003	AABBBAA
8706	Surat Thani	Karnjanadit District (Site 2)	10/21/2003	AABBBAA
8707	Surat Thani	Karnjanadit District (Site 2)	10/21/2003	BBBBABA
8708	Surat Thani	Karnjanadit District (Site 2)	10/21/2003	BBBBABA
8709	Surat Thani	Karnjanadit District (Site 2)	10/21/2003	BBBBABA
8710	Surat Thani	Karnjanadit District (Site 2)	10/21/2003	BBBBABA
8711	Surat Thani	Karnjanadit District (Site 2)	10/21/2003	BBBBABA
8712	Surat Thani	Karnjanadit District (Site 2)	10/21/2003	AABBBAA
8713	Surat Thani	Karnjanadit District (Site 2)	10/21/2003	BCBBABA
8714	Surat Thani	Karnjanadit District (Site 3)	10/21/2003	BCBBABA

#DNA	Province	Location	Date(mm/dd/yy)	Haplotypes
8715	Surat Thani	Karnjanadit District (Site 3)	10/21/2003	BCBBABA
8716	Surat Thani	Karnjanadit District (Site 3)	10/21/2003	BBBBABA
8717	Surat Thani	Karnjanadit District (Site 3)	10/21/2003	BBBBABA
8718	Surat Thani	Karnjanadit District (Site 3)	10/21/2003	BBBBABA
8719	Surat Thani	Karnjanadit District (Site 3)	10/21/2003	BBBBABA
8720	Nakhon Srithammarat	Chang Klang District	10/21/2003	AABBBAA
8721	Nakhon Srithammarat	Chang Klang District	10/21/2003	BBBBABA
8722	Nakhon Srithammarat	Chang Klang District	10/21/2003	BBBBABA
8723	Nakhon Srithammarat	Chang Klang District	10/21/2003	BBBBABA
8724	Nakhon Srithammarat	Chang Klang District	10/21/2003	BBBBABA
8725	Nakhon Srithammarat	Chang Klang District	10/21/2003	BBBBABA
8726	Nakhon Srithammarat	Chang Klang District	10/21/2003	BBBBABA
8727	Nakhon Srithammarat	Chang Klang District	10/21/2003	AABBBAA
8728	Nakhon Srithammarat	Chang Klang District	10/21/2003	BBBBABA
8729	Nakhon Srithammarat	Chang Klang District	10/21/2003	BBBBBAA
8730	Nakhon Srithammarat	Chang Klang District	10/21/2003	AABBBAA
8731	Nakhon Srithammarat	Chang Klang District	10/21/2003	BBBBABA
8732	Nakhon Srithammarat	Chang Klang District	10/21/2003	BBBBABA
8733	Nakhon Srithammarat	Chang Klang District	10/21/2003	BCBBABA
8734	Nakhon Srithammarat	Chang Klang District	10/21/2003	BBBBABA
8735	Song-Khla	Kra Say Sin District	10/24/2003	BBBBABA
8736	Song-Khla	Kra Say Sin District	10/24/2003	BBBBBAA
8737	Song-Khla	Kra Say Sin District	10/24/2003	BBBBABA
8738	Song-Khla	Kra Say Sin District	10/24/2003	AABBBAA
8739	Song-Khla	Kra Say Sin District	10/24/2003	AABBBAA
8740	Song-Khla	Kra Say Sin District	10/24/2003	AABBBAA
8741	Song-Khla	Kra Say Sin District	10/24/2003	AABBBAA
8742	Song-Khla	Kra Say Sin District	10/24/2003	BBBBABA
8743	Song-Khla	Kra Say Sin District	10/24/2003	BBBBABA
8744	Song-Khla	Kra Say Sin District	10/24/2003	BBBBABA
8745	Song-Khla	Kra Say Sin District	10/24/2003	AABBBAA
8746	Song-Khla	Kra Say Sin District	10/24/2003	AABBBAA
8747	Song-Khla	Kra Say Sin District	10/24/2003	BBBBABA
8748	Song-Khla	Kra Say Sin District	10/24/2003	BBBBABA

#DNA	Province	Location	Date(mm/dd/yy)	Haplotypes
8749	Song-Khla	Kra Say Sin District	10/24/2003	BBBBABA
8750	Song-Khla	Kra Say Sin District	10/24/2003	BBBBABA
8751	Song-Khla	Kra Say Sin District	10/24/2003	AABBBAA
8752	Song-Khla	Kra Say Sin District	10/24/2003	AABBBAA
8753	Song-Khla	Kra Say Sin District	10/24/2003	BBBBABA
8754	Song-Khla	Kra Say Sin District	10/24/2003	BCBBABA
8755	Australia		10/--/2003	BCBAABA
8756	Australia		10/--/2003	BCBAABA
8757	Australia		10/--/2003	BCBAABA
8758	Australia		10/--/2003	BCBAABA
8759	Australia		10/--/2003	BCBAABA
8760	New Zealand		11/--/2003	BCBAABA
8761	Nan	Poa District	01/15/2004	BCBAABA
8762	Nan	Poa District	01/15/2004	BCBAABA
8763	Nan	Poa District	01/15/2004	BCBAABA
8764	Nan	Poa District	01/15/2004	BCBAABA
8765	Nan	Poa District	01/15/2004	BBBBABA
8766	Nan	Poa District	01/15/2004	BCBBABA
8767	Nan	Poa District	01/15/2004	BCBAABA
8768	Nan	Poa District	01/15/2004	BCBAABA
8769	Nan	Poa District	01/15/2004	BCBAABA
8770	Nan	Poa District	01/15/2004	BCBAABA
8771	Nan	Poa District	01/15/2004	BCBAABA
8772	Nan	Poa District	01/15/2004	BCBAABA
8773	Nan	Poa District	01/15/2004	BCBAABA
8774	Nan	Poa District	01/15/2004	BCBAABA
8775	Nan	Poa District	01/15/2004	BCBAABA
8776	Nan	Poa District	01/15/2004	BCBAABA
8777	Nan	Poa District	01/15/2004	BCBAABA
8778	Nan	Poa District	01/15/2004	BCBAABA
8779	Nan	Poa District	01/15/2004	BCBAABA
8780	Nan	Poa District	01/15/2004	BCBAABA
8781	Utaradit	Meuang District	01/17/2004	BBBBABA
8782	Utaradit	Meuang District	01/17/2004	BBBBABA

