การระบุสปีชีส์และความหลากหลายทางพันธุกรรมของชันโรง *Tetragonilla collina* ในประเทศไทยโดยการวิเคราะห์ด้วยเอเอฟแอลพีและพีซีอาร์-เอสเอสซีพี

นางสาว มณฑลี ธีรอภิศักดิ์กุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเคมี ภาควิชาชีวเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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SPECIES IDENTIFICATION AND GENETIC DIVERSITY OF STINGLESS BEE *Tetragonilla collina* IN THAILAND USING AFLP AND PCR-SSCP ANALYSES

Miss Montalee Theeraapisakkun

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biochemistry
Department of Biochemistry
Faculty of Science
Chulalongkorn University
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Thesis Title	SPECIES IDENTIFICATION AND GENETIC DIVERSITY OF STINGLESS BEE *Tetragonilla collina* IN THAILAND USING AFLP AND PCR-SSCP ANALYSES
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มณฑลี ธีรอภิศักดิ์กุล : การระบุสปีชีส์และความหลากหลายทางพันธุกรรมของชันโรง *Tetragonilla collina* ในประเทศไทยโดยการวิเคราะห์ด้วยเอเอฟแอลพีและพีซีอาร์-เอสเอสซีพี (SPECIES IDENTIFICATION AND GENETIC DIVERSITY OF STINGLESS BEE *Tetragonilla collina* IN THAILAND USING AFLP AND PCR-SSCP ANALYSES) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร. ศิริพร สิทธิประณีต, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร. ศิราวุธ กลิ่นบุหงา และ Professor Deborah R. Smith, Ph.D., 133 หน้า.

การค้นหาเครื่องหมายพันธุกรรมที่จำเพาะต่อชนิดชันโรง *Tetragonilla collina* โดยเทคนิคเอเอฟแอลพี เมื่อวิเคราะห์ตัวอย่างดีเอ็นเอของชันโรง 11 ชนิดด้วยไพรเมอร์ทั้งหมด 64 คู่ผสม พบแถบดีเอ็นเอขนาด 316 คู่เบสที่ปรากฏเฉพาะใน *T. collina* จึงทำการโคลนและหาลำดับนิวคลีโอไทด์เพื่อออกแบบคู่ไพรเมอร์ (CUTc1-F/R) และทดสอบความจำเพาะของคู่ไพรเมอร์กับชันโรง 15 ชนิด (239 รัง) พบผลิตภัณฑ์จากปฏิกิริยาลูกโซ่โพลิเมอเรสขนาด 259 คู่เบสปรากฏใน *T. collina* (134/134 รัง, 100%) และ *T. pagdeni* (43/51 รัง, 84.3%) แต่ไม่พบในชันโรงชนิดอื่น เมื่อนำเทคนิค SSCP มาใช้ค้นหาความแตกต่างของผลิตภัณฑ์ที่ได้ พบรูปแบบ SSCP ของ *T. collina* ต่างจากรูปแบบของ *T. pagdeni* อย่างชัดเจน นอกจากนี้ยังพบผลิตภัณฑ์จากปฏิกิริยาลูกโซ่โพลิเมอเรสของชันโรง *T. collina* แสดงจีโนไทป์ 3 แบบ โดยจีโนไทป์แบบ AA (259/259 คู่เบส) พบในชันโรงทุกตัวจากภาคเหนือ (21 รัง) และภาคตะวันออกเฉียงเหนือ (32 รัง) และพบในชันโรงจากภาคกลาง 23 รังจากทั้งหมด 28 รัง ขณะที่ชันโรงส่วนใหญ่จากภาคใต้ (42/53 รัง) พบจีโนไทป์แบบ BB (253/253 คู่เบส) ส่วนจีโนไทป์แบบผสม AB (259/253 คู่เบส) พบในชันโรงจากประจวบคีรีขันธ์ซึ่งอยู่ใกล้ Kra ecotone (แนวเปลี่ยนของสังคมพืชและสัตว์) จำนวน 5 รังจาก 28 รังและอีก 11 รังจาก 53 รังซึ่งอยู่ห่างจาก Kra ecotone ลงไปทางใต้ ผลจากการกระจายตัวของจีโนไทป์ของเครื่องหมายพันธุกรรม CUTc1 สามารถจำแนกกลุ่มประชากรของชันโรง *T. collina* ในประเทศไทยได้อย่างชัดเจน เมื่อทำการศึกษาความหลากหลายทางพันธุกรรมของชันโรง *T. collina* โดยเทคนิคลายพิมพ์ดีเอ็นเอ (TE-AFLP) พบความหลากหลายทางพันธุกรรมสูงภายในแต่ละกลุ่มประชากรของ *T. collina* เมื่อใช้ AMOVA คำนวณค่า Φ_{PT} เพื่อเปรียบเทียบความแตกต่างทางพันธุกรรมระหว่างกลุ่มประชากรชันโรง พบความแตกต่างทางพันธุกรรมระหว่างกลุ่มประชากรชันโรงภาคเหนือ กลาง ตะวันออกเฉียงเหนือ และใต้ของประเทศไทยอย่างมีนัยสำคัญ (Φ_{PT} = 0.258, P = 0.001) และพบความแตกต่างทางพันธุกรรมที่น้อยกว่าแต่มีนัยสำคัญระหว่างกลุ่มประชากรชันโรงทางตอนเหนือและตอนใต้ของ Kra ecotone (Φ_{PT} = 0.207, P = 0.001) นอกจากนี้เมื่อศึกษาความหลากหลายทางพันธุกรรมของชันโรง *T. collina* โดยใช้เครื่องหมายพันธุกรรม TECU ซึ่งได้จากบริเวณหนึ่งของลายพิมพ์ดีเอ็นเอ (TE-AFLP) พบความแตกต่างทางพันธุกรรมสูงสุดระหว่างกลุ่มประชากรชันโรง 4 กลุ่ม ได้แก่ กลุ่มประชากรซึ่งรวมชันโรงภาคเหนือ กลาง ตะวันออกเฉียงเหนือเข้าด้วยกัน กลุ่มประชากรจากประจวบคีรีขันธ์ กลุ่มประชากรจากชุมพร และกลุ่มประชากรภาคใต้ (Φ_{PT} = 0.903, P = 0.001) ในทำนองเดียวกัน การศึกษาความหลากหลายทางพันธุกรรมในยีน 16S rRNA, COI และ cytb โดยเทคนิค SSCP พบความหลากหลายทางพันธุกรรมสูงภายในแต่ละกลุ่มประชากรของ *T. collina* เมื่อคำนวณค่า Φ_{PT} ของแต่ละยีน (16S rRNA, COI และ cytb) โดย AMOVA พบความแตกต่างทางพันธุกรรมสูงระหว่างกลุ่มประชากรชันโรงภาคเหนือ กลาง ตะวันออกเฉียงเหนือ ประจวบคีรีขันธ์ ชุมพร และภาคใต้ของประเทศไทย (Φ_{PT} = 0.563, Φ_{PT} = 0.204 และ Φ_{PT} = 0.294, P = 0.001 ตามลำดับ) ขณะที่พบความแตกต่างทางพันธุกรรมอย่างมีนัยสำคัญระหว่างกลุ่มประชากรชันโรงทางตอนเหนือและตอนใต้ของ Kra ecotone (Φ_{PT} = 0.334, Φ_{PT} = 0.106 และ Φ_{PT} = 0.133, P = 0.001 ตามลำดับ) เช่นกัน

ภาควิชา..... ชีวเคมี..... ลายมือชื่อนิสิต มณฑลี ธีรอภิศักดิ์กุล

สาขาวิชา..... ชีวเคมี..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา..... 2553..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

4773829823: MAJOR BIOCHEMISTRY
KEYWORDS : STINGLESS BEES / *Tetragonilla collina* / SPECIES-SPECIFIC MARKER / GENETIC DIVERSITY / POPULATION DIFFERENTIATION

MONTALEE THEERAAPISAKKUN : SPECIES IDENTIFICATION AND GENETIC DIVERSITY OF STINGLESS BEE *Tetragonilla collina* IN THAILAND USING AFLP AND PCR-SSCP ANALYSES. THESIS ADVISOR : ASSOC. PROF. SIRIPORN SITTIPRANEED, Ph.D., THESIS CO-ADVISOR : SIRAWUT KLINBUNGA, Ph.D., PROF. DEBORAH R. SMITH, Ph.D., 133 pp.

A molecular maker for authenticating species origin of the stingless bee (*Tetragonilla collina*) was developed. Initially, amplified fragment length polymorphism analysis was made of 11 stingless bee species using 64 primer combinations. A 316-bp band found only in *T. collina* was cloned and sequenced. A primer pair (CUTc1-F/R) was designed and tested for specificity in 15 stingless bee species (239 nests). The expected 259-bp fragment was consistently amplified in all *T. collina* individuals (134/134 nests, 100%). Cross-species amplification was observed in *T. pagdeni* (43/51 nests; 84.3%), but not in other species. SSCP analysis of CUTc1 unambiguously differentiated *T. collina* from *T. pagdeni*. CUTc1 generated three genotypes in Thai *T. collina* (134 nests). An AA (259/259 bp) genotype was found in all stingless bees from the north (21 nests) and northeast (32 nests), and 23/28 nests from the Central region, whereas a BB (253/253 bp) genotype was observed in most samples from peninsular Thailand (42/53 nests). Heterozygotes exhibiting the AB (259/253 bp) genotype were observed in 5 of 28 nests from Prachuap Khiri Khan located slightly above the Kra ecotone and 11 of 53 nests originated further south of the Kra ecotone. Genotype distribution patterns of CUTc1 clearly indicated intraspecific population differentiation of Thai *T. collina*. The regional genetic variation of Thai *T. collina* was investigated using DNA fingerprinting technique, three-enzyme amplified fragment length polymorphisms (TE-AFLPs) and Analysis of Molecular Variance (AMOVA). High levels of genetic variation among individuals in each population were detected. AMOVA analysis indicated significant genetic differentiation among the four geographic regions; North, Central, Northeast, and Peninsular Thailand (Φ_{PT} = 0.258, P = 0.001). The smaller but significant differentiation between samples from North and South of Isthmus of Kra was also detected (Φ_{PT} = 0.207, P = 0.001). The genetic differentiation in *T. collina* was also analyzed by TE-AFLP derived SCAR marker (TECU marker) using SSCP analysis. The greatest differentiation was detected among 4 populations; North+Central+Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular Thailand (Φ_{PT} = 0.903, P = 0.001). The SSCP analysis of the 16S rRNA, COI, and cytb genes was also used to clarify the mtDNA diversity of *T. collina*. High levels of genetic variation among individuals within each population were observed. AMOVA analysis of the 16S rRNA, COI, and cytb genes showed high genetic differentiation among 6 populations; North, Central, Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular Thailand (Φ_{PT} = 0.563, Φ_{PT} = 0.204, and Φ_{PT} = 0.294, P = 0.001, respectively) while the significant genetic differentiations between samples from North and South of Isthmus of Kra were also found (Φ_{PT} = 0.334, Φ_{PT} = 0.106, and Φ_{PT} = 0.133, P = 0.001, respectively).

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Co-Advisor's Signature S. Klinbunga
Co-Advisor's Signature

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Finally, I wish to dedicate this dissertation to my parents for their love, care, understanding, encouragement, and doing whatever they can do for me throughout my study.

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LIST OF ABBREVIATIONS

^{o}C	degree celcius
^{o}N	degree of north latitude
µl	microlitre
µM	micromolar
bp	base pair
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
mtDNA	mitochondrial deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid (disodium salt)
EtBr	ethidium bromide
IPTG	isopropyl-thiogalactoside
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mmol	millimole
pmol	picomole
ng	nanogram
OD	optical density
PCR	polymerase chain reaction
RNA	Ribonucleic acid
RNase A	Ribonuclease A
SDS	Sodium dodecyl sulfate
TE	Tris EDTA
Tris	Tris(hydroxyl methyl) aminomethane

CHAPTER I

INTRODUCTION

1.1 Stingless bee biology

1.1.1 Division of labor

Social life in the stingless bees involves division of labor which is similar to that in honeybees: very young bees start to work with wax and cerumen, away from the brood nest, then some of them shifts towards building and provisioning brood cells, during which period eggs may be laid. From brood care they go to foraging: first they receive incoming nectar and dehydrate it before becoming actual foragers (Bassindale, 1955; Velthuis, 1997). Only the females are divided into castes which are queens and workers. They are morphologically very different. Stingless bees are highly eusocial. The queen is unable to live alone nor do workers alone form viable colonies because the queen never forages and workers can not mate and produce female off-spring (Michener, 2007). The different activities among workers are related to ages. The sequence of tasks in workers is divided into five stages as follows: (1) self-grooming (during the first hours after emergence from the pupae); (2) incubation and repairs in the brood chamber; (3) construction and provisioning of cells, cleaning of the nest, and feeding young adults and the queen; (4) further cleaning of the nest, reconstruction of the involucrum, reception of nectar, and guard duty at the entrance of nest; (5) collection of pollen, nectar, and propolis. The duration of of each stage depends on the species and on the condition of the colony (Wille, 1983).

1.1.2 Nest architecture of stingless bees

The nests of stingless bees are more elaborate and complex than those of most other bees. Their nesting sites are diverse. Nests of most species are built within protective cavities such as hollow trees or in the ground. Some species establish nests within nests of termites or ants. Few species build their nests in exposed positions (Roubik, 1979; Sakagami et al., 1983; Sakagami et al., 1989; Sommeijer, 1999). In general term, stingless bees may build solid batumen plates to shield and protect the colony. Nests are then constructed using wax in a mixture with resins or gum, mud,

feces, or other materials collected by the bees. The nest entrance provides access into the nest where the brood is located. A mixture of wax with resins, which is called cerumen, is used to build involucrum sheaths as a protective layer or sheath around the brood chamber, called brood involucrum, or around the whole colony, called external involucrum, including the storage vessels for honey and pollen (Figure 1.1). In principle, there are two cell types in nest of stingless bees: brood cell and storage pots (Figure 1.2). Brood cells can be clustered or arranged in combs that are usually positioned in a horizontal plane. Outside the brood involucrum, small pots with food provisions are built in clusters. All of these nest characteristics are variable across the stingless bees (Michener, 1961; Wille and Michener, 1973; Sakagami, 1982; Wille, 1983; Roubik, 2006).

1.1.3 Foundation of new colonies and mating

Stingless bees produce swarms which are different process from that of honeybees (Kerr, 1951; Kerr et al., 1962). The process of swarming starts with transferring nest material and food from the old nest. When the new queens emerge, they together with swarms of workers leave to new nest sites, and males wait there in anticipation (Velthuis et al., 2005). Afterward, the new queens fly out for a single mating, followed by hundreds of males (Peters et al., 1999). Then, brood cell construction and oviposition are started in the new nest (Moure et al., 1958, Sakagami 1982, Inoue et al., 1984). The recent report reveals that males are produced by workers (Tóth et al., 2004). Subsequently, the bond between the mother and daughter colony slowly degrades (Wille, 1983).

1.2 Distribution of stingless bees

Stingless bees belong to the tribe Meliponini in the family Apidae along with honeybees, carpenter bees, orchid bees, and bumblebees. Relevant evidence suggests that the stingless bees have a center of origin and dispersion in Africa and migrated to tropical and subtropical regions around the world, such as Southeast Asia, Australia, parst of Mexico and Brazil (Michener, 1974; Sakagami, 1982; Michener, 1990; 2007) (Figure 1.3). Stingless bees are the most diverse in morphology and behavior of the eusocial bees. They live in permanent colonies and multiply through a process of

swarming. Colony size is diverse and ranges from a few dozen to 100,000 or more individuals (Michener, 2007).

Today, over 600 species in 56 named genera live in tropical and subtropical areas of the world (Cortopassi-Laurino et al., 2006). *Melipona* and *Trigona* are the most important genera. *Melipona*, including approximately 50 species restricted to the neotropic regions, has more complex communication systems (Nieh and Roubik, 1995). It is able to buzz pollinate (ejecting pollen grains by vibration of the pollen-bearing anthers of flowers that dehisce pollen through pores) (Buchmann, 1995). *Trigona* is the largest and most widely distributed genus more than 120 species in ten subgenera from the Indo-Malayan/Australasian and Neotropical regions (Michener, 2007). Recently, Rasmussen (2008) provides a catalog of Indo-Malayan/Australasian stingless bees that is an index to previous studies in taxonomy, behavioral research, and pollination ecology. This study divides the stingless bees in *Trigona* into several genera. Following this catalog, the former subgenera of *Trigona* are raised to genera (e.g. *Trigona collina* changed to *Tetragonilla collina*).

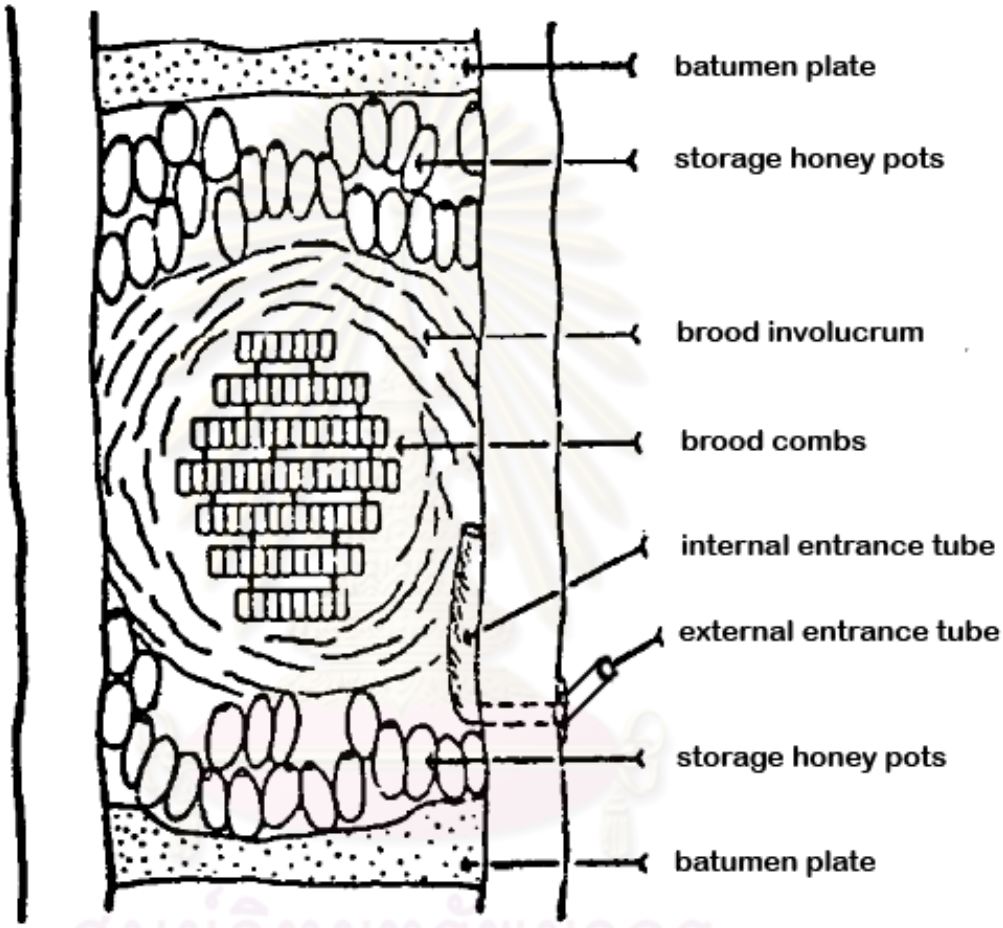
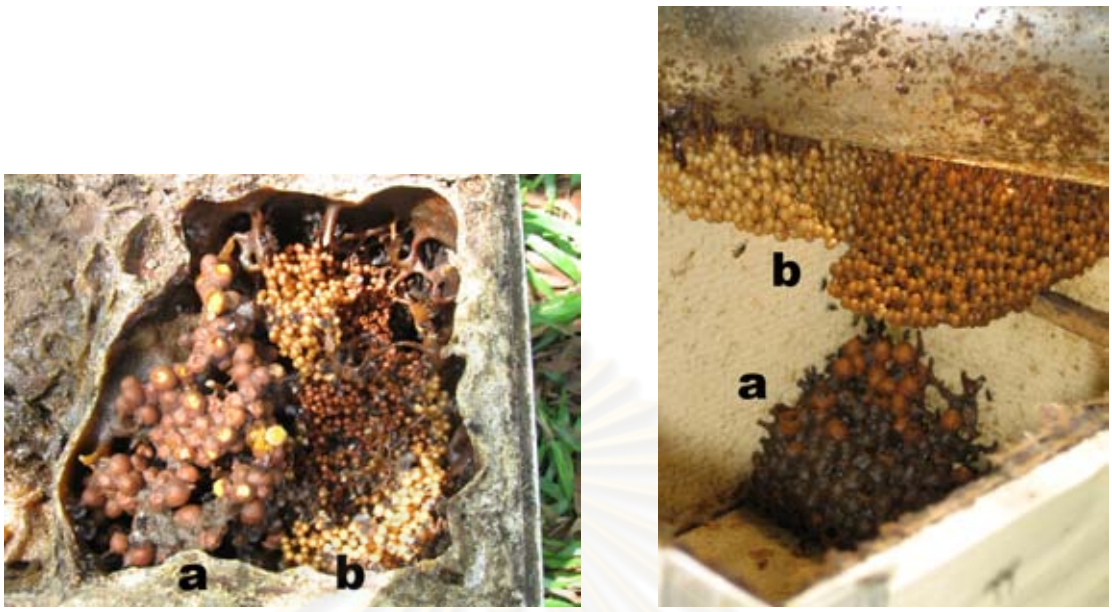


Figure 1.1 Diagram of stingless bees nest with the structure labeled (modified from Wille, 1983).



(A) **(B)**

Figure 1.2 Nest of stingless bees; *Tetragonilla collina* (A), *Tetragonula laeviceps* (B). There are two cell types: storage honey pots (a) and brood cell (b) in the nest of stingless bees.

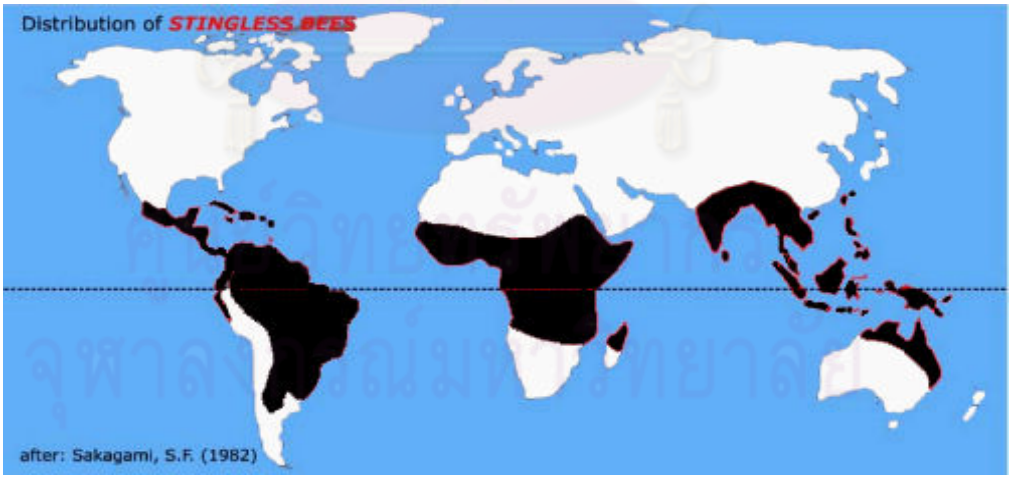


Figure 1.3 Distribution of stingless bees (marked in black). (http://www.blab.at/Bilderchens/Distribution-of-stingless-bees.jpg)

1.3 Exploitation of native species

Stingless bees play an important ecological role as pollinators of many wild plant species and seem good candidates for future alternatives in commercial pollination (Slaa et al., 2006). They are true generalists, collecting nectar and pollen from a vast array of plants (Heithaus, 1979). The fact that they lack a functional sting makes them especially suitable for pollination in enclosures (Slaa et al., 2000). Stingless bees are used widely as crop pollinators under greenhouse conditions for the following reasons: they are harmless to beekeepers and greenhouse workers, visit a wide range of crops (polylecty), are tolerant of high temperatures, are active throughout the year, can be transported easily (Amano, 2004). They also show some characteristics that support the ability as pollinators; for example, workers usually visit only one plant species on a single trip (Ramalho et al., 1994), workers collect food beyond immediate needs and store in nests allowing colonies to survive long periods (Roubik et al., 1986), and workers can help nest mates by providing information on the position of those floral resources. Moreover, many species of stingless bees can be managed in hives boxes for using as crop pollinators and for commercial production (Slaa et al., 2006). *Tetragonula laeviceps* is one of the commonest for using as tropical fruit pollinators in Thailand (Oldroyd and Wongsiri, 2006).

Stingless bee beekeeping is known as meliponiculture. This activity has local characteristics according to regional and traditional knowledge. Honey, wax, and resin are the traditional products of stingless bees and they are an important income source for the stingless bee beekeeper. The extraction of honey is often the income for keepers of stingless bees, followed by cerumen and resin (Cortopassi-Laurino et al., 2006). Propolis is made from plant exudates that workers forage and accumulate for construction, protection, and adaptation of their nests (Velikova et al., 2000). Subsequently, the study of Meliponinae propolis provides information on the chemical composition of stingless bee propolis, as well as the plants which they use as a resource for collecting propolis to support the medicinal properties of stingless bee propolis (Bankova and Popova, 2007).

1.4 Stingless bees in Thailand

Several genera of stingless bees have been recognized in Thailand, where 32 species have been recorded (Schwarz, 1939; Sakagami et al., 1983; Michener and Boongird, 2004; Klakasikorn et al., 2005; Rasmussen, 2008) (Table 1.1). Of these, the stingless bee *Tetragonilla collina* Smith, which is one of the commonest and most widespread species in Southeast Asia, is distributed throughout Thailand (Sakagami, 1975; Jongjitvimol et al., 2005). Like most stingless bee species, the nests of *T. collina* are usually constructed in small to large hollows in trees or in cavities in the soil (Velthuis, 1997; Michener, 2007). Colonies of *T. collina* have an elongate entrance tube leading to an underground nest (Figure 1.4). Their nests are often built in the roots of large trees and they have an aggregated distribution, with many colonies nesting in close proximity (i.e., nesting under the same tree) with an average of 2 nests per occupied nest tree (Eltz et al., 2002 and 2003).

Figure 1.4 Pictures of nest entrances of stingless bees *T. collina*

Table 1.1 Stingless bee species found in Thailand (modified Klakasikorn et al., 2005)

Stingless bee species	Klakasikorn et al. (2005)	Michener and Boongird (2004)	Rajitparinya et al. (2000)	Sakagami et al. (1985)	Schwarz (1939)
Tetragonula sirindhornae Michener and Boongird, 2004		*			
Tetragonilla collina Smith, 1857	*		*	*	*
Lepidotrigona terminata Smith, 1878	*		*	*	*
Tetrigona apicalis Smith, 1857	*		*	*	*
Lepidotrigona doipaensis Schwarz, 1939	*				*
Tetragonula laeviceps Smith, 1857	*		*	*	
Tetragonula minor Sakagami, 1978	*				
Geniotrigona thoracica Smith, 1857	*			*	*
Tetrigona binghami Schwarz, 1939	*				
Homotrigona fimbriata Smith, 1857	*		*	*	
Tetragonula fuscobalteata Cameron, 1908	*			*	*
Heterotrigona itama Cockerell, 1918				*	*
Tetrigona melanoleuca Cockerell, 1929			*	*	*
Tetrigona peninsularis Cockerell, 1927				*	*
Lophotrigona canifrons Smith, 1857				*	*
Homotrigona aliceae Cockerell, 1929					*
Homotrigona lutea Bingham, 1897					*
Tetragonula pagdeni Schwarz, 1939				*	*
Tetragonula geissleri Cockerell, 1918				*	*
Tetragonula iridipennis Smith, 1854					*
Tetragonula valdezi Cockerell, 1918					*
Tetragonula melina Gribodo, 1893				*	*
Tetragonula sarawakesis Schwarz, 1937					*
Lepidotrigona flavibasis Cockerell, 1929					*
Lepidotrigona ventralis Smith, 1857			*	*	*
Lisotrigona cacciae Nurse, 1907			*	*	*
Lepidotrigona nitidiventris Smith, 1857				*	
Tetragonilla atripes Smith, 1857				*	
Tetragonilla fuscibasis Cockerell, 1920				*	
Tetragonula hirashimai Sakagami, 1978				*	
Tetragonula pagdeniformis Sakagami, 1978				*	
Tetragonula reepeni Friese, 1918				*	

1.5 *Tetragonilla collina* Smith in Thailand

The taxonomy of *T. collina* Smith was identified according to Michener (2007) and Rasmussen (2008).

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Hymenoptera

Superfamily: Apoidae

Tribe: Meliponini

Genus: Tetragonilla

Subgenus: Tetragonilla

Scientific name: *Tetragonilla collina* (Smith, 1857)

Common name: Stingless bee

Morphological structures of stingless bees are shown in Figure 1.5 and 1.6. *Tetragonilla collina* Smith, an indigenous stingless bee, is distributed covering vast geographic locations in Thailand. Body coloration of *T. collina* ranges from blackish to dark brown. Clypeus varies from nearly as dark as the face above to distinctly pale. The tegulae are dark brown to black. Fore wing basal is distinctly darker, contrasting to milky white apical half and veins are basally dark brown while apically pale. The northward increase of body size is detected from Malaya and Southern Thailand to Northern Thailand (Sakagami, 1975; Sakagami et al., 1985).

Jongjitvimol and Wattanachaiyingcharoen (2007) report the distribution, nesting sites and nest structures of *T. collina* in Thailand. Most nests of *T. collina* are found in mixed deciduous forests. Their nesting sites are divided into 4 groups; cavities in tree trunks, cavities in termite mounds, underground cavities, and cavities in buildings. Moreover, the first record of an assassin bug, *Pahabengkakia piliceps* is reported as a specialized predator of the stingless bee *T. collina* (Wattanachaiyingcharoen and Jongjitvimol, 2007).

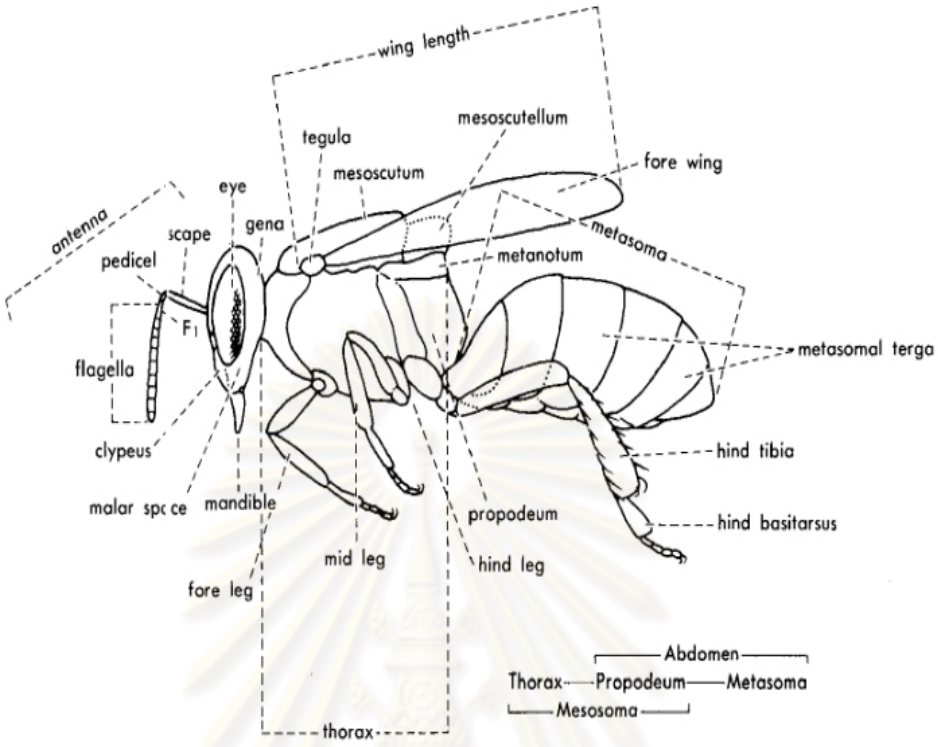


Figure 1.5 Morphological structures of stingless bees (Sakagami et al., 1985)

Figure 1.6 Morphological structure of *T. collina* (Wattanachaiyingcharoen et al., 2004)

1.5.1 Identification of *T. collina*

Today, the stingless bees are exploited as pollinators in agriculture and their products are also valuable. Therefore, the knowledge of stingless bees is continually increased and identification of stingless bees has also been reported over the years (Michener, 1961; Sakagami, 1978; Dollin et al., 1997). They are classified based on morphology and nest architecture. However, taxonomic identification of stingless bees remains unclear and requires experienced scientists.