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

#DNA	Province	Location	Date(mm/dd/yy)	Haplotypes
8783	Utaradit	Meuang District	01/17/2004	BBBBABA
8784	Utaradit	Meuang District	01/17/2004	BBBBABA
8785	Utaradit	Meuang District	01/17/2004	BBBBABA
8786	Utaradit	Meuang District	01/17/2004	BBBBABA
8787	Utaradit	Meuang District	01/17/2004	BBBBABA
8788	Utaradit	Meuang District	01/17/2004	BBBBABA
8789	Utaradit	Meuang District	01/17/2004	BBBBABA
8790	Loi Ed	Prathumrat District	04/10/2004	BBBBABA
8791	Loi Ed	Prathumrat District	04/10/2004	BBBBBAA
8792	Loi Ed	Prathumrat District	04/10/2004	BBBBABA
8793	Loi Ed	Prathumrat District	04/10/2004	BBBBABA
8794	Loi Ed	Prathumrat District	04/10/2004	BBBBABA
8795	Loi Ed	Prathumrat District	04/10/2004	BBBBABA
8796	Loi Ed	Prathumrat District	04/10/2004	BBBBABA
8797	Loi Ed	Prathumrat District	04/10/2004	BBBBBAA
8798	Loi Ed	Prathumrat District	04/10/2004	BBBBABA
8799	Loi Ed	Prathumrat District	04/10/2004	BBBBABA
8800	Loi Ed	Prathumrat District	04/10/2004	AABBBAA
8801	Loi Ed	Prathumrat District	04/10/2004	AABBBAA
8802	Loi Ed	Prathumrat District	04/10/2004	BBBBABA
8803	Loi Ed	Prathumrat District	04/10/2004	BBBBABA
8804	Loi Ed	Prathumrat District	04/10/2004	BBBBABA
8805	Loi Ed	Prathumrat District	04/10/2004	BBBBABA
8806	Loi Ed	Prathumrat District	04/10/2004	BBBBABA
8807	Loi Ed	Prathumrat District	04/10/2004	AABBBAA
8808	Loi Ed	Prathumrat District	04/10/2004	BBBBABA
8809	Loi Ed	Prathumrat District	04/10/2004	AABABAA
8810	Udorntani	Khud Jub District	04/20/2004	BBBBABA
8811	Udorntani	Khud Jub District	04/20/2004	BBBBABA
8812	Udorntani	Khud Jub District	04/20/2004	BCBAABA
8813	Udorntani	Khud Jub District	04/20/2004	BCBAABA
8814	Udorntani	Khud Jub District	04/20/2004	BCBAABA
8815	Udorntani	Khud Jub District	04/20/2004	BCBAABA
8816	Udorntani	Khud Jub District	04/20/2004	BBBBABA

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

#DNA	Province	Location	Date(mm/dd/yy)	Haplotypes
8817	Udorntani	Khud Jub District	04/20/2004	BBBBABA
8818	Udorntani	Khud Jub District	04/20/2004	BBBBABA
8819	Udorntani	Khud Jub District	04/20/2004	BBBBABA
8820	Udorntani	Khud Jub District	04/20/2004	BCBAABA
8821	Udorntani	Khud Jub District	04/20/2004	AABBBAA
8822	Udorntani	Khud Jub District	04/20/2004	BCBAABA
8823	Udorntani	Khud Jub District	04/20/2004	BCBAABA
8824	Udorntani	Khud Jub District	04/20/2004	BBBBABA
8825	Udorntani	Khud Jub District	04/20/2004	BBBBABA
8826	Udorntani	Khud Jub District	04/20/2004	BBBBABA
8827	Udorntani	Khud Jub District	04/20/2004	AABBBAA
8828	Udorntani	Khud Jub District	04/20/2004	BBBBABA
8829	Udorntani	Khud Jub District	04/20/2004	AABBBAA
8830	Udorntani	Khud Jub District	04/20/2004	AABBBAA
8831	Udorntani	Khud Jub District	04/20/2004	BBBBABA
8832	Udorntani	Khud Jub District	04/20/2004	BCBAABA
8833	Udorntani	Khud Jub District	04/20/2004	BBBBABA
8834	Udorntani	Khud Jub District	04/20/2004	BBBBABA
8835	Udorntani	Khud Jub District	04/20/2004	BCBAABA
8836	Udorntani	Khud Jub District	04/20/2004	BCBAABA
8837	Udorntani	Khud Jub District	04/20/2004	BCBAABA
8838	Udorntani	Khud Jub District	04/20/2004	BBBBABA
8839	Udorntani	Khud Jub District	04/20/2004	BCBAABA
8840	Khonkaen	AEF, Meuang District	05/10/2004	BBBBABA
8841	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8842	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8843	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8844	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8845	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8846	Khonkaen	AEF, Meuang District	05/10/2004	BCBAABA
8847	Khonkaen	AEF, Meuang District	05/10/2004	BBBBABA
8848	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8849	Khonkaen	AEF, Meuang District	05/10/2004	BCBAABA
8850	Khonkaen	AEF, Meuang District	05/10/2004	AABAABA

#DNA	Province	Location	Date(mm/dd/yy)	Haplotypes
8851	Khonkaen	AEF, Meuang District	05/10/2004	BCBAABA
8852	Khonkaen	AEF, Meuang District	05/10/2004	BBBBABA
8853	Khonkaen	AEF, Meuang District	05/10/2004	BBBBABA
8854	Khonkaen	AEF, Meuang District	05/10/2004	AABABAA
8855	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8856	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8857	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8858	Khonkaen	AEF, Meuang District	05/10/2004	BCBBABA
8859	Khonkaen	AEF, Meuang District	05/10/2004	BCBBABA
8860	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8861	Khonkaen	AEF, Meuang District	05/10/2004	BCBBABA
8862	Khonkaen	AEF, Meuang District	05/10/2004	BBBBABA
8863	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8864	Khonkaen	AEF, Meuang District	05/10/2004	BCBBABA
8865	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8866	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8867	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8868	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8869	Khonkaen	AEF, Meuang District	05/10/2004	BCBBABA
8870	Khonkaen	AEF, Meuang District	05/10/2004	BCBBABA
8871	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8872	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8873	Khonkaen	AEF, Meuang District	05/10/2004	BBBBABA
8874	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8875	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8876	Khonkaen	AEF, Meuang District	05/10/2004	AABBBAA
8877	Khonkaen	AEF, Meuang District	05/10/2004	BCBAABA
8878	Khonkaen	Num Pong District	05/10/2004	AABAABA
8879	Khonkaen	Num Pong District	05/10/2004	AABBBAA
8880	Khonkaen	Num Pong District	05/10/2004	AABBBAA
8881	Khonkaen	Num Pong District	05/10/2004	AABBBAA
8882	Khonkaen	Num Pong District	05/10/2004	AABBBAA
8883	Khonkaen	Num Pong District	05/10/2004	BBBBABA

#DNA	Province	Location	Date(mm/dd/yy)	Haplotypes
8884	Khonkaen	Num Pong District	05/10/2004	AABBBAA
8885	Khonkaen	Num Pong District	05/10/2004	AABBBAA
8886	Khonkaen	Num Pong District	05/10/2004	AABBBAA
8887	Khonkaen	Num Pong District	05/10/2004	AABBBAA
8888	Khonkaen	Num Pong District	05/10/2004	AABBBAA
8889	Khonkaen	Num Pong District	05/10/2004	AABBBAA
8890	Khonkaen	Num Pong District	05/10/2004	BBBBABA
8891	Khonkaen	Num Pong District	05/10/2004	AABBBAA
8892	Khonkaen	Num Pong District	05/10/2004	BCBAABA
8893	Khonkaen	Num Pong District	05/10/2004	AABBBAA
8894	Khonkaen	Num Pong District	05/10/2004	BCBAABA
8895	Khonkaen	Num Pong District	05/10/2004	BCBAABA
8896	Chiangmai	AEF, Hangdong District	01/14/2003	BBBBABA
8897	Chiangmai	AEF, Hangdong District	01/14/2003	BBBBABA
8898	Chiangmai	AEF, Hangdong District	01/14/2003	BBBBABA
8899	Chiangmai	AEF, Hangdong District	01/14/2003	BCBAABA
8900	Chiangmai	AEF, Hangdong District	01/14/2003	BBBBABA
8901	Chiangmai	AEF, Hangdong District	01/14/2003	AABBBAA
8902	Chiangmai	AEF, Hangdong District	01/14/2003	BBBBABA
8903	Chiangmai	AEF, Hangdong District	01/14/2003	BBBBABA
8904	Chiangmai	AEF, Hangdong District	01/14/2003	AABBBAA
8905	Chiangmai	AEF, Hangdong District	01/14/2003	BBBBABA
8906	Chiangmai	AEF, Hangdong District	01/14/2003	BBBBABA
8907	Chiangmai	AEF, Hangdong District	01/14/2003	AABBBAA
8908	Chiangmai	AEF, Hangdong District	01/14/2003	BBBBABA
8909	Chiangmai	AEF, Hangdong District	01/14/2003	BBBBABA
8910	Chiangmai	AEF, Hangdong District	01/14/2003	BBBBABA
8911	Chiangmai	AEF, Hangdong District	01/14/2003	BBBBABA
8912	Chiangmai	AEF, Hangdong District	01/14/2003	BBBBABA
8913	Chiangmai	AEF, Hangdong District	01/14/2003	BBBBABA
8914	Chiangmai	AEF, Hangdong District	01/14/2003	BBBBABA
8915	Chiangmai	AEF, Hangdong District	01/14/2003	BCBAABA
8916	Chiangmai	AEF, Hangdong District	01/14/2003	BBBBABA

#DNA	Province	Location	Date(mm/dd/yy)	Haplotypes
8917	Chiangmai	AEF, Hangdong District	01/14/2003	BBBBABA
8918	Chiangmai	AEF, Hangdong District	01/14/2003	BBBBABA
8919	Chiangmai	AEF, Hangdong District	01/14/2003	BBBBABA
8920	Utaradit	Meuang District (Site 1)	08/14/2005	BBBBABA
8921	Utaradit	Meuang District (Site 1)	08/14/2005	BBBBABA
8922	Utaradit	Meuang District (Site 1)	08/14/2005	BBBBABA
8923	Utaradit	Meuang District (Site 1)	08/14/2005	BBBBABA
8924	Utaradit	Meuang District (Site 1)	08/14/2005	BBBBABA
8926	Utaradit	Meuang District (Site 1)	08/14/2005	BBBBABA
8929	Utaradit	Meuang District (Site 1)	08/14/2005	BBBBABA
8930	Utaradit	Meuang District (Site 2)	08/14/2005	BBBBABA
8931	Utaradit	Meuang District (Site 2)	08/14/2005	AABBBAA
8932	Utaradit	Meuang District (Site 2)	08/14/2005	BBBBABA
8933	Utaradit	Meuang District (Site 2)	08/14/2005	BBBBABA
8934	Utaradit	Meuang District (Site 2)	08/14/2005	BBBBABA
8935	Utaradit	Meuang District (Site 2)	08/14/2005	BBBBABA
8936	Utaradit	Meuang District (Site 2)	08/14/2005	BBBBABA
8937	Utaradit	Meuang District (Site 2)	08/14/2005	BBBBABA
8940	Utaradit	Meuang District (Site 2)	08/14/2005	BBBBABA
8941	Utaradit	Meuang District (Site 2)	08/14/2005	BBBBABA
8943	Chiangmai	AEF,Hangdong District	08/13/2005	AABABAA
8944	Chiangmai	AEF,Hangdong District	08/25/2005	AABBBAA
8945	Chiangmai	AEF,Hangdong District	08/25/2005	BBBBABA
8946	Chiangmai	AEF,Hangdong District	08/25/2005	BBBBABA
8948	Chiangmai	AEF,Hangdong District	08/25/2005	BBBBABA
8953	Nan	Poa District	10/29/2005	BCBAABA
8958	Nan	Poa District	10/29/2005	BCBAABA
8959	Phitsanulok	AEF, Meuang District	12/19/2005	BBBBABA
8960	Phitsanulok	AEF, Meuang District	12/19/2005	BBBBABA
8961	Phitsanulok	AEF, Meuang District	12/19/2005	BBBBABA
8962	Phitsanulok	AEF, Meuang District	01/18/2006	BCBAABA
8963	Phitsanulok	AEF, Meuang District	01/18/2006	BCBAABA
8964	Phitsanulok	AEF, Meuang District	01/18/2006	BCBAABA