Morphology is the most commonly used method of classification but many stingless bee species are sympatric species (e.g. between *T. pagdeni* and *T. fuscobalteata*; Sakagami 1978) and can not be preliminary distinguished based on geographic distribution. In addition, species recognition of stingless bees is more complicated by the existence of cryptic species (e.g. between *T. carbonaria* and *T. hockingsi* and between *T. iridipennis* and *T. laviceps*; Starr and Sakagami 1987). The external characteristics are thus unstable and not reproducible because of a variety of habitats and environmental conditions. Nevertheless, nest architecture characters are usually relevant but they are reported that they are not sufficient criteria for authenticating species origins of Australian stingless bees (i.e. *T. hockingsi* and *T. davenporti*; Franck et al. 2004).

The use of specimens with correct species origin is one of main factors that affect further molecular genetic studies of stingless bees. Therefore, species-diagnostic markers for reliable differentiation of abundantly distributed species such as *T. collina* are a prerequisite for eliminating confusion of similar species in genetic diversity and population structure analyses of this species. The development of molecular biology techniques, such as DNA-based markers, has given a new opportunity for genetic characterization, allowing the direct comparison of different genetic material without environmental influences. Various molecular marker techniques, such as DNA fingerprinting are available to detect diversity at the DNA level. One of these techniques, AFLP, has been proven to be valuable to genotype characterization in many crop species (Vos et al., 1995). Likewise, AFLP has been widely used to study polymorphism among populations and species (Blears et al., 1998; Mueller and Wolfenbarger, 1999) and to identify species-diagnostic markers in

various taxa (Liu and Cordes, 2004; Klinbunga et al., 2007). AFLP can generate high-resolution markers that exhibit such a purpose, where no data in stingless bees are reported at present.

Recently, a species-diagnostic AFLP-derived marker for identification of Thai *T. pagdeni* is successfully developed. The CUTp1 marker can discriminate *T. pagdeni* from 10 stingless bee species in Thailand. The further analysis (SSCP analysis) then differentiates *T. pagdeni* from two more species (*T. fuscobalteata* and *T. collina*) while the remaining 2 species (*T. laeviceps* and *T. fimbriata*) can be clearly identified by morphology (Thummajitsakul et al., 2010).

After 3 years of this study, the catalog of published literature on stingless bees from the Indo-Malayan/Australasian region is recompiled (Rasmussen, 2008). The subgenus of Indo-Malayan stingless bees in *Trigona* genus is treated as genus. All collected samples in this study are in *Trigona* genus. Therefore, they are separated to several genera such as *Geniotrigona* (*T. thoracica*), *Heterotrigona* (*T. itama*), *Homotrigona* (*T. fimbriata*), *Lepidatrigona* (*T. terminata* and *T. doipaensis*), *Lophotrigona* (*T. canifrons*), *Tetrigona* (*T. apicalis* and *T. melanoleuca*), *Tetragonilla* (*T. collina*) and *Tetragonula* including *T. laeviceps*, *T. pagdeni*, *T. melina*, *T. minor*, and *T. fuscobalteata*.

1.5.2 Genetic diversity of *T. collina* in Thaialnd

Sustainable conservation and the construction of effective genetic management of important natural resource species require basic knowledge of the genetic population structure of that species (Avise, 1994). Genetic diversity is a level of the variation of the nucleotides, genes, chromosomes within the cells or organelles of any organism. Genetic diversity enables them to survive and adapt to changing in their environment including new pests, diseases and new climatic conditions. The variation is introduced through harmless mutation of gene or the result of sexual reproduction. These may provide the evolution of new characteristics within a single species for survival and adaptation in their environment. Genetic diversity has been studied in several social insects. These studies reveal the useful knowledge from the insects for further studies; for example, genetic diversity can help to prevent severe infections and promote colony growth in social insects because of the evolution of

polyandry (many females mate with more than one male) in social insects (Arnqvist and Nilsson, 2000). The polyandrous queen can produce genetically diverse workers that carry different genes for resistance to a particular disease (Tarpy, 2002).

Genetic diversity can be determined by many different ways. Traditionally, protein marker had been used to survey genetic diversity within several organisms such as in insects (Sánchez and Keena, 2009). A DNA marker is also used as a marker of genetic diversity within and among individuals of any organisms such as in Thai honey bee, *Apis cerana* (Sitttipraneed et al., 2001) and Thai stingless bee, *T. pagdeni* (Thummajitsakul et al., 2008). In Thailand, Cameron et al. (2004) showed that colonies of *T. collina* within nest aggregations are not genetically related-that is, queens in a nest aggregation are not related as sisters or mother and daughter-and suggested that new nests are established in an unrelated nest aggregation. However, population genetic structure of *T. collina* at larger scales has not been reported. Information about the intraspecific genetic variation of this native species is fundamental to designing appropriate management strategies for genetic improvement and efficient conservation programs.

1.6 Molecular marker

Molecular markers can reveal genetic variation (polymorphism) at the protein level (protein marker) or at the DNA level (DNA marker) without environmental factors. At the DNA level, types of genetic variation include: base substitutions, commonly referred to as single nucleotide polymorphisms (SNPs), insertions or deletions of nucleotide sequences (indels) within a locus, inversion of a segment of DNA within a locus, and rearrangement of DNA segments around a locus of interest. Through long evolutionary accumulation, many different instances of each types of mutation should exist in any interest species and the number and degree of the various types of mutations define the genetic variation within species. DNA marker can be applied to reveal these mutations (Liu et al., 2004).

Numerous molecular markers have been characterized e.g. allozyme, restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellite, etc. These molecular markers are classified into 2 groups: markers that require prior

molecular information (e.g. allozyme, RFLP, microsatellite) and markers that no need prior molecular information (e.g. RAPD, AFLP).

Fernandes-Salomao et al. (2005) examined phylogenetic relationships of eight stingless bee species (*Melipona quadrifasciata anthidioides, M. mandacata, M. bicolor bicolor, M. quinquefasciata, M. rufiventis, M. scutellaris, M. compressipes, M. marginata*) by using RNA Intergenic Transcribed Spacer 1 sequences. Likewise, Franck et al. (2004) studied genetic diversity of the *cabonaria* species (*Trigona carbonaria, T. hockingsi,* and *T. davenporti*) from eastern Australia using 13 microsatellite loci. These reports investigated polymorphisms of stingless bees that have already been reported the molecular information. Recently, Thummajitsakul et al. (2008, 2010) revealed the studies of Thai *T. pagdeni* that lack their molecular information. They developed a species-diagnostic AFLP-derived marker and also investigated genetic diversity of this species in Thailand based on three enzymes amplified fragment length polymorphism (TE-AFLP).

Mitochondrial DNA (mtDNA) is also widely employed as a molecular marker in systematic, species characterization, population structure, and phylogenetic studies. Animal mtDNA is a circular molecule and maternally inherited in most animals without recombination, so the whole set of genes is inherited as one unit. MtDNA is used in study of honey bees, *A. cerana* for example, to study the genetic polymorphisms of *A. cerana* based on PCR-RFLP method (Sittipraneed et al., 2001; Songram et al., 2006; Warrit et al., 2006).

Amplified fragment length polymorphism (AFLP)

Amplified Fragment Length Polymorphism (AFLP) is a DNA fingerprinting approach that combines advantages from both RFLP (cutting of genomic DNA with restriction endonucleases) and RAPD (the amplification of particular DNA sequences using arbitrary primers). It has the potential to screen many different DNA regions randomly distributed throughout the entire genome without the need for knowledge of sequences of the genome under investigation (Blears, 1998; Vos et al., 1995). The main disadvantages of AFLP is the difficulty in identifying homologous markers (alleles), rendering this method less useful for studies that require precise assignment of allelic states. However, it is advantageous because of the rapidity and ease with

which reliable, reproducible, high-resolution markers can be generated. According to Vos et al. (1995), AFLP analysis involves the digestion of genomic DNA with a combination of rare cutter and frequent cutter restriction enzymes. Then, double-stranded oligonucleotide adaptors are ligated to both sides of the restriction fragments to provide templates for PCR amplification. The PCR amplification is twice performed by the primers containing the sequences that are able to anneal to the sequences of the adapters and one additional base at the 3' ends which complementary to the restriction sites for preamplification while the primers with two or three additional bases at the 3' ends are used for the selective amplification (Figure 1.7).

Three enzyme amplified fragment length polymorphism (TE-AFLP)

Three enzyme amplified fragment length polymorphism (TE-AFLP) is a type of fingerprinting technique based on AFLP. This technique is the use of three endonucleases instead of two enzymes as in AFLP. The use of three enzymes provides highly discriminating fingerprinting because the addition of third endonuclease reduces the number of bands amplified. According to van der Wurff et al. (2000), the digestion and ligation reaction were processed by adding three restriction endonucleases together with only two sets of adapters in a single reaction. This method can simplify the two-step amplification to one-step amplification in fingerprinting complex genomes. Therefore, TE-AFLP technique is one of the most common techniques used for genetic variation analysis or marker detection.

PCR-Single strand conformational polymorphism (PCR-SSCP)

PCR single-strand conformational polymorphism (PCR-SSCP) is one of popular techniques extensively used to identify a sequence variation or a polymorphism in a known gene. SSCP is the electrophoretic separation of single strand DNA (ssDNA) on non-denaturing polyacrylamide gel. The mobility of ssDNA depends on the secondary structure of ssDNA. The differences in DNA sequence result in a different secondary structure and mobility of DNA although they are the same size. SSCP has high sensitivity in detection of mutations because a single base change of the sequence can cause a radical change in nucleic acid migration (Orita et al., 1989) (Figure 1.8). SSCP bands can be visualized by using autoradiograms

(radioactive detection), or silver staining or fluorescent labels. Because of its high sensitivity, SSCP experimental conditions can be optimized by alteration of the gel temperature or the degree of cross-liking or by the addition of glycerol or sucrose, to maximize differential migration among fragments. Thus, the SSCP technique is considered to be a method that reveals inexpensive cost, convenience, highly efficiency and sensitivity for detecting mutation or sequence variation (Sheffield et al., 1993).

1.7 Objectives of this dissertation

The aims of this dissertation are to develop species diagnostic markers to distinguish and the first time according to Michener (2007) identified *T. collina* from other stingless bee species in Thailand based on AFLP analysis, to estimate genetic variation of *T. collina* in Thailand using nuclear DNA marker analysis; TE-AFLP analysis and TE-AFLP derived markers, and to estimate mitochondrial DNA diversity of *T. collina* in Thailand using PCR-SSCP analysis

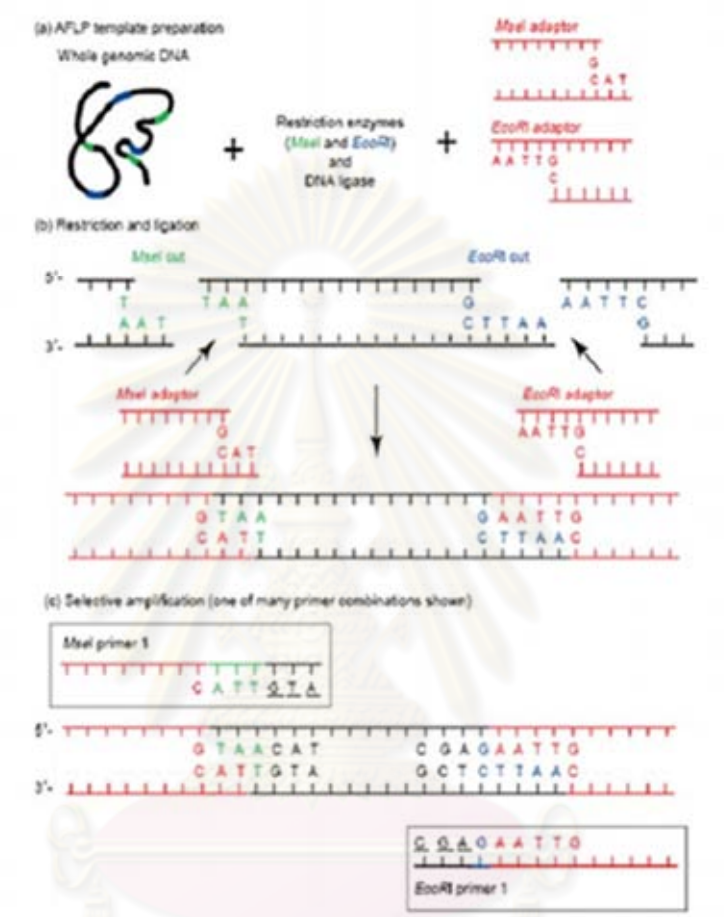


Figure 1.7 AFLP procedures: Genomic DNA is digested with two restriction enzymes and adaptors are ligated to the end of restriction fragments. The adaptor sequence was marked in red and the remaining part of the restriction sequence was marked in blue and green. For PCR amplifications, the primers extending selective base into the unknown part of the fragments (in black and underlined base pairs) were needed. The first PCR amplification is performed with a 1-bp extension, followed by a more selective primer with a 3-bp extension.

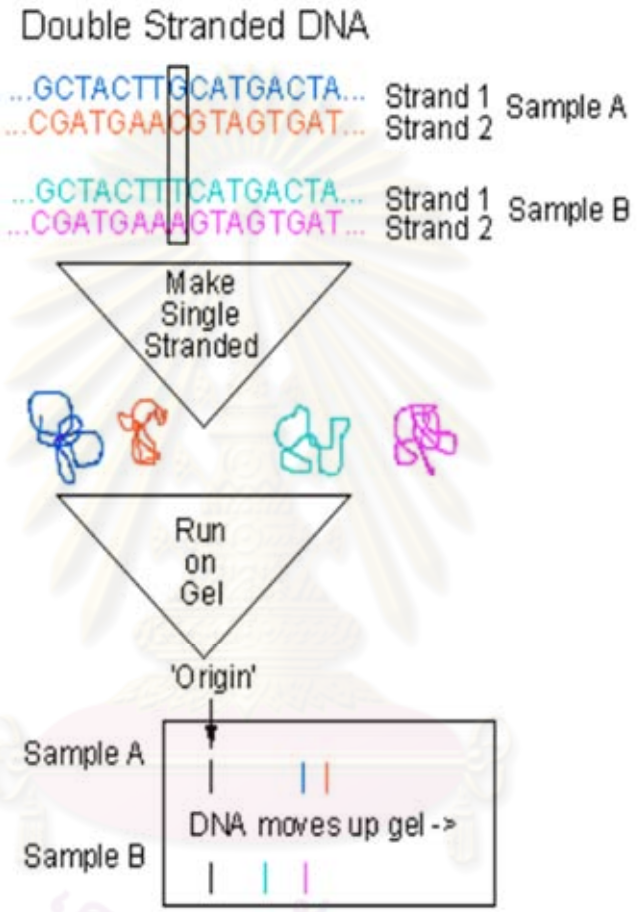


Figure 1.8 Diagram shows the principal diagram of SSCP analysis

(http://www.austmus.gov.au/evolutionary_biology/images/sscp.gif)

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

- Autoclave MLS-3020 (Sanyo, Japan)
- Automatic micropipette P2, P20, P100, P200, P1000 (Gilson Medical Electrical S.A., France)
- Centrifuge, Microfuge® 22R (Beckmen Coulter, USA)
- Dry bath incubator MD-01N (Major Science, Taiwan)
- -20 ^{o}C freezer
- Horizontal agarose gel electrophoresis apparatus, GelMate 2000 (Toyobo, Japan)
- Microcentrifuge tubes 0.6, 1.5 ml (Axygen Harward, USA)
- Micro Pulser (Bio-RAD Laboratories, USA)
- Pipette tips (Axygen Harward, USA)
- Power supply (Bio-RAD Laboratories, USA)
- Thermal cycler, Mastercycler gradient (Eppendorf, Germany)
- Thin-wall microcentrifuge tubes 0.2 ml (Axygen Harward, USA)
- UV transilluminator model M-20 (UVP, UK)
- Vertical gel electrophoresis apparatus for AFLP and TE-AFLP analysis, Sequencing system Model SA or Model S2 (GibcoBRL Life Technologies, Inc., USA)
- Vertical gel electrophoresis apparatus for SSCP analysis, Protean II xi Cell (Bio-RAD Laboratories, USA)
- X-ray film, X-O1000 mat film (Eastman Kodak Company Rochester, USA)

2.2 Chemicals

- Absolute ethanol (Merck, Germany)
- Acetic acid, glacial (Merck, Germany)
- Acrylamide (Merck, Germany)
- Agarose, GenePure LE (ISC BioExpress, USA)
- Ammonium persulfate (Promega, USA)
- Bind silane, PlusOne (Amersham Biosciences, Sweden)
- Boric acid (BDH, England)
- Bromophenol blue (Sigma, USA)
- Chloroform (Merck, Germany)
- Ethylene diamine tetraacetic acid, disodium salt dihydrate (Fluka, Switzerland)
- Ethidium bromide (Sigma, USA)
- Formaldehyde (Carlo Erba Reagent, Italy)
- Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan)
- High-Speed Plasmid Mini Kit (Geneaid, Taiwan)
- *N, N*-methylene-bis-acrylamide (Promega, USA)
- *N, N, N', N'*- tetramethylenediamine, TEMED (USB Corporation, USA)
- pGem®-T Easy Vector (50 ng/µl; Promega, USA)
- Phenol, Equilibrated (USB Corporation, USA)
- Repel silane, PlusOne (Amersham Biosciences, Sweden)
- Silver nitrate (Merck, Germany)
- Sodium carbonate (Merck, Germany)
- Sodium chloride (Merck, Germany)
- Sodium dodecyl sulfate (SDS) (Sigma, USA)
- Sodium thiosulfate (Merck, Germany)

- Tris-(hydroxyl methyl)-amminomethane (USB Corporation, USA)
- Urea (Fluka, Switzerland)
- Xylene cyanol (Sigma, USA)

2.3 Oligonucleotide primers

Oligonucleotides used for PCR were purchased from Bio Basic Inc., Canada or from 1st BASE Holdings, Singapore.

2.4 Enzymes and Restriction enzymes

- *Taq* DNA polymerase, DyNazyme™ II DNA polymerase (Finnzymes, Finland) and GoTaq® Flexi DNA polymerase (Promega, USA)
- Proteinase K (Sigma, USA)
- 2X Rapid Ligation Buffer, T4 DNA Ligase (Promega, USA)
- Restriction endonucleases; *Bam*HI, *Eco*RI, *Pst*I, *Rsa*I, *Tru*9I, *Xba*I (Promega, USA)
- RNaseA (Sigma, USA)
- T4 DNA ligase (Promega, USA)
- T4 Polynucleotide Kinase (Promega, USA)

2.5 Radioactive

- [γ-^{32}P] dATP specific activity 100 μCi/mmol (Perkin Elmer, USA)

2.6 Samples

Adult workers of *Tetragonilla collina* from 159 colonies were collected from geographically different locations in Thailand. Other species of stingless bees ;*Tetrigona apicalis* (n = 12), *Lophotrigona caniform* (n = 1), *Lepidatrigona doipaensis* (n = 1), *Homotrigona fimbriata* (n = 3), *Tetragonula fuscobalteata* (n = 6), *Heterotrigona itama* (n = 4), *Tetragonula laeviceps* (n = 6), *Tetrigona melanoleuca* (n = 1), *Tetragonula melina* (n = 1), *Tetragonula minor* (n = 8), *Tetragonula pagdeni* (n = 51), *Lepidatrigona terminata* (n = 7), *Geniotrigona thoracica* (n = 3), *Lisotrigona furva* (n = 2), were included in the experiment (appendix A). Specimens were placed in 95% ethanol and kept at 4 ^{o}C until required. Taxonomic identification of collected

stingless bees was examined based on the nest architecture and morphology according to Sakagami (1978) and Sakagami et al. (1983). Species identifications of specimens were kindly confirmed based on external morphology by Dr. Charles D. Michener (University of Kansas). These specimens were used to develop species-specific AFLP marker, to study genetic diversity and population structure of *T. collina* in Thailand using nuclear DNA and mitochondrial DNA polymorphisms.

2.7 DNA preparation

2.7.1 DNA extraction

Genomic DNA was extracted from each stingless bee per nest using a phenol-chloroform-SDS method (Smith and Hagen, 1996). A stingless bee was homogenized in 1.5 ml microcentrifuge tube containing 500 µl of STE extraction buffer (100 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA), then 20 % SDS solution was added to a final concentration of 1.0 %. A proteinase K solution (10 mg/ml) was added to a final concentration of 500 µg/ml and incubated at 65 ^{o}C for 3 hours. After that, 25 µl of RNase A (10 mg/ml) was added and incubated at 37 ^{o}C for 1 hour. Then, the supernatant was extracted twice with an equal volume of phenol/chloroform (1:1v/v) gently and once with an equal volume of chloroform. After each extraction, the mixture was then centrifuged at 10,000 rpm for 10 minutes at room temperature. The upper aqueous phase was carefully transferred to a new microcentrifuge tube and mixed with double volume of chilled absolute ethanol and kept at -20 ^{o}C overnight to precipitate DNA. The DNA pellet was recovered by centrifugation at 12,000 rpm for 20 minutes at 4 ^{o}C and washed twice with 70 % ethanol (v/v). The pellet was dried and dissolved with 1X TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA). Genomic DNA was kept at 4 ^{o}C until use.

2.7.2 Measurement of DNA concentration / Agarose gel electrophoresis

The concentration of DNA samples was estimated by comparison with the intensity of ethidium bromide fluorescent DNA standards (e.g. λ/*Hin*dIII standard DNA) on agarose gel electrophoresis (Sambrook and Russell, 2001). After staining with ethidium bromide, the intensity of orange-red fluorescence of DNA bands was observed under UV light.

Agarose gel was prepared by weighting out an appropriate amount of agarose and mixing with 1X TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2.5 mM Na_2EDTA, pH 8.3). The agarose was heated in a microwave oven until complete solubilization and cooled at room temperature before pouring gel into a gel tray containing a comb. The agarose gel was completely set at room temperature. Before sample was loaded into the well of agarose gel, sample was mixed with one-fifth volume of the loading dye (0.25 % bromophenol blue and 25 % Ficoll in water). A 100 bp DNA ladder or λ/*Hin*dIII was used as the standard marker.

The extracted total DNA was electrophoresed on 0.8 % agarose gel in 1X TBE buffer at 100 volts whereas λ/*Hin*dIII standard DNA was used to compare for size and concentration of extracted total DNA. When electrophoresis is complete, the gel was stained with ethidium bromide solution and then destained in distilled water to remove unbound ethidium bromide from the gel. DNA bands were visualized under a UV transilluminator and photographed.

2.8 Development of *T. collina*-specific marker using AFLP (Amplified Fragment Length Polymorphism) and SSCP (Single Strand Conformational Polymorphism) analysis

2.8.1 AFLP analysis

2.8.1.1 Digestion and adaptor ligation

The AFLP procedure was carried out as described by Vos et al. (1995) with a few modifications. Each genomic DNA (250 ng) of different *Trigona* species was digested with 5 units of *Pst*I in a 25 µl reaction mixture consist of 1X O-Phor-All buffer (10 mM Tris-acetate, pH 7.5, 10 mM magnesium acetate, and 50 mM potassium acetate) at 37 ^{o}C for 3 hours. Then, the digestion was inactivated at 65 ^{o}C for 15 minutes. After that, *Tru*9I (3 units) was added in a final volume of 40 µl and incubated at 65 ^{o}C for 3 hours. The double stranded adaptors (Table 2.1) were ligated to the restriction fragments in a total volume of 50 µl consist of 5 µM *Pst*I adaptor and 50 µM *Mse*I adaptor, 1X O-Phor-All buffer, 0.4 mM ATP and 1 unit of T4 DNA ligase at 12 ^{o}C for 16 hours.

Table 2.1 Adaptor sequences and AFLP primers used for the ligation and PCR amplification

Primer	**Sequences**
Adaptor sequences	
*Pst*I adaptor	5’-CTCGTAGACTGCGTACATGCA-3’
	5’-TGTACACAGTCTAC-3’
*Mse*I adaptor	5’-GACGATGAGTCCTGAG-3’
	5’-TACTCAGGACTCAT-3’
Preamplification primers	
P_{+A}	5’-GACTGCGTACATGCAGA-3’
M_{+C}	5’-GATGAGTCCTGAGTAAC-3’
Selective amplification primers	
P_{+3}-1	P_{+A}-AG
P_{+3}-2	P_{+A}-AC
P_{+3}-3	P_{+A}-GA
P_{+3}-4	P_{+A}-GT
P_{+3}-5	P_{+A}-CG
P_{+3}-6	P_{+A}-CT
P_{+3}-7	P_{+A}-TC
P_{+3}-8	P_{+A}-TT
M_{+3}-1	M_{+C}-AA
M_{+3}-2	M_{+C}-AC
M_{+3}-3	M_{+C}-AG
M_{+3}-4	M_{+C}-AT
M_{+3}-5	M_{+C}-TA
M_{+3}-6	M_{+C}-TC
M_{+3}-7	M_{+C}-TG
M_{+3}-8	M_{+C}-TT

2.8.1.2 Pre-amplification

The ligated DNA was used as a template. Preamplification was carried out utilizing adaptor-specific primers with a single selective base at 3' end on each primer (5'-GACTGCGTACATGCAG**A**-3' and 5'-GATGAGTCCTGAGTAA**C**-3'). Each 25 µl of reaction contained 1X PCR buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM each dNTP, 1.5 mM $MgCl_2$, 30 ng of each primer, 1.5 units of DyNazyme™ II DNA polymerase and 1 µl of ligated DNA. PCR was performed consisting 20 cycles of denaturation at 94 ^oC for 30 seconds, annealing at 56 ^oC for 1 minute and extension at 72 ^oC for 1 minute. The final extension was 72 ^oC for 5 minutes.

2.8.1.3 Selective amplification

The pre-amplification product was diluted 25-fold with sterile deionized water and selectively amplified with primer combinations having three selective bases at the 3′ end of each primer shown in Table 2.1. The selective amplification was performed in a 25 µl reaction volume including 1X PCR buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM each dNTP, 1.5 mM $MgCl_2$, 30 ng of P_{+3} and M_{+3} primers, 1.5 units of DyNazyme™ II DNA polymerase and 5 µl of the diluted preamplification product. PCR was carried out consisting 2 cycles of denaturation at 94 ºC for 45 s, annealing at 65 ºC for 60 s, and extension at 72 ºC for 90 s, followed by 10 cycles of a touchdown phase with lowering of the annealing temperature 0.7 ºC in every cycle and additional 25 cycles of 94 ºC for 45 s, 56 ºC for 60 s, and 72 ºC for 90 s. The final extension was carried out at 72 ºC for 5 min.

2.8.2 Preparation of polyacrylamide gel and gel electrophoresis

The AFLP fragments were size-fractionated through denaturing polyacrylamide gel. The gel was run at constant power and then the banding pattern was revealed with silver staining.

A pair of glass plates (the long and the short glass plates) was cleaned with deionized water to eliminate impurities, twice washed with 2 ml of 95% ethanol in one plane of glass. Then, the long plate was coated with 1 ml of freshly prepared binding solution consisting 4 µl of bind silane, 995 µl ethanol and 5 µl glacial acetic acid, and left for 10 minutes. The excess binding solution was eliminated by cleaning

the coated long glass with 95% ethanol for 3 times. The short glass plate was also treated as the long one except for coating step. It was coated by the Rapel silane (2% dimethyldichlorosilane in octamethylcyclotetrasitoxone). The coated glass plates were assembled to each other with a pair of spacer in between. The bottom and both sides of assembled glass plates were sealed with tape.

The gel was prepared by 40 ml of 6% denaturing polyacrylamide gel including 19% acrylamide, 1% bisacrylamide, 7 M urea and 10X TBE (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3). The solution was degassed for 20 minutes, then added 240 µl of freshly prepared 10% ammonium persulfate and 24 µl TEMED, gently mixed and poured between the glass plated using a 50 ml syringe. The gel comb was inserted and allowed to polymerize at room temperature for 1 hour. The gel was covered by water-soaked tissue paper after it had polymerized. To complete polymerization, the gel was left at room temperature for 4 hours or overnight. The sealing tape and gel comb were removed when gel was required.

The assembled gel was placed in the gel running apparatus. The upper and lower buffer chambers were added with 1X TBE buffer. The comb was reinserted with the teeth on the top of gel. The gel was prerun at 35 W for 15 minutes.

The amplified products (6 µl) was mixed with 3 µl of a loading buffer (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol) and heated at 95 ^{o}C for 5 minutes, immediately cooled on ice. For electrophoresis, 6 µl of the denatured mixtures were loaded. The gel was run at 35 W for 2 hours.

2.8.3 Silver staining

After electrophoresis, the short glass plate was removed out of gel. The gel on the long glass plate was soaked and agitated in 2 litres of the fix/stop solution (20% glacial acetic acid) for 30 minutes. The gel was placed in deionized water and agitated 3 times for 2 minutes. The gel was incubated in 0.1% silver nitrate (1.5 liters) with agitation at room temperature for 30 minutes. The gel was immersed in deionized water (1.5 liters) and shaken no longer than 10 seconds and quickly transferred to 1.5 liters of the chilled developing solution (3% sodium carbonate, 0.15% formaldehyde, 0.02% sodium thiosulphate). The gel was well agitated until first band was observed and then placed in another chilled developer and shaken until all bands were

visualized (usually 2-3 minutes). One liter of the fix/stop solution was directly added to the developing solution and continuously shaken for 3 minutes. The stained gel was soaked in deionized water at least 3 minutes. The gel was left at 80 ^{o}C for 2-3 hours (for AFLP gels) or at room temperature (for SSCP gels).

2.8.4 Cloning of species-specific AFLP fragment

2.8.4.1 Elution of DNA from polyacrylamide gels

AFLP fragment found in *T. collina* but not in other screened species was excised from the gel using a sterile razor blade. The gel fragment was twice washed with 500 µl of sterile deionized water for 2 hours at room temperature. Twenty microliters of water was added and incubated at 50 ^{o}C for 30 minutes and at 37 ^{o}C overnight. Reamplification of the target fragment was carried out using the original primer pairs used in selective amplification according to the same PCR recipes with the exception that 100 ng of each primer and 5 µl of the eluted AFLP product were used. The PCR conditions were performed consisting 5 cycles of 94 ^{o}C for 30 seconds, 42 ^{o}C for 45 seconds and 72 ^{o}C for 1 minute followed by additional 35 cycles at a higher stringent annealing temperature at 50 ^{o}C. The final extension was performed at 72 ^{o}C for 7 minutes. The reamplified product was electrophoresed through 1.5% agarose gel at 100 volt for approximately 40 minutes.

2.8.4.2 Elution of DNA from agarose gels

The required DNA fragment was run through agarose gels in duplication to avoid contamination of ethidium bromide and UV damage. One was run side-by-side with a 100 bp DNA marker and the other was loaded into the distal well of gel. After electrophoresis, lanes of DNA standard and its proximal DNA sample were cut and stained with EtBr for 1 minute. Positions of DNA markers and the EtBr-stained fragment were used to align the position of the non-stained target DNA fragment.

The DNA fragment was excised from gel and eluted out from agarose gel using Gel/PCR DNA Fragments Extraction Kit (Geneaid) according to the protocol recommended by the manufacture. The purified sample was stored at -20 ^{o}C until required.