#DNA	Province	Location	Date(mm/dd/yy)	Haplotypes
8965	Phitsanulok	AEF, Meuang District	01/18/2006	BCBAABA
8966	Phitsanulok	AEF, Meuang District	01/18/2006	BBBBABA
8967	Phitsanulok	AEF, Meuang District	01/18/2006	BCBAABA
8968	Phitsanulok	AEF, Meuang District	01/18/2006	BCBAABA
8969	Phitsanulok	AEF, Meuang District	01/18/2006	BCBAABA
8970	Phitsanulok	AEF, Meuang District	01/18/2006	BCBAABA
8971	Phitsanulok	AEF, Meuang District	01/18/2006	BCBAABA
8972	Phitsanulok	AEF, Meuang District	01/18/2006	BCBAABA
8973	Phitsanulok	AEF, Meuang District	01/18/2006	BCBAABA
8974	Phitsanulok	AEF, Meuang District	01/18/2006	BBBBABA
8975	Phitsanulok	AEF, Meuang District	01/18/2006	BBBBABA
8976	Phitsanulok	AEF, Meuang District	01/18/2006	BBBBABA
8977	Phitsanulok	AEF, Meuang District	01/18/2006	BCBAABA
8978	Phitsanulok	AEF, Meuang District	01/18/2006	BCBAABA
8979	Phitsanulok	AEF, Meuang District	01/18/2006	BCBAABA
8980	Phitsanulok	AEF, Meuang District	01/18/2006	BCBAABA
8981	Phitsanulok	AEF, Meuang District	01/18/2006	BCBAABA
8982	Chiangrai	Meuang District	01/15/2006	BBBBABA
8988	Loi Ed	Prathumrat District	08/31/2005	BBBBABA
8989	Udonthani	Khud Jub District	08/31/2005	BBBBABA
8990	Udonthani	Khud Jub District	08/31/2005	AABBBAA
9019	Udonthani	Khud Jub District	08/31/2005	BBBBABA
9029	Khonkaen	AEF, Meuang District	10/10/2005	BBBBABA
9056	Srakaew	Kaow Chakun District	10/03/2005	BBBBABA
9057	Srakaew	Kaow Chakun District	10/03/2005	BBBBABA
9058	Srakaew	Kaow Chakun District	10/03/2005	BBBBABA
9059	Srakaew	Kaow Chakun District	10/03/2005	BBBBABA
9060	Srakaew	Kaow Chakun District	10/03/2005	BBBBABA
9061	Srakaew	Kaow Chakun District	10/03/2005	BBBBABA
9062	Srakaew	Kaow Chakun District	10/03/2005	BCBAABA
9063	Srakaew	Kaow Chakun District	10/03/2005	BBBBABA
9064	Srakaew	Kaow Chakun District	10/03/2005	BCBAABA
9065	Srakaew	Kaow Chakun District	10/03/2005	BCBAABA

#DNA	Province	Location	Date(mm/dd/yy)	Haplotypes
9066	Srakaew	Kaow Chakun District	10/03/2005	BCBAABA
9067	Srakaew	Kaow Chakun District	10/03/2005	BBBBABA
9068	Srakaew	Kaow Chakun District	10/03/2005	BCBAABA
9069	Srakaew	Kaow Chakun District	10/03/2005	BCBAABA
9070	Srakaew	Kaow Chakun District	10/03/2005	BBBBABA
9071	Srakaew	Kaow Chakun District	10/03/2005	BBBBABA
9072	Srakaew	Kaow Chakun District	10/03/2005	BCBAABA
9073	Srakaew	Kaow Chakun District	10/03/2005	BCBAABA
9075	Srakaew	Kaow Chakun District	10/03/2005	AABBBAA
9076	Srakaew	Kaow Chakun District	10/03/2005	BCBAABA
9077	Srakaew	Kaow Chakun District	10/03/2005	BCBAABA
9078	Srakaew	Kaow Chakun District	10/03/2005	AABBBAA
9079	Srakaew	Kaow Chakun District	10/03/2005	BBBAABA
9080	Srakaew	Kaow Chakun District	10/03/2005	BBBAABA
9081	Srakaew	Kaow Chakun District	10/03/2005	BBBBABA
9082	Srakaew	Kaow Chakun District	10/03/2005	BBBBABA
9083	Srakaew	Kaow Chakun District	10/03/2005	BBBBABA
9084	Srakaew	Kaow Chakun District	10/03/2005	BBBBABA
9085	Srakaew	Kaow Chakun District	10/03/2005	BBBBABA
9151	Graz2	DRS lab.		BBBBABA
9152	Graz5	DRS lab.		BBBBABA
9153	Lunz1	DRS lab.		BBBBABA
9154	Hambruge1	DRS lab.		BBBBBAA
9155	Slovania1	DRS lab.		BCBBABA
9156	Klagenf5	DRS lab.		BBBBABA
9157	East Austria3	DRS lab.		BBBBBAA
9158	East Austria11	DRS lab.		BCBBABA
9159	CA2	DRS lab.		BBBBBAA
9160	CA3	DRS lab.		BCBBABA
9240	A.m. mel	DRS lab.		DABBCAA
9241	A.m. scu1	DRS lab.		ABAABAA
9242	A.m. scu2	DRS lab.		ABAABAA

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

II. Total of 184 *A. cerana* were selected to investigate in genomic DNA

#DNA	Population	Province	Locality	mm/dd/yy	Collector*
#6051	North	Chiang Rai	Chiang San	--/--/2001	W. N.
#5649	North	Chiang Rai	Chiang San	--/--/2001	W. N.
#5648	North	Chiang Rai	Chiang San	--/--/2001	W. N.
#5741	North	Chiang Rai	Mae Sruy	--/--/2001	W. N.
#6047	North	Chiang Rai	Mae Sruy	--/--/2001	W. N.
#5745	North	Chiang Rai	Meaung	--/--/2001	W. N.
#5644	North	Chiang Rai	Meaung	--/--/2001	W. N.
#5646	North	Chiang Rai	Meaung	--/--/2001	W. N.
#5647	North	Chiang Rai	Meaung	--/--/2001	W. N.
#8508	North	Chiang Rai	Meaung	01/15/2006	S. T.
#8509	North	Chiang Rai	Meaung	01/15/2006	S. T.
#8498	North	Prae	Sung Men	--/--/1996	S. D.
#8510	North	Nan	Pao	01/22/2006	S. T.
#8511	North	Nan	Pao	01/22/2006	S. T.
#8493	North	Chiang Mai	Doi Saked	--/--/1996	S. D.
#8499	North	Chiang Mai	Chiang Dow	--/--/1996	S. D.
#5744	North	Chiang Mai	San Pa Tong	--/--/2001	W. N.
#5650	North	Chiang Mai	Sara Pae	--/--/2001	W. N.
#8506	North	Chiang Mai	Mae Rim	01/13/2006	S. T.
#8507	North	Chiang Mai	Mae Rim	01/13/2006	S. T.
#5742	North	Chiang Mai	Meaung	--/--/2001	W. N.
#8502	North	Chiang Mai	Meaung	01/13/2006	S. T.
#8503	North	Chiang Mai	Meaung	01/13/2006	S. T.
#8504	North	Chiang Mai	Meaung	01/13/2006	S. T.
#8505	North	Chiang Mai	Meaung	01/13/2006	S. T.
#8492	North	Lumpoon	Mae Tha	--/--/1996	S. D.
#8478	North	Lumpoon	Meaung	09/07/1996	S. D.
#8496	North	Lumpang	Ngaw	--/--/1996	S. D.
#8500	North	Uttaradit	Meaung	08/14/2005	S. T.
#8501	North	Uttaradit	Meaung	08/14/2005	S. T.
#8490	North	Tak	Samkao	--/--/1996	S. D.
#8475	Northeast	Udonthani	Kud Jub	01/07/2006	S. T.
#8458	Northeast	Udonthani	Nong Voew Saw	12/07/1996	S. D.

#DNA	Population	Province	Locality	mm/dd/yy	Collector*
#8459	Northeast	Udonthani	Meaung	12/07/1996	S. D.
#8460	Northeast	Nong Khay	Meaung	12/08/1996	S. D.
#8461	Northeast	Nong Khay	Meaung	12/08/1996	S. D.
#8470	Northeast	Nong Khay	Tha Bo	08/31/2005	S. T.
#8463	Northeast	Nakhonratchasrima	Nong Boon Nak	12/10/1996	S. D.
#8454	Northeast	Khonkaen	Chum Prae	12/06/1996	S. D.
#8467	Northeast	Srisaket	Pra Sart	03/27/2004	S. T.
#8468	Northeast	Yasothorn	Pa Tiw	08/29/2005	S. T.
#8472	Northeast	Mukdaharn	Pa Yai	01/06/2006	S. T.
#8473	Northeast	Sakhulnakorn	Meaung	01/07/2006	S. T.
#8469	Northeast	Loi Ed	Prathumrat	08/30/2005	S. T.
#8464	Northeast	Loi Ed	Jaturapatpimarn	03/26/2004	S. T.
#8465	Northeast	Loi Ed	Prathumrat	03/26/2004	S. T.
#8466	Northeast	Loi Ed	Jaturapatpimarn	03/26/2004	S. T.
#8476	Northeast	Chaiyaphum	Pukeaw	01/08/2006	S. T.
#8471	Northeast	Khonkaen	Lum Pra Pleng	10/15/2003	S. T.
#8455	Northeast	Khonkaen	Chum Prae	12/06/1996	S. D.
#8456	Northeast	Nhongbualumpoo	Non Lung	12/07/1996	S. D.
#8457	Northeast	Nhongbualumpoo	Non Lung	12/07/1996	S. D.
#8474	Northeast	Sakhulnakorn	Punnanikum	01/07/2006	S. T.
#8462	Northeast	Sakhulnakorn	Phung Khon	12/09/1996	S. D.
#8477	Northeast	Ubolratchathani	Chong Mek	01/06/2006	S. T.
#8479	Central	Phitsanulok	Meaung	09/10/1996	S. D.
#8481	Central	Phitsanulok	Meaung	09/10/1996	S. D.
#8480	Central	Phitsanulok	Meaung	09/10/1996	S. D.
#5746	Central	Phitsanulok	Bang La Ka	--/--/2001	W. N.
#8482	Central	Nakhonsawan	Payuhakiri	02/09/1996	S. D.
#8486	Central	Nakhonsawan	Bunpotpisai	06/05/1996	S. D.
#5748	Central	Nakhonsawan	Kork Pra	--/--/2001	W. N.
#8487	Central	Nakhonsawan	Bunpotpisai	06/05/1997	S. D.
#6053	Central	Nakhonsawan	Kork Pra	--/--/2001	W. N.
#8483	Central	Chainat	Manorom	06/05/1997	S. D.
#8484	Central	Chainat	Wat Shing	06/06/1997	S. D.
#8485	Central	Chainat	Meaung	06/06/1997	S. D.