2.8.4.3 Ligation of PCR product to vector

The ligation reaction was set up in the total volume of 10 µl containing 3 µl of the gel eluted PCR product, 25 ng of pGem-T easy vector (Promega), 5 µl of 2X Rapid ligation buffer (60 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10% PEG 8000) and 3 units of T4 DNA ligase. The ligation mixture was incubated at 4 ^{o}C overnight.

2.8.4.4 Preparation of competent cells

A single colony of *E. coli* JM109 was inoculated in 10 ml of LB broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl) and incubated at 37 ^{o}C with 250 rpm shaking overnight. A half of the starter was inoculated to 250 ml of LB broth and then incubated at 37 ^{o}C with 250 rpm shaking until the optical density at 600 nm of cells reached 0.5-0.8. The culture was chilled on ice for 30 minutes and then centrifuged at 8,000X g for 15 minutes at 4 ^{o}C. The supernatant was discarded. The pellets were washed twice with 1 volume and 0.5 volume of cold sterile water, respectively and centrifuged as above. The supernatant was removed and the pellet was washed with 10 ml of 10% (v/v) ice cold sterile glycerol and finally resuspend in a final volume of 1-2 ml of 10% ice cold sterile glycerol. The cell suspension was divided into 40 µl aliquots and stored at -80 ^{o}C until used.

2.8.4.5 Electroporation

The 0.2 cm cuvettes and sliding cuvette holder were chilled on ice. The competent cells were thawed on ice. One microliter of the ligation mixture was added in 40 µl of the competent cells and placed on ice for 1 minute. This mixture was transferred to a chilled cuvette and one pulse was applied. One milliliter of LB broth was immediately added to the cuvette and quickly resuspended the mixture. The mixture was transferred to new microcentrifuge tube and incubated at 37 ^{o}C for 1 hour with 250 rpm shaking. Finally, this suspension was spreaded onto the LB agar plate containing 50 µg/ml ampicillin, 25 µg/ml IPTG and 20 µg/ml X-gal and incubated at 37 ^{o}C for 16-18 hours. The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

2.8.4.6 Detection of recombinant clone by colony PCR

The colony PCR was performed in a 25 µl reaction volume consisting of 1X PCR buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM each dNTP, 1.5 mM $MgCl_2$, 0.2 µM of primers PUCI (5'-TCC GGC TCG TAT GTT GTG TGG A-3') and PUCII (5'-GTG GTG CAA GGC GAT TAA GTT GG-3') and 0.5 units of DyNazyme™ II DNA polymerase. A recombinant clone was picked up and mixed well in the amplification reaction. The PCR profile was predenaturation at 94 ^{o}C for 3 minutes, followed by 35 cycles of 94 ^{o}C for 1 minute, 50 ^{o}C for 1 minute and 72 ^{o}C for 1 minute. The final extension was carried out 72 ^{o}C for 7 minutes. The amplified product was analyzed through agarose gel.

2.8.4.7 Plasmid extraction

The recombinant clone was inoculated into 3 ml of LB broth containing 50 µg/ml ampicillin and incubated at 37 ^{o}C with 250 rpm shaking overnight. The culture was transferred into 1.5 ml microcentrifuge tube and centrifuged at 10,000X g for 1 minute. The cell pellet was collected and extracted the recombinant plasmid using High-Speed Plasmid Mini Kit (Geneaid) according to the protocol recommended by the manufacture.

2.8.5 DNA sequencing and primer design

The sequence of recombinant plasmid was analyzed by DNA sequencing service (Macrogen, Inc, Korea) with the M13 forward or reverse primers (universal primer) under BigDye™ terminator cycling conditions on automatic sequencer 3730x1. The sequences were analyzed and compared for the homology search using BlastN (nucleotide similarity) and BlastX (translated protein similarity) available at www.ncbi.nlm.nih.gov. A pair of designed primers is shown in Table 2.2.

2.8.6 PCR amplification of candidate *T. collina*-specific AFLP marker and species-specific test

PCR was carried out using primers designed from the sequences of candidate *T. collina*-specific AFLP marker (Table 2.2). The amplification reaction was performed in a 25 µl reaction volume containing 1X PCR buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM each dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer, 50 ng of genomic DNA and 1 unit of DyNazyme™ II DNA

polymerase. The PCR profile consisted of predenaturation at 94 ^{o}C for 3 minutes followed by 35 cycles of denaturation at 94 ^{o}C for 30 seconds, annealing at 56 ^{o}C for 1 minute and extension at 72 ^{o}C for 30 seconds. The final extension was performed at 72 ^{o}C for 7 minutes. For species-specific test, the primer pairs were used to test the positive amplified product across the representative individuals of *T. collina* and other *Trigona* species. The amplification product was analyzed by 1.5% agarose gel electrophoresis (Sambrook and Russell, 2001). The expected amplified products were purified and further analyzed by single strand conformation polymorphism (SSCP) to determine polymorphisms of the products.

Table 2.2 Primers designed from a candidate *T. collina*-specific AFLP marker

Primer	**Sequence**	**Annealing temperature (^{o}C)**	**Expected size (bp)**
CUTc1-F	5′-GGTTCGGATTTGGTTGGCATTG-3′	56	259
CUTc1-R	5′-CGGTGTACGAAGCGCCAG-3′		

2.8.7 SSCP analysis

Non-denaturing polyacrylamide gel was used for fractionation of single stranded DNA secondary structure conformation. A pair of glass plates was prepared as described in 2.8.2. A 40% stock solution (37.5:1 crosslink) was diluted to prepare 12.5% polyacrylamide solution. The acrylamide gel solution (40 ml) was added with 300 µl 10% ammonium persulfate and 30 µl TEMED and poured between the glass plates. The gel was left to polymerize at least for 4 hours.

Six microliters of the purified amplification was mixed with 4 volumes of the loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH). After denaturing at 95 ºC for 5 minutes, it was immediately cooled on ice for 2 minutes and electrophoretically analyzed through non-denaturing polyacrylamide gels at 12.5 V/cm for 16 hours at 4 ºC. The gels were then visualized by silver staining as decribed in 2.8.3.

2.9 Genetic diversity and population structure analysis of *T. collina* using TE-AFLP (Three Enzymes-Amplified Fragment Length Polymorphism) and TE-AFLP derived markers

2.9.1 TE-AFLP analysis

2.9.1.1 Digestion and adaptor ligation

TE-AFLP procedure was carried out as essentially described in van der Wuff et al. (2000). Genomic DNA (~30 ng) of each stingless bee was simultaneously digested with *Rsa*I, *Xba*I and *Bam*HI and ligated to adaptors having ends complementary to the restricted DNA fragments in a single reaction. Digestion/ligation was carried out in a 20 µl reaction volume containing 1X digestion-ligation buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM ATP), 4 picomol of both *Xba*I and *Bam*HI adaptors (Table 2.3), 0.5 units T4 DNA ligase, 6 units *Xba*I, 1.25 units *Bam*HI and 1 unit *Rsa*I then incubated at 30 ^{o}C for 1.5 hours.

2.9.1.2 PCR amplification

The sequence of *Xba*I and *Bam*HI primer for amplification (Table 2.3) are complementary to one strand of each adaptor with arbitrary extensions. The *Bam*HI primer with arbitrary extension-C was end labeled with ^{32}P at the 5' end of primer. The labeling reaction was prepared by 40 µl of 1X T4 kinase buffer (70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT), 10 µM *Bam*HI-C primer, 100 µCi [γ-^{32}P] dATP, and 10 units T4 polynucleotide kinase. The mixture was incubated at 37 ^{o}C for 30 minutes and then inactivated at 90 ^{o}C for 2minutes (Vos et al., 1995).

Amplification was carried out in a 12.75 µl reaction volume containing 1X PCR buffer (10 mM Tris-HCl, pH 7.6, 50 mM KCl, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 µM each of unlabelled-*Xba*I primer and ^{32}P-labeled *Bam*HI primer and 0.6 units GoTaq® DNA polymerase (Promega). The digestion-ligation mixture of 0.5 µl was used as DNA template. PCR was performed using the following thermal profile: predenaturation at 95 ºC for 3 minutes followed by 10 cycles of denaturation at 95 ºC for 30 seconds, annealing at 70 ºC for 30 seconds and extension at 72 ºC for 1 minute, and an additional 40 cycles of denaturation at 95 ºC for 30 seconds, annealing at 60 ºC for 30 seconds and extension at 72 ºC for 1 minute. The final extension was carried out at 72 ºC for 20 minutes.

Each reaction was then electrophoresed through 8% denaturing polyacrylamide gel. The PCR product was mixed to 3 µl loading dye and denatured for 5 minutes at 95 ^{o}C. The denatured mixture was loaded on the gel with 0.6X TBE electrophoresis buffer and run for 3 hours at 500 V. At the end of the run, gels were dried on filter paper and exposed X-ray film overnight at room temperature.

2.9.2 Scoring TE-AFLP variation

After autoradiography, the bands were read and recorded manually. AFLP bands were treated as biallelic dominant markers; present (homozygotes or heterozygotes for amplification of the band) or absent (homozygote for lack of amplification) was scored as 1 or 0, respectively, generating a multi-band pattern for each individual. These data were used to calculate genetic diversity and population structure statistics.

2.9.3 Cloning of TE-AFLP derived marker

The AFLP bands from TE-AFLP analysis (as described in 2.9.1) were investigated for polymorphic AFLP band that was not uniquely or commonly found in investigated *T. collina*. It was excised and eluted out from the polyacrylamide gel. It was then reamplified and the target fragment was eluted from agarose gel (2.8.4.1 and 2.8.4.2). The fragment was ligated to vector and transformed into host cell (2.8.4.3, 2.8.4.5 and 2.8.4.6). The plasmid of recombinant clone was extracted as described earlier (2.8.4.7).

Table 2.3 Adaptor sequences and TE-AFLP primers used for the ligation and PCR amplification

Primer	**Sequences**
Adaptor sequences	
*Bam*HI adaptor	5’-ACGAAGTCCCGCGCCAGCAA-3’
	5’-GATCTTGCTGGCGCGGG-3’
*Xba*I adaptor	5’-ACGTTGTGGCGGCGTCGAGA-3’
	5’-CTAGTCTCGACGCCGCC-3’
Selective amplification primers	
B_{+C}	5’-GTTTCGCGCCAGCAAGATCCC-3’
X_{+CC}	5’-GGCGTCGAGACTAGACC-3’
X_{+CT}	5’-GGCGTCGAGACTAGACT-3’

Table 2.4 Primers of TE-AFLP derived marker

Primer	**Sequence**	**Annealing temperature (^{o}C)**	**Expected size (bp)**
TECU-F	5′-CGTATCAGTGTCGTTCATGGC-3′	56.9	222
TECU-R	5′-CGAGCGCGTGGAATCTC-3′		

2.9.4 DNA sequencing and primer design

The sequence of recombinant plasmid was analyzed by DNA sequencing service (Macrogen, Inc, Korea) with the M13 forward or reverse primers (universal primer) under BigDye™ terminator cycling conditions on automatic sequencer 3730x1. The sequences were analyzed and compared for the homology search using BlastN (nucleotide similarity) and BlastX (translated protein similarity) available at www.ncbi.nlm.nih.gov. A pair of designed primers was shown in Table 2.4.

2.9.5 PCR amplification of TE-AFLP derived marker and SSCP analysis

PCR was carried out using TECU primer (Table 2.4). The amplification reaction was performed in a 25 µl reaction volume containing 1X PCR buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM each dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer, 50 ng of genomic DNA and 1 unit of DyNazyme™ II DNA polymerase. The PCR profile consisted of predenaturation at 94 ^{o}C for 3 minutes followed by 35 cycles of denaturation at 94 ^{o}C for 30 seconds, annealing at 56.9 ^{o}C for 1 minute and extension at 72 ^{o}C for 30 seconds. The final extension was performed at 72 ^{o}C for 7 minutes. The primer pair was tested against genomic DNA of representative individual *T. collina*. The expected amplified product was purified and further determined on non-denaturing polyacrylamide gel by SSCP analysis as described in 2.8.7.

2.9.6 Scoring SSCP variation

The visible bands in each lane in SSCP gels were read and recorded manually. The bands were treated as present or absent bands and scored as 1 or 0 respectively, generating 0/1 matrix.

2.9.7 Data analysis

The data of scoring TE-AFLP or SSCP variation were used for analysis. Genetic diversity, genetic distance among populations and population structure statistics were calculated using Genetic Analysis in Excel (GenAlEx6; Peakall and Smouse, 2006).

Genetic diversity was estimated as the proportion of polymorphic loci (P) and expected heterozygosity (H_e). A band was considered polymorphic band if samples showed any variation for presence or absence although bands were present or absent in only a single individual. The expected heterozygosity (H_e) was estimated following Lynch and Milligan (1994), which observed each band position as a different locus with two alleles, band amplified (dominant) and band not amplified (recessive); absence of band indicates a recessive homozygote. At each locus, the frequency of the recessive allele (q) is estimated from the frequency of recessive homozygotes (q^2) and the frequency of dominant allele is estimated as p = 1 - q. Expected heterozygosity at each locus is $h = 1 - \sum x_i^2$, where x_i is the frequency of the i^{th} allele or $1 – (p^2+q^2)$. Expected heterozygosity averaged over all loci is calculated as:

$$H_e = 1 – 1/m \sum_{y=1}^{m} \sum x_i^2$$

where *y* represents loci or bands 1 through *m*

Genetic heterogeneity in allele distribution frequencies between compared geographic samples was examined using the exact test (Guo and Thompson, 1992). F_{ST}-based statistics (Φ_{PT}) between pairs of geographic populations, which generated by Analysis of Molecular Variance (AMOVA; Excoffier et al., 1992 implemented in GenAlEx6.1), were calculated and tested to determine whether Φ_{PT} was statistically different from zero (Weir and Cockerham 1984; Peakall and Smouse, 2006). The significances performing 999 permutations in which individuals are randomly assigned to regions of the same size, was calculated. Unbiased genetic distance between pairs of geographic samples was determined (Nei 1978). The investigated *T. collina* samples from TE-AFLP analysis were divided into hierarchical groups in three ways: (A) geographic region (North, Central, Northeast, and Peninsular Thailand); (B) populations north (North, Central and Northeast) and south (Peninsular Thailand) of the Isthmus of Kra; and (C) Northeast population versus the remaining populations (North, Central, and Peninsular Thailand) while those from TE-AFLP derived marker were grouped into two ways: (A) six populations (North, Central, Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular); and (B) four populations (North+Central+Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular Thailand).

2.10 Mitochondrial DNA diversity of *T. collina* using PCR-SSCP

2.10.1 PCR amplification of the mtDNA gene segments and SSCP analysis

The primers were designed from the sequences of mtDNA genes which were previously deposited in GenBank. The large ribosomal RNA (16S) and cytochrome b (cytb) mitochondrial DNA genes were known from GenBank Accession no. DQ790412 and AY575081, respectively. On the other hand, the primer pair of cytochrome c oxidase I gene (COI) was obtained from sequences of *T. amalthea* (GenBank Accession no. AF214669). These primers (Table 2.5) were tested against genomic DNA of representative individual *T. collina*. The amplification reaction was performed in a 25 µl reaction volume containing 1X PCR buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM each dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer, 50 ng of genomic DNA and 1 unit of DyNazyme™ II DNA polymerase. The PCR profiles of each amplification reaction are shown in Table 2.6. All PCR products of each mtDNA region from each *T. collina* were analyzed by SSCP analysis as described in 2.8.7 whereas the amplified products of 16S and COI gene were run on 11% non-denaturing polyacrylamide gel (75:1 crosslink).

Table 2.5 Sequences of primers and size of the expected amplified product mtDNA segment

mtDNA region	Primer	Sequence	Expected size (bp)
16S rRNA	16S-F	5′-ATGGCTGCAGTATAACTGAC-3′	478
	16S-R	5′-ACTTACGTCGATTTGAACTC-3′	
COI	COI-F	5′-CATTCATCTCCTTCTGTTG-3′	497
	COI-R	5′-GCTCGTGTATCAATATCTAATC-3′	
Cyt b	cytb-F	5′-TTGTAGAGTGATTATGAGGAG-3′	316
	cytb-R	5′-GGAGTAACTATAGGATCAGC-3′	

Table 2.6 PCR profile used for amplification of mtDNA segment using primers in Table 2.5

Main genes	PCR profile
16S rRNA	94 °C for 3 minutes 5 cycles of 94 °C for 30 seconds 40 °C for 60 seconds 72 °C for 60 seconds 35 cycles of 94 °C for 30 seconds 56 °C for 60 seconds 72 °C for 60 second 72 °C for 7 minutes
COI	94 °C for 3 minutes 5 cycles of 94 °C for 30 seconds 40 °C for 60 seconds 72 °C for 60 seconds 35 cycles of 94 °C for 30 seconds 50 °C for 60 seconds 72 °C for 60 second 72 °C for 7 minutes
Cyt b	94 °C for 3 minutes 35 cycles of 94 °C for 30 seconds 55 °C for 60 seconds 72 °C for 60 second 72 °C for 7 minutes

2.10.2 Scoring SSCP variation

The bands visible in each lane in SSCP gels were read and recorded manually. The bands for each amplified mtDNA region were treated as present or absent bands and scored as 1 or 0, respectively, generating 0/1 matrix.

2.10.3 Data analysis

Genetic Analysis in Excel (GenAlEx6) was used to calculate genetic diversity, genetic distance among populations and population structure statistics as described in 2.9.7. The investigated *T. collina* samples were divided into 6 hierarchical groups; North, Central, Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular Thailand.

CHAPTER III

RESULT

3.1 DNA extraction

Genomic DNA was extracted from each stingless bee individual using the extraction protocol described in 2.7.1. High molecular weight DNA obtained was at least 23.1 kb. The DNA concentration was estimated by comparison the intensity of EtBr-DNA complex with a known amount of λ/*Hin*dIII marker in 0.8% agarose gel electrophoresis (Figure 3.1). The extracted DNA was used in subsequent analysis.

Figure 3.1 High molecular weight DNA of *Tetragonilla collina* worker extracted from one bee per nest. Lane M is λ/*Hin*dIII markers.

3.2 Development of *T. collina*-specific marker using AFLP (Amplified Fragment Length Polymorphism) and SSCP (Single Strand Conformational Polymorphism) analysis

3.2.1 AFLP analysis

The AFLP analysis consisted of several steps: genomic DNA digestion, adaptor ligation, preamplification, and selective amplification of the digested/ligated fragments. One primer combination (*Pst*I$_{+A}$ and *Mse*I$_{+C}$ primer) was used in the preamplification step. The preamplified products were then amplified with selective primers having three selective bases at the 3′ end of each primer. There were 64 primer combinations which were used to test against genomic DNA of 11 stingless bee species including *Tetragonilla collina*. The products of selective amplification showed different band patterns in each stingless bee species using the same primer combination (Figure 3.2). The products were then size-fractionated through denaturing polyacrylamide gel. The primer combinations provided a low level of polymorphism in *Tetragonilla collina* and different band patterns from other species were screened to search species-specific bands in *T. collina*. The *Pst*I$_{+AGT}$ and *Mse*I$_{+CAG}$ primer combination provided a 316 bp fragment found only in *T. collina* (Figure 3.3).

3.2.2 Cloning and characterization of a species-specific AFLP fragment

A 316 bp fragment only found in *T. collina* was successfully reamplified (Figure 3.4). The purified product was cloned. Colony PCR was performed to verify the inserted fragment of 316 bp (Figure 3.5). The recombinant plasmid was sequenced in both directions. The nucleotide sequence (Figure 3.6) did not match any sequence in the GenBank (*E*-value >1e-04) and was regarded as an anonymous DNA segment.

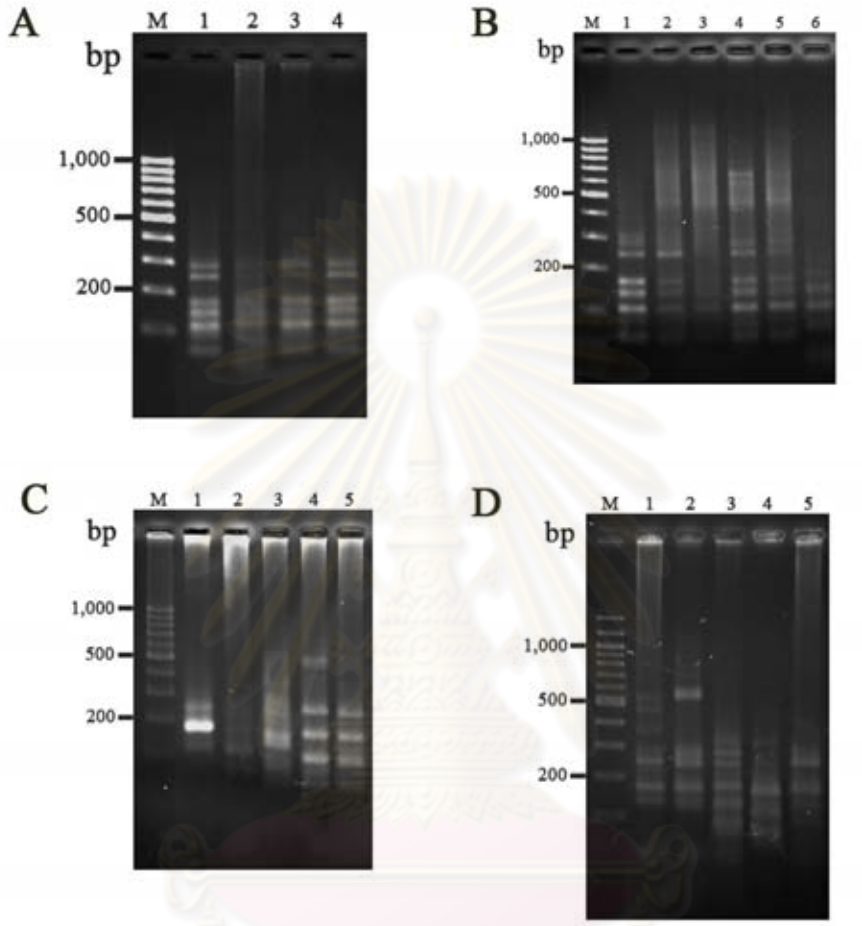


Figure 3.2 The selective amplification products of each stingless bee species on agarose gel; *Tetragonilla collina* (lanes 1-4 A and lanes 1-6 B), *Heterotrigona itama* (lane 1C), *Tetrigona apicalis* (lane 2C), *Lophotrigona canifrons* (lane 3C), *Tetragonula fuscobalteata* (lane 4C), *Tetragonula pagdeni* (lane 5C), *Tetragonula minor* (lane 1D), *Lepidotrigona terminata* (lane 2D), *Geniotrigona thoracica* (lane 3D), *Homotrigona fimbriata* (lane 4D), and *Tetragonula melina* (lane 5D) amplified by *Pst*I_{+AGT}/*Mse*I_{+CAG}. Lanes M (A, B, C, and D) are a 100 bp DNA ladder.

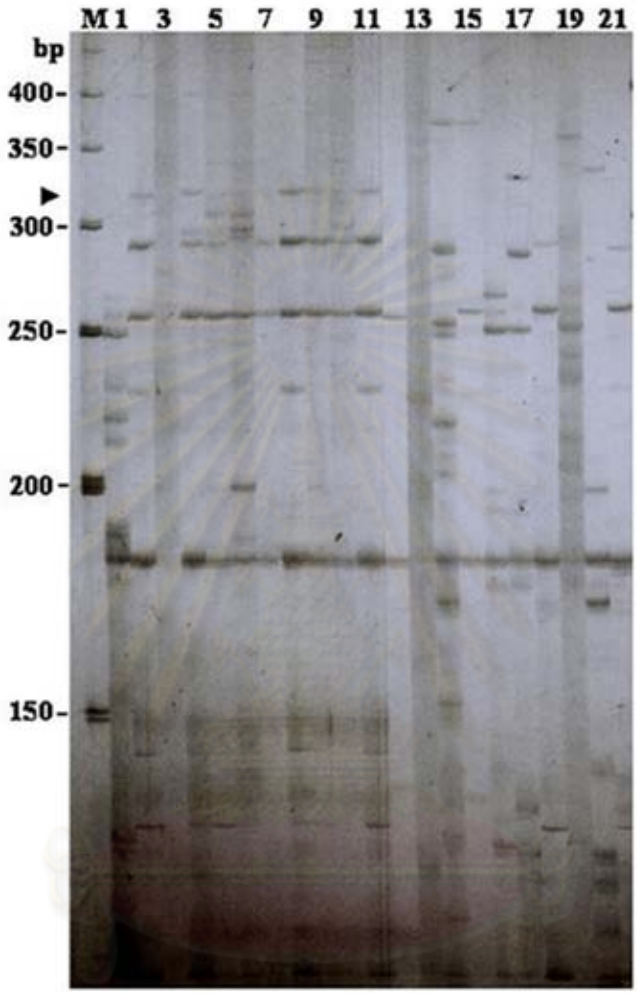


Figure 3.3 AFLP patterns of various stingless bees; *Heterotrigona itama* (lanes 1 and 19), *Tetragonilla collina* (lanes 2-11), *Tetragonula pagdeni* (lane 12), *Tetrigona apicalis* (lane 13), *Lophotrigona caniforns* (lane 14), *Tetragonula minor* (lane 15), *Lepidotrigona doipaensis* (lane 16), *Geniotrigona thoracica* (lane 17), *Tetragonula fuscobalteata* (lane 18), *Homotrigona fimbriata* (lane 20), *Tetragonula melina* (lane 21) genotyped by *Pst*I_{+AGT}/*Mse*I_{+CAG}. Lane M is a 50 bp DNA ladder. An arrow indicates an AFLP band of 316 bp found only in *T. collina*.

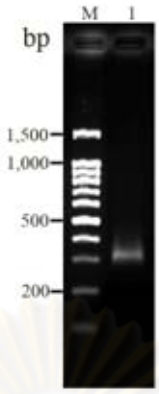


Figure 3.4 Reamplification of the species-specific marker of *T. collina*. Lame M is a 100 bp ladder

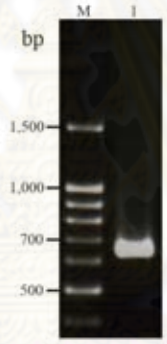


Figure 3.5 Colony PCR product of the recombinant clone containing the targeted insert (the species-specific AFLP marker in *T. collina*). Lame M is a 100 bp DNA ladder

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5'GACTGCGTAC ATGCAGAGTG GTTCGGATTT GGTTGGCATT GGAACCCTGA
  TCAGCGCGAA TTCTCACTGG AGTTCCCAGC ACACGGCGGT CGGAGAGTGA
  TCAAATCTCA AGTCGTTGGC CGTCTGAGGG CCGCAGTGTC GGAGCACTAC
  ATGTCCGTGC TTATCGCGAT ATCAGACCAG GGCAAGGTCT TCGATGCGAT
  CTCTCGCCAT AAGGCGAGCA ACTACTTCAT GCGGACGGGT CAGTACCTAC
  GCTTGGTCGA CTGGCGCTTC GTACACCGCG CGCGCCTAGA CGTGCTCCTG
  TTACTCAGGA CTCATC 3'
```

Figure 3.6 Nucleotide sequence of a *T. collina*-specific AFLP fragment (316 bp). The locations and sequences of a forward primer (CUTc1-F) and those complementary to a reverse primer (CUTc1-R) for species-diagnostic SCAR marker (CUTc1) are illustrated in boldface and underlined.

3.2.3 Development of species-diagnostic SCAR (sequence-characterized amplified region) marker in *T. collina*

A pair of primers (CUTc1-F and CUTc1-R) was developed from the nucleotide sequences of candidate *T. collina*-specific AFLP fragment (Figure 3.6). The developed SCAR marker (hereafter called CUTc1) was tested against one individual per nest of *T. collina* (134 nests) and 14 other stingless bee species (Figure 3.7). The expected amplification product (259 bp) was found in all *Tetragonilla collina* individuals (134/134 nests accounting for 100% of investigated specimens) but not in the other genus and species of investigated specimens, *Tetrigona apicalis*, *Lophotrigona canifrons*, *Lepidotrigona doipaensis*, *Homotrigona fimbriata*, *Tetragonula fuscobalteata*, *Heterotrigona itama*, *Tetragonula laeviceps*, *Tetrigona melanoleuca*, *Tetragonula melina*, *Tetragonula minor*, *Geniotrigona thoracica*, *Lepidotrigona terminata* and *Lisotrigona furva*. Nevertheless, cross-species amplification was found in *Tetragonula pagdeni* (43/51 nests, 84.3%). It indicated that species-specific PCR of CUTc1 marker unsuccessfully discriminated *Tetragonilla collina* from *Tetragonula pagdeni*.

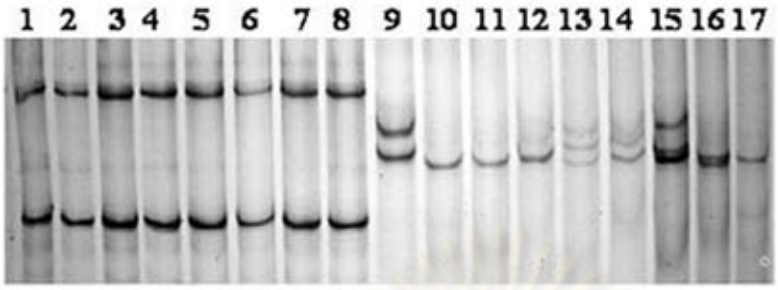
3.2.4 Characterization of the SCAR marker using SSCP analysis

SSCP analysis was performed to characterize the amplified CUTc1 marker found in *Tetragonilla collina* and *Tetragonula pagdeni*. Non-overlapping SSCP patterns between *T. collina* and *T. pagdeni* were observed (Figure 3.8A). Nucleotide sequences of representative individuals of these species were different, owing to a 15-bp indel (CGGCCGCCAAGCGGC) and several single nucleotide polymorphisms (SNPs). In addition, within species SNPs were also observed in *T. pagdeni* (Figure 3.8B). Moreover, the polymorphic SSCP patterns of CUTc1 marker in *T. collina* were observed (Figure 3.9A). An AA (259/259 bp) genotype was found in all *T. collina* from north (21 nests) and northeast (32 nests), and 23/28 nests from central region, whereas a BB (253/253 bp) genotype was observed in most individuals from peninsular Thailand (42/53 nests). In addition, heterozygotes exhibiting the AB (253/259 bp) genotype were observed in some individuals from central region (Prachuap Khiri Khan, 5/28 nests) and those from peninsular Thailand (Chumphon, Ranong, Surat Thani, and Nakon Si Thammarat, 11/53 nests). Genotypic differences between these specimens were consistent when the amplified product of CUTc1 from

representative individuals carrying AA, AB and BB genotypes were re-examined by denaturing gel electrophoresis (Figure 3.9B). Nucleotide sequences of stingless bees carrying different homozygotic genotypes indicated allelic polymorphism owing to a 6-bp indel (GACCAG) present in AA but absent in BB genotypes (Figure 3.9C).

Figure 3.7 Amplification results of species-diagnostic SCAR marker CUTc1 against genomic DNA of *Tetragonilla collina* (lanes 2-6, A and 1-4, B), *Tetrigona apicalis* (lanes 7-9, A), *Lepidotrigona doipaensis* (lane 10, A), *Homotrigona fimbriata* (lane 11, A), *Heterotrigona itama* (lane 12, A), *Tetragonula minor* (lane 13, A), *Tetragonula fuscobalteata* (lane 14, A), *Tetragonula pagdeni* (lanes 15, A and 8-9, B), *Tetrigona melanoleuca* (lane 16, A), *Tetragonula laeviceps* (lane 5, B), *Lisotrigona furva* (lane 6, B), *Tetragonula melina* (lane 7, B), *Lepidotrigona terminata* (lane 10, B), *Geniotrigona thoracica* (lane 11, B), and *Lophotrigona canifrons* (lane 12, B). Lanes M (A and B) and 1 (A) are a 100 bp DNA ladder and the negative control (without genomic DNA template), respectively.

A



B