#DNA	Population	Province	Locality	mm/dd/yy	Collector*
#5749	North	Kranjanaburi	Si Yok	--/--/2001	W. N.
#6034	Central	Kranjanaburi	Si Yok	--/--/2001	W. N.
#6033	Central	Ang Thong	Po Thong	--/--/2001	W. N.
#6052	Central	Ang Thong	Po Thong	--/--/2001	W. N.
#5747	Central	Ang Thong	Po Thong	--/--/2001	W. N.
#8428	Central	Supanburi	Dan Chang	01/23/1996	S. D.
#8434	Central	Trat	Kaow Saming	03/07/1996	S. D.
#6262	Central	Trat	Kaow Saming	--/--/2001	W. N.
#6263	Central	Trat	Kaow Saming	--/--/2001	W. N.
#5751	Central	Trat	Kaow Saming	--/--/2001	W. N.
#6035	Central	Chanthaburi	Khlung	--/--/2001	W. N.
#8447	Central	Chanthaburi	Khlung	06/28/2004	S. T.
#8446	Central	Samurtsongkhram	Meaung	09/12/2005	S. T.
#5621	Central	Samurtsongkhram	Meaung	--/--/2001	W. N.
#8448	Central	Samurtsongkhram	Meaung	11/09/2005	S. T.
#8449	Central	Samurtsongkhram	Meaung	11/09/2005	S. T.
#8450	Central	Samurtsongkhram	Meaung	11/09/2005	S. T.
#8451	Central	Samurtsongkhram	Meaung	11/09/2005	S. T.
#6045	Central	Samurtsongkhram	Meaung	--/--/2001	W. N.
#5622	Central	Samurtsongkhram	Meaung	--/--/2001	W. N.
#5625	Central	Petchaburi	Ban Lad	--/--/2001	W. N.
#5629	Central	Petchaburi	Ban Lad	--/--/2001	W. N.
#8425	Prachuap	Prachuap kerekhun	Sam Roi Yoth	12/26/2005	S. T.
#8424	Prachuap	Prachuap kerekhun	Sam Roi Yoth	12/26/2005	S. T.
#5627	Prachuap	Prachuap kerekhun	Pranburi	--/--/2001	W. N.
#5626	Prachuap	Prachuap kerekhun	Pranburi	--/--/2001	W. N.
#5630	Prachuap	Prachuap kerekhun	Bang Sa Pan	--/--/2001	W. N.
#5752	Prachuap	Prachuap kerekhun	Meaung	--/--/2001	W. N.
#5753	Prachuap	Prachuap kerekhun	Meaung	--/--/2001	W. N.
#6036	Prachuap	Prachuap kerekhun	Meaung	--/--/2001	W. N.
#8412	Prachuap	Prachuap kerekhun	Meaung	12/24/2005	S. T.
#8413	Prachuap	Prachuap kerekhun	Meaung	12/24/2005	S. T.
#8414	Prachuap	Prachuap kerekhun	Meaung	12/24/2005	S. T.
#8415	Prachuap	Prachuap kerekhun	Meaung	12/24/2005	S. T.

#DNA	Population	Province	Locality	mm/dd/yy	Collector*
#8416	Prachuap	Prachuap Khiri Khan	Meaung	12/24/2005	S. T.
#8417	Prachuap	Prachuap Khiri Khan	Meaung	12/24/2005	S. T.
#8418	Prachuap	Prachuap Khiri Khan	Meaung	12/24/2005	S. T.
#8419	Prachuap	Prachuap Khiri Khan	Meaung	12/24/2005	S. T.
#8420	Prachuap	Prachuap Khiri Khan	Meaung	12/24/2005	S. T.
#8421	Prachuap	Prachuap Khiri Khan	Meaung	12/24/2005	S. T.
#5632	Prachuap	Prachuap Khiri Khan	Bang Sa Pan	--/--/2001	W. N.
#5634	Prachuap	Prachuap Khiri Khan	Bang Sa Pan	--/--/2001	W. N.
#5633	Prachuap	Prachuap Khiri Khan	Bang Sa Pan	--/--/2001	W. N.
#5635	Prachuap	Prachuap Khiri Khan	Bang Sa Pan	--/--/2001	W. N.
#5637	Prachuap	Prachuap Khiri Khan	Bang Sa Pan	--/--/2001	W. N.
#8441	Prachuap	Prachuap Khiri Khan	Bang Sa Pan	--/--/1999	S. D.
#5636	Prachuap	Prachuap Khiri Khan	Bang Sa Pan	--/--/2001	W. N.
#8442	Prachuap	Prachuap Khiri Khan	Tab Sa Kae	05/13/1999	S. D.
#8440	Prachuap	Prachuap Khiri Khan	Tab Sa Kae	--/--/1999	S. D.
#8422	Chumporn	Chumporn	Meaung	12/25/2005	S. T.
#8423	Chumporn	Chumporn	Meaung	12/25/2005	S. T.
#8403	Chumporn	Chumporn	Meuang	10/18/2003	S. T.
#8371	Chumporn	Chumporn	Meuang	05/16/1996	S. D.
#8372	Chumporn	Chumporn	Meuang	05/16/1996	S. D.
#8364	Chumporn	Chumporn	Meaung	03/25/1996	S. D.
#8406	Chumporn	Chumporn	Tha-sae	10/19/2003	S. T.
#8405	Chumporn	Chumporn	Tha-sae	10/19/2003	S. T.
#5639	Chumporn	Chumporn	Tha-sae	--/--/2001	W. N.
#5755	Chumporn	Chumporn	Sawee	--/--/2001	W. N.
#5756	Chumporn	Chumporn	Sawee	--/--/2001	W. N.
#5638	Chumporn	Chumporn	Sawee	--/--/2001	W. N.
#5757	Chumporn	Chumporn	Sawee	--/--/2001	W. N.
#6266	Chumporn	Chumporn	Sawee	--/--/2001	W. N.
#6267	Chumporn	Chumporn	Sawee	--/--/2001	W. N.
#6038	Chumporn	Chumporn	Sawee	--/--/2001	W. N.
#6040	Chumporn	Chumporn	Sawee	--/--/2001	W. N.
#6269	Chumporn	Chumporn	Sawee	--/--/2001	W. N.
#6270	Chumporn	Chumporn	Sawee	--/--/2001	W. N.

#DNA	Population	Province	Locality	mm/dd/yy	Collector*
#6271	Chumporn	Chumporn	Sawee	--/--/2001	W. N.
#6272	Chumporn	Chumporn	Sawee	--/--/2001	W. N.
#8370	Chumporn	Chumporn	Sawee	05/16/1996	S. D.
#6273	Chumporn	Chumporn	Sawee	--/--/2001	W. N.
#5754	Chumporn	Chumporn	Suan Pheng	--/--/2001	W. N.
#6037	Chumporn	Chumporn	Suan Pheng	--/--/2001	W. N.
#8397	Chumporn	Chumporn	Pra-Til	08/--/2004	S. W.
#5758	Chumporn	Ra-Nong	Krapher	--/--/2001	W. N.
#6041	Chumporn	Ra-Nong	Krapher	--/--/2001	W. N.
#8373	Chumporn	Ra-Nong	Krapher	03/21/1996	S. D.
#8374	Chumporn	Ra-Nong	Krapher	03/22/1996	S. D.
#8375	Chumporn	Ra-Nong	Rathchakrut,	03/23/1996	S. D.
#8365	Peninsular	Surat-thani	Thachana	05/12/1996	S. D.
#8366	Peninsular	Surat-thani	Thachana	05/12/1996	S. D.
#8377	Peninsular	Surat-thani	Phanum	03/24/1996	S. D.
#5641	Peninsular	Surat-thani	Ko Samui	--/--/2001	W. N.
#5642	Peninsular	Surat-thani	Ko Samui	--/--/2001	W. N.
#6258	Peninsular	Surat-thani	Ko Samui	--/--/2001	W. N.
#6259	Peninsular	Surat-thani	Ko Samui	--/--/2001	W. N.
#6260	Peninsular	Surat-thani	Ko Samui	--/--/2001	W. N.
#5640	Peninsular	Surat-thani	Ko Samui	--/--/2001	W. N.
#5643	Peninsular	Surat-thani	Ko Samui	--/--/2001	W. N.
#8389	Peninsular	Nakorn Sri Thammasrat	Chalermphakret	03/28/1997	S. D.
#5759	Peninsular	Nakorn Sri Thammasrat	Sichon	--/--/2001	W. N.
#6042	Peninsular	Nakorn Sri Thammasrat	Sichon	--/--/2001	W. N.
#6043	Peninsular	Nakorn Sri Thammasrat	Sichon	--/--/2001	W. N.
#6054	Peninsular	Nakorn Sri Thammasrat	Sichon	--/--/2001	W. N.
#6055	Peninsular	Nakorn Sri Thammasrat	Thasala	--/--/2001	W. N.
#6056	Peninsular	Nakorn Sri Thammasrat	Thasala	--/--/2001	W. N.
#6057	Peninsular	Nakorn Sri Thammasrat	Thasala	--/--/2001	W. N.
#6058	Peninsular	Nakorn Sri Thammasrat	Lansaka	--/--/2001	W. N.
#5760	Peninsular	Nakorn Sri Thammasrat	Lansaka	--/--/2001	W. N.
#6059	Peninsular	Nakorn Sri Thammasrat	Lansaka	--/--/2001	W. N.

#8407	Peninsular	Patthalung	Meuang	10/21/2003	S. T.

#DNA	**Population**	**Province**	**Locality**	**mm/dd/yy**	**Collector***
#8376	Peninsular	Phung-Pha	Khuyburi	03/23/1997	S. D.
#8363	Peninsular	Phuket	Ta Lang	01/22/1996	S. D.
#6256	Peninsular	Phuket	Kra Tu	--/--/2001	W. N.
#5743	Peninsular	Phuket	Kra Tu	--/--/2001	W. N.
#8367	Peninsular	Phuket	Meaung	05/13/1996	S. D.
#8368	Peninsular	Phuket	Meaung	05/13/1996	S. D.
#8369	Peninsular	Phuket	Ta-lang	05/14/1996	S. D.
#8379	Peninsular	Krabi	Meaung	03/25/1997	S. D.
#8378	Peninsular	Krabi	Oua Lek	03/24/1997	S. D.
#6044	Peninsular	Trang	Si Kao	--/--/2001	W. N.
#6060	Peninsular	Trang	Si Kao	--/--/2001	W. N.
#8388	Peninsular	Song-kla	Meuang	03/27/1997	S. D.
#8386	Peninsular	Song-kla	Hat Yai	03/27/1997	S. D.
#8408	Peninsular	Song-Kla	Sa Daew	10/22/2003	S. T.
#5762	Peninsular	Song-Kla	Kra Say Sin	--/--/2001	W. N.

- Collecteors* referred to W. N. = Warrit, N.; S. D. = Sihanunthavong, D.;

S. W. = Sukthawornjaroenporn, W. and S. T. = Suppasat, T.

- #DNA referred running series number of honey bees DNA into DRS laboratory

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

III. Total of 73 *Varroa* mite collections were selected to investigation.

1.) Total of 42 *Varroa* samples parasitized on *A. mellifera* colonies.

#DNA (*Varroa*)	*Varroa* attached on their host	Locality (Provinces)	#DNA (host)	Date mm/dd/yy	MtDNA Haplotypes
#9161	Phoretic/adult	Surat Thani	#8705	10/20/2003	'ThaiV3'
#9162	Phoretic/adult	Song-Kla	#8736	10/24/2003	'ThaiV3'
#9163	Phoretic/adult	Song-Kla	#8741	10/24/2003	'ThaiV3'
#9164	Phoretic/adult	Song-Kla	#8742	10/24/2003	'ThaiV3'
#9165	Phoretic/adult	Song-Kla	#8743	10/24/2003	'ThaiV3'
#9166	Phoretic/adult	Song-Kla	#8750	10/24/2003	'ThaiV3'
#9167	Phoretic/adult	Khonkaen	#8885	5/15/2004	'ThaiV3'
#9168	Phoretic/adult	Khonkaen	#8889	5/15/2004	'ThaiV3'
#9169	Bottom hive*	Utaradit	#8920	8/14/2005	'ThaiV1'
#9170	Bottom hive*	Utaradit	#8921	8/14/2005	'ThaiV3'
#9171	worker brood	Utaradit	#8922	8/14/2005	'ThaiV1'
#9172	Phoretic/adult	Utaradit	#8923	8/14/2005	'ThaiV3'
#9173	worker brood	Utaradit	#8924	8/14/2005	'ThaiV3'
#9174	Phoretic/adult	Utaradit	#8926	8/14/2005	'ThaiV3'
#9175	Phoretic/adult	Utaradit	#8929	8/14/2005	'ThaiV3'
#9176	worker brood	Utaradit	#8930	8/14/2005	'ThaiV2'
#9177	Bottom hive*	Utaradit	#8931	8/14/2005	'ThaiV3'
#9178	Bottom hive*	Utaradit	#8932	8/14/2005	'ThaiV3'
#9179	Phoretic/adult	Utaradit	#8933	8/14/2005	'ThaiV3'
#9180	Bottom hive*	Utaradit	#8934	8/14/2005	'ThaiV3'
#9181	Bottom hive*	Utaradit	#8935	8/14/2005	'ThaiV1'
#9182	Bottom hive*	Utaradit	#8936	8/14/2005	'ThaiV3'
#9183	Phoretic/adult	Utaradit	#8937	8/14/2005	'ThaiV3'
#9184	worker brood	Utaradit	#8940	8/14/2005	'ThaiV3'
#9185	worker brood	Utaradit	#8941	8/14/2005	'ThaiV3'
#9186	worker brood	Chiangmai	#8943	8/25/2005	'ThaiV3'
#9187	worker brood	Chiangmai	#8944	8/25/2005	'ThaiV3'
#9188	Bottom hive*	Chiangmai	#8945	8/25/2005	'ThaiV3'
#9189	Bottom hive*	Chiangmai	#8946	8/25/2005	'ThaiV3'
#9190	Bottom hive*	Chiangmai	#8948	8/25/2005	'ThaiV3'