```
T. pagdeni-C1   GGTTCGGATTTGGTTGGCATTGGAACCCTGATCAGTACGAACTCTCACTGGAGTGCCCAG 60
T. pagdeni-C2   GGTTCGGATTTGGTTGGCATTGGAACCCTGATCAGTGCGAATTCTCACTGGAGTGCCCAG 60
T. collina-N    GGTTCGGATTTGGTTGGCATTGGAACCCTGATCAGCGCGAATTCTCACTGGAGTTCCCAG 60
T. collina-NE   GGTTCGGATTTGGTTGGCATTGGAACCCTGATCAGCGCGAATTCTCACTGGAGTTCCCAG 60
                ********************************  **** *********** *****

T. pagdeni-C1   CACGTGGCGGTCGGAGAGTGATCATACCTCCGGCCGCCAAGCGGCAAGTCGTTGGACATC 120
T. pagdeni-C2   CACGCGGCGGTCGGAGAGTGATCATACCTCCGGCCGCCAAGCGGCAAGTCGTTGGACATC 120
T. collina-N    CACACGGCGGTCGGAGAGTGATCAAATCTC---------------AAGTCGTTGGCCGTC 105
T. collina-NE   CACACGGCGGTCGGAGAGTGATCAAATCTC---------------AAGTCGTTGGCCGTC 105
                ***  ****************** * ***               ********** * **

T. pagdeni-C1   TGAGGGCCGCAGTGTCGGAGCACTACATGTCCGTGCTTATCGCGAAGCCGGACCAGGGCA 180
T. pagdeni-C2   TGAGGGCCGCAGTGTCGGAGCACTACATGTCCGTGCTTATCGCGAAGCCGGACCAGGGCA 180
T. collina-N    TGAGGGCCGCAGTGTCGGAGCACTACATGTCCGTGCTTATCGCGAAATCGGACCAGGGCA 165
T. collina-NE   TGAGGGCCGCAGTGTCGGAGCACTACATGTCCGTGCTTATCGCGAAATCGGACCAGGGCA 165
                ******************************************  ************

T. pagdeni-C1   AGGTCTTCGATGCGACCTTCCGTCATGGAGCGAGTAATCGCTTCATACGGACAGGTCAGT 240
T. pagdeni-C2   AGGTCTTCGATGCGACCTTCCGTCATGGAGCGAGTAATCGCTTCATACGGACAGGTCAGT 240
T. collina-N    AGGTCTTCGATGCGATCTCTCGCCATAAGGCGAGCAACTACTTCATGCGGACGGGTCAGT 225
T. collina-NE   AGGTCTTCGATGCGATCTCTCGCCATAAGGCGAGCAACTACTTCATGCGGACGGGTCAGT 225
                ************** **  ** ***   ***** **   ****** ***** *******

T. pagdeni-C1   ACCTGCGCTTTGCCGACTGGCGCTTCGTACACCG 274
T. pagdeni-C2   ACCTGCGCTTTGCCGACTGGCGCTTCGTACACCG 274
T. collina-N    ACCTACGCTTGGTCGACTGGCGCTTCGTACACCG 259
T. collina-NE   ACCTACGCTTGGTCGACTGGCGCTTCGTACACCG 259
                **** ***** * **********************
```


Figure 3.8 SSCP patterns of the amplified CUTc1 of *Tetragonilla collina* (lanes 1-8, A) and *Tetragonula pagdeni* (lanes 9-17, A) and nucleotide sequences of CUTc1 (B) in representative individuals of *Tetragonilla collina* originating from the north (N) and northeast (NE) and *Tetragonula pagdeni* originating from the central region (C), respectively.

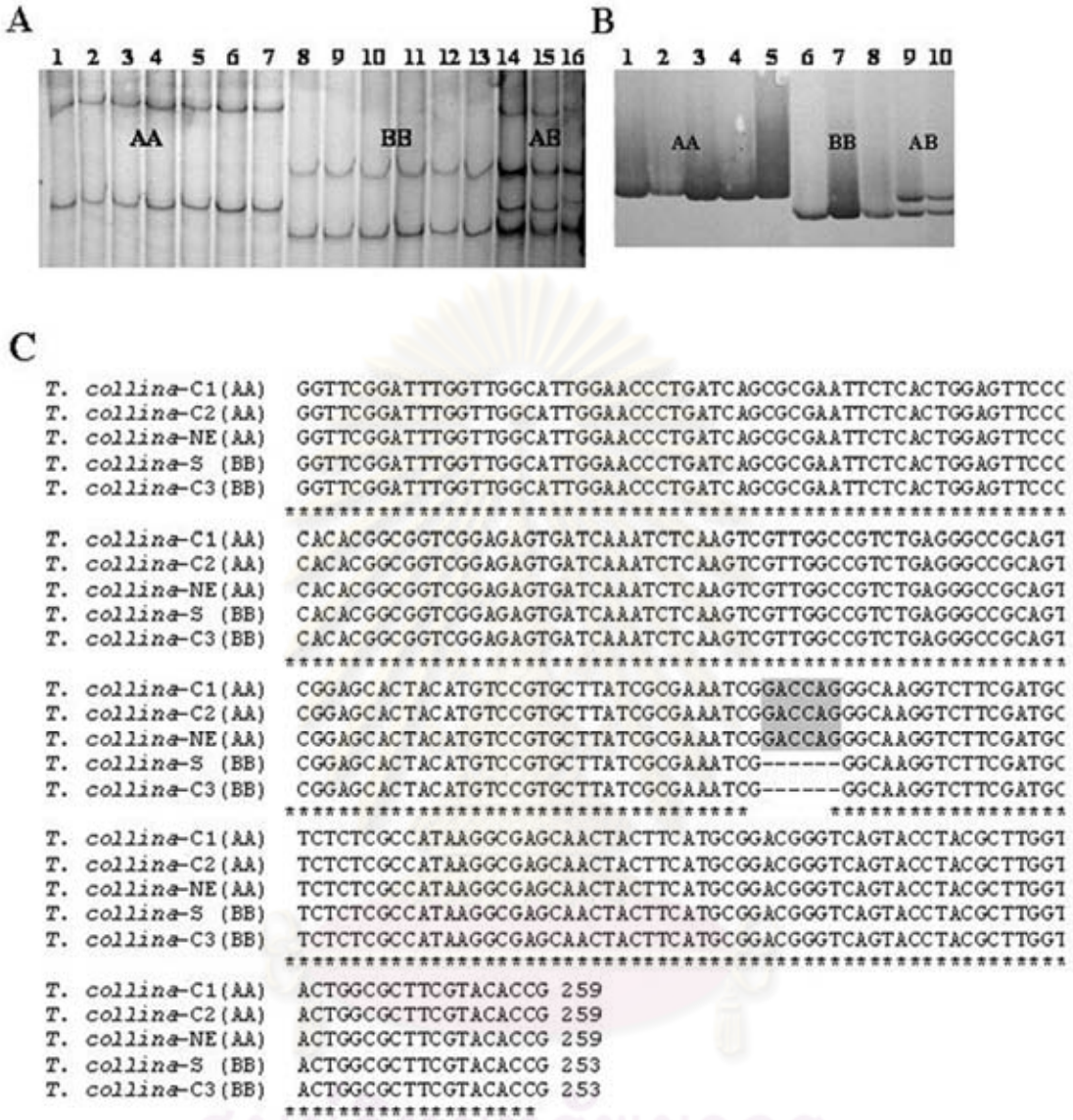


Figure 3.9 The amplified CUTc1 against genomic DNA of *T. collina* fractionated in 12.5% non-denaturing polyacrylamide (SSCP) gel electrophoresis (A) and 6% denaturing (B). Three genotypes; AA (lanes 1-7, A and 1-5, B), BB (lanes 8-13, A and 6-8, B) and AB (lanes 14-16, A and 9-10, B) were observed. Nucleotide sequence of CUTc1 in *T. collina* possessing genotype AA (259/259 bp alleles; *T. collina*-C1, *T. collina*-C2 and *T. collina*-NE) and BB (253/253 bp alleles; *T. collina*-S and *T. collina*-C3) are illustrated (C).

3.3 Genetic diversity and population structure of *T. collina* using TE-AFLP (Three Enzymes-Amplified Fragment Length Polymorphism) and TE-AFLP derived markers

3.3.1 TE-AFLP analysis

Genomic DNA of individuals representing 98 nests of *T. collina* was carried out by TE-AFLP procedure: genomic DNA digestion, adaptor ligation, and amplification of the digested/ligated fragments. Two sets of primer combinations (*Xba*I-CC/*Bam*HI-C and *Xba*I-CT/*Bam*HI-C) were used for generating fingerprints of each specimen. The *Bam*HI-C primer was end labeled with ^{32}P. The fingerprint patterns were visualized by autoradiography (Figure 3.10).

3.3.2 Scoring TE-AFLP variation

A total of 53 bands were scored from analysis of *T. collina* using TE-AFLP with two sets of primers (*Xba*I-CC/*Bam*HI-C and *Xba*I-CT/*Bam*HI-C). Thirty bands (57%) were variable (absent in at least one individual), while the rest 23 bands were present in all 98 individuals. We observed 47 unique banding patterns or phenotypes for the primer pair *Xba*I-CC/*Bam*HI-C, and 79 phenotypes for the primer pair *Xba*I-CT/*Bam*HI-C (appendix B). Relatively high genetic diversity was observed in all geographic samples (Table 3.1). The percentage number of polymorphic bands and H_e for each region varied from 42-55% and 0.090-0.141, respectively. The greatest level of within region diversity was found in the Central region.

The unbiased genetic distance between pairs of geographic regions was 0.022-0.094. Significant geographic heterogeneity was observed between all pairwise comparisons of regions analyzed by F_{ST}-based statistics, Φ_{PT} ($P < 0.001$, Table 3.2). The exact test revealed significant genetic differences in most comparisons except between *T. collina* from Central and Peninsular Thailand ($P = 0.6202$) and between North and Northeast ($P = 0.1875$). The results indicated stronger degrees of differentiation between North+Northeast and Peninsular Thailand ($\Phi_{PT} = 0.334$ and 0.359) than other comparisons ($\Phi_{PT} = 0.076$-0.242). Lower (but significant) levels of geographic differentiation were observed between the Central and each of the other populations ($\Phi_{PT} = 0.076$-0.199).

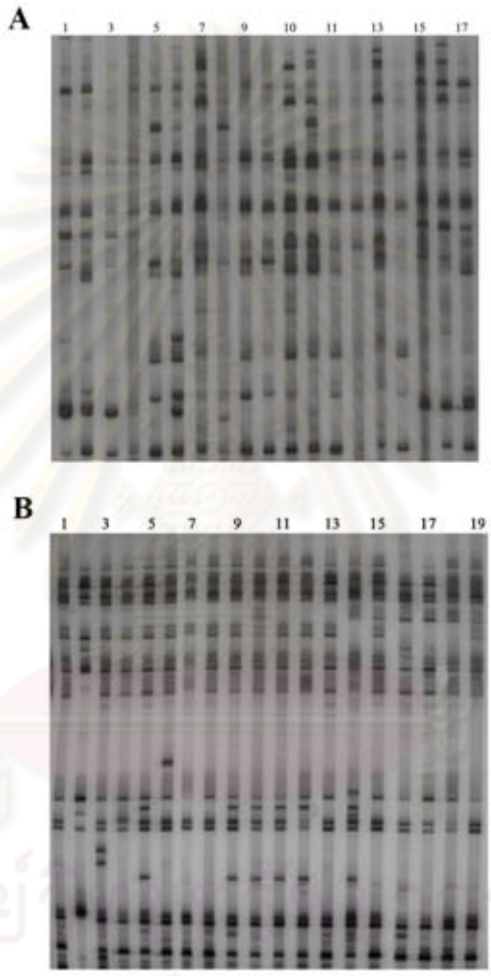


Figure 3.10 Autoradiogram of some TE-AFLP patterns generated by two sets of primer pairs; *Xba*I-CC and *Bam*HI-C (panel A), and *Xba*I-CT and *Bam*HI-C (panel B). The amplification was obtained by *T. collina* DNA from each of four geographic regions (Central, lanes 1-4 A, 1-6 B; North, lanes 5-7 A, 7-12 B; Northeast, lanes 8-14 A, lanes 13-16 B; and Peninsular Thailand, lanes 15-17 A, 17-19 B).

Table 3.1 Comparision of TE-AFLP bands and the expected heterozygosity (H_e) generated by *Bam*HI-C and *Xba*I-CC, and *Bam*HI-C and *Xba*I-CT primer pairs in *T. collina* from 4 geographic regions in Thailand

TE-AFLP bands (***N*** **= 98)**	**Central** (***N*** **= 20)**	**North** (***N*** **= 18)**	**Northeast** (***N*** **= 23)**	**Peninsular** (***N*** **= 37)**
Number scored	51	43	45	48
Number fixed	23	25	24	23
Number absent	2	10	8	5
# polymorphic	28	18	21	25
% polymorphic	55% of 51* (53% of 53)**	42% of 43 (34% of 53)	47% of 45 (40% of 53)	52% of 48 (47% of 53)
Mean H_e (± SD)	0.141 (0.025)	0.095 (0.021)	0.090 (0.020)	0.139 (0.026)

*Total number of TE-AFLP bands in a particular regions; ** Total number of scorable TE-AFLP bands across all regions.

Table 3.2 Pairwise F_{ST}-based statistics (Φ_{PT}, below diagonal), Nei's (1972) genetic distance (in brackets) and the exact test (above diagonal) among populations of *T. collina* in Thailand

	Central	**North**	**Northeast**	**Peninsular**
Central (*N* = 20)	-	$P = 0.0104$	$P < 0.0001$	$P = 0.6202^{ns}$
North (*N* = 18)	0.199* (0.050)	-	$P = 0.1835^{ns}$	$P < 0.0001$
Northeast (*N* = 23)	0.242* (0.059)	0.242* (0.046)	-	$P < 0.0001$
Peninsular (*N* = 37)	0.076* (0.022)	0.334* (0.087)	0.359* (0.094)	-

*significant differences at $P < 0.05$; ns, not significant

AMOVA illustrated significant variance components among the four geographic regions, North, Central, Northeast, and Peninsular Thailand (Φ_{PT} = 0.258, P = 0.001), between North-to-Central and Peninsular Thailand (Φ_{PT} = 0.207, P = 0.001) and between Northeast and other populations (North, Central and Peninsular Thailand; Φ_{PT} = 0.172, P = 0.001, Table 3.3).

Table 3.3 AMOVA for genetic differentiation of *T. collina* among four geographic regions (A), between the North-to-Central and Peninsular populations (B), and between Northeast and other populations (C)

A. Four geographic regions

Component	df	Estimate Variance	% of total variance	Φ_{PT}	*P*-value
Among population	3	1.253	26	0.258	0.001
Within population	94	3.603	74		

B. North-to-Central (North+Northeast+Central) vs. Peninsular Thailand

Component	df	Estimate Variance	% of total variance	Φ_{PT}	*P*-value
Among population	1	1.052	21	0.207	0.001
Within population	96	4.023	79		

C. Northeast vs. other populations

Component	df	Estimate Variance	% of total variance	Φ_{PT}	*P*-value
Among population	1	0.871	17	0.172	0.001
Within population	96	4.207	83		

3.3.3 Cloning and characterization of a TE-AFLP derived fragment in *T. collina*

A polymorphic AFLP band (420 bp) that was variably found in investigated *T. collina* was successfully reamplified by *Bam*HI-C and *Xba*I-CT primer pairs (Figure 3.11). The purified product was cloned. The inserted fragment was verified by colony PCR. The recombinant plasmid was sequenced in both directions. The nucleotide sequence (Figure 3.12) did not match any sequence in the GenBank (*E*-value >1e-04) and was regarded as an anonymous DNA segment.

3.3.4 Development and characterization of TE-AFLP derived SCAR marker using SSCP analysis

A pair of primers (TECU-F and TECU-R) was developed from the nucleotide sequences of polymorphic AFLP fragment (Figure 3.12). The developed SCAR marker (hereafter called TECU) was tested against one individual per nest of *T. collina* (Figure 3.13). The expected amplification product (222 bp) was found in all individuals representing 96 nests of *T. collina*.

SSCP analysis was performed to characterize the amplified TECU marker in 96 investigated specimens. Three SSCP patterns (pattern I, II, III) were observed (Figure 3.14). The nucleotide sequences of representative individuals of each SSCP pattern (Figure 3.15) were different due to a 4-bp indel (GACA) and 6 single nucleotide polymorphisms (SNPs). The pattern I was commonly distributed in all *T. collina* from North (15 nests), Central (12 nests), and Northeast (23 nests) while the pattern II was found in *T. collina* from Prachuap Khiri Khan (3/8 nests, 37.5%), Chumphon (10/13 nests, 77%), and Peninsular Thailand (20/25 nests, 80%). In addition, the pattern III was also observed in those from Prachuap Khiri Khan (5/8 nests, 62.5%), Chumphon (3/13 nests, 23%), and Peninsular Thailand (5/25 nests, 20%). The phylogeographic pattern among 3 patterns based on number of mutation was illustrated in Figure 3.16. Seven mutation steps were found between pattern I and III. The pattern II was an intermediate pattern between pattern I and III.

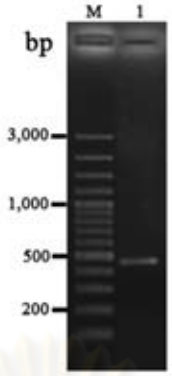


Figure 3.11 Reamplification of the TE-AFLP derived marker of *T. collina.* Lane M is a 100 bp ladder.

```
5′GTTTCGCGCC AGCAAGATCC CACAGTTTTT TATCGATACT GGGATATTTC
  TTTAATCCTC TGTGCAAGCA GCGATGGTTG CTCGATAACG ACGTATCAGT
  GTCGTTCATG GCAATGAAGA GGACTTCTTT TTTTTTTTCT TTATCGAAAT
  ATCACCGAAC AACACAATTA CGAGACATCT TGCAAAATAC AAAGTGTAGT
  TTACACTTTC ACACCATCAT AGACCACGAA CAGTCTTCAT TATGGCACAG
  CTAAATATAT CTTCTCGCAT CCTTTTGCAT TTACACGATA TCAATCGAGA
  TTCCACGCGC TCGAGAAGTC AATAGTTCGT CAAAGTTATT AACGAGAAAC
  AAAAGATATT CGCTTGGAGA AAAGTGAAAA CTCGCGGCTC ACGTTTCTTC
  TCGAGTCTAG TCTCGACGCC 3′
```

Figure 3.12 Nucleotide sequence of the TE-AFLP derived fragment (420 bp). The locations and sequences of a forward primer (TECU-F) and those complementary to a reverse primer (TECU-R) for developed SCAR marker (TECU) are illustrated in boldface and underlined.

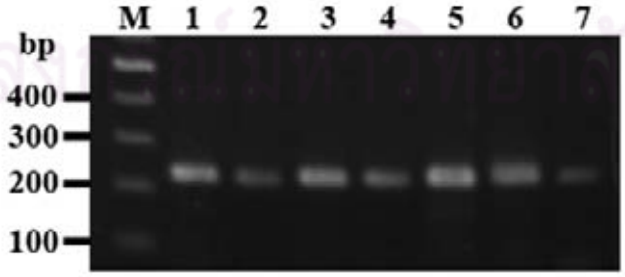


Figure 3.13 Amplification results of TECU marker against genomic DNA of *T. collina*. Lane M is a 100 bp DNA ladder.

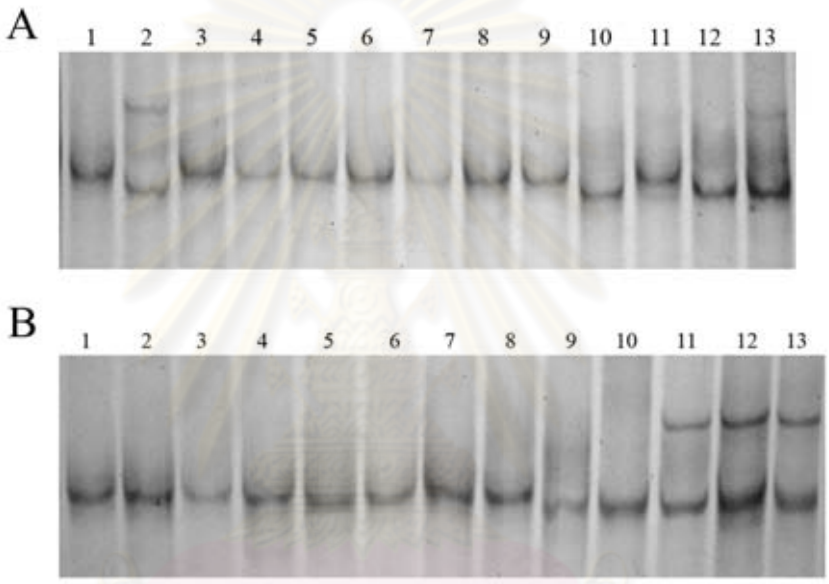


Figure 3.14 The SSCP pattern of the amplified TECU marker of *T. collina* from different geographic regions in Thailand. Three patterns; pattern I (lanes 1, 3-9, 11, A and lanes 1-8, B), pattern II (lanes 10, 12, A and lanes 9, 10, B), and pattern III (lanes 2, 13, A and lanes 11-13, B) were observed.

```
T. collina-NE(I)    CGTATCAGTGTCGTTCATGGCAATGAAGAGGACTTTTTTTTTTTTTTTCTTTATCGAAATA 60
T. collina-C (II)   CGTATCAGTGTCGTTCATGGCAATGAAGAGGACTTTTTTTTTTTTTTTCTTTATCGAAATA 60
T. collina-S (III)  CGTATCAGTGTCGTTCATGGCAATGAAGAGGACTTTTTTTTTTTTTTTCTTTATCGAAATA 60
                    ************************************************************

T. collina-NE(I)    TCACCGAACAACACAATTACGAGACATCTTGCAAAATACAAAGTGTAGTTTACACTTTCA 120
T. collina-C (II)   TCACCGAACAACACAATTACGAGACATCTTGCAAAATACAAAGTGTAGTTTACACTTTCA 120
T. collina-S (III)  TCACCGAATAACACAATTACGAGACATCTTGCAAAATACAAAGTGTAGTTTACACTTTCA 120
                    ******** ***************************************************

T. collina-NE(I)    CACCATCATAGACCACGAAGACACGGTCTTCATTATGGCACAGCTAAATATATCTTCTCG 180
T. collina-C (II)   CACCATCATAGACCACGAA----CAGTCTTCATTATGGCACAGCTAAATATATCTTCTCG 176
T. collina-S (III)  CACCATCATAGACCACGAA----CAGTCTTCATTATGGCACAGCTAAATATATCTTCTCG 176
                    ******************    * ***********************************

T. collina-NE(I)    CATCCTTTTGCATTTACACAATATCAATCGAAATTCCACGCGCTCG 226
T. collina-C (II)   CATCCTTTTGCATTTACACGATATCAATCGAGATTCCACGCGCTGG 222
T. collina-S (III)  CATCTTTTTGCATTTACACGATATCAATCGAGATTCCACGCGCTGG 222
                    **** ************** *********** ************ *
```

Figure 3.15 Nucleotide sequences of TECU marker in representative individuals of *T. collina* possessing the pattern I (*T. collina*-NE), pattern II (*T. collina*-C), and pattern III (*T. collina*-S).

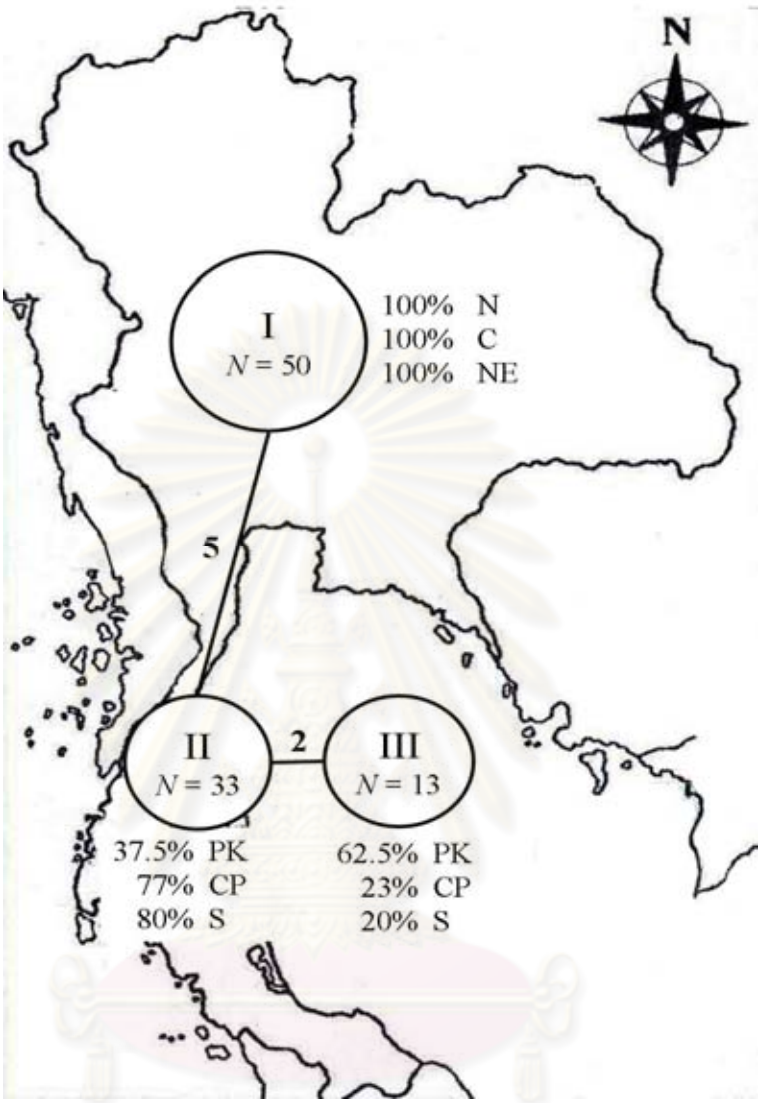


Figure 3.16 Phylogeographic pattern deduced from TECU marker (pattern I, II, and III). Numbers along connected lines indicated inferred mutation steps. Letter *N* represented the amount of *T. collina* possessing each SSCP pattern. The percentages were calculated from the total number of *T. collina* in a particular region; North (15 nests), Central (12 nests), Northeast (23 nests), Prachuap Khiri Khan (8 nests), Chumphon (13 nests), and Peninsular Thailand (25 nests). Capital letters behind percentage were abbreviations of Central (C), Northeast (NE), Prachuap Khiri Khan (PK), Chumphon (CP), and Peninsular Thailand (S).

A total of 3 bands were scored from SSCP analysis of TECU marker in *T. collina* and all bands were variable (absent in at least one individual).Genetic diversity was only observed in Prachuap Khiri Khan, Chumphon, and Peninsular Thailand populations (Table 3.4). The percentage of polymorphic bands for these 3 regions was 50% while the mean expected heterozygosity, H_e varied from 0.063 – 0.158. No polymorphic bands and H_e were observed in North, Central, and Northeast populations.

The unbiased genetic distance between pairs of geographic regions was 0.000-1.994. Significant geographic heterogeneity was observed in most pairwise comparisons of regions analyzed by F_{ST}-based statistics, Φ_{PT} ($P < 0.05$) and the exact test ($P < 0.0001$) except the pairwise comparison of North, Central, and Northeast and the pairwise comparison of Chumphon versus either Prachuap Khiri Khan or Peninsular Thailand and (Table 3.5). The results indicated no degrees of differentiation between North, Central, and Northeast and between Chumphon and Peninsular Thailand ($\Phi_{PT} = 0.000$) while the strong degrees of differentiation were observed in other comparisons ($\Phi_{PT} = 0.899$-0.948).

AMOVA analysis revealed significant molecular variability among 6 population groupings; North, Central, Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular Thailand (88%, $\Phi_{PT} = 0.877$, $P = 0.001$). Higher variance component was observed among 4 populations; North-to-central, Prachuap Khiri Khan, Chumphon, and Peninsular Thailand (90%, $\Phi_{PT} = 0.903$, $P = 0.001$, Table 3.6).

Table 3.4 Comparison of SSCP bands and the expected heterozygosity (H_e) of TECU marker from 6 populations of *T. collina* in Thailand

SSCP Bands (***N*** **= 96)**	**Central** (***N*** **= 12)**	**North** (***N*** **= 15)**	**Northeast** (***N*** **= 23)**	**Peninsular Thailand** (***N*** **= 25)**	**Prachuap Khiri Khan** (***N*** **= 8)**	**Chumphon** (***N*** **= 13)**
Number scored	1	1	1	2	2	2
Number fixed	1	1	1	1	1	1
Number absent	2	2	2	0	0	0
# polymorphic	0	0	0	1	1	1
% polymorphic	0% of 1* (0% of 3)**	0% of 1 (0% of 3)	0% of 1 (0% of 3)	50% of 2 (33.3% of 3)	50% of 2 (33.3% of 3)	50% of 2 (33.3% of 3)
Mean H_e (± SD)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.063 (0.063)	0.158 (0.158)	0.072 (0.072)

*Total number of scorable SSCP bands in a particular regions; ** Total number of scorable SSCP bands across all region

Table 3.5 Pairwise F_{ST}-based statistics (Φ_{PT}, below diagonal), Nei's (1972) genetic distance (in brackets) and the exact test (above diagonal) of TECU marker among populations of *T. collina* in Thailand

	Central	**North**	**Northeast**	**Peninsular Thailand**	**Prachuap Khiri Khan**	**Chumphon**
Central (*N* = 12)	-	*P* = 1.0000	*P* = 1.0000	*P* < 0.0001	*P* < 0.0001	*P* < 0.0001
North (*N* = 15)	0.000^{ns} (0.000)	-	*P* = 1.0000	*P* < 0.0001	*P* < 0.0001	*P* < 0.0001
Northeast (*N* = 23)	0.000^{ns} (0.000	0.000^{ns} (0.000)	-	*P* < 0.0001	*P* < 0.0001	*P* < 0.0001
Peninsular Thailand (*N* = 25)	0.899* (1.265)	0.906* (1.265)	0.921* (1.265)	-	*P* = 0.3529	*P* = 1.0000
Prachuap Khiri Khan (*N* = 8)	0.919* (1.994)	0.930* (1.994)	0.948* (1.994)	0.283* (0.071)	-	*P* = 0.7298
Chumphon (*N* = 13)	0.910* (1.298)	0.920* (1.298)	0.938* (1.298)	0.000^{ns} (0.000)	0.201^{ns} (0.062)	-

*significant differences at $P < 0.05$; ns, not significant

Table 3.6 Analysis of Molecular Variance (AMOVA) of TECU marker for genetic differentiation of *T. collina* among 6 population groupings; North, Central, Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular Thailand (A), and among 4 populations; North-to-central (North+Central+Northeast), Prachuap Khiri Khan, Chumphon, and Peninsular Thailand (B)

A. Six population groupings

Component	df	Estimate Variance	% of total variance	Φ_{PT}	*P*-value
Among population	5	0.650	88	0.877	0.001
Within population	90	0.091	12		

B. Four population groupings

Component	df	Estimate Variance	% of total variance	Φ_{PT}	*P*-value
Among population	3	0.831	90	0.903	0.001
Within population	92	0.089	10		

3.4 Mitochondrial DNA diversity of *T. collina* using PCR-SSCP

The three mitochondrial DNA gene segments; large subunit ribosomal RNA (16S rRNA), cytochrome oxidase I (COI), and cytochrome b (cytb), were used for genetic diversity and population structure studies of *T. collina*. The specific primer pairs of these 3 genes were successfully developed and amplified against 103 individual colonies of *T. collina*. The amplified products were then characterized by SSCP analysis to examine mtDNA polymorphisms.

3.4.1 Analysis of 16S ribosomal RNA (16S rRNA) gene polymorphism

The expected products (478 bp) were found in all investigated *T. collina* (103/103 nests accounting for 100% of investigated specimens). The products were identified on the 11% non-denaturing polyacrylamide gel (75:1 crosslink) to characterize the 16S rRNA gene polymorphisms (Figure 3.17). Seventeen SSCP phenotypes were observed (appendix B). The nucleotide sequences of 6 common phenotypes (pattern A, B, C, D, E, and F, Figure 3.18), which observed in at least 4 individuals, were different due to a 1-bp indel (T) and several SNPs. The phylogeographic pattern of 6 haplotypes was shown in Figure 3.19. The pattern A was found in all *T. collina* from Prachuap Khiri Khan (12/12 nests, 100%) and most individuals from Central (16/19 nests, 84.2%). The pattern B, C, and D were observed in samples from Northeast (6/25 nests, 24%; 5/25 nests, 20%; 4/25 nests, 16%, respectively). The pattern E, which was different from pattern F by 3 point mutations was distributed in all individuals from Chumphon (11/11 nests, 100%) and some individuals from Peninsular Thailand (5/25 nests, 20%) whereas most of them (20/25 nests, 80%) were observed in pattern F.

Twenty-nine scored fragments were considered polymorphic. Gene diversity was observed in North, Central, Northeast, and Peninsular Thailand (Table 3.7). All scorable bands of each population were polymorphic bands except those from Prachuap Khiri Khan and Chumphon had only fixed bands (present in all samples of particular region). The mean expected heterozygosity (H_e) varied from 0.043-0.065 in North, Central, Northeast, and Peninsular Thailand.

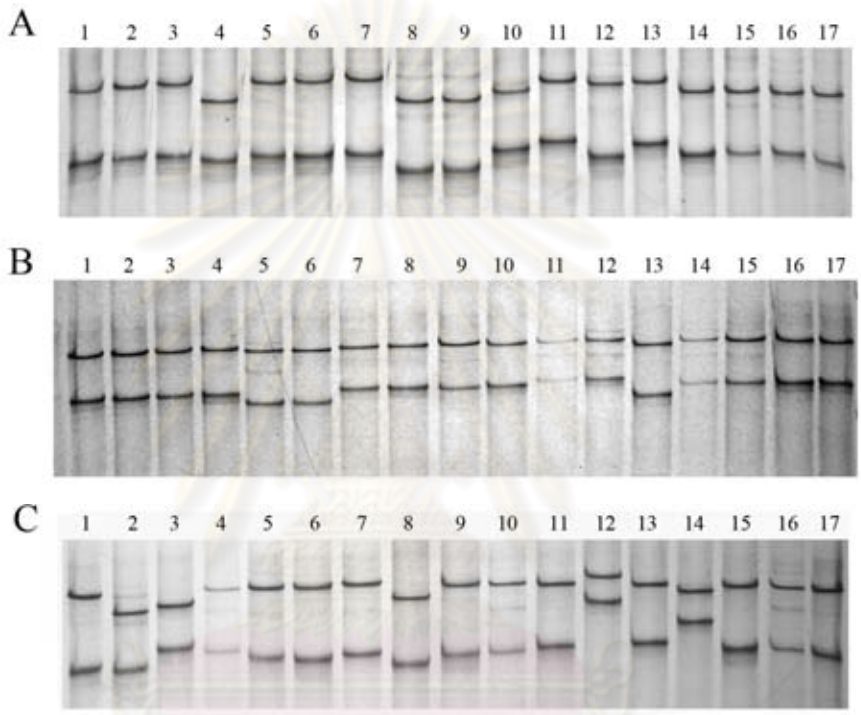


Figure 3.17 The SSCP pattern of the amplified 16S rRNA gene of *T. collina* from different geographic regions in Thailand; North population (lanes 6-9 A, 5-9 B, and 2-3, 7 C), Central population (lanes 2-5 A, 4 B, and 5-6 C), Northeast population (lanes 10-13 A, 10-13 B, and 4, 8-14 C), Peninsular population (lanes 14-17 A and 14-15 B), Prachuap Khiri Khan population (lanes 1 A, 1-3 B, and 1 C), and Chumphon population (lanes 16-17 B and 15-17 C).

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Pattern A    ATGGCTGCAGTATAACTGACTGTACAAAGGTAGCATAATCAATTGATTTTTAAATGAAAT 60
Pattern B    ATGGCTGCAGTATAACTGACTGTACAAAGGTAGCATAATCAATTGATTTTTAAATGAAAT 60
Pattern C    ATGGCTGCAGTATAACTGACTGTACAAAGGTAGCATAATCAATTGATTTTTAAATGAAAT 60
Pattern D    ATGGCTGCAGTATAACTGACTGTACAAAGGTAGCATAATCAATTGGTTTTTAAATGAAAT 60
Pattern E    ATGGCTGCAGTATAACTGACTGTACAAAGGTAGCATAATCAATTGGTTTTTAAATGAAAT 60
Pattern F    ATGGCTGCAGTATAACTGACTGTACAAAGGTAGCATAATCAATTGGTTTTTAAATGAAAT 60
             ************************************************ **************

Pattern A    CTGGAATGAAAGGATTAATGAAATATATACTGTCTCATGTATATAAAGTGAATTTAAAAT 120
Pattern B    CTGGAATGAAAGGATTAATGAAATATATACTGTCTCATGTATATAAAATGAACTTAAAAT 120
Pattern C    CTGGAATGAAAGGATTAATGAAATATATACTGTCTCATGTATATAAAATGAACTTAAAAT 120
Pattern D    CTGGAATGAAAGGATTAATGAAATATATACTGTCTCATGTATATAGAGTGAATTTAAAAT 120
Pattern E    CTGGAATGAAAGGATTAATGAAATATATACTGTCTCATGTATATAAAATGAACTTAAAAT 120
Pattern F    CTGGAATGAAAGGATTAATGAAATATATACTGTCTCATGTATATAAAATGAACTTAAAAT 120
             ************************************************ * **** *******

Pattern A    TTTAATTAAAATGTTAAAATTTTTTTATGGGACGATAAGACCCTATAGAATTTTATATTG 180
Pattern B    TTTAATTAAAATGTTAAAATTTTTTTATGGGACGATAAGACCCTATAGAATTTTATATTG 180
Pattern C    TTTAATTAAAATGTTAAAATTTTTTTATGGGACGATAAGACCCTATAGAATTTTATATTG 180
Pattern D    TTTAATTAAAATGTTAAAATTTTTTTATGGGACGATAAGACCCTATAGAATTTTATATTG 180
Pattern E    TTTAATTAAAATGTTAAAATTTTTTTATGGGACGATAAGACCCTATAGAATTTTATATTG 180
Pattern F    TTTAATTAAAATGTTAAAATTTTTTTATGGGACGATAAGACCCTATAGAATTTTATATTG 180
             ************************************************************

Pattern A    CTACATTTTAATACTTAATATAAAATATGTAGTAATATTTGGTTGGGAGGACTATTAAAT 240
Pattern B    CTACATTTTAATACTTAATATAAAATATGTAGTAATATTTGGTTGGGAGGACTATTAAAT 240
Pattern C    CTACATTTTAATACTTAATATAAAATATGTAGTAATATTTGGTTGGGAGGACTATTAAAT 240
Pattern D    CTACATTTTAATACTTAATATAAAATATGTAGTGATATTTGGTTGGGAGGACTATTAAAT 240
Pattern E    CTACATTTTAATACTTAATATAAAATATGTAGTAATATTTGGTTGGGAGGACTATTAAAT 240
Pattern F    CTGCATTTTAATACTTAATATAAAATATGTAGTAATATTTGGTTGGGAGGACTATTAAAT 240
             ** ***************************** *************************

Pattern A    TTGATTAACTTTAATTTTT-GTTTACTTTGATTTAAGAAAGTACAATGATCTTTAAATTA 299
Pattern B    TTGACTAACTTTAATTTTT-GATTACTTTAATTTAAGAAAATACAATGATCTTTAAATTA 299
Pattern C    TTGACTAACTTTAATTTTT-GATTACTTTAATTTAAGAAAATACAATGATCTTTAAATTA 299
Pattern D    TTGACTAACTTTAATTTTT-GATTACTTTGATTTAAGAAAATACAATGATCTTTAAATTA 299
Pattern E    TTGACTAACTTTAATTTTT-GATTACTTTGATTAAAGAAAATACAGTGATCTTTAAATTA 299
Pattern F    TTGACTAACTTTAATTTTTTGATTACTTTGATTAAAGAAAATACAATGATCTTTAAATTA 300
             **** ************** * ******* *** ****** **** **************

Pattern A    AAATATCTAGATTAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCGTATA 359
Pattern B    AAATATCTAGATTAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCGTATA 359
Pattern C    AAATAGCTAGATTAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCGTATA 359
Pattern D    AAATATCTAGACTAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCGTATA 359
Pattern E    AAATATCTAGATTAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCGTATA 359
Pattern F    AAATATCTAGATTAAATTACCTTAGGGATAACAGCGTAATATTTTTTTATAGATCGTATA 360
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Figure 3.18 Alignment of 6 patterns of 16S rRNA gene from *T. collina* in Thailand. Asterisk indicated the same nucleotides among 6 patterns.

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Pattern A    GAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAACGCAGGAGTTTAA 419
Pattern B    GAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAACGCAGGAGTTTAA 419
Pattern C    GAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAACGCAGGAGTTTAA 419
Pattern D    GAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAACGCAGGAGTTTAA 419
Pattern E    GAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAACGCAGGAGTTTAA 419
Pattern F    GAAAAAAATGATTGCGACCTCGATGTTGAATTAAGATAAATTTTAAACGCAGGAGTTTAA 420
             ************************************************************

Pattern A    TGATTAAGTCTGTTCGACTTTTAAAATCTTACATGATTTGAGTTCAAATCGACGTAAGT 478
Pattern B    TGATTAAGTCTGTTCGACTTTTAAAATCTTACATGATTTGAGTTCAAATCGACGTAAGT 478
Pattern C    TGATTAAGTCTGTTCGACTTTTAAAATCTTACATGATTTGAGTTCAAATCGACGTAAGT 478
Pattern D    TGATTAAGTCTGTTCGACTTTTAAAATCTTACATGATTTGAGTTCAAATCGACGTAAGT 478
Pattern E    TGATTAAGTCTGTTCGACTTTTAAAATCTTACATGATTTGAGTTCAAATCGACGTAAGT 478
Pattern F    TGATTAAGTCTGTTCGACTTTTAAAATCTTACATGATTTGAGTTCAAATCGACGTAAGT 479
             ************************************************************
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Figure 3.18 continue

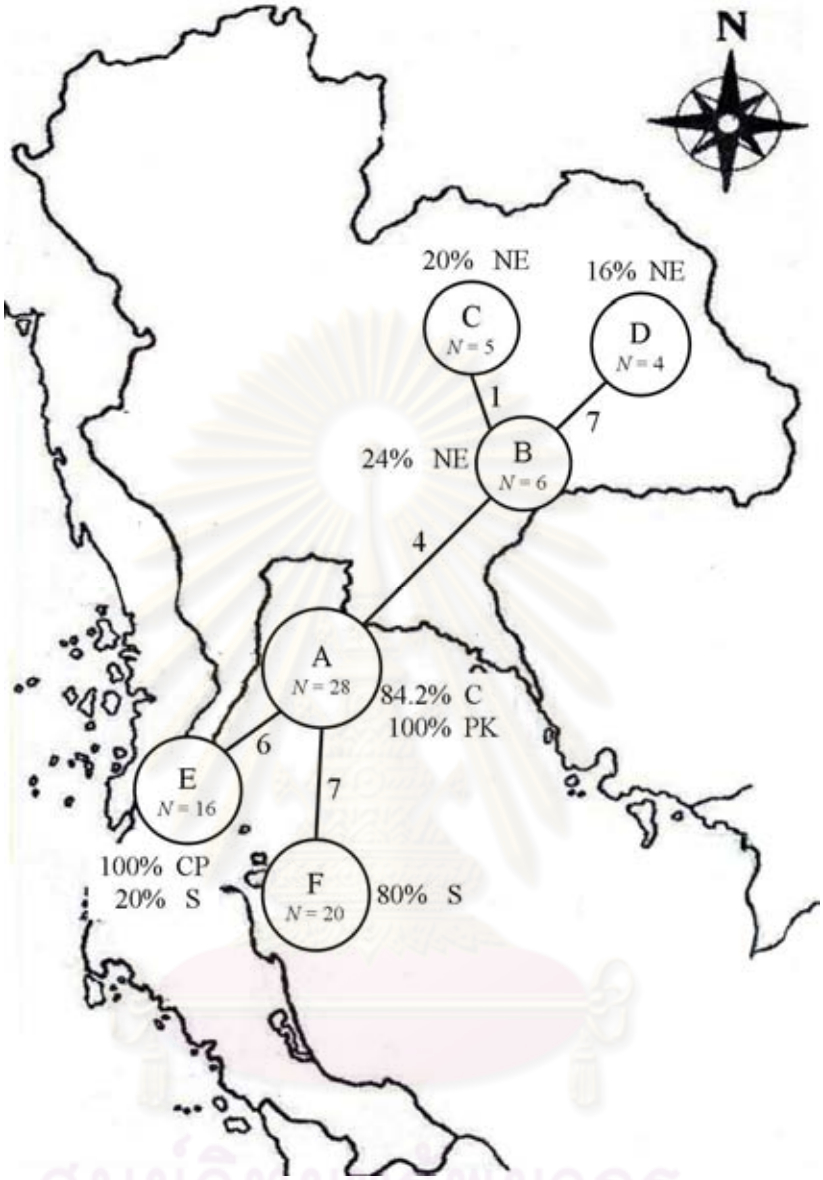

Figure 3.19 Phylogeographic pattern deduced from 16S rRNA gene haplotypes of *T. collina* (pattern A, B, C, D, E, and F). Numbers along connected lines indicated inferred mutation steps. Letter *N* represented the amount of *T. collina* possessing each pattern. The percentages were calculated from the total number of *T. collina* in a particular region; North (11 nests), Central (19 nests), Northeast (25 nests), Prachuap Khiri Khan (12 nests), Chumphon (11 nests), and Peninsular Thailand (25 nests). Capital letters behind percentage were abbreviations of Central (C), Northeast (NE), Prachuap Khiri Khan (PK), Chumphon (CP), and Peninsular Thailand (S).

Table 3.7 Comparison of genetic diversity in six populations of 16S rRNA gene in *T. collina* in Thailand

SSCP Bands (***N*** **= 103)**	**Central (19)**	**North (11)**	**Northeast (25)**	**Peninsular Thailand (25)**	**Prachuap Khiri Khan (12)**	**Chumphon (11)**
Number scored	4	7	15	4	2	2
Number private	2	6	15	2	0	0
Number fixed	0	0	0	0	2	2
Number absent	25	22	14	25	27	27
# polymorphic	4	7	15	4	0	0
% polymorphic	100% of 4* (14% of 29)**	100% of 7 (24% of 29)	100% of 15 (52% of 29)	100% of 4 (14% of 29)	0% of 2 (0% of 29)	0% of 2 (0% of 29)
Mean H_e (± SD)	0.043 (0.024)	0.061 (0.023)	0.065 (0.016)	0.047 (0.025)	0.000 (0.000)	0.000 (0.000)

*Total number of scorable SSCP bands in a particular regions; ** Total number of scorable SSCP bands across all region

The unbiased genetic distance between pairs of populations was 0.003-0.148 (Table 3.8). Significant geographic heterogeneity was observed in most pairwise comparisons of regions analyzed by F_{ST}-based statistics, Φ_{PT} ($P < 0.05$). The exact test revealed significant genetic differences in comparison between Peninsular Thailand and each population. The results indicated strong degree of differentiation in each comparison (Φ_{PT} = 0.070-0.826) whereas no degrees of differentiation was found between Prachuap Khiri Khan and Chumphon.

AMOVA analysis showed high molecular variance components among 6 populations; North, Central, Northeast, Prachuap Khiri Khan, Chumphon and Peninsular Thailand (56%, Φ_{PT} = 0.563, P = 0.001), among 5 population groupings; North, Central, Northeast, Prachuap Khiri Khan+Chumphon, and Peninsular Thailand (44%, Φ_{PT} = 0.437, P = 0.001), and between North and South of Isthmus of Kra (33%, Φ_{PT} = 0.334, P = 0.001, Table 3.9).

3.4.2 Analysis of cytochrome oxidase I (COI) gene polymorphism

The expected products (497 bp) were found in 73 nests from total 103 investigated *T. collina*, accounting for 71% of investigated specimens. To characterize the polymorphisms, the products were run on the 11% non-denaturing polyacrylamide gel (Figure 3.20). Four common SSCP patterns (pattern A, B, C, and D) of all 34 patterns were sequenced (Figure 3.21, appendix B). Their nucleotide sequences were unlike by several SNPs. The pattern A was found in samples from Prachuap Khiri Khan (6/10 nests) and one individual from Central (1/10 nest). Both pattern B and C were observed in samples from Chumphon (2/11 nests for B, 2/11 nests for C) and Peninsular Thailand (6/22 nests for B, 3/22 nests for C) while pattern D was distributed in those from Peninsular Thailand (6/22 nests).

Fifty-seven SSCP bands were scored as polymorphic. Gene diversity was observed in all 6 populations, North, Central, Northeast, Prachuap Khiri Khan, Chumphon and Peninsular Thailand (Table 3.10). No fixed bands were present in all populations of *T. collina*. The percentage of polymorphic bands for all populations was 100%. The mean expected heterozygosity (H_e) for 6 populations was in the vicinity (H_e = 0.029-0.039).

Table 3.8 Pairwise F_{ST}-based statistics (Φ_{PT}, below diagonal), Nei's (1972) genetic distance (in brackets) and the exact test (above diagonal) of 16S rRNA gene among six populations of *T. collina* in Thailand

	Central	**North**	**Northeast**	**Peninsular Thailand**	**Prachuap Khiri Khan**	**Chumphon**
Central (*N* = 19)	-	$P = 0.0015$	$P < 0.0001$	$P < 0.0001$	$P = 1.0000$	$P = 0.0001$
North (*N* = 11)	0.527* (0.078)	-	$P = 0.0153$	$P < 0.0001$	$P = 0.0075$	$P = 0.0821$
Northeast (*N* = 25)	0.419* (0.071)	0.219* (0.043)	-	$P < 0.0001$	$P = 0.0304$	$P = 0.0321$
Peninsular Thailand (*N* = 25)	0.690* (0.107)	0.528* (0.080)	0.412* (0.067)	-	$P < 0.0001$	$P = 0.0008$
Prachuap Khiri Khan (*N* = 12)	0.070 (0.003)	0.646* (0.095)	0.492* (0.089)	0.783* (0.126)	-	$P = 0.0005$
Chumphon (*N* = 11)	0.826* (0.130)	0.650* (0.102)	0.483* (0.089)	0.726* (0.094)	0.000 ns (0.148)	-

*significant differences at $P < 0.05$; ns, not significant

Table 3.9 Analysis of Molecular Variance (AMOVA) of 16S rRNA gene for genetic differentiation of *T. collina* among 6 population groupings; North, Central, Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular Thailand (A), among 5 populations; North, Central, Northeast, Prachuap Khiri Khan+Chumphon, and Peninsular Thailand (B), and between North (North+Central+Northeast+Prachuap Khiri Khan) and South (Chumphon and Peninsular Thailand) of Isthmus of Kra (C)

A. Six population groupings

Component	df	Estimate Variance	% of total variance	Φ_{PT}	*P*-value
Among population	5	1.069	56	0.563	0.001
Within population	97	0.831	44		

B. Five population groupings

Component	df	Estimate Variance	% of total variance	Φ_{PT}	*P*-value
Among population	4	0.819	44	0.437	0.001
Within population	98	1.057	56		

C. North and South of Isthmus of Kra

Component	df	Estimate Variance	% of total variance	Φ_{PT}	*P*-value
Among population	1	0.696	33	0.334	0.001
Within population	101	1.388	67		

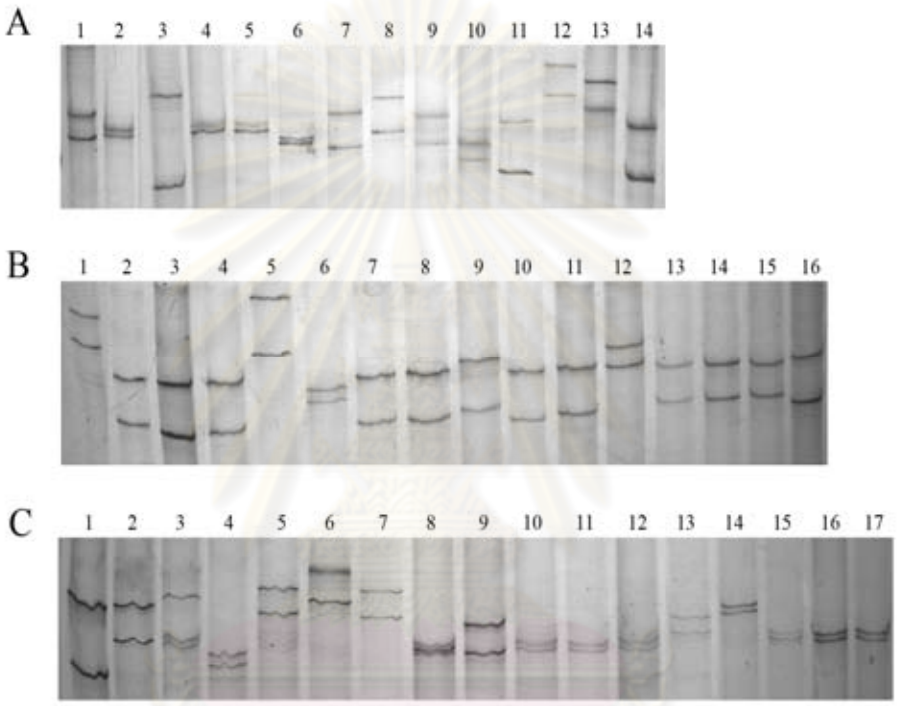

Figure 3.20 The SSCP pattern of the amplified COI gene of *T. collina* from different geographic regions in Thailand; North population (lanes 8-10 A and 1-7 C), Central population (lanes 11-13 A and 1, 4-6 B), Northeast population (lanes 6-7 A and 8-17 C), Peninsular population (lanes 2-5 A and 10, 16 B), Prachuap Khiri Khan population (lanes 14 A and 2-3, 7 B), and Chumphon population (lanes 1 A and 8-9, 11-15 B).

```
Pattern A    CATTCATCTCCTTCTGTTGATTTTACTATTTTTTCAATTCATATGACTGGGGTTTCATCT 60
Pattern B    CATTCATCTCCTTCTGTTGATTTTACTATTTTTTCAATTCATATGACTGGGGTTTCATCT 60
Pattern C    CATTCATCTCCTTCTGTTGATTTTACTATTTTTTCAATTCATATGACTGGGGTTTCATCT 60
Pattern D    CATTCATCTCCTTCTGTTGATTTTACTATTTTTTCAATTCATATGACTGGGGTTTCATCT 60
             ************************************************************

Pattern A    ATTTTAGGATCACTAAATTTTATTGTAACAATTTTTATAATAAAAAATTTTTCAATTAGG 120
Pattern B    ATTTTAGGGTCACTAAATTTTATTGTAACAATTTTTATAATAAAAAATTTTTCAATTAGC 120
Pattern C    ATTTTAGGGTCACTAAATTTCATTGTAACAATTTTTATAATAAAAAATTTTTCAATTAAC 120
Pattern D    ATTTTAGGGTCACTAAATTTTATTGTAACAATTTTTATAATAAAAAATTTTTCAATTAAC 120
             ******** *********** ************************************

Pattern A    TATGATCAAATTACACTATTTTCTTGATCTATTTCAATTACTGTAATTCTTTTAATCATC 180
Pattern B    TATGATCAAATTACACTATTTTCTTGATCTATTTCAATTACTGTAATTCTTTTAATTGTC 180
Pattern C    TATGATCAAATTACACTATTTTCTTGGTCTATTTCAATTACTGTAATTCTTTTAATCATC 180
Pattern D    TATGATCAAATTACACTATTTTCGTGATCTATTTCAATTACTGTAATTCTTTTAATCATT 180
             ********************** ** ****************************  *

Pattern A    TCTCTTCCTGTTTTGGCTGGAGCTATTACAATACTTTTATTTGATCGAAACTTTAATACT 240
Pattern B    TCTCTTCCTGTCTTGGCTGGAGCTATTACAATACTTTTATTTGATCGAAATTTTAATACT 240
Pattern C    TCTCTTCCTGTTTTGGCTGGAGCTATTACAATACTTTTATTTGATCGAAACTTCAATACT 240
Pattern D    TCTCTTCCTGTTTTAGCTGGAGCTATTACAATACTTTTATTTGATCGAAATTTTAATACT 240
             *********** ** ********************************** ** ******

Pattern A    TCATTTTTTGATCCGGTAGGAGGAGGTGATCCAATTCTTTACCAACATCTATTTTGATTC 300
Pattern B    TCATTTTTTGATCCAGTAGGAGGAGGTGATCCAATTCTTTACCAACATCTATTTTGATTC 300
Pattern C    TCATTTTTTGATCCGGTGGGAGGAGGTGATCCAATTCTTTACCAACATCTATTTTGATTC 300
Pattern D    TCATTTTTTGATCCAGTGGGAGGGGGTGATCCAATTCTTTACCAACATCTATTTTGATTC 300
             ************** ** ***** ************************************

Pattern A    TTTGGACATCCTGAAGTTTATATTCTTATTCTTCCTGGATTTGGATTAATTTCCCAGATT 360
Pattern B    TTTGGACATCCTGAAGTTTATATTCTTATTCTTCCTGGATTTGGATTAATTTCCCAGATT 360
Pattern C    TTTGGGCATCCTGAAGTTTATATTCTCATTCTTCCTGGATTTGGATTAATTTCCCAGATT 360
Pattern D    TTTGGACATCCTGAAGTTTATATTCTTATTCTTCCTGGATTTGGATTAATTTCTCAGATT 360
             ***** ******************** ************************* ******

Pattern A    ATTATGAATGAAAGGGGAAAGAAGGAGGTTTTTGGGAATTTAAGAATAATTTATGCAATA 420
Pattern B    ATTATAAATGAAAGGGGAAAGAAGGAGGTTTTTGGGAATTTAAGAATAATTTATGCAATA 420
Pattern C    ATTATAAATGAAAGAGGAAAGAAGGAGGTTTTCGGAAATTTAAGAATAATTTACGCAATA 420
Pattern D    ATTATAAATGAAAGGGGGAAGAAGGAGGTTTTTGGAAATTTGAGAATAATTTATGCAATG 420
             ***** ******** ** ************** ** ***** *********** *****

Pattern A    ATTGGTATTGGATTTCTTGGTTTTATTGTTTGAGCTCATCATATATTTACTGTAGGATTA 480
Pattern B    ATTGGTATTGGATTTCTTGGTTTTATTGTTTGAGCTCATCATATATTTACTGTAGGATTA 480
Pattern C    ATTGGTATTGGATTTCTTGGTTTTATTGTTTGAGCTCATCATATATTTACTGTGGGATTA 480
Pattern D    ATTGGTATTGGATTTCTTGGTTTTATTGTTTGAGCTCATCATATATTTACTGTGGGATTA 480
             ************************************************** ******

Pattern A    GATATTGATACACGAGC 497
Pattern B    GATATTGATACACGAGC 497
Pattern C    GATATTGATACACGAGC 497
Pattern D    GATATTGATACACGAGC 497
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Figure 3.21 Alignment of 4 patterns of COI gene from *T. collina* in Thailand. Asterisk indicated the same nucleotides among 4 patterns.

Table 3.10 Comparison of genetic diversity in six populations of COI gene in *T. collina* in Thailand

SSCP Bands (***N*** **= 73)**	**Central (10)**	**North (10)**	**Northeast (10)**	**Peninsular Thailand (22)**	**Prachuap Khiri Khan (10)**	**Chumphon (11)**
Number scored	17	13	10	13	7	12
Number private	10	11	9	7	1	6
Number fixed	0	0	0	0	0	0
Number absent	40	44	47	44	50	45
# polymorphic	17	13	10	13	7	12
% polymorphic	100% of 17* (30% of 57)**	100% of 13 (23% of 57)	100% of 10 (18% of 57)	100% of 13 (23% of 57)	100% of 7 (12% of 57)	100% of 12 (21% of 57)
Mean H_e (± SD)	0.034 (0.008)	0.039 (0.012)	0.030 (0.012)	0.034 (0.010)	0.029 (0.013)	0.033 (0.010)

*Total number of scorable SSCP bands in a particular regions; ** Total number of scorable SSCP bands across all region

The unbiased genetic distance between pairs of populations was 0.009-0.033 (Table 3.11). Significant geographic heterogeneity was observed in almost all pairwise comparisons of regions analyzed by F_{ST}-based statistics, Φ_{PT} ($P < 0.05$) except between Chumphon and Peninsular Thailand. The degree of differentiation in each comparison varied from 0.062 (between Chumphon and Peninsular Thailand) to 0.378 (between Northeast and Prachuap Khiri Khan).

AMOVA results revealed significance molecular variance components among 6 populations; North, Central, Northeast, Prachuap Khiri Khan, Chumphon and Peninsular Thailand (20%, $\Phi_{PT} = 0.204$, $P = 0.001$), among 5 population groupings; North, Central, Northeast, Prachuap Khiri Khan, and Chumphon+Peninsular Thailand (21%, $\Phi_{PT} = 0.208$, $P = 0.001$), and between North and South of Isthmus of Kra (11%, $\Phi_{PT} = 0.106$, $P = 0.001$, Table 3.12).

3.4.3 Analysis of cytochrome b (cytb) gene polymorphism

The expected products (316 bp) were found in 91 investigated *T. collina* (91/103 nests accounting for 88% of investigated specimens). Thirty-four SSCP phenotypes were obtained when the products were characterized on the 12.5% non-denaturing polyacrylamide gel (Figure 3.22, appendix B). The nucleotide sequences of 8 common SSCP phenotypes (pattern A, B, C, D, E, F, G, and H) were dissimilar by several SNPs (Figure 3.23). The phylogeographic pattern of them was shown in Figure 3.24. Both pattern A and B were found in specimens from Central (10/15 nests, 66.7% for A, 4/15 nests, 26.7% for B). Pattern C was observed in those from Prachuap Khiri Khan (6/8 nests, 75%). The pattern D and E, which were different by 1 point mutation were observed in Northeast population (5/24 nests, 20.8% and 7/24 nests, 29%, respectively). Pattern F, G, and H were distributed in individuals from Peninsular Thailand (7/23 nests, 30.4%, 10/23 nests, 43.5%, 4/23 nests, 17.4%, respectively). In addition, one individual from Chumphon (1/11 nests, 12.5%) was found in pattern F.

Table 3.11 Pairwise F_{ST}-based statistics (Φ_{PT}, below diagonal), Nei's (1972) genetic distance (in brackets) and the exact test (above diagonal) of COI gene among six populations of *T. collina* in Thailand

	Central	**North**	**Northeast**	**Peninsular Thailand**	**Prachuap Khiri Khan**	**Chumphon**
Central (*N* = 10)	-	*P* = 1.0000	*P* = 1.0000	*P* = 0.9998	*P* = 1.0000	*P* = 1.0000
North (*N* = 10)	0.144* (0.019)	-	*P* = 1.0000	*P* = 0.9729	*P* = 1.0000	*P* = 1.0000
Northeast (*N* = 10)	0.189* (0.020)	0.257* (0.027)	-	*P* = 0.9976	*P* = 1.0000	*P* = 1.0000
Peninsular Thailand (*N* = 22)	0.110* (0.013)	0.217* (0.023)	0.246* (0.024)	-	*P* = 0.9947	*P* = 1.0000
Prachuap Khiri Khan (*N* = 10)	0.153* (0.016)	0.313* (0.032)	0.378* (0.033)	0.281* (0.027)	-	*P* = 1.0000
Chumphon (*N* = 11)	0.083* (0.013)	0.190* (0.023)	0.233* (0.023)	0.062 ns (0.009)	0.276* (0.026)	-

*significant differences at $P < 0.05$; ns, not significant

Table 3.12 Analysis of Molecular Variance (AMOVA) of COI gene for genetic differentiation of *T. collina* among 6 population groupings; North, Central, Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular Thailand (A), among 5 populations; North, Central, Northeast, Prachuap Khiri Khan, and Chumphon+Peninsular Thailand (B), and between North (North+Central+Northeast+Prachuap Khiri Khan) and South (Chumphon and Peninsular Thailand) of Isthmus of Kra (C)

A. Six population groupings

Component	df	Estimate Variance	% of total variance	Φ_{PT}	*P*-value
Among population	5	0.419	20	0.204	0.001
Within population	67	1.640	80		

B. Five population groupings

Component	df	Estimate Variance	% of total variance	Φ_{PT}	*P*-value
Among population	4	0.437	21	0.208	0.001
Within population	68	1.666	79		

C. North and South of Isthmus of Kra

Component	df	Estimate Variance	% of total variance	Φ_{PT}	*P*-value
Among population	1	0.223	11	0.106	0.001
Within population	71	1.873	89		

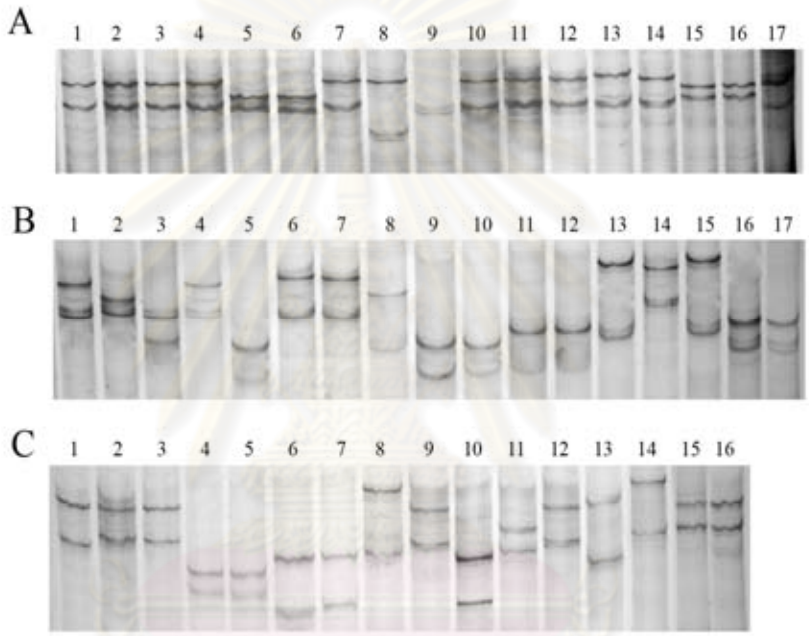

Figure 3.22 The SSCP pattern of the amplified cytb gene of *T. collina* from different geographic regions in Thailand; North population (lanes 5-8 A, 13-17 B, and 6-8 C), Central population (lanes 1-4 A, 6-7, 11-12 B, and 1-3 C), Northeast population (lanes 9-12 A, and 9-12 C), Peninsular population (lanes 13-16 A, 1-2, 4 B, and 16 C), Prachuap Khiri Khan population (lanes 5, 8-10 B and 4-5 C), and Chumphon population (lanes 17 A, 3 B, and 13-15 C).

```
Pattern A    TTGTAGAGTGATTATGAGGAGGATTTTCTATTAATAATTCTACTCTTAATCGATTTTTTT 60
Pattern B    TTGTAGAGTGATTATGAGGAGGATTTTCTATTAATAATTCTACTCTTAATCGATTTTTTT 60
Pattern C    TTGTAGAGTGATTATGAGGAGGATTTTCTATTAATAATTCTACTCTTAATCGATTTTTTT 60
Pattern D    TTGTAGAGTGATTATGAGGAGGATTCTCAATTAATAATTCTACTCTTAATCGATTTTTTT 60
Pattern E    TTGTAGAGTGATTATGAGGAGGATTCTCAATTAATAATTCTACTCTTAACCGATTTTTTT 60
Pattern F    TTGTAGAGTGATTATGAGGAGGATTTTCTATTAATAATTCTACTCTTAACCGATTTTTTT 60
Pattern G    TTGTAGAGTGATTATGAGGAGGATTTTCTATTAATAATTCTACTCTTAATCGATTCTTTT 60
Pattern H    TTGTAGAGTGATTATGAGGAGGATTTTCTATTAATAATTCTACTCTTAATCGATTCTTTT 60
             ************************* ** ******************** ***** ****

Pattern A    CACTACATTTTATTTTACCATTTATTATTCTTATTTTAGTATTTATTCATATTATAATAT 120
Pattern B    CACTACATTTTATTTTACCATTTATTATTCTTATTTTAGTATTTATTCATATTATAATAT 120
Pattern C    CATTACATTTTATTTTACCATTTATTATTCTTATTTTAGTATTTATTCATATCATAGTAT 120
Pattern D    CATTACATTTTATTTTACCATTTATTATTCTTATTTTAGTATTTATTCATATTATAATAT 120
Pattern E    CATTACATTTTATTTTACCATTTATTATTCTTATTTTAGTATTTATTCATATTATAATAT 120
Pattern F    CATTACATTTTATTTTACCATTTATTATTCTTATTTTAGTATTTATTCATATCATAGTAT 120
Pattern G    CATTACATTTTATTTTACCATTTATTATTCTTATTTTAGTATTTATTCATATCATAGTAT 120
Pattern H    CGTTACATTTTATTTTACCATTTATTATTCTTATTTTAGTATTTATTCATATTATAGTAT 120
             *  ************************************************ *** ***

Pattern A    TGCATACATCTGGATCTTCAAATCCAATCCACTCAAAAATAAATATCTATAAAATTTCAT 180
Pattern B    TGCATACATCTGGATCTTCAAATCCAATCCATTCAAAAATAAATATCTATAAAATTTCAT 180
Pattern C    TGCATATATCTGGATCTTCAAACCCAATTCATTCTAAAATAAATATTTATAAAATTTCAT 180
Pattern D    TACACATATCTGGATCTTCAAATCCAATTCATTCAAAAATAAATATTTACAAAATTTCAT 180
Pattern E    TACACATATCTGGATCTTCAAATCCAATTCATTCAAAAATAAATATTTACAAAATTTCAT 180
Pattern F    TGCATATATCCGGATCTTCAAATCCAATTCATTCTAAAATAAATATTTATAAAATTTCAT 180
Pattern G    TGCATATATCTGGATCTTCAAACCCAATTCATTCTAAAATAAATATTTATAAAATTTCAT 180
Pattern H    TGCATATATCTGGATCTTCAAATCCAATTCATTCTAAAATAAATATTTATAAAATTTCAT 180
             * ** * *** *********** ***** ** ** *********** ** **********

Pattern A    TTTATCCATATTTTGTAATTAAAGATATAGTAACTATTTTATTTACTATTTTATTATTTA 240
Pattern B    TTCATCCATATTTTGTAATTAAAGATATAGTAACTATTTTATTTACTATTTTATTATTTA 240
Pattern C    TTTACCCGTACTTTGCAATTAAAGATATAGTAACTATTTTGTTTATTATTTTATTATTTA 240
Pattern D    TTTATCCATATTTTATAATTAAAGATATAGTAACTATTTTATTTACTATCTTATTATTTA 240
Pattern E    TTTATCCATATTTTATAATTAAAGATATAGTAACTATTTTATTTACTATCTTATTATTTA 240
Pattern F    TTTATCCATACTTCGCAATTAAAGATATAGTAACTATTTTGTTTACTATTTTATTATTTA 240
Pattern G    TTTATCCATACTTCGCAATTAAAGATATAGTAACTATTTTGTTTACTATTTTATTATTTA 240
Pattern H    TTTATCCATACTTTGCAATTAAAGATATAGTAACTATTTTGTTTACTATTTTATTATTTA 240
             ** * ** ** **   ********************** **** *** **********

Pattern A    TACTTTTAAATTTTCAGATACCTTATTTTTTAAGAGATCCTGATAATTTTAAAATAGCTG 300
Pattern B    TACTTTTAAATTTTCAGATACCTTATTTTTTAAGAGATCCTGATAATTTTAAAATAGCTG 300
Pattern C    TATTTTTAAATCTTCAGGCACCCTATATTTTAAGTGATCCAGATAATTTTAAAATAGCTG 300
Pattern D    TACTTTTAAATTTTCAGATACCTTATTTTTTAAGAGATCCTGATAATTTTAAAATAGCTG 300
Pattern E    TACTTTTAAATTTTCAGATACCTTATTTTTTAAGAGATCCTGATAATTTTAAAATAGCTG 300
Pattern F    TATTTTTAAATTTTCAGGCACCCTATATTTTAAGTGATCCAGATAATTTTAAAATAGCTG 300
Pattern G    TATTTTTAAATTTTCAGGCACCCTATATTCTAAGTGATCCAGATAATTTTAAAATAGCTG 300
Pattern H    TATTTTTAAATTTTCAGGCACCCTATATTTTAAGTGATCCAGACAATTTTAAAATAGCTG 300
             ** ******** *****  *** *** ** **** ***** ** ***************

Pattern A    ATCCTATAGTTACTCC 316
Pattern B    ATCCTATAGTTACTCC 316
Pattern C    ATCCTATAGTTACTCC 316
Pattern D    ATCCTATAGTTACTCC 316
Pattern E    ATCCTATAGTTACTCC 316
Pattern F    ATCCTATAGTTACTCC 316
Pattern G    ATCCTATAGTTACTCC 316
Pattern H    ATCCTATAGTTACTCC 316
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Figure 3.23 Alignment of 8 patterns of cytb gene from *T. collina* in Thailand. Asterisk indicated the same nucleotides among 8 patterns.

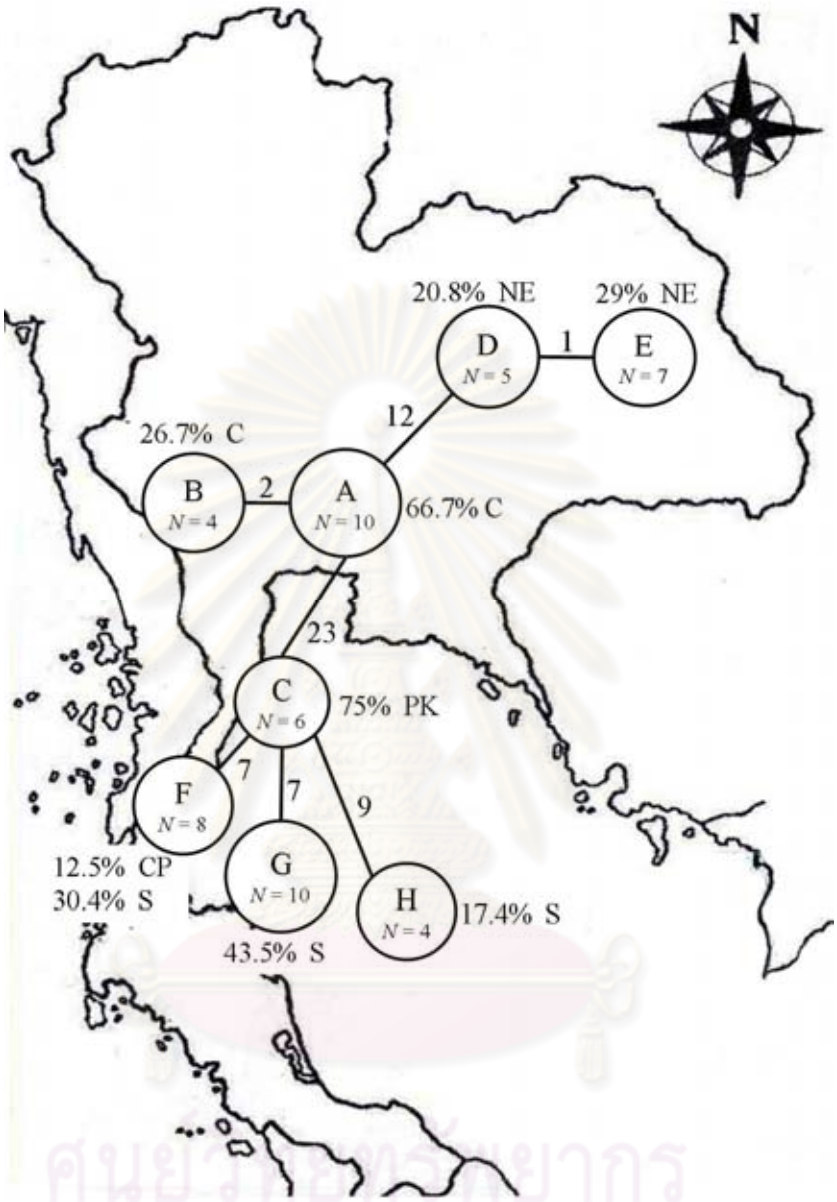

Figure 3.24 Phylogeographic pattern deduced from cytb gene of *T. collina* (pattern A, B, C, D, E, F, G, and H). Numbers along connected lines indicated inferred mutation steps. Letter *N* represented the amount of *T. collina* possessing each pattern. The percentages were calculated from the total number of *T. collina* in a particular region; North (10 nests), Central (15 nests), Northeast (24 nests), Prachuap Khiri Khan (8 nests), Chumphon (11 nests), and Peninsular Thailand (23 nests). Capital letters behind percentage were abbreviations of Central (C), Northeast (NE), Prachuap Khiri Khan (PK), Chumphon (CP), and Peninsular Thailand (S).

A total of 38 scored SSCP bands were considered polymorphic. Genetic diversity was observed in all 6 populations, North, Central, Northeast, Prachuap Khiri Khan, Chumphon and Peninsular Thailand (Table 3.13). The percentage of polymorphic bands for all populations was 100% and the mean expected heterozygosity (H_e) varied from 0.033-0.050.

The unbiased genetic distance between pairs of populations was 0.015-0.072 (Table 3.14). Significant geographic heterogeneity was observed in all pairwise comparisons of regions analyzed by F_{ST}-based statistics, Φ_{PT} ($P < 0.05$). The results indicated high degree of differentiation in each comparison ranged from 0.079 (between Northeast and Chumphon) to 0.627 (between Central and Prachuap Khiri Khan).

AMOVA analysis showed high molecular variance components among 6 populations; North, Central, Northeast, Prachuap Khiri Khan, Chumphon and Peninsular Thailand (29%, $\Phi_{PT} = 0.294$, $P = 0.001$), and between North and South of Isthmus of Kra (13%, ΦPT = 0.133, $P = 0.001$, Table 3.15).

Table 3.13 Comparison of genetic diversity in six populations of cytb gene in *T. collina* in Thailand

SSCP Bands (***N*** **= 91)**	**Central (15)**	**North (10)**	**Northeast (24)**	**Peninsular Thailand (23)**	**Prachuap Khiri Khan (8)**	**Chumphon (11)**
Number scored	5	13	14	8	4	14
Number private	1	6	6	3	1	6
Number fixed	0	0	0	0	0	0
Number absent	33	25	24	30	34	24
# polymorphic	5	13	14	8	4	14
% polymorphic	100% of 5* (13% of 38)**	100% of 13 (34% of 38)	100% of 14 (37% of 38)	100% of 8 (21% of 38)	100% of 4 (11% of 38)	100% of 14 (37% of 38)
Mean H_e (± SD)	0.033 (0.017)	0.050 (0.013)	0.049 (0.013)	0.042 (0.018)	0.039 (0.020)	0.050 (0.012)

*Total number of scorable SSCP bands in a particular regions; ** Total number of scorable SSCP bands across all region

Table 3.14 Pairwise F_{ST}-based statistics (Φ_{PT}, below diagonal), Nei's (1972) genetic distance (in brackets) and the exact test (above diagonal) of cytb gene among six populations of *T. collina* in Thailand

	Central	**North**	**Northeast**	**Peninsular Thailand**	**Prachuap Khiri Khan**	**Chumphon**
Central (*N* = 15)	-	*P* = 0.5389	*P* = 0.6540	*P* = 0.2885	*P* = 0.5338	*P* = 0.6770
North (*N* = 10)	0.411* (0.048)	-	*P* = 0.8613	*P* = 0.1845	*P* = 1.0000	*P* = 0.9992
Northeast (*N* = 24)	0.286* (0.031)	0.111* (0.019)	-	*P* = 0.0288	*P* = 0.9038	*P* = 0.8864
Peninsular Thailand (*N* = 23)	0.431* (0.041)	0.304* (0.037)	0.284* (0.034)	-	*P* = 0.3312	*P* = 0.8609
Prachuap Khiri Khan (*N* = 8)	0.627* (0.070)	0.281* (0.039)	0.305* (0.042)	0.497* (0.061)	-	*P* = 0.9979
Chumphon (*N* = 11)	0.346* (0.038)	0.082* (0.019)	0.079* (0.015)	0.229* (0.027)	0.289* (0.040)	-

*significant differences at $P < 0.05$

Table 3.15 Analysis of Molecular Variance (AMOVA) of cytb gene for genetic differentiation of *T. collina* among 6 population groupings; North, Central, Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular Thailand (A), and between North (North+Central+Northeast+Prachuap Khiri Khan) and South (Chumphon and Peninsular Thailand) of Isthmus of Kra (B)

A. Six population groupings

Component	df	Estimate Variance	% of total variance	Φ_{PT}	*P*-value
Among population	5	0.547	29	0.294	0.001
Within population	85	1.311	71		

B. North and South of Isthmus of Kra

Component	df	Estimate Variance	% of total variance	Φ_{PT}	*P*-value
Among population	1	0.252	13	0.133	0.001
Within population	89	1.637	87		

3.4.4 Analysis of geographic population structure based on all 3 mtDNA gene polymorphisms

Although the specimens used to examine the data were the same sample set but the number of amplified specimens was inconsistent in each mtDNA gene. Therefore, the positively amplified specimens by the primers of 3 mtDNA genes were used to analyze (61 nests).

A total of 113 SSCP bands were generated from the data of 3 mtDNA genes. Genetic diversity was observed in all 6 populations, North, Central, Northeast, Prachuap Khiri Khan, Chumphon and Peninsular Thailand (Table 3.16). No fixed bands were present in samples from North, Central, Northeast, and Peninsular Thailand except those from Prachuap Khiri Khan and Chumphon. The mean expected heterozygosity (H_e) varied from 0.030-0.052.

The unbiased genetic distance between pairs of populations was 0.029-0.067 (Table 3.17). Significant geographic heterogeneity was observed in all pairwise comparisons of regions analyzed by F_{ST}-based statistics, Φ_{PT} ($P < 0.05$). The degree of differentiation in each comparison varied from 0.231 (between North and Northeast) to 0.515 (between Prachuap Khiri Khan and Peninsular Thailand).

AMOVA analysis showed significance molecular variance components among 6 populations; North, Central, Northeast, Prachuap Khiri Khan, Chumphon and Peninsular Thailand (37%, $\Phi_{PT} = 0.369$, $P = 0.001$, Table 3.18).

Table 3.16 Comparison of genetic diversity in six populations of 16S rRNA, COI, and cytb gene in *T. collina* in Thailand

SSCP Bands	**Central**	**North**	**Northeast**	**Peninsular Thailand**	**Prachuap Khiri Khan**	**Chumphon**
(*N* = 61)	**(6)**	**(8)**	**(10)**	**(20)**	**(6)**	**(11)**
Number scored	20	30	31	25	13	28
Number private	10	21	25	12	3	13
Number fixed	0	0	0	0	2	2
Number absent	93	83	82	88	100	85
# polymorphic	20	30	31	25	11	26
% polymorphic	100% of 20*	100% of 30	100% of 31	100% of 25	85% of 13	93% of 28
	(18% of 113)**	(26.5% of 113)	(27% of 113)	(22% of 113)	(10% of 113)	(23% of 113)
Mean H_e	0.043	0.052	0.047	0.040	0.030	0.033
(± SD)	(0.010)	(0.010)	(0.009)	(0.009)	(0.009)	(0.007)

*Total number of scorable SSCP bands in a particular regions; ** Total number of scorable SSCP bands across all region

Table 3.17 Pairwise F_{ST}-based statistics (Φ_{PT}, below diagonal), Nei's (1972) genetic distance (in brackets) and the exact test (above diagonal) of 16S rRNA, COI, and cytb gene among six populations of *T. collina* in Thailand

	Central	**North**	**Northeast**	**Peninsular Thailand**	**Prachuap Khiri Khan**	**Chumphon**
Central (*N* = 6)	-	*P* = 1.0000	*P* = 1.0000	*P* = 1.0000	*P* = 1.0000	*P* = 1.0000
North (*N* = 8)	0.264* (0.045)	-	*P* = 1.0000	*P* = 0.7046	*P* = 1.0000	*P* = 1.0000
Northeast (*N* = 10)	0.244* (0.038)	0.231* (0.039)	-	*P* = 0.2239	*P* = 1.0000	*P* = 1.0000
Peninsular Thailand (*N* = 20)	0.399* (0.048)	0.396* (0.052)	0.389* (0.049)	-	*P* = 0.9907	*P* = 0.9645
Prachuap Khiri Khan (*N* = 6)	0.234* (0.029)	0.341* (0.052)	0.371* (0.054)	0.515* (0.067)	-	*P* = 1.0000
Chumphon (*N* = 11)	0.363* (0.049)	0.324* (0.048)	0.309* (0.042)	0.402* (0.046)	0.462* (0.062)	-

*significant differences at $P < 0.05$

Table 3.18 Analysis of Molecular Variance (AMOVA) of 16S rRNA, COI, and cytb gene for genetic differentiation of *T. collina* among 6 population groupings; North, Central, Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular Thailand

Component	df	Estimate Variance	% of total variance	Φ_{PT}	*P*-value
Among population	5	2.157	37	0.369	0.001
Within population	55	3.696	63		

CHAPTER IV

DISCUSSION

The Indo-Malayan stingless bees have divided into those which easily distinguished from others and those which required studies to discriminate from others (Sakagami, 1978). Morphological and nest architecture characters were usually used for taxonomic identification. Most Indo-Malayan stingless bees could be unambiguously classified based on morphology whereas those belonging to the subgenus *Tetragonula* are taxonomic problematic (Sakagami, 1975; 1978). Moreover, Franck et al. (2004) investigated the taxonomic significance of nest architecture in Australian *Trigona* by 13 microsatellite loci. They reported that nest architectures were relevant but not sufficient to discriminate *T. hockingsi* and *T. davenporti* but they could be differentiated at the genetic level.

In Brazil, *Melipona quadrifasciata quadrifasciata*, which possesses 3-5 continuous yellow stripes and *M. quadrifasciata anthidiodes*, which possesses 2-5 interrupted stripes on the terga of the 3rd and 6th segments in workers and males, have been found. Inter-subspecific hybrids exhibiting intermediate stripe patterns were found in some areas of Brazil. A 750 bp RAPD marker from OPE07 was present in the former, except stingless bees from northern Minas Gerais but was absent in the latter subspecies (Waldschmidt et al., 2000). Subsequently, Souza et al. (2008) reported restriction analysis of cytochrome b with *Vap*I and present the first population analysis from a large number of *M. quadrifasciata* colonies (155 colonies) from different geographical origin. The mitochondrial DNA molecular marker could successfully differentiate these *M. quadrifasciata* subspecies.

Thus, species identification is necessary, particularly when species could be misidentified morphologically. AFLP is a multiple-locus fingerprinting, enabling the identification of genetic markers at different taxonomic levels, without the need for knowledge of sequences of the genome under investigation (Vos et al., 1995). It has been widely used to study polymorphism among populations and species (Blear et al., 1998; Mueller et al., 1999) and to identify species-diagnostic markers in various taxa (Lui and Cordes, 2004; Klinbunga et al., 2007).

Thummajitsakul et al. (2010) successfully developed a species-diagnostic AFLP derived marker for identification of *T. pagdeni*. The expected 163 bp fragment (CUTp1) was amplified in all examined individuals of *T. pagdeni* (129/129 nests). Nevertheless, cross-species amplification was also observed in *T. fimbriata* (1/3 nests), *T. collina* (11/112 nests), *T. laeviceps* (1/12 nests) and *T. fuscobalteata* (15/15 nests) but not in *T. apicalis*, *T. canifrons*, *T. itama*, *T. melina*, *T. minor*, *T. terminata*, *T. doipaensis*, *T. melanoleuca* and *T. thoracica* and *L. furva*. SSCP analysis of CUTp1 further differentiated *T. fuscobalteata* and *T. collina* from *T. pagdeni*. Although, *T. laeviceps*, *T. fimbriata* and *T. pagdeni* shared an identical SSCP genotype but they are not taxonomically problematic species.

The AFLP technique was applied in this study. Using 64 primer combinations against 11 stingless bee species, a 316 bp fragment generated by *Pst*I$_{+AGT}$/*Mse*I$_{+CAG}$ was found in *Tetragonilla collina* but not in the other genus and species of investigated stingless bees. Basically, an AFLP approach is composed of several steps (i.e., genomic DNA digestion, adaptor ligation, preselective, and selective amplification of the digested/ligated fragments, PAGE and silver-staining; Muller and Wolfenbarger, 1999), limiting the ability to authenticate a large number of specimens within a short period of time. As a result, species-diagnostic sequence-characterized amplified region (SCAR) markers were further developed from candidate species-specific AFLP fragments found in *T. collina* (called CUTc1). Nucleotide sequence of CUTc1 was regarded as an anonymous DNA segment because they did not match any sequence in the GenBank. The developed SCAR marker was tested in larger sample sizes of previously examined species and additional four stingless bee species (239 nests) that were not analyzed in screening of AFLP primer (*Tetragonula laeviceps*, *Tetrigona melanoleuca*, *Lepidotrigona terminata* and *Lisotrigona furva*).

The expected amplification product (259 bp) was found in all *Tetragonilla collina* individuals (134/134 nests accounting for 100% of investigated specimens) but not in the other genus and species of investigated bees, *Tetrigona apicalis*, *Lophotrigona canifrons*, *Lepidotrigona doipaensis*, *Homotrigona fimbriata*, *Tetragonula fuscobalteata*, *Heterotrigona itama*, *Tetragonula laeviceps*, *Tetrigona melanoleuca*, *Tetragonula melina*, *Tetragonula minor*, *Geniotrigona thoracica*, *Lepidotrigona terminata* and *Lisotrigona furva*. Nevertheless, cross-species

amplification was found in *Tetragonula pagdeni* (275 bp, 43/51 nests, 84.3%). Thus, species-specific PCR of the CUTc1 marker successfully discriminated *Tetragonilla collina* from 13 other stingless bee species. However, the differentiation between *Tetragonilla collina* and *Tetragonula pagdeni* can also be carried out based on nest architecture and external morphology (Sakagami, 1978; Sakagami et al., 1985).

SSCP analysis, which is favored for identifying species origins of various taxa, due to its convenience and cost-effectiveness (Orita et al., 1989; Weder et al., 2001; Klinbunga et al., 2007), was then applied to determine whether nucleotide sequences of CUTc1 in *Tetragonula pagdeni* and *Tetragonilla collina* were different. Non-overlapping SSCP patterns between *Tetragonilla collina* and *Tetragonula pagdeni* were observed and Nucleotide sequences of representative individuals of these species were different (Figure 3.8). Therefore, a species-diagnostic marker for *Tetragonilla collina* was successfully developed.

The CUTc1 SCAR marker is convenient and cost effective for differentiation of *T. collina* from other stingless bees in Thailand. This is convenient for molecular geneticists who are not familiar with species differentiation based on nest architecture and external morphology of stingless bees. Moreover, both CUTc1 (this study) and CUTp1 (Thummajitsakul et al., 2010) should be concurrently used to eliminate possible misidentification problems between *Tetragonilla collina* and *Tetragonula pagdeni* when new geographic populations of these species are examined.

Interestingly, the differentiation of CUTc1 marker in *T. collina* was observed when examined by SSCP analysis. Genotypic distribution patterns of CUTc1 were different in stingless bee from the north-to-central region (259/259 bp alleles corresponding to the AA genotype found in 76/81 nests) sample and most individuals of *T. collina* from peninsular Thailand (253/253 bp alleles corresponding to the BB genotype found in 42/53 nests). Moreover, heterozygotes exhibiting 253/259 bp alleles (AB genotype) were observed in stingless bees from Prachuap Khiri Khan located slightly above the Kra ecotone (5/28 nests) and those from peninsular Thailand (Chumphon, Ranong, Surat Thani and Nakon Si Thammarat, 11/53 nests). The re-examination of representative individual carrying AA, AB, and BB genotypes by denaturing gel electrophoresis and their nucleotide sequences confirmed the genotypic differences of these species (Figure 3.9). Genotype distribution patterns of

CUTc1 strongly suggested biogeographic differentiation between *T. collina* originating from north and south of the Kra ecotone.

Analysis of genetic diversity and population differentiation is essential for genetic research (population genetics, phylogenetics, molecular taxonomy and systematics, and evolutionary studies) of various organisms (Avise, 1994). In addition, Basic knowledge of genetic population structure is required for effective management of native bee species. TE-AFLP method provides a high discriminatory fingerprinting. It can reduce the number of bands, making it suitable for analysis of complex genomes (van der Wurff et al., 2000).

DNA fingerprints were used in this study to analyze the geographic pattern of genetic variation and differentiation in Thai *T. collina*. Individuals representing 98 nests were collected from four geographic regions: the North, Northeast, Central and Peninsular Thailand. Relatively high genetic diversity within each geographic sample of Thai *T. collina* was observed from TE-AFLP analysis (74 -83% of total variance occurs within region; Table 3.3), suggesting that inbreeding is not a major concern for this species at present. Significant genetic differentiation among the four geographic regions was detected with approximately 26% of observed variance explained by differentiation among the geographic regions (Φ_{PT} = 0.258, P = 0.001). Although we also investigated other ways to partition our samples by combining them into two or three larger geographic units, such as samples north and south of the Kra ecotone, none partitioned variance as effectively as grouping nests into the North, Central, Northeast and South geographic regions (values of Φ_{PT} were lower, ranging from 0.172 to 0.207; Table 3.3).

The pattern of genetic differentiation in *T. collina* was different from that of honey bees, *Apis cerana* (Sihanunthavong et al., 1999; Sittipraneed et al., 2001b; Songram et al., 2006) and *Apis dorsata* (Insuan et al., 2007) where there was clear differentiation between the North-to-Central group (North, Northeast and Central regions) and South group (Peninsular Thailand), but no significant differentiation was detected among North, Northeast and Central honey bee. In Thai *T. pagdeni*, genetic diversity and biogeography based on TE-AFLP analysis were reported. The differentiation between all pairs of populations was clearly observed, as in *T. collina*, but the strongest differentiation levels were found between Northeast and other

populations (Φ_{PT} = 0.21, P = 0.001; Thummajitsakul et al., 2008) while the high differentiation levels in *T. collina* were observed among 4 geographic regions.

In *T. collina*, Peninsular Thailand-Northeast (Φ_{PT} = 0.359) and Peninsular Thailand-North (Φ_{PT} = 0.334) revealed stronger degrees of geographic differentiation than other comparisons (Φ_{PT} = 0.076 - 0.242) when pairwise comparisons were considered by AMOVA (Table 3.2). Interestingly, the Central region showed the greatest diversity (55% of bands are polymorphic and H_e = 0.141; Table 3.1), and lower degrees of genetic differentiation was found between this and other populations (Table 3.2). The information suggests that *T. collina* from the Central region may have ancestrally colonized other parts of Thailand or alternatively colonization of *T. collina* may have occurred in the opposite direction, from surrounding regions into the central area. The use of additional nuclear and mitochondrial DNA markers on large sample sizes of *T. collina* from different geographic regions in Thailand would clarify this speculation.

However, genetic variation and differentiation in *T. collina* was also analyzed by TE-AFLP derived SCAR marker (TECU marker) using SSCP analysis. Individuals representing 96 nests were the same set of samples in genetic variation studies by TE-AFLP. Genotypic distribution of TECU marker showed 3 different patterns. Pattern I was found in all *T. collina* from the north-to-central region while pattern II and III were distributed in stingless bees from Prachuap Khiri Khan, Chumphon, and Peninsular Thailand. Nucleotide sequences of each pattern showed their phylogeographic pattern. Based on number of mutations, pattern I and III were different by seven mutation steps, and pattern II was intermediate between pattern I and III (Figure 3.16).

As a result, population genetic studies of the Asian honey bee (*A. cerana*) in Thailand using mitochondrial DNA and microsatellite polymorphism revealed the biogeographical transition area between Mainland and Sundaland populations located at the Kra ecotone (at Tup Sa Kae, Prachup Kiri Khan, 11°31'N, 99°35'E, and Bang Sapan, Prachup Kiri Khan, 11°24'N, 99°31'E; Deowanish et al., 1996; Smith and Hagen, 1996 and 1999; Sihanuntavong et al., 1999; Warrit et al., 2006). A pattern of geographic differentiation of the giant honey bee (*A. dorsata*) in Thailand between north-to-central and peninsular Thailand populations was also noticed found, based on

microsatellite analysis (Insuan et al., 2007). In addition, the small but significant differentiation between bees from north and south of the Isthmus of Kra was also detected in Thai *T. pagdeni* (Thummajitsakul et al., 2008). The sample grouping for population genetic studies except those by TE-AFLP analysis was North, Central, Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular population (6 populations).

Analysis of *T. collina* using TECU marker, the North, Central, and Northeast regions showed no genetic diversity within each region (0% of polymorphic bands and H_e = 0.000; Table 3.4), and no significant differentiation was detected among Northern populations (North, Central, and Northest), between Chumphon and Prachuap Khiri Khan, and between Chumphon and Peninsular Thailand (Table 3.5). The level of differentiation among 4 populations (North+Central+Northest, Prachuap Khiri Khan, Chumphon, and Peninsular Thailand) was higher than those among 6 populations (Table 3.6). The pattern of genetic differentiation strongly indicated differentiation between bees from north and south of Kra ecotone, as previously genotypic distribution patterns of CUTc1, whereas it was different from the study of *T. collina* based on TE-AFLP analysis. However, the lower differentiation (but significance) between bees from north and south of Kra ecotone was also found in analysis by TE-AFLP (Table 3.3B). These might occur from the difference of investigated position. The analysis of CUTc1 and TECU markers was determination in particular region while TE-AFLP analysis was in whole genomic DNA (more than one region).

Like honey bees, *A. cerana and A. dorsata* as aforementioned, biogeographic differentiation between bees from north and south of the Kra ecotone was also observed in Thai *T. pagdeni* based on mtDNA polymorphism (cytb, ATPase(6, 8), and 16S rRNA gene; Thummajitsakul, 2008). The patterns of mtDNA variation in *T. collina* were examined to estimate genetic diversity and population structure, and determine if it shows a break in mtDNA haplotypes north and south of the Kra ecotone.

The mtDNA diversity of 16Sr RNA, COI, and cytb gene was investigated in this study. Since there was inconsistent amplification of *T. collina* in each mtDNA gene, the results were first analyzed separately for each gene. Then, those specimens

for which all 3 mtDNA genes were successfully amplified were used in the combined analyses. For investigation of each gene, high levels of polymorphisms for 16S rRNA, COI, and cytb (%P: 0 to 52%; H_e: 0.000 to 0.065 (Table 3.7); %P: 12 to 30%; H_e: 0.029 to 0.039 (Table 3.10); %P: 11 to 37%; H_e: 0.033 to 0.050 (Table 3.13), respectively) were detected which indicated SSCP analysis is a powerful tool for estimating genetic diversity in *T. collina*. In addition, the number of haplotypes from polymorphisms of COI and cytb were 34 patterns and the lower haplotypes of 16S rRNA polymorphism was 17 patterns. However, the statistic values for 16S rRNA polymorphism were the highest values.

In polymorphism of 16S rRNA gene, the values of Φ_{PT} for pairwise comparisons (considered by AMOVA) revealed stronger degree of geographic differentiation in Central-Chumphon (Φ_{PT} = 0.826) than other comparisons (Φ_{PT} = 0.070-0.783; Table 3.8). For polymorphism of COI gene, the Φ_{PT} values for pairwise comparisons showed higher degree of differentiation in Prachuap Khiri Khan-Northeast (Φ_{PT} = 0.378) than others (Φ_{PT} = 0.062-0.313; Table 3.11). The pairwise genetic distance values of cytb detected the highest degree of differentiation in Prachuap Khiri Khan-Central (Φ_{PT} = 0.627) and the other comparisons ranges in 0.079-0.497 (Table 3.14). When overall data of 3 genes were combined, Prachuap Khiri Khan-Peninsular (Φ_{PT} = 0.515) also revealed stronger degrees of differentiation than others (Φ_{PT} = 0.231-0.462; Table 3.17). These results indicated a similar trend of biogeographic differentiation between *T. collina* from north and south of Kra ecotone.

High levels of genetic variation among individuals within each population were observed in all 3 genes (44-67% of total variance occurs within region for 16S rRNA polymorphism, Table 3.9; 79-89% of total variance for COI polymorphism, Table 3.12; and 71-87% of total variance for cytb polymorphism, Table 3.15). AMOVA analysis for 16S rRNA, COI, and cytb genes of *T. collina* samples detected genetic differentiation among 6 populations (North, Central, Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular population), with Φ_{PT} = 0.563, 0.204, and 0.294 respectively (P = 0.001, Table 3.9A, 3.12A, 3.15A). The overall analysis of 3 genes also showed significant genetic differentiation among the 6 populations with 37% of observed variance explained by differentiation among populations (Φ_{PT} = 0.369, P = 0.001; Table 3.18). However, no degree of differentiation was observed

between Prachuap Khiri Khan and Chumphon in 16S rRNA polymorphism (Table 3.8) and between Chumphon and Peninsular in COI polymorphism (Table 3.11) when pairwise comparisons were considered by AMOVA. As the result, the other partitions for genetic variance of 16S rRNA and COI genes were estimated by combining any groups that showed no significance between pairwise comparisons together. AMOVA analysis showed slightly higher molecular variance components among 5 populations for COI polymorphism (North, Central, Northeast, Prachuap Khiri Khan, and Chumphon+ Peninsular Thailand; Φ_{PT} = 0.208, P = 0.001; Table 3.12B) while the molecular variance components among 5 populations for 16S rRNA polymorphism; North, Central, Northeast, Prachuap Khiri Khan+Chumphon, and Peninsular Thailand, were lower (Φ_{PT} = 0.437, P = 0.001; Table 3.9B). Additionally, the significant genetic differentiations between samples from north and south of Isthmus of Kra were also found in polymorphism of 16S rRNA, COI, and cytb genes (Φ_{PT} = 0.334, 0.106, and 0.133 respectively, P = 0.001; Table 3.9C, 3.12C, 3.15B).

The haplotype pattern of each gene that was observed in at least 4 individuals were chosen to sequence in this study. The number of mutational steps between each haplotype was estimated from the sequence data to illustrate the phylogeographic patterns of 16s rRNA and cytb gene haplotypes while the pattern of COI gene was not considered because there was no pattern of samples from northern Thailand. The phylogeographic pattern of 16S rRNA indicated differentiated groups (Figure 3.19): Northeast (pattern B, C, D), Central and Prachuap Khiri Khan (pattern A), Chumphon and a few from Peninsular (pattern E), and the rest Peninsular (pattern F). The geographic differentiation was also observed from the phylogeographic pattern of cytb (Figure 3.24): Northeast (pattern D and E), Central (pattern A and B), Prachuap Khiri Khan (pattern C), Chumphon and some from Peninsular Thailand (pattern F), and the rest Peninsular (pattern G and H). These followed the AMOVA result of 16S rRNA and cytb gene which showed the genetic differentiation among each population. However, the phylogeographic pattern of 16S rRNA and cytb genes present the trend of differentiation between samples from north and south of Isthmus of Kra, as the results considered by AMOVA that found the significant genetic differentiations between samples from north and south of Isthmus of Kra.

The mtDNA polymorphism studies of *A. cerana* indicated that *A. cerana* from the north-to-central region was recognized to the Asian mainland group, whereas bees from peninsular Thailand and Samui Island were recognized to the Sundaland group. The shift from the Asian mainland to the Sundaland mitotypes of honey bees, *A. cerana* in Thailand had been proposed to occur at the Isthmus of Kra at Bang Sapan, Prachuap Khiri Khan (11^{o}24'N, 99^{o}31'E) to Tha Sae, Chumphon (10^{o}34'N, 99^{o}06'E) which corresponds to the Kra ecotone (Sihanuntavong et al., 1999; Smith et al., 1999; 2005; Sittipraneed et al., 2001b; Warrit et al., 2006).

Thummajitsakul (2008) examined the population structure within Thai *T. pagdeni* using mitochondrial diversity of cytb, ATP(6,8), and 16S rRNA genes. The result indicated differentiation among North, Central, Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular populations and also found significant differentiation between bees from north and south of the Isthmus of Kra.

In the present study, the analysis of mtDNA polymorphism in *T. collina* revealed the high differentiation level among 6 populations (North, Central, Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular Thailand). The smaller but significant differentiation between bees from North and South of Isthmus of Kra was also observed, as previously reported in *A. cerana* and *T. pagdeni*.

The Central region showed the greatest diversity and lower degrees of genetic differentiation between this and other populations were found by using TE-AFLP but this were not observed by mtDNA analysis. This observation that can be detected using nuclear DNA but not mtDNA may be explained by the effect of the male component because the study of mtDNA diversity provides the relative importance of female component (tracked with maternally inherited mtDNA). The rate of gene flow may vary among male and female lineages as previously reported in *A. cerana* by microsatellite (Sittipraneed et al., 2001a). They reported the separate group of Northeast population from North and Central population while this was not observed by mtDNA studies.

Basic knowledge of genetic population structure is required for effective management of native bee species (Thummajitsakul et al., 2008). The ability to identify population differentiation within *T. collina* is also the important for establishing natural management of resources and conservation programs for this

native species. The information indicated that *T. collina* from North and South of the Isthmus of Kra should be treated and genetically managed separately but they also showed the variation among individuals within each region. This may help in maintaining out-breeding population structure of this species.

Since there was no significant differentiation among Northern population (North, Northeast, and Central) when population structure of this species were investigated by CUTc1 and TECU marker while this significance was observed in analysis by using TE-AFLP although these analysis were the investigation of nuclear DNA. Therefore, more nuclear DNA and mtDNA genes markers should be further studied for understanding genetic relationships of this species accurately and the exact area for biogeographic boundary in Thai *T. collina* should be studied further.

CHAPTER V

CONCLUSION

1. In AFLP analysis, a 316 bp fragment generated by *Pst*I$_{+AGT}$/*Mse*I$_{+CAG}$ was found in *Tetragonilla collina* but not in 11 other stingless bee species. It was cloned and sequenced to develop *T. collina*-specific marker (called CUTc1).
2. The CUTc1 marker was successfully discriminated *Tetragonilla collina* from the 13 other stingless bee species and SSCP analysis of CUTc1 marker could further differentiate *Tetragonula pagdeni* from *Tetragonilla collina*.
3. This marker is convenient for differentiation of *Tetragonilla collina* from other stingless bees in Thailand and will help to prevent misidentification species for molecular systematic of stingless bees and for population studies of *T. collina*.
4. CUTc1 marker also generated 3 genotypes in Thai *T. collina* (genotype AA, BB, and AB) and these distribution patterns strongly suggested biogeographic differentiation between *T. collina* originating from North and South of Isthmus of Kra.
5. The DNA fingerprints from TE-AFLP analysis, to analyze genetic variation in *T. collina* revealed high genetic diversity among individuals in each population.
6. AMOVA analysis indicated the significant genetic differentiation among four geographic regions; North, Central, Northeast, and Peninsular Thailand. The smaller but significant differentiation was detected between samples from North and South of Isthmus of Kra.
7. The greatest genetic differentiation of *T. collina* based on TE-AFLP derived marker (TECU marker) using SSCP analysis was observed among four populations; North+Central+Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular Thailand.

8. The mtDNA diversity of *T. collina* by using SSCP analysis of 16S rRNA, COI, and cytb genes showed high genetic variation among individuals within each population.

9. AMOVA analysis of the 16S rRNA, COI, and cytb genes revealed high genetic differentiation among 6 populations; North, Central, Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular Thailand. The significant genetic differentiations were also found between samples from North and South of Isthmus of Kra.

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APPENDICES

APPENDIX A

Sample collections

Collection sites of stingless bees used in this study

Abbreviations corresponding to table of sample locations; N = North, NE = Northeast, C = Central region and S = Peninsular Thailand

Sample locations and map abbreviation of stingless bee nests used in this study

Location	Region	Abbreviation
Ayutthaya	Central	AY
Bangkok	Central	BK
Chonburi	Central	CB
Chanthaburi	Central	CT
Kanchanaburi	Central	KB
Lopburi	Central	LB
Nonthaburi	Central	NB
Phetchaburi	Central	PB
Prachuap Khiri Khan	Central	PK
Ratchaburi	Central	RB
Singburi	Central	SI
Suphanburi	Central	SP
Trat	Central	TR
Chiang Mai	North	CM
Kamphaeng Phet	North	KP
Lampang	North	LP
Nan	North	NA
Phichit	North	PC
Phitsanulok	North	PN
Phrae	North	PR
Sukho Thai	North	ST
Uthai Thani	North	UH
Uttaradit	North	UT
Buriram	Northeast	BR
Chaiyaphum	Northeast	CY
Khon Kaen	Northeast	KK
Maha Sarakham	Northeast	MK
Mukdahan	Northeast	MD
Nakhon Ratchasima	Northeast	NR
Roi Et	Northeast	RE
Sakhon Nakhon	Northeast	SN
Sisaket	Northeast	SS
Surin	Northeast	SU
Ubon Rachathani	Northeast	UB
Udon Thani	Northeast	UD
Chumphon	Peninsular	CP
Nakhon Si Thammarat	Peninsular	NT
Phatthalung	Peninsular	PT
Ranong	Peninsular	RN
Songkhla	Peninsular	SK
Surat Thani	Peninsular	SR

Total of 159 *Tetragonilla collina* were used in this study

Code	Population	Province	Locality
COL 1C	Central	Ratchaburi	Muang district
COL 3C	Central	Kanchanaburi	Sai Yok district
COL 4C	Central	Kanchanaburi	Sai Yok district
COL 5C	Central	Kanchanaburi	Sai Yok district
COL 6C	Central	Kanchanaburi	Sai Yok district
COL 7C	Central	Kanchanaburi	Sai Yok district
COL 8C	Central	Kanchanaburi	Sai Yok district
COL 9C	Central	Kanchanaburi	Sai Yok district
COL 10C	Central	Kanchanaburi	Thong Pha Phum district
COL 11C	Central	Kanchanaburi	Thong Pha Phum district
COL 12C	Central	Kanchanaburi	Thong Pha Phum district
COL 13C	Central	Kanchanaburi	Thong Pha Phum district
COL 14C	Central	Kanchanaburi	Thong Pha Phum district
COL 15C	Central	Kanchanaburi	Thong Pha Phum district
COL 16C	Central	Kanchanaburi	Thong Pha Phum district
COL 17C	Central	Kanchanaburi	Thong Pha Phum district
COL 18C	Central	Kanchanaburi	Thong Pha Phum district
COL 19C	Central	Kanchanaburi	Thong Pha Phum district
COL 20C	Central	Kanchanaburi	Thong Pha Phum district
COL 21C	Central	Prechuap Khiri Khan	Bang Saphan Noi district
COL 22C	Central	Prechuap Khiri Khan	Bang Saphan Noi district
COL 23C	Central	Prechuap Khiri Khan	Bang Saphan Noi district
COL 24C	Central	Prechuap Khiri Khan	Bang Saphan Noi district
COL 25C	Central	Prechuap Khiri Khan	Bang Saphan Noi district
COL 26C	Central	Prechuap Khiri Khan	Bang Saphan Noi district
COL 27C	Central	Prechuap Khiri Khan	Bang Saphan Noi district
COL 28C	Central	Prechuap Khiri Khan	Bang Saphan Noi district
COL 29C	Central	Prechuap Khiri Khan	Bang Saphan Noi district
COL 30C	Central	Prechuap Khiri Khan	Bang Saphan Noi district
COL 31C	Central	Prechuap Khiri Khan	Bang Saphan district
COL 32C	Central	Prechuap Khiri Khan	Bang Saphan district
COL 33C	Central	Chanthaburi	Makham district
COL 34C	Central	Chanthaburi	Makham district
COL 2N	North	Nan	Pua district
COL 3N	North	Nan	Pua district
COL 5N	North	Phrae	Muang district
COL 6N	North	Phrae	Muang district
COL 7N	North	Phrae	Muang district
COL 8N	North	Phrae	Muang district

Code	Population	Province	Locality
COL 9N	North	Uttaradit	Laplae district
COL 10N	North	Uttaradit	Laplae district
COL 11N	North	Uthai Thani	Nong Chang district,
COL 12N	North	Uthai Thani	Nong Chang district,
COL 14N	North	Uthai Thani	Nong Chang district,
COL 17N	North	Uthai Thani	Lan Sak district
COL 19N	North	Uthai Thani	Lan Sak district
COL 21N	North	Uthai Thani	Lan Sak district
COL 23N	North	Uthai Thani	Lan Sak district
COL 24N	North	Uthai Thani	Lan Sak district
COL 26N	North	Kamphaeng Phet	Muang district
COL 27N	North	Kamphaeng Phet	Muang district
COL 28N	North	Kamphaeng Phet	Muang district
COL 30N	North	Chiang Mai	San Sai district
COL 31N	North	Phichit	Muang district
COL 32N	North	Phichit	Muang district
COL 33N	North	Phichit	Pho Thale district
COL 34N	North	Phichit	Pho Thale district
COL 35N	North	Phichit	Pho Thale district
COL 1NE	Northeast	Khon Kaen	Nong Song Hong district
COL 2NE	Northeast	Khon Kaen	Phra Yuen district
COL 3NE	Northeast	Khon Kaen	Phra Yuen district
COL 6NE	Northeast	Khon Kaen	Phra Yuen district
COL 7NE	Northeast	Khon Kaen	Phra Yuen district
COL 9NE	Northeast	Khon Kaen	Phra Yuen district
COL 10NE	Northeast	Khon Kaen	Phra Yuen district
COL 12NE	Northeast	Khon Kaen	Phra Yuen district
COL 13NE	Northeast	Maha Sarakham	Wapi Pathum district
COL 14NE	Northeast	Maha Sarakham	Wapi Pathum district
COL 15NE	Northeast	Maha Sarakham	Wapi Pathum district
COL 16NE	Northeast	Maha Sarakham	Wapi Pathum district
COL 17NE	Northeast	Roi Et	Muang district
COL 19NE	Northeast	Roi Et	Chaturakphak Phiman district
COL 20NE	Northeast	Sisaket	Kanthararom district
COL 22NE	Northeast	Sisaket	Kanthararom district
COL 26NE	Northeast	Sisaket	Kanthararom district
COL 29NE	Northeast	Sisaket	Kanthararom district
COL 30NE	Northeast	Sisaket	Kanthararom district
COL 31NE	Northeast	Sisaket	Kanthararom district
COL 35NE	Northeast	Surin	Tha Tum district
COL 39NE	Northeast	Surin	Tha Tum district

Code	Population	Province	Locality
COL 43NE	Northeast	Buriram	Nang Rong district
COL 44NE	Northeast	Buriram	Nang Rong district
COL 46NE	Northeast	Buriram	Nang Rong district
COL 48NE	Northeast	Buriram	Nang Rong district
COL 49NE	Northeast	Buriram	Nang Rong district
COL 50NE	Northeast	Sakhon Nakhon	Phanna Nikhom district
COL 51NE	Northeast	Sakhon Nakhon	Phanna Nikhom district
COL 52NE	Northeast	Sakhon Nakhon	Phanna Nikhom district
COL 55NE	Northeast	Sakhon Nakhon	Phanna Nikhom district
COL 56NE	Northeast	Chaiyaphum	Phu Khiao district
COL 57NE	Northeast	Chaiyaphum	Kaeng Khro district
COL 58NE	Northeast	Chaiyaphum	Kaeng Khro district
COL 59NE	Northeast	Udon Thani	Kut Chap district
COL 61NE	Northeast	Udon Thani	Kut Chap district
COL 63NE	Northeast	Ubon Rachathani	Phibun Mangsahan district
COL 64NE	Northeast	Ubon Rachathani	Phibun Mangsahan district
COL 65NE	Northeast	Ubon Rachathani	Phibun Mangsahan district
COL 66NE	Northeast	Ubon Rachathani	Phibun Mangsahan district
COL 69NE	Northeast	Mukdahan	Loeng Nok Tha district
COL 70NE	Northeast	Ubon Rachathani	Sirinthon district
COL 71NE	Northeast	Khon Kaen	Muang district
COL 2S	Peninsular	Chumphon	Sawi district
COL 3S	Peninsular	Chumphon	Sawi district
COL 5S	Peninsular	Surat Thani	Kanchanadit district
COL 6S	Peninsular	Surat Thani	Chaiya district
COL 7S	Peninsular	Surat Thani	Chaiya district
COL 11S	Peninsular	Surat Thani	Tha Chang district
COL 14S	Peninsular	Ranong	Muang district
COL 15S	Peninsular	Ranong	Muang district
COL 16S	Peninsular	Ranong	Muang district
COL 17S	Peninsular	Ranong	Muang district
COL 18S	Peninsular	Chumphon	Muang district
COL 19S	Peninsular	Chumphon	Muang district
COL 20S	Peninsular	Chumphon	Muang district
COL 21S	Peninsular	Chumphon	Lang Suan district
COL 22S	Peninsular	Chumphon	Lang Suan district
COL 23S	Peninsular	Chumphon	Lang Suan district
COL 24S	Peninsular	Chumphon	Lang Suan district
COL 25S	Peninsular	Chumphon	Lang Suan district
COL 26S	Peninsular	Chumphon	Lang Suan district
COL 27S	Peninsular	Chumphon	Thung Tako district

Code	Population	Province	Locality
COL 28S	Peninsular	Chumphon	Sawi district
COL 29S	Peninsular	Chumphon	Sawi district
COL 30S	Peninsular	Chumphon	Sawi district
COL 31S	Peninsular	Chumphon	Sawi district
COL 32S	Peninsular	Chumphon	Muang district
COL 33S	Peninsular	Chumphon	Muang district
COL 34S	Peninsular	Chumphon	Muang district
COL 35S	Peninsular	Chumphon	Phato district
COL 36S	Peninsular	Ranong	Muang district
COL 37S	Peninsular	Ranong	Muang district
COL 38S	Peninsular	Ranong	Kra Buri district
COL 39S	Peninsular	Chumphon	Muang district
COL 40S	Peninsular	Chumphon	Muang district
COL 41S	Peninsular	Chumphon	Tha Sae district
COL 42S	Peninsular	Surat Thani	Chaiya district
COL 43S	Peninsular	Surat Thani	Chaiya district
COL 44S	Peninsular	Surat Thani	Chaiya district
COL 45S	Peninsular	Surat Thani	Kanchanadit district
COL 47S	Peninsular	Surat Thani	Phun Phin district
COL 48S	Peninsular	Surat Thani	Phun Phin district
COL 49S	Peninsular	Surat Thani	Khiri Rat Nikhom district
COL 50S	Peninsular	Surat Thani	Khiri Rat Nikhom district
COL 52S	Peninsular	Surat Thani	Vibhavadi district
COL 53S	Peninsular	Surat Thani	Vibhavadi district
COL 54S	Peninsular	Surat Thani	Vibhavadi district
COL 55S	Peninsular	Surat Thani	Vibhavadi district
COL 56S	Peninsular	Surat Thani	Vibhavadi district
COL 57S	Peninsular	Surat Thani	Vibhavadi district
COL 58S	Peninsular	Surat Thani	Tha Chana district
COL 59S	Peninsular	Nakhon Si Thammarat	Sichon district
COL 60S	Peninsular	Nakhon Si Thammarat	Sichon district
COL 61S	Peninsular	Nakhon Si Thammarat	Muang district
COL 62S	Peninsular	Nakhon Si Thammarat	Muang district
COL 63S	Peninsular	Nakhon Si Thammarat	Muang district
COL 64S	Peninsular	Nakhon Si Thammarat	Thung Song district
COL 66S	Peninsular	Nakhon Si Thammarat	Thung Song district
COL 67S	Peninsular	Nakhon Si Thammarat	Thung Song district
COL 68S	Peninsular	Nakhon Si Thammarat	Thung Yai district

Total of 106 other stingless bees were used in this study

Code	Species	Population	Province	Locality
API05C	*Tetrigona apicalis*	Central	Ratchaburi	Chom Bueng district
API06C	*Tetrigona apicalis*	Central	Ratchaburi	Chom Bueng district
API07C	*Tetrigona apicalis*	Central	Ratchaburi	Chom Bueng district
API08C	*Tetrigona apicalis*	Central	Ratchaburi	Suan Phueng district
API02N	*Tetrigona apicalis*	North	Chiang Mai	Hang Dong district
API03N	*Tetrigona apicalis*	North	Chiang Mai	Muang district
API15N	*Tetrigona apicalis*	North	Kamphaeng Phet	Muang district
API16N	*Tetrigona apicalis*	North	Kamphaeng Phet	Muang district
API01S	*Tetrigona apicalis*	Peninsular	Nakhon Si Thammarat	Chang Klang district
API10S	*Tetrigona apicalis*	Peninsular	Chumphon	Sawi district
API12S	*Tetrigona apicalis*	Peninsular	Chumphon	Muang district
API13S	*Tetrigona apicalis*	Peninsular	Chumphon	Muang district
CAN01S	*Lophotrigona canifrons*	Peninsular	Surat Thani	Chaiya district
DOI14N	*Lepidatrigona doipaensis*	North	Chiang Mai	Mae Rim district
FIM01N	*Homotrigona fimbriata*	North	Chiang Mai	Muang district
FIM02NE	*Homotrigona fimbriata*	Northeast	Sisaket	Muang district
FIM04NE	*Homotrigona fimbriata*	Northeast	Ubon Rachathani	Phibun Mangsahan district
FUS02C	*Tetragonula fuscobalteata*	Central	Kanchanaburi	Sai Yok district
FUS05C	*Tetragonula fuscobalteata*	Central	Kanchanaburi	Sai Yok district
FUS08C	*Tetragonula fuscobalteata*	Central	Kanchanaburi	Sai Yok district
FUS01N	*Tetragonula fuscobalteata*	North	Uttaradit	Laplae district
FUS11N	*Tetragonula fuscobalteata*	North	Chiang Mai	Mae Rim district
FUS15N	*Tetragonula fuscobalteata*	North	Phrae	Wang Chin district
ITM01S	*Heterotrigona itama*	Peninsular	Songkhla	Hat Yai district
ITM03S	*Heterotrigona itama*	Peninsular	Surat Thani	Singhanakhon district
ITM04S	*Heterotrigona itama*	Peninsular	Surat Thani	Chaiya district
ITM05S	*Heterotrigona itama*	Peninsular	Surat Thani	Chaiya district
L003N	*Tetragonula laeviceps*	North	Phitsanulok	Muang district
L004N	*Tetragonula laeviceps*	North	Phitsanulok	Muang district
L006N	*Tetragonula laeviceps*	North	Phichit	Muang district
L007N	*Tetragonula laeviceps*	North	Phichit	Muang district
L008N	*Tetragonula laeviceps*	North	Phichit	Muang district
L019N	*Tetragonula laeviceps*	North	Phrae	Wang Chin district
MELA01N	*Tetrigona melanoleuca*	North	Chiang Mai	Muang district
MEL01S	*Tetragonula melina*	Peninsular	Surat Thani	Kanchanadit district
MIN02N	*Tetragonula minor*	North	Nan	Pua district
MIN04N	*Tetragonula minor*	North	Uttaradit	Laplae district
MIN07N	*Tetragonula minor*	North	Uttaradit	Laplae district
MIN08N	*Tetragonula minor*	North	Uttaradit	Laplae district
MIN10NE	*Tetragonula minor*	Northeast	Ubon Rachathani	Muang district
MIN11NE	*Tetragonula minor*	Northeast	Ubon Rachathani	Muang district

Code	Species	Population	Province	Locality
MIN14NE	*Tetragonula minor*	Northeast	Sisaket	Huai Thap Than district
MIN01S	*Tetragonula minor*	Peninsular	Songkhla	Muang district
PAG003E	*Tetragonula pagdeni*	Central	Trat	Khao Saming district
PAG004E	*Tetragonula pagdeni*	Central	Trat	Bo Rai district
PAG009E	*Tetragonula pagdeni*	Central	Chanthaburi	Khlung district
PAG010E	*Tetragonula pagdeni*	Central	Chanthaburi	Khlung district
PAG012E	*Tetragonula pagdeni*	Central	Chanthaburi	Makham district
PAG015E	*Tetragonula pagdeni*	Central	Chonburi	Muang district
PAG016E	*Tetragonula pagdeni*	Central	Chonburi	Sriracha district
PAG001C	*Tetragonula pagdeni*	Central	Nonthaburi	Pak Kret district
PAG003C	*Tetragonula pagdeni*	Central	Nonthaburi	Pak Kret district
PAG005C	*Tetragonula pagdeni*	Central	Lopburi	Muang district
PAG007C	*Tetragonula pagdeni*	Central	Lopburi	Muang district
PAG010C	*Tetragonula pagdeni*	Central	Suphanburi	Muang district
PAG013C	*Tetragonula pagdeni*	Central	Singburi	Muang district
PAG014C	*Tetragonula pagdeni*	Central	Singburi	Muang district
PAG015C	*Tetragonula pagdeni*	Central	Nakhon Nayok	Banna district
PAG016C	*Tetragonula pagdeni*	Central	Bangkok	Pathumwan district
PAG017C	*Tetragonula pagdeni*	Central	Bangkok	Pathumwan district
PAG022C	*Tetragonula pagdeni*	Central	Bangkok	Chatuchak district
PAG023C	*Tetragonula pagdeni*	Central	Bangkok	Chatuchak district
PAG025C	*Tetragonula pagdeni*	Central	Phetchaburi	Banlad district
PAG026C	*Tetragonula pagdeni*	Central	Phetchaburi	Banlad district
PAG027C	*Tetragonula pagdeni*	Central	Phetchaburi	Banlad district
PAG029C	*Tetragonula pagdeni*	Central	Prachuap Khiri Khan	Muang district
PAG030C	*Tetragonula pagdeni*	Central	Prachuap Khiri Khan	Muang district
PAG032C	*Tetragonula pagdeni*	Central	Prachuap Khiri Khan	Kui Buri district
PAG038C	*Tetragonula pagdeni*	Central	Prachuap Khiri Khan	Sila Loi district
PAG049C	*Tetragonula pagdeni*	Central	Ayutthaya	Muang district
PAG053C	*Tetragonula pagdeni*	Central	Kanchanaburi	Sangklaburi district
PAG056C	*Tetragonula pagdeni*	Central	Kanchanaburi	Sai Yok district
PAG057C	*Tetragonula pagdeni*	Central	Kanchanaburi	Sai Yok district
PAG059C	*Tetragonula pagdeni*	Central	Kanchanaburi	Muang district
PAG069C	*Tetragonula pagdeni*	Central	Prachuap Khiri Khan	Bang Saphan district
PAG01N	*Tetragonula pagdeni*	North	Chiang Mai	Hang Dong district
PAG02N	*Tetragonula pagdeni*	North	Chiang Mai	Hang Dong district
PAG03N	*Tetragonula pagdeni*	North	Chiang Mai	Hang Dong district
PAG04N	*Tetragonula pagdeni*	North	Chiang Mai	San Sai district
PAG024N	*Tetragonula pagdeni*	North	Nan	Muang district
PAG027N	*Tetragonula pagdeni*	North	Sukho Thai	Muang district
PAG028N	*Tetragonula pagdeni*	North	Sukho Thai	Muang district
PAG031N	*Tetragonula pagdeni*	North	Sukho Thai	Sri Sum Rong district
PAG002NE	*Tetragonula pagdeni*	Northeast	Ubon Rachathani	Muang district

Code	Species	Population	Province	Locality
PAG014NE	*Tetragonula pagdeni*	Northeast	Surin	Kra Pho district
PAG019NE	*Tetragonula pagdeni*	Northeast	Khon Kaen	Nong Wag district
PAG023NE	*Tetragonula pagdeni*	Northeast	Roi Et	Chaturakphak Phiman district
PAG026NE	*Tetragonula pagdeni*	Northeast	Surin	Kra Pho district
PAG028S	*Tetragonula pagdeni*	Peninsular	Chumphon	Muang district
PAG034S	*Tetragonula pagdeni*	Peninsular	Chumphon	Tha Sae district
PAG035S	*Tetragonula pagdeni*	Peninsular	Chumphon	Muang district
PAG039S	*Tetragonula pagdeni*	Peninsular	Surat Thani	Muang district
PAG077S	*Tetragonula pagdeni*	Peninsular	Surat Thani	Tha Chang district
PAG092S	*Tetragonula pagdeni*	Peninsular	Chumphon	Thung Tako district
TER01N	*Lepidatrigona terminata*	North	Uttaradit	Laplae district
TER09C	*Lepidatrigona terminata*	Central	Ratchaburi	Chom Bueng district
TER11N	*Lepidatrigona terminata*	North	Lampang	Some Ngam district
TER13N	*Lepidatrigona terminata*	North	Lampang	Some Ngam district
TER02NE	*Lepidatrigona terminata*	Northeast	Roi Et	Chaturakphak Phiman district
TER04NE	*Lepidatrigona terminata*	Northeast	Roi Et	Chaturakphak Phiman district
TER06NE	*Lepidatrigona terminata*	Northeast	Ubon Rachathani	Muang district
THO04C	*Geniotrigona thoracica*	Central	Ratchaburi	Muang district
THO01S	*Geniotrigona thoracica*	Peninsular	Songkhla	Hat Yai district
THO02S	*Geniotrigona thoracica*	Peninsular	Surat Thani	Chaiya district
Lfur02NE	*Lisotrigona furva*	Northeast	Nakhon Ratchasima	Chaloem Phra Kiat district
Lfur03C	*Lisotrigona furva*	Central	Ratchaburi	Suan Phueng district

APPENDIX B

TE-AFLP and SSCP Haplotypes

Forty- seven TE-AFLP patterns for the primer pair *Xba*I-CC/*Bam*HI-C

Sample	Population	Haplotype Code	Haplotype
COL 1C	Central	29	0110110101h
COL 3C	Central	9	0010010100h
COL 5C	Central	44	1110110001h
COL 6C	Central	40	1110010001h
COL 8C	Central	45	1110110101h
COL 10C	Central	4	0000010001h
COL 13C	Central	22	0110010001h
COL 14C	Central	27	0110110001h
COL 16C	Central	40	1110010001h
COL 20C	Central	35	1010010001h
COL 21C	Central	9	0010010100h
COL 24C	Central	7	0010000001h
COL 26C	Central	39	1110000001h
COL 27C	Central	35	1010010001h
COL 28C	Central	3	0000010000h
COL 30C	Central	34	1010010000h
COL 31C	Central	33	1000010101h
COL 32C	Central	38	1100010101h
COL 33C	Central	20	0110000010h
COL 34C	Central	16	0100000100h
COL 2N	North	43	1110100000h
COL 3N	North	24	0110100000h
COL 5N	North	24	0110100000h
COL 8N	North	47	1111100010h
COL 9N	North	22	0110010001h
COL 10N	North	46	1111100000h
COL 11N	North	27	0110110001h
COL 12N	North	26	0110100100h
COL 14N	North	24	0110100000h
COL 23N	North	30	0110110111h
COL 24N	North	17	0100010001h
COL 26N	North	12	0010100000h
COL 27N	North	31	0111100000h
COL 30N	North	15	0010110001h
COL 31N	North	21	0110000100h
COL 32N	North	21	0110000100h

Sample	Population	Haplotype Code	Haplotype
COL 33N	North	26	0110100100h
COL 34N	North	21	0110000100h
COL 1NE	Northeast	25	0110100010h
COL 2NE	Northeast	13	0010100010h
COL 10NE	Northeast	22	0110010001h
COL 13NE	Northeast	11	0010011100h
COL 14NE	Northeast	26	0110100100h
COL 17NE	Northeast	24	0110100000h
COL 19NE	Northeast	27	0110110001h
COL 20NE	Northeast	15	0010110001h
COL 31NE	Northeast	22	0110010001h
COL 35NE	Northeast	25	0110100010h
COL 43NE	Northeast	31	0111100000h
COL 49NE	Northeast	6	0000010110h
COL 51NE	Northeast	24	0110100000h
COL 52NE	Northeast	28	0110110011h
COL 56NE	Northeast	19	0110000000h
COL 58NE	Northeast	24	0110100000h
COL 59NE	Northeast	27	0110110001h
COL 61NE	Northeast	14	0010100100h
COL 64NE	Northeast	14	0010100100h
COL 65NE	Northeast	8	0010000100h
COL 69NE	Northeast	2	0000000100h
COL 70NE	Northeast	23	0110010101h
COL 71NE	Northeast	10	0010010101h
COL 5S	Peninsular	42	1110011100h
COL 11S	Peninsular	18	0100010100h
COL 16S	Peninsular	8	0010000100h
COL 17S	Peninsular	9	0010010100h
COL 18S	Peninsular	38	1100010101h
COL 22S	Peninsular	8	0010000100h
COL 26S	Peninsular	4	0000010001h
COL 27S	Peninsular	32	1000010100h
COL 28S	Peninsular	32	1000010100h
COL 30S	Peninsular	35	1010010001h
COL 32S	Peninsular	36	1010010101h
COL 35S	Peninsular	41	1110010101h
COL 36S	Peninsular	5	0000010101h
COL 37S	Peninsular	37	1100010001h
COL 38S	Peninsular	37	1100010001h
COL 39S	Peninsular	35	1010010001h
COL 40S	Peninsular	37	1100010001h
COL 41S	Peninsular	41	1110010101h

Sample	Population	Haplotype Code	Haplotype
COL 42S	Peninsular	35	1010010001h
COL 43S	Peninsular	40	1110010001h
COL 45S	Peninsular	1	0000000000h
COL 47S	Peninsular	1	0000000000h
COL 48S	Peninsular	36	1010010101h
COL 49S	Peninsular	36	1010010101h
COL 50S	Peninsular	36	1010010101h
COL 52S	Peninsular	35	1010010001h
COL 53S	Peninsular	34	1010010000h
COL 55S	Peninsular	34	1010010000h
COL 57S	Peninsular	37	1100010001h
COL 58S	Peninsular	37	1100010001h
COL 59S	Peninsular	40	1110010001h
COL 60S	Peninsular	38	1100010101h
COL 61S	Peninsular	38	1100010101h
COL 62S	Peninsular	41	1110010101h
COL 66S	Peninsular	40	1110010001h
COL 67S	Peninsular	40	1110010001h
COL 68S	Peninsular	38	1100010101h

Seventy-nine TE-AFLP patterns for the primer pair *Xba*I-CT/*Bam*HI-C

Sample	Population	Haplotype Code	Haplotype
COL 1C	Central	55	0101000000000000111h
COL 3C	Central	28	0001101100000010101h
COL 5C	Central	20	0001000100011001101h
COL 6C	Central	18	0001000100000010101h
COL 8C	Central	21	0001000101000101010 1h
COL 10C	Central	63	0101000110000010101h
COL 13C	Central	23	0001001100000010001h
COL 14C	Central	16	0001000010000011101h
COL 16C	Central	22	0001001000011001 1101h
COL 20C	Central	6	0001000000000010101h
COL 21C	Central	70	0111000000000000100h
COL 24C	Central	56	0101000000000001001h
COL 26C	Central	64	0101001000000001001h
COL 27C	Central	77	1101000101011001 1001h
COL 28C	Central	54	0100010100100000 1001h
COL 30C	Central	57	0101000000000001101h
COL 31C	Central	65	0101001000000101101h
COL 32C	Central	78	1101001000000101101h
COL 33C	Central	30	0010000000000110000h
COL 34C	Central	32	0010000000100011 0000h
COL 2N	North	11	0001000000000110101h
COL 3N	North	11	0001000000000110101h
COL 5N	North	21	0001000101000101010 1h
COL 8N	North	45	0011000001000101001 1h
COL 9N	North	14	0001000001000101010 1h
COL 10N	North	15	0001000001000111010 1h
COL 11N	North	59	0101000000000010101h
COL 12N	North	6	0001000000000010101h
COL 14N	North	7	0001000000000010110h
COL 23N	North	46	0011000001000111010 1h
COL 24N	North	43	0011000000000110101h
COL 26N	North	41	0011000000000010101h
COL 27N	North	19	0001000100000110101h
COL 30N	North	18	0001000100000010101h
COL 31N	North	15	0001000001000111010 1h
COL 32N	North	11	0001000000000110101h
COL 33N	North	44	0011000001000101000 1h
COL 34N	North	11	0001000000000110101h
COL 1NE	Northeast	42	0011000000000110001h
COL 2NE	Northeast	34	0010000001000101000 1h
COL 10NE	Northeast	40	0011000000000010001h

Sample	Population	Haplotype Code	Haplotype
COL 13NE	Northeast	69	01101000000000110001h
COL 14NE	Northeast	35	00100001000000010001h
COL 17NE	Northeast	36	00100001000000110001h
COL 19NE	Northeast	49	00110001000000110001h
COL 20NE	Northeast	58	01010000000000010001h
COL 31NE	Northeast	37	00100011000000010001h
COL 35NE	Northeast	50	00110011000000010101h
COL 43NE	Northeast	38	00100011000000110001h
COL 49NE	Northeast	68	01100001000000111001h
COL 51NE	Northeast	35	00100001000000010001h
COL 52NE	Northeast	47	00110001000000010001h
COL 56NE	Northeast	31	00100000000000110001h
COL 58NE	Northeast	29	00100000000000010001h
COL 59NE	Northeast	29	00100000000000010001h
COL 61NE	Northeast	33	00100000010000010001h
COL 64NE	Northeast	40	00110000000000010001h
COL 65NE	Northeast	29	00100000000000010001h
COL 69NE	Northeast	29	00100000000000010001h
COL 70NE	Northeast	39	00110000000000010000h
COL 71NE	Northeast	29	00100000000000010001h
COL 5S	Peninsular	8	00010000000000011001h
COL 11S	Peninsular	27	00010101000000001001h
COL 16S	Peninsular	57	01010000000000001101h
COL 17S	Peninsular	67	01010100010000101000h
COL 18S	Peninsular	71	01110000000110011001h
COL 22S	Peninsular	75	11010000100000001110h
COL 26S	Peninsular	48	00110001000000011101h
COL 27S	Peninsular	79	11110100001000011000h
COL 28S	Peninsular	4	00000010100000010000h
COL 30S	Peninsular	76	11010000110110111101h
COL 32S	Peninsular	17	00010000110110111101h
COL 35S	Peninsular	61	01010000001110111101h
COL 36S	Peninsular	62	01010000010110111001h
COL 37S	Peninsular	12	00010000000110111001h
COL 38S	Peninsular	73	10010000000110011001h
COL 39S	Peninsular	5	00010000000000001001h
COL 40S	Peninsular	13	00010000000110111101h
COL 41S	Peninsular	74	11010000000000001001h
COL 42S	Peninsular	5	00010000000000001001h
COL 43S	Peninsular	9	00010000000000101001h
COL 45S	Peninsular	11	00010000000000110101h
COL 47S	Peninsular	66	01010100000110111001h
COL 48S	Peninsular	9	00010000000000101001h

Sample	Population	Haplotype Code	Haplotype
COL 49S	Peninsular	5	000100000000000001001h
COL 50S	Peninsular	10	000100000000000110001h
COL 52S	Peninsular	72	100100000000000110001h
COL 53S	Peninsular	26	000101001000000110101h
COL 55S	Peninsular	25	000101000001101111101h
COL 57S	Peninsular	60	010100000001101111001h
COL 58S	Peninsular	60	010100000001101111001h
COL 59S	Peninsular	1	000000000000000101001h
COL 60S	Peninsular	51	010000000001101111101h
COL 61S	Peninsular	3	000000001000000101001h
COL 62S	Peninsular	24	000101000000000101101h
COL 66S	Peninsular	53	010001000001101111101h
COL 67S	Peninsular	52	010000001000000101001h
COL 68S	Peninsular	2	000000000001101111101h

Seventeen SSCP haplotypes for 16S rRNA gene

Sample	Population	Haplotype Code	Haplotype
COL 1C	Central	15	00100000010000000000000000000h
COL 3C	Central	17	11000000000000000000000000000h
COL 4C	Central	17	11000000000000000000000000000h
COL 5C	Central	17	11000000000000000000000000000h
COL 6C	Central	17	11000000000000000000000000000h
COL 7C	Central	17	11000000000000000000000000000h
COL 8C	Central	17	11000000000000000000000000000h
COL 9C	Central	17	11000000000000000000000000000h
COL 10C	Central	17	11000000000000000000000000000h
COL 11C	Central	17	11000000000000000000000000000h
COL 12C	Central	15	00100000010000000000000000000h
COL 13C	Central	15	00100000010000000000000000000h
COL 14C	Central	17	11000000000000000000000000000h
COL 15C	Central	17	11000000000000000000000000000h
COL 16C	Central	17	11000000000000000000000000000h
COL 17C	Central	17	11000000000000000000000000000h
COL 18C	Central	17	11000000000000000000000000000h
COL 19C	Central	17	11000000000000000000000000000h
COL 20C	Central	17	11000000000000000000000000000h
COL 2N	North	14	00011000000000000000000000000h
COL 3N	North	14	00011000000000000000000000000h
COL 5N	North	11	00000100100000000000000000000h
COL 8N	North	11	00000100100000000000000000000h
COL 9N	North	13	00010010000000000000000000000h
COL 10N	North	13	00010010000000000000000000000h
COL 11N	North	13	00010010000000000000000000000h
COL 12N	North	12	00001001000000000000000000000h
COL 14N	North	12	00001001000000000000000000000h
COL 17N	North	12	00001001000000000000000000000h
COL 32N	North	16	10001000000000000000000000000h
COL 1NE	Northeast	8	00000000000001100000000000000h
COL 2NE	Northeast	9	00000000000011000000000000000h
COL 3NE	Northeast	9	00000000000011000000000000000h
COL 9NE	Northeast	9	00000000000011000000000000000h
COL 10NE	Northeast	9	00000000000011000000000000000h
COL 13NE	Northeast	7	00000000000000011000000000000h
COL 14NE	Northeast	7	00000000000000011000000000000h
COL 17NE	Northeast	3	00000000000000000000000110000h
COL 19NE	Northeast	7	00000000000000011000000000000h
COL 20NE	Northeast	8	00000000000001100000000000000h
COL 22NE	Northeast	8	00000000000001100000000000000h

Sample	Population	Haplotype Code	Haplotype
COL 26NE	Northeast	4	00000000000000000000011000000h
COL 31NE	Northeast	8	00000000000000110000000000000h
COL 43NE	Northeast	6	00000000000000000110000000000h
COL 44NE	Northeast	6	00000000000000000110000000000h
COL 49NE	Northeast	6	00000000000000000110000000000h
COL 51NE	Northeast	7	00000000000000001100000000000h
COL 52NE	Northeast	3	00000000000000000000000110000h
COL 56NE	Northeast	5	00000000000000000001100000000h
COL 61NE	Northeast	7	00000000000000001100000000000h
COL 64NE	Northeast	4	00000000000000000000011000000h
COL 65NE	Northeast	4	00000000000000000000011000000h
COL 69NE	Northeast	7	00000000000000001100000000000h
COL 70NE	Northeast	10	00000000011000000000000000000h
COL 71NE	Northeast	9	00000000000011000000000000000h
COL 5S	Peninsular	2	00000000000000000000000001100h
COL 11S	Peninsular	2	00000000000000000000000001100h
COL 16S	Peninsular	1	00000000000000000000000000011h
COL 17S	Peninsular	1	00000000000000000000000000011h
COL 36S	Peninsular	1	00000000000000000000000000011h
COL 37S	Peninsular	1	00000000000000000000000000011h
COL 38S	Peninsular	1	00000000000000000000000000011h
COL 42S	Peninsular	2	00000000000000000000000001100h
COL 43S	Peninsular	2	00000000000000000000000001100h
COL 45S	Peninsular	2	00000000000000000000000001100h
COL 47S	Peninsular	2	00000000000000000000000001100h
COL 48S	Peninsular	2	00000000000000000000000001100h
COL 49S	Peninsular	2	00000000000000000000000001100h
COL 50S	Peninsular	2	00000000000000000000000001100h
COL 52S	Peninsular	2	00000000000000000000000001100h
COL 53S	Peninsular	2	00000000000000000000000001100h
COL 55S	Peninsular	2	00000000000000000000000001100h
COL 56S	Peninsular	2	00000000000000000000000001100h
COL 57S	Peninsular	2	00000000000000000000000001100h
COL 58S	Peninsular	2	00000000000000000000000001100h
COL 59S	Peninsular	2	00000000000000000000000001100h
COL 60S	Peninsular	2	00000000000000000000000001100h
COL 61S	Peninsular	2	00000000000000000000000001100h
COL 62S	Peninsular	2	00000000000000000000000001100h
COL 67S	Peninsular	2	00000000000000000000000001100h
COL 21C	Prachuap Khiri Khan	17	11000000000000000000000000000h
COL 22C	Prachuap Khiri Khan	17	11000000000000000000000000000h
COL 23C	Prachuap Khiri Khan	17	11000000000000000000000000000h
COL 24C	Prachuap Khiri Khan	17	11000000000000000000000000000h

Sample	Population	Haplotype Code	Haplotype
COL 25C	Prachuap Khiri Khan	17	11000000000000000000000000000h
COL 26C	Prachuap Khiri Khan	17	11000000000000000000000000000h
COL 27C	Prachuap Khiri Khan	17	11000000000000000000000000000h
COL 28C	Prachuap Khiri Khan	17	11000000000000000000000000000h
COL 29C	Prachuap Khiri Khan	17	11000000000000000000000000000h
COL 30C	Prachuap Khiri Khan	17	11000000000000000000000000000h
COL 31C	Prachuap Khiri Khan	17	11000000000000000000000000000h
COL 32C	Prachuap Khiri Khan	17	11000000000000000000000000000h
COL 18S	Chumphon	1	00000000000000000000000000011h
COL 20S	Chumphon	1	00000000000000000000000000011h
COL 22S	Chumphon	1	00000000000000000000000000011h
COL 26S	Chumphon	1	00000000000000000000000000011h
COL 27S	Chumphon	1	00000000000000000000000000011h
COL 28S	Chumphon	1	00000000000000000000000000011h
COL 30S	Chumphon	1	00000000000000000000000000011h
COL 32S	Chumphon	1	00000000000000000000000000011h
COL 39S	Chumphon	1	00000000000000000000000000011h
COL 40S	Chumphon	1	00000000000000000000000000011h
COL 41S	Chumphon	1	00000000000000000000000000011h

Thirty-four SSCP haplotypes for COI gene

Sample	Population	Haplotype Code	Haplotype
COL 1C	Central	20	00000000000000000001100000000000000000000000000000000000000h
COL 4C	Central	20	00000000000000000001100000000000000000000000000000000000000h
COL 7C	Central	4	00000000000000000000000000000000000000000000000011000000000h
COL 9C	Central	15	00000000000000000000000011000000000000000000000000000000000h
COL 10C	Central	5	00000000000000000000000000000000000000000000011000000000000h
COL 11C	Central	22	00000000000000001100000000000000000000000000000000000000000h
COL 13C	Central	17	00000000000000000000011000000000000000000000000000000000000h
COL 15C	Central	9	00000000000000000000000000000000000010000000000000000010h
COL 17C	Central	19	00000000000000000010000001000000000000000000000000000000000h
COL 34C	Central	18	00000000000000000000110000000000000000000000000000000000000h
COL 2N	North	26	00000000010000000000000000000000000000000000000000000000000h
COL 3N	North	24	00000000000001100000000000000000000000000000000000000000000h
COL 5N	North	30	00000001000000000000000000000001000000000000000000000000000h
COL 8N	North	27	00000000011000000000000000000000000000000000000000000000000h
COL 9N	North	21	00000000000000001000000000000000000000000000000000000000100h
COL 10N	North	25	00000000001000000000000000000001000000000000000000000000000h
COL 11N	North	28	00000000011110000000000000000000000000000000000000000000000h
COL 14N	North	28	00000000011110000000000000000000000000000000000000000000000h
COL 17N	North	28	00000000011110000000000000000000000000000000000000000000000h
COL 32N	North	3	00000000000000000000000000000000000000000000000110000000000h
COL 1NE	Northeast	29	00000000110000000000000000000000000000000000000000000000000h
COL 9NE	Northeast	31	00000011000000000000000000000000000000000000000000000000000h
COL 10NE	Northeast	31	00000011000000000000000000000000000000000000000000000000000h
COL 17NE	Northeast	31	00000011000000000000000000000000000000000000000000000000000h
COL 19NE	Northeast	2	00000000000000000000000000000000000000000000000001100000000h
COL 26NE	Northeast	1	00000000000000000000000000000000000000000000000011000000000h
COL 31NE	Northeast	11	00000000000000000000000000000000110000000000000000000000000h
COL 44NE	Northeast	31	00000011000000000000000000000000000000000000000000000000000h
COL 51NE	Northeast	31	00000011000000000000000000000000000000000000000000000000000h
COL 61NE	Northeast	31	00000011000000000000000000000000000000000000000000000000000h
COL 5S	Peninsular	8	00000000000000000000000000000000000011000000000000000000000h
COL 11S	Peninsular	12	00000000000000000000000000011000000000000000000000000000000h
COL 17S	Peninsular	32	00001100000000000000000000001000000000000000000000000000000h
COL 38S	Peninsular	32	00001100000000000000000000001000000000000000000000000000000h
COL 42S	Peninsular	33	00110000000000000000000000000000000000000000000000000000000h
COL 43S	Peninsular	33	00110000000000000000000000000000000000000000000000000000000h
COL 45S	Peninsular	14	00000000000000000000000000011000000000000000000000000000000h
COL 47S	Peninsular	23	00000000000000001000000000000000000000000010000000000000000h
COL 48S	Peninsular	10	00000000000000000000000000000000011000000000000000000000000h
COL 49S	Peninsular	10	00000000000000000000000000000000011000000000000000000000000h
COL 50S	Peninsular	10	00000000000000000000000000000000011000000000000000000000000h

Sample	Population	Haplotype Code	Haplotype
COL 52S	Peninsular	14	00000000000000000000000000000011000000000000000000000000000000h
COL 53S	Peninsular	14	00000000000000000000000000000011000000000000000000000000000000h
COL 55S	Peninsular	33	00110000000000000000000000000000000000000000000000000000000000h
COL 56S	Peninsular	10	00000000000000000000000000000000000000110000000000000000000000h
COL 57S	Peninsular	10	00000000000000000000000000000000000000110000000000000000000000h
COL 58S	Peninsular	10	00000000000000000000000000000000000000110000000000000000000000h
COL 59S	Peninsular	12	00000000000000000000000000000011000000000000000000000000000000h
COL 60S	Peninsular	12	00000000000000000000000000000011000000000000000000000000000000h
COL 61S	Peninsular	33	00110000000000000000000000000000000000000000000000000000000000h
COL 62S	Peninsular	33	00110000000000000000000000000000000000000000000000000000000000h
COL 67S	Peninsular	33	00110000000000000000000000000000000000000000000000000000000000h
COL 21C	Prachuap Khiri Khan	15	00000000000000000000000011000000000000000000000000000000000000h
COL 22C	Prachuap Khiri Khan	17	00000000000000000000000110000000000000000000000000000000000000h
COL 23C	Prachuap Khiri Khan	17	00000000000000000000000110000000000000000000000000000000000000h
COL 24C	Prachuap Khiri Khan	16	00000000000000000000000100000000000000000000000000000000000001h
COL 26C	Prachuap Khiri Khan	17	00000000000000000000000110000000000000000000000000000000000000h
COL 27C	Prachuap Khiri Khan	17	00000000000000000000000110000000000000000000000000000000000000h
COL 29C	Prachuap Khiri Khan	17	00000000000000000000000110000000000000000000000000000000000000h
COL 30C	Prachuap Khiri Khan	17	00000000000000000000000110000000000000000000000000000000000000h
COL 31C	Prachuap Khiri Khan	22	00000000000000000110000000000000000000000000000000000000000000h
COL 32C	Prachuap Khiri Khan	15	00000000000000000000000011000000000000000000000000000000000000h
COL 18S	Chumphon	10	00000000000000000000000000000000000000110000000000000000000000h
COL 20S	Chumphon	8	00000000000000000000000000000000000000011000000000000000000000h
COL 22S	Chumphon	6	00000000000000000000000000000000000000001100000000000000000000h
COL 26S	Chumphon	34	11000000000000000000000000000000000000000000000000000000000000h
COL 27S	Chumphon	13	00000000000000000000000000001001000000000000000000000000000000h
COL 28S	Chumphon	7	00000000000000000000000000000000000000011000000000000000000000h
COL 30S	Chumphon	7	00000000000000000000000000000000000000011000000000000000000000h
COL 32S	Chumphon	14	00000000000000000000000000011000000000000000000000000000000000h
COL 39S	Chumphon	7	00000000000000000000000000000000000000011000000000000000000000h
COL 40S	Chumphon	10	00000000000000000000000000000000000000110000000000000000000000h
COL 41S	Chumphon	14	00000000000000000000000000011000000000000000000000000000000000h

Thirty-four SSCP haplotypes for cytb gene

Sample	Population	Haplotype Code	Haplotype
COL 1C	Central	2	00000000000000000000000000000010000100h
COL 3C	Central	13	00000000000010000000000000000010000000h
COL 5C	Central	13	00000000000010000000000000000010000000h
COL 6C	Central	13	00000000000010000000000000000010000000h
COL 8C	Central	13	00000000000010000000000000000010000000h
COL 9C	Central	13	00000000000010000000000000000010000000h
COL 10C	Central	13	00000000000010000000000000000010000000h
COL 12C	Central	13	00000000000010000000000000000010000000h
COL 13C	Central	13	00000000000010000000000000000010000000h
COL 14C	Central	13	00000000000010000000000000000010000000h
COL 16C	Central	2	00000000000000000000000000000010000100h
COL 17C	Central	13	00000000000010000000000000000010000000h
COL 18C	Central	2	00000000000000000000000000000010000100h
COL 20C	Central	2	00000000000000000000000000000010000100h
COL 34C	Central	31	01000010000000000000000000000000000000h
COL 2N	North	23	00000100000000001000000000000000000000h
COL 3N	North	23	00000100000000001000000000000000000000h
COL 7N	North	11	00000000000000000001000000000100000h
COL 8N	North	11	00000000000000000001000000000100000h
COL 9N	North	25	00010000000000000000000000000100000h
COL 11N	North	30	01000001000000000000000000000000000000h
COL 12N	North	1	00000000000000000000000000000000000011h
COL 14N	North	1	00000000000000000000000000000000000011h
COL 17N	North	33	10000000100000000000000000000000000000h
COL 32N	North	29	00100010000000000000000000000000000000h
COL 1NE	Northeast	21	00000000100000000000001000000000000000h
COL 2NE	Northeast	17	00000000000010000100000000000000000000h
COL 3NE	Northeast	17	00000000000010000100000000000000000000h
COL 9NE	Northeast	17	00000000000010000100000000000000000000h
COL 10NE	Northeast	17	00000000000010000100000000000000000000h
COL 13NE	Northeast	7	00000000000000000000000100000001000000h
COL 15NE	Northeast	7	00000000000000000000000100000001000000h
COL 17NE	Northeast	17	00000000000010000100000000000000000000h
COL 19NE	Northeast	17	00000000000010000100000000000000000000h
COL 20NE	Northeast	27	00010000000001000000000000000000000000h
COL 22NE	Northeast	27	00010000000001000000000000000000000000h
COL 26NE	Northeast	34	10000100000000000000000000000000000000h
COL 31NE	Northeast	27	00010000000001000000000000000000000000h
COL 44NE	Northeast	7	00000000000000000000000100000001000000h
COL 49NE	Northeast	7	00000000000000000000000100000001000000h

Sample	Population	Haplotype Code	Haplotype
COL 51NE	Northeast	7	00000000000000000000000100000001000000h
COL 52NE	Northeast	8	00000000000000000000001000000010000000h
COL 56NE	Northeast	19	00000000010000000000000000000000000000h
COL 61NE	Northeast	17	00000000000010000100000000000000000000h
COL 64NE	Northeast	28	00010000000001000000000000000000000000h
COL 65NE	Northeast	28	00010000000001000000000000000000000000h
COL 69NE	Northeast	8	00000000000000000000001000000010000000h
COL 70NE	Northeast	20	00000000010100000000000000000000000000h
COL 71NE	Northeast	8	00000000000000000000001000000010000000h
COL 5S	Peninsular	4	00000000000000000000000010000000010000h
COL 11S	Peninsular	5	00000000000000000000000010000010000000h
COL 16S	Peninsular	3	00000000000000000000000001000000001000h
COL 17S	Peninsular	3	00000000000000000000000001000000001000h
COL 36S	Peninsular	6	00000000000000000000000010000100000000h
COL 37S	Peninsular	3	00000000000000000000000001000000001000h
COL 38S	Peninsular	3	00000000000000000000000001000000001000h
COL 42S	Peninsular	6	00000000000000000000000010000100000000h
COL 43S	Peninsular	6	00000000000000000000000010000100000000h
COL 47S	Peninsular	5	00000000000000000000000010000010000000h
COL 48S	Peninsular	5	00000000000000000000000010000010000000h
COL 49S	Peninsular	5	00000000000000000000000010000010000000h
COL 50S	Peninsular	5	00000000000000000000000010000010000000h
COL 52S	Peninsular	5	00000000000000000000000010000010000000h
COL 53S	Peninsular	5	00000000000000000000000010000010000000h
COL 55S	Peninsular	5	00000000000000000000000010000010000000h
COL 56S	Peninsular	5	00000000000000000000000010000010000000h
COL 57S	Peninsular	5	00000000000000000000000010000010000000h
COL 59S	Peninsular	6	00000000000000000000000010000100000000h
COL 60S	Peninsular	6	00000000000000000000000010000100000000h
COL 61S	Peninsular	15	00000000000010000000000000000001000000h
COL 62S	Peninsular	6	00000000000000000000000010000100000000h
COL 67S	Peninsular	6	00000000000000000000000010000100000000h
COL 21C	Prachuap Khiri Khan	32	01001000000000000000000000000000000000h
COL 24C	Prachuap Khiri Khan	32	01001000000000000000000000000000000000h
COL 25C	Prachuap Khiri Khan	32	01001000000000000000000000000000000000h
COL 27C	Prachuap Khiri Khan	32	01001000000000000000000000000000000000h
COL 28C	Prachuap Khiri Khan	32	01001000000000000000000000000000000000h
COL 29C	Prachuap Khiri Khan	32	01001000000000000000000000000000000000h
COL 31C	Prachuap Khiri Khan	26	00010000000000010000000000000000000000h
COL 32C	Prachuap Khiri Khan	26	00010000000000010000000000000000000000h
COL 18S	Chumphon	14	00000000000010000000000000000100000000h
COL 20S	Chumphon	14	00000000000010000000000000000100000000h
COL 22S	Chumphon	22	00000001000000000000000000000000010000h

Sample	Population	Haplotype Code	Haplotype
COL 26S	Chumphon	22	00000001000000000000000000000000010000h
COL 27S	Chumphon	9	00000000000000000000010000000001000000h
COL 28S	Chumphon	10	00000000000000000000010000010000000000h
COL 30S	Chumphon	16	00000000000010000000000000100000000000h
COL 32S	Chumphon	12	00000000000000000001000000100000000000h
COL 39S	Chumphon	18	00000000001000000010000000000000000000h
COL 40S	Chumphon	24	00001000010000000000000000000000000000h
COL 41S	Chumphon	6	00000000000000000000000010000100000000h

BIOGRAPHY

Miss Montalee Theeraapisakkun was born on Sep 20, 1982 in Bangkok, Thailand. She graduated with a Bachelor of Science degree in Biochemistry, Faculty of Science, Chulalongkorn University in 2004. She has further studied for the Doctor of Philosophy (Ph.D.) degree in Biochemistry, Faculty of Science, Chulalongkorn University since 2004.