#DNA (*Varroa*)	*Varroa* attached on their host	Locality (Provinces)	#DNA (host)	Date mm/dd/yy	MtDNA Haplotypes
#9191	Bottom hive*	Nan	#8953	10/29/2005	'ThaiV3'
#9192	Bottom hive*	Nan	#8958	10/29/2005	'ThaiV3'
#9193	worker brood	Phitsanulok	#8959	1/18/2006	'ThaiV3'
#9194	Phoretic/adult	Phitsanulok	#8961	1/18/2006	'ThaiV3'
#9195	worker brood	Phitsanulok	#8962	1/18/2006	'ThaiV3'
#9196	worker brood	Phitsanulok	#8975	1/18/2006	'ThaiV3'
#9197	Bottom hive*	Phitsanulok	#8976	1/18/2006	'ThaiV3'
#9198	worker brood	Phitsanulok	#8977	1/18/2006	'ThaiV3'
#9199	worker brood	Phitsanulok	#8978	1/18/2006	'ThaiV3'
#9200	worker brood	Phitsanulok	#8979	1/18/2006	'ThaiV3'
#9201	worker brood	Phitsanulok	#8980	1/18/2006	'ThaiV3'
#9202	worker brood	Phitsanulok	#8981	1/18/2006	'ThaiV3'
#9203	Phoretic/adult	Loi Ed	#8988	8/30/2005	'ThaiV3'
#9204	worker brood	Udonthani	#8989	8/31/2005	'ThaiV3'
#9205	worker brood	Udonthani	#8990	8/31/2005	'ThaiV3'
#9206	drone brood	Udonthani	#9019	8/31/2005	'ThaiV3'
#9207	worker brood	Khonkaen	#9029	10/10/2005	'ThaiV3'
#9208	worker brood	Srakaew	#9066	10/3/2005	'ThaiV3'

2.) Total of 31 *Varroa* samples parasitized on *A. cerana* colonies.

#DNA (*Varroa*)	*Varroa* attached on their host	Locality (Provinces)	#DNA (host)	Date mm/dd/yy	MtDNA Haplotypes
#9209	drone brood	Loi Ed	#8464	8/29/2005	'NothThai'
#9210	drone brood	Loi Ed	#8466	8/30/2005	'NothThai'
#9211	worker brood	Yasothron	#8468	8/29/2005	'NothThai'
#9212	drone brood	Loi Ed	#8469	8/30/2005	'NothThai'
#9213	worker brood	Sakhulnakorn	#8474	1/7/2006	'NothThai'
#9214	drone brood	Udonthani	#8475	1/7/2006	'NothThai'
#9215	drone brood	Samurtsongkhram	#8446	11/19/2005	'NothThai'
#9216	worker brood	Samurtsongkhram	#8448	11/19/2005	'NothThai'
#9217	drone brood	Samurtsongkhram	#8449	11/19/2005	'NothThai'
#9218	worker brood	Samurtsongkhram	#8450	11/19/2005	'NothThai'

#DNA (*Varroa*)	*Varroa* attached on their host	Locality (Provinces)	#DNA (host)	Date mm/dd/yy	MtDNA Haplotypes
#9219	worker brood	Samurtsongkhram	#8451	11/19/2005	'NothThai'
#9220	drone brood	Chumporn	No	2/10/2004	'Malaysia'
#9221	drone brood	Chumporn	No	2/10/2004	'Malaysia'
#9222	drone brood	Samurtsongkhram	No	2/10/2004	'Malaysia'
#9223	drone brood	Chumporn	No	2/10/2004	'Malaysia'
#9224	drone brood	Petchaburi	#8409	12/24/2005	'NothThai'
#9225	drone brood	Prachuap Khiri Khan	#8411	12/24/2005	'NothThai'
#9226	drone brood	Prachuap Khiri Khan	#8412	12/24/2005	'NothThai'
#9227	worker brood	Prachuap Khiri Khan	#8413	12/24/2005	'NothThai'
#9228	drone brood	Prachuap Khiri Khan	#8414	12/24/2005	'NothThai'
#9229	worker brood	Prachuap Khiri Khan	#8416	12/24/2005	'NothThai'
#9230	drone brood	Prachuap Khiri Khan	#8417	12/24/2005	'NothThai'
#9231	drone brood	Prachuap Khiri Khan	#8418	12/24/2005	'NothThai'
#9232	drone brood	Prachuap Khiri Khan	#8419	12/24/2005	'NothThai'
#9233	drone brood	Prachuap Khiri Khan	#8420	12/24/2005	'NothThai'
#9234	worker brood	Utaradit	#8500	8/14/2005	'NothThai'
#9235	drone brood	Chiangmai	#8502	1/13/2006	'NothThai'
#9236	drone brood	Chiangmai	#8507	1/14/2006	'Vietnam'
#9237	worker brood	Chiangrai	#8508	1/15/2006	'Vietnam'
#9238	drone brood	Nan	#8510	1/22/2006	'Vietnam'
#9239	drone brood	Samurtsongkhram	No	8/29/2004	'NothThai'

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

BIOGRAPHY

Miss Tipwan Suppasat was born on October 12th, 1977 at Chantaburi Province, Thailand. She graduated her Bacheler's Degree (B.Sc.) in Biology on 1999 and received her Master's Degree (M.S.) in Zoology on 2002 from Chulalongkorn University. Among 2001-2006, she was supported scholarship from Naresuan University for her study Master and Doctoral Degrees. Also, she was supported from RTA45008-Siriwat Wongsiri grant from Thailand Research Fund (TRF) to Center of Excellence in Entomology, Department of Biology, Faculty of Science, Chulalongkorn University for her study and experience. In 2004, she awarded the research fund of Royal Golden Jubilee Ph. D. Program of Thailand Research Fund for her study Ph.D. in Biological Sciences Program, Faculty of Science, Chulalongkorn University. In 2006, she went to The University of Kansas, Lawrence, Kansas, USA for her Ph.D. research. At present she works at School of Science and Technology, Naresuan University (Payao Campus), Payao Province, Thailand.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย