สัญญาเลขที่ MRG6080272

รายงานวิจัยฉบับสมบูรณ์

โครงการ

ลักษณะทางพันธุศาสตร์และจุดกำเนิดลูกผสมของผึ้งพันธุ์ *Apis mellifera* ในประเทศไทย

Genetic Characterization and Hybrid Origins of the Commercial Honeybee, *Apis mellifera*, in Thailand

โดย

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บทคัดย่อ

การปรับตัวเป็นสัตว์เลี้ยงของสัตว์หลายชนิดมักมีความเกี่ยวเนื่องกับการลดลงของความหลากหลายทางพันธุกรรม โดยผึ้งพันธุ์ (*Apis mellifera*) นับเป็นหนึ่งในสัตว์ที่มนุษย์นำมาปรับปรุงสายพันธุ์ให้เป็นสัตว์เลี้ยงมาหลายศตวรรษเพื่อผลิตน้ำผึ้งและไขผึ้งซึ่งเป็นผลิตภัณฑ์หลักที่ได้จากการเลี้ยงผึ้ง และนอกจากนี้มนุษย์ยังใช้ผึ้งพันธุ์ในการเป็นแมลงผสมเกสรของพืชเศรษฐกิจหลากหลายชนิดอีกด้วย การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อวิเคราะห์ความแปรผันทางพันธุกรรมและจุดกำเนิดลูกผสมของประชากรผึ้งพันธุ์ *A. mellifera* ที่เลี้ยงในอุตสาหกรรมการเลี้ยงผึ้งของประเทศไทยด้วยเครื่องหมายดีเอ็นเอแบบไมโครแซทเทลไลท์ การวิเคราะห์ลำดับเบสของยีน COI ในไมโตคอนเดรียดีเอ็นเอ และวิธีพีซีอาร์-อาร์เอฟแอลพีของบางส่วนของยีนในไมโตคอนเดรียดีเอ็นเอ ผลการวิเคราะห์เครื่องหมายดีเอ็นเอแบบไมโครแซทเทลไลท์พบค่าเฉลี่ยความหลากหลายทางพันธุกรรม (expected hetrozygosity, H_e) ของประชากรผึ้งพันธุ์ *A. Mellifera* ที่เลี้ยงในอุตสาหกรรมการเลี้ยงผึ้งของประเทศไทยมีค่าสูงโดยมีค่าอยู่ระหว่าง 0.620±0.184 ถึง 0.734±0.071 นอกจากนี้ภายใต้สภาวะสมดุลของฮาร์ดี–ไวน์เบิร์ก พบค่า Observed heterozygosity (H_o) มีค่าน้อยกว่าค่า H_e ในขณะที่ผลการวิเคราะห์ลำดับเบสบางส่วนของยีน COI และการวิเคราะห์ด้วยวิธีพีซีอาร์-อาร์เอฟแอลพีพบว่าผึ้งพันธุ์ *A. mellifera* ในประเทศไทยประกอบด้วยผึ้งสายพันธุ์วิวัฒนาการ C และ O (evolutionary linages C and O) ซึ่งยังคงเหมือนเดิมเมื่อประมาณสิบปีที่ผ่านมา นอกจากนี้ยังพบว่าประชากผึ้งพันธุ์ในประเทศไทยผ่านช่วงการเปลี่ยนแปลงขนาดของประชากร (Tajima's D = –0.25, P > 0.05; Fu's Fs = –3.75, P > 0.05) แต่ไม่ส่งผลต่อความหลากหลายทางพันธุกรรมของประชากร ดังนั้นจากผลการศึกษาครั้งนี้แสดงให้เห็นว่าเกษตรกรผู้เลี้ยงผึ้งในประเทศไทยยังคงรักษาระดับความหลากหลายทางพันธุกรรมของประชากรผึ้งพันธุ์ได้เป็นอย่างดี แต่เนื่องจากยังพบสายพันธุ์วิวัฒนาของผึ้งพันธุ์ในประเทศไทยเพียงสองสายพันธุ์ ซึ่งอาจจะส่งผลให้มีการลดลงของควาหลากหลายทางพันธุกรรมของประชากรได้ในอนาคต ดังนั้นเพื่อให้สามารถเพิ่มและรักษาความหลากหลายทางพันธุกรรมของประชากรหากมีสายพันธุ์วิวัฒนาของผึ้งพันธุ์ *A. mellifera* ใหม่ๆ เข้ามาเพิ่มเติมในอุตสหกรรมการเลี้ยงผึ้งของประเทศไทยในอนาคตต่อไป

คำหลัก : ผึ้งพันธุ์, ความแปรผันทางพันธุกรรม, ไมโครแซทเทลไลท์, ไมโตคอนเดรีย ดีเอ็นเอ, พีซีอาร์-อาร์เอฟแอลพี

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Abstract

Domestication is of animal species is often associated with a reduction in genetic diversity. The honey bee, *Apis mellifera*, has been managed by beekeepers for centuries for both honey and wax production and for crop pollination. Here we use microsatellite markers, sequence data from the mitochondrial COI gene, and PCR–RFLP of four mitochondrial gene fracments to evaluate temporal genetic variation and hybrid origin of managed *A. mellifera* in Thailand where the species is introduced. Microsatellite analysis revealed high average genetic diversity (expected hetrozygosity, H_e), ranging from 0.620±0.184 to 0.734±0.071. Observed heterozygosity was generally lower than expected heterozygosity under Hardy–Weinberg equilibrium. Mitochondrial sequencing and PCR–RFLP revealed two evolutionary linages (C and O) have remained constant over the last decade. There is evidence of recent changes in effective population size (Tajima's D = –0.25, P > 0.05; Fu's Fs = –3.75, P > 0.05). Our results suggest that Thai beekeepers are managing their *A. mellifera* in ways that retain overall genetic diversity, but will likely reduce genetic diversity between apiaries over time.

Keywords : Honeybee, Genetic variation, Microsatellites, Mitochondrial DNA, PCR-RFLP

Executive Summary

Animal domestication often results in dramatic morphological and behavioral modifications over short time scales and a reduction in genetic diversity relative to their wild progenitors. Loss of genetic diversity probably arises as a result of a small initial population sizes and intense artificial selection for economic traits. The former effect, also known as the 'domestication bottleneck', is often evidenced by genome-wide loss of genetic diversity during the founding of a new domestic lineage. Both the magnitude and duration of the bottleneck affects levels of genetic variation in domesticated plants and animals. The Western honeybee, *Apis mellifera*, is managed by beekeepers for honey and wax production and crop pollination. Selective breeding is believed to have caused reductions in genetic variation in many populations of commercial *A. mellifera*. Indeed, several authors have speculated that unusually high rates of mortality among commercial colonies over the last decade are due in part to reduction in genetic diversity within commercial populations. Genetic diversity is important to colony fitness and productivity because it increases nest homeostasis, disease resistance and honey production. Therefore, maintenance of genetic diversity within commercial populations is essential for ongoing productivity.

A. mellifera was first imported to Thailand on a large scale from Taiwan in the 1970s. In later years *A. mellifera* is thought to have been imported from Russia, Europe and Australia though the frequency and success of these latter imports is unknown. *A. mellifera* beekeeping is now practiced throughout Thailand with more than 300,000 managed colonies. However no feral population has established. The ancestry and genetic diversity of the commercial population is unclear. There are beekeeper concerns that increasing homogenization both within and between Thai honeybee populations may be impacting production and disease resistance, leading to calls for further imports of genetic material. In this study we assess genetic variation, population structure and hybrid origin of commercial *A. mellifera* stocks of Thailand using microsatellites, mitochondrial cytochrome oxidase subunit I sequences, and PCR–RFLP of four mitochondrial gene fragments ($tRNA^{leu}$–COII, COI, Ls rRNA, and Cytb). Our goal is to assess whether there is sufficient genetic variation to prevent the effects of inbreeding or whether importation of additional genetic material is warranted.

Microsatellite analysis revealed high average genetic diversity with expected heterozygosities, ranging from 0.620±0.184 to 0.734±0.071 per locus per province. Observed heterozygosities were generally lower than those expected under Hardy–Weinberg equilibrium, both locally and across the population as a whole. Mitochondrial COI sequencing and restriction patterns of five gene/enzyme combinations demonstrated that the frequency of two evolutionary linages (C - Eastern European and O – Middle Eastern) are similar to those observed in a previous survey 10 years ago. Our results suggest that Thai beekeepers are managing their *A. mellifera* in ways that retain overall genetic diversity, but reduce genetic diversity between apiaries.

Chapter 1

Introduction

Domestication has provided good examples of dramatically morphological and genetic modification existing on a short evolutionary time scale (Haudry et al., 2007). Varying degree of reduction of genetic diversity in domesticated organisms relative to their progenitors occurred during the process of domestication (Tanksley and McCouch, 1997; Eyre-Walker et al., 1998). This reduction in genetic variation is probably a product of a small initial population sizes relative to their ancestors and intense artificial selection for agronomic traits (Eyre-Walker et al., 1998; Clark et al., 2005; Zeder et al., 2006). The former effect, also known as domestication bottleneck, is featured by a genome-wide loss of genetic diversity that occurs during the founding of a new domestic lineage (Eyre-Walker et al. 1998; Clark et al. 2005). Both the magnitude and length of time of the bottleneck affect contemporary level of genetic variation in domesticated plants and animals (Wright et al., 2005). Most domesticated crop plants such as rice, soybeans and maize have remained 30 – 67% of the genetic variation relative to their progenitors (Eyre-Walker et al. 1998; Hyten et al., 2006; Zhu et al., 2007). The same is true for domesticated animals (Bruford et al., 2003). For instance, chickens and silk worms have lost more than 50% of the genetic variation of their progenitors (Muira et al., 2008; Guo et al. 2011). Domestication also allows human selection that is also expected to influence levels of genetic variation at specific loci affecting traits of interest (Zeder et al., 2006; Guo et al., 2011). Therefore, domesticated plants and animals are expected to decrease in genome-wide genetic diversity relative to their ancestors because of the bottleneck effect and artificial selection may further reduce genetic diversity level at loci affect desirable traits (Eyre-Walker et al. 1998; Zeder et al., 2006; Zhu et al., 2007).

In recent decades, molecular genetic markers are becoming increasingly important and widely used to reveal the patterns of genetic diversity within and between populations (Heyen et al., 1997; Savolainen et al., 2000; Heaton et al., 2002; Ramos-Onsin et al., 2004; Zhu et al., 2007; Fernández

et al., 2013). Among the molecular techniques available, microsatellites have been widely used in analyses of intraspecific variation, identification in parentage testing, established relationship between two or more individuals, bottlenecks in the population history, migration and gene flow of various groups of animals (Heyen et al., 1997; Williams et al., 1997; Beaumont and Bruford, 1999; Heaton et al., 2002; Arias et al., 2006). In addition, mitochondrial DNA sequencing combine with restriction fragment length polymorphism (RFLP) are also succeeded to determine which subspecies and mitochondrial lineages of *A. mellifera* (Diniz et al., 2003; Pinto et al., 2005; Oxley and Oldroyd, 2009).

The European honeybee, *Apis mellifera*, is often considered to be a partially domesticated animal, managed by beekeepers for honey and wax production and crop pollination. Africa, Europe and the Middle East were once the natural distribution areas of the species (Seeley, 1985; Ruttner, 1988). Today, the distribution of *A. mellifera* has now extended over most of the world. Based on morphology and ecological traits, *A. mellfera* has been grouped into 4 main evolutionary lineages (Ruttner, 1988): an African lineage (A), an Eastern Europe lineage (C), a Western Europe lineage (M), and a Middle Eastern lineage (O), and at least 26 named subspecies (Whitfield et al., 2006; Wallberg et al., 2014). Recently, Ruttner's classification has been supported by molecular genetic analysis of mitochondrial DNA (Estoup et al., 1995; Franck et al., 2000). According to mitochondrial DNA analysis data, each lineage is classifiable from both the pattern and variation of sequence motifs that make up the cytochrome oxidase subunit I and II intergenic region (Franck et al. 2000). The honeybees of C lineage are identifiable because they lack of the P motif, and contain only a single copy of the Q motif (Susnik et al., 2004, Kozmus et al., 2007). Honeybees of M lineage contain a shorter 54 – 56 bp P motif, compared with the longer 62 – 69 bp sequence in lineages A and lineages O (Franck et al. 2000; Oxley and Oldroyd, 2009).

Human management and selective breeding are believed to have caused reductions in genetic variation in *A. mellifera* population, thereby contributing to the global population declines (Delaney et al., 2009; Mexiner et al., 2010; Harpur et al., 2012). Several studies hypothesized that high rates of *A. mellifera* colony mortality and population decline over the past several decades due

to reductions in genetic diversity in managed honeybees (van Engelsdorp and Meixner, 2010; Sheppard, 2012). Genetic diversity within colony is clearly important, which are headed by multiple mating by queens (Palmer and Oldroyd, 2000; Crozier and Fjerdingstad, 2001). Various studies have convincingly revealed that multiple mating by honeybees queen results in increased intra-colonial genetic diversity (Tarpy and Seeley, 2006; Tarpy et al., 2013), which increases nest homeostasis (Oldroyd and Fewell, 2007), disease resistant (Tarpy, 2003) and honey production (Oldroyd et al. 1992; Mattila and Seeley 2007). In breeding within honey populations leads to significant negative impact to colony productivity. Woyke (1963) show that homozygosity at the sex determining locus leads to production of diploid males that are eliminated by female workers, making it necessary to maintain a high level of diversity at this locus to prevent significant loss of brood viability. In 1986, Moritz demonstrated that there is a rapid increase in the inbreeding coefficient in breeding schemes composed of less than 5 unrelated queens, resulting to a loss of colony's productivity in only a few generations. In addition, several studies have shown a strong fitness benefit of within-colony genetic diversity that is unrelated to inbreeding at the sex locus (Mattila and Seeley 2007, Oldroyd and Fewell 2007). Thus, in any honeybee-breeding program, maintenance of genetic diversity among breeding lines is an important factor in selective breeding and stock management.

In Thailand, *A. mellifera* was first imported from Australia in the early 1950s for research purposes (Wongsiri, 1988; Akratanakul, 2000). Then, in the 1970s a large number of *A. mellifera* colonies were introduced from Taiwan to Chiang Mai and Lampoon in north of Thailand for apiculture industry purposes (Wongsiri, et al., 1995). *Apis mellifera* may have additionally imported from Russia, Europe and Australia (Kavinseksan et al., 2004). Since then, they have been spread throughout Thailand by local beekeepers (Thapa and Wongsiri, 1997). Nowadays, more than 300,000 colonies are managed in Thailand (Wongsiri et al., 2000) but the ancestry and hybrid origin of *A. mellifera* in the country is unclear. Furthermore, there are concerns over increasing homogenization both within and between Thai honeybee populations, and the maintenance of genetic diversity. The origins and levels of genetic diversity in Thai *A. mellifera* populations is therefore of interest and concern for

improvement of Thai stocks. This study, I used the 6-base restriction enzymes (Suppasat et al., 2007; Wu et al., 2017), DNA sequencing of mitochondrial genes (Oxley and Oldroyd, 2009; Delanney et al., 2009) and variable microsatellite loci (Garnery et al., 1998; Solignac et al., 2007; Jensen et al., 2005; Bourgeois et al., 2008; Delanney et al., 2009) to determine the maternal ancestry, neutrality test, bottleneck intensity, and intra- and inter-locus linkage disequilibrium of commercial *A. mellifera* stocks in Thailand.

Many Thai's beekeepers have produced very good queen daughters of imported stock or daughters of some selected queen that they obtained from other beekeepers. Then, the new stocks queens usually mate with drones (males) of the beekeeper's own stock. If the new stocks queens are genetically different from the stock that beekeeper already has, the imported queens produce hybrid progeny and high quality brood within colony. Therefore, the genetic profile collected will be become very useful basic information for breeding improvement strategies and maintenance of genetic variation of the European honey bee, *A. mellifera*, in Thailand. In this study we aimed to (1) assess genetic variation and population structure of commercial *A. mellifera* stocks of Thailand using microsatellites and mitochondrial cytochrome oxidase subunit I (mtCOI) sequences, and (2) identify mitochondrial lineages and subspecies presented by using PCR-RFLP techniques. Our goal is to assess whether there is sufficient genetic variation to prevent the effects of inbreeding or whether importation of additional genetic material is warranted.

Chapter 2

Literature Reviews

2.1 The European or Western honeybees, *Apis mellifera*

The western honeybee or European honeybee (*Apis mellfera* Linnaeus, 1758) is native to Africa, Europe, and some parts of western Asia, ranging from Kirgizia in the east to the west of Europe; from the south of Africa to the north of Europe (Seeley, 1985; Ruttner, 1988). In this large distribution range, the species can be in a vast range of habitats (Ruttner, 1988; Sheppard and Meixner, 2003), ranging from desert to tropical rain forest and from plains to mountainous (Smith, 1991). The European honeybee is often considered to be a partially domesticated animal, managed by beekeepers for honey and wax production and crop pollination. Today, the distribution of *A. mellifera* has now extended over most of the world (Sheppard and Meixner, 2003).

Generally, *A. mellifera* is regarded as the medium-sized honeybees, against which other honeybee species are judged as “large” or “small” (Gupta, 2014). In the wild, the natural nesting sites of *A. mellifera* are caves, rock cavities and hollow trees. The nests are composed of multiple combs, parallel to each other, with a relatively uniform bee space. The nest usually has a single entrance. The temperate races prefer nest cavities of about 45 liters in volume and avoid those smaller than 10, or larger than 100 liters. Colonies of the *A. mellfera* are composed of relatively large populations, usually between 15,000 and 60,000 worker bees (Crane, 1999; Gupta, 2014).

Like all Hymenopterans, *A. mellifera* have haplo-diploid sex determination. Unfertilized eggs develop into males (drones), whereas fertilized eggs develop into females (queens and workers). Female larvae that are fed with pollen, nectar, and brood food become adult workers (non-reproductive females). Whilst, female larvae fed a rich diet of royal jelly, pollen, and nectar develop into queens (Seeley, 1985; Ruttner, 1988; Crane, 1999).

Female worker honeybees are the smallest in physical size of the three castes and their bodies are specialized for pollen and nectar collection (Fig. 1) (Ruttner, 1988). Each worker will perform different tasks exclusively in a predictable order based on their age (Seeley, 1985; Ruttner, 1988). This is called age-related polytheism (Crane, 1999). The youngest workers tend the brood (eggs, larvae, and pupae) and the queen, while older workers build wax comb, handle food stores within the colony, and guard the colony entrance (Crane, 1999). The oldest female workers are foragers (Crane, 1999). The female queen honeybee is the only reproductive female in the colony during normal circumstances (Fig. 1). Her head and thorax are similar in size to that of the worker. However, the queen has a longer and plumper abdomen than does a worker (Seeley, 1985; Ruttner, 1988).

The queen is the sole egg layer in the colony and is responsible for producing all of the colony's offspring (up to 1500 eggs/day) (Crane, 1999). Drones are the male caste of honeybees (Fig. 2). The drone's head and thorax are larger than those of the female castes, and their large eyes appear more 'y-like' touching in the top center of the head. Their abdomen is thick and blunt at the end, appearing bullet-shaped rather than pointy at the end as with the female castes. A drone's only purpose is to mate with a virgin queen from another colony (Crane, 1999).

Figure 1 *Apis mellifera* queen with a retinue of young female workers round her (Photo by Preecha Rod-Im)

Figure 2 Drone of *Apis mellifera*

2.2 *Apis mellifera* lineages and subspecies

Ruttner *et al.* (1978) hypothesized that *A. mellifera* spread out from the Near East into three branches; (M) the branch that distributed in Western Europe and Northern Africa, (A) the branch of Southern and Central Africa, and (C) the branch of Northern Mediterranean. Later, Ruttner (1988) added a fourth branch which includes Middle Eastern subspecies, known as the (O) lineage. Therefore, based on morphology and ecological traits, *A. mellfera* has been grouped into 4 main evolutionary lineages (Ruttner, 1988): an African lineage (A), an Eastern Europe lineage (C), a Western Europe lineage (M), and a Middle Eastern lineage (O). These four lineages were confirmed by using restriction fragment length polymorphism (RFLP) and mitochondrial DNA sequencing.

Today, at least 28 named subspecies were described (Whitfield et al., 2006; Miguel et al. 2011; Wallberg et al., 2014). According to mitochondrial DNA analysis data, each lineage is classifiable from both the pattern and variation of sequence motifs that make up the cytochrome oxidase subunit I and II intergenic region (Franck et al. 2000). The honeybees of C lineage are identifiable because they lack of the P motif, and contain only a single copy of the Q motif (Susnik et al., 2004, Kozmus et al., 2007). Honeybees of M lineage contain a shorter 54 – 56 bp P motif, compared with the longer 62 – 69 bp sequence in lineages A and lineages O (Franck et al. 2000; Oxley and Oldroyd, 2009). Recently, a fifth lineage (Y lineage) has been described by Frank et al. (2001) comprising the subspecies of *A. m. jemenitica*.

There are currently 26 recognized subspecies of *A. mellifera* based largely geographic variations as follow (Engel, 1999):

- Subspecies originating in Europe: *A. m. ligustica, A. m. carnica, A. m. caucasia, A. m. remipes, A. m. mellifera, A. m. iberiensis, A. m. cecropia, A. m. adami, A. m. cypria, A. m. ruttneri, and A. m. siciliana*
- Subspecies originated in Africa: *A. m. scutellata*, *A. m. capensis, A. m. monticola*, *A. m.* sahariensis, *A. m. intermissa, A. m. adansonii*, *A. m. unicolor, A. m. lamarkii, A. m. littorea,*

and *A. m. jemenitica*

- Subspecies originated in the Middle East and Asia: *A. m. macedinica, A. m. meda, A. m. anatiliaca, A. m. syriaca, and A. m. pomonella*

These subspecies of *A. mellifera* have been found to have specific behavioral and morphological characteristics (Crane, 1999; Gupta, 2014). All subspecies are cross fertile. Geographic isolation led to numerous local adaptations as this species spread after the last ice age. These adaptations include brood cycles synchronized with the bloom period of local flora, forming a winter cluster in colder climates, migratory swarming in Africa, enhanced foraging behavior in desert areas, and numerous other inherited traits (Gupta, 2014).

2.3 Domestication of honeybee, *Apis mellifera*

The honeybee, *A. mellifera*, has a long relationship with human, most likely somewhere in Egypt, although the earliest representations of bees are illustrated in rock art in southern France and northern Spain (Crane 1999). Dating back to at least 7,000 BC, Egyptian and Mesopotamian beekeepers used honeybees to produce honey and wax (Bloch et al., 2010). As *A. mellifera* domestication spread into Europe, artificially selective pressures from human drastically changed the development of the once uniform species (Weber, 2012). These differences primarily stemmed from specific characteristics desired by beekeepers. Generally, this list is confined to six main factors: colony survival during dearth periods, colony survival when honey flow is poor, resistance to disease, maximum amount of honey storage, tendency to sting, and ease of pacification by smoke (Crane, 1999). While these characteristics benefitted honey-gatherers, they however acted to remove natural defences of honeybees. Differences in selection arose depending on the degree of desirability in each location (Weber, 2012). For instance, in Africa, honey-hunters were in direct competition with native animals, and therefore bees that showed increased aggression to deter competitors were selected (Crane, 1999). In Europe where areas with a more temperate climate, beekeepers were often less concerned with temperament and focused on selecting honeybees lines that could survive

in cold winter periods (Crane, 1999). Thus, honeybees more suitable for changing temperatures would have shown greater fitness in Europe, whereas bees more capable of migration and surviving drought, heat, and excessive rain would have been artificially selected for in tropical regions (Weber, 2012).

Modern techniques and equipment have been developed to facilitate the management of honeybees resulting in modern apiculture (Harpur et al., 2012), which based on hive systems and removable combs allowing for collecting honey and a colony transport. The development of modern transportation for long distance allowed beekeepers carry *A. mellifera* colonies to the new habitats, where no natural *A. mellifera* populations. For instance, African and European races of *A. mellifera* have been introduced to America (Engel et al., 2009). In 1622, beekeepers from Virginia in Northern America imported Black German honeybee from England. Then, through natural swarming and migratory beekeeping, *A. mellfera* spread in throughout the continent (Crane, 1999; Engel et al., 2009; Weber, 2012). The spreading of *A. mellifera* was completed around the world when English beekeepers carried honeybees to Australia, Tasmania and New Zealand (Crane, 1999; Oxley and Oldroyd, 2006; Chapman et al., 2016).

In Thailand, the first colonies of *A. mellifera* were imported to the country between the 1940s - 1950s for scientific research propose at Kasetsart University and Chulalongkorn University in Bangkok (Wongsiri, 1988; Akratanakul, 2000). However, these early importation appears to have not established lasting *A. mellifera* population in Thailand. After that, *A. mellifera* colonies were introduced from Tiwan, Russia, Europe and Australia (Wongsiri et al., 1995; Kavinseksan et al., 2004) to the northern part of the country for commercial propose. Since then, the species were spread rapidly in northern Thailand by beekeepers, especially, Chiang Mai and Lampoon (Thapa and Wongsiri, 1997). *Apis mellifera* beekeeping was later extended throughout the rest of the country (Figure 3). Today, more than 300,000 *A. mellifera* colonies are well managed (Wongsiri et al., 2000), but no such research regarding wild or feral population of *A. mellfera* in Thailand has been reported. Currently in Thailand, the matrilineal origin of *A. mellifera* is unknown, but could include a mixture of many

subspecies (Suppasat, 2007). Therefore, the identity of *A. mellifera* population in Thailand is potentially of interest for improvement and maintenance of Thai honey bee stocks.

Figure 3 Apiary of *Apis mellifera* in Nan province, Northern Thailand.

2.4 Genetic diversity and molecular diversity indices

Genetic diversity is the total genetic characteristics in the genetic makeup of the species (Freeland, 2005; Frankham et al., 2010). This serves as a way for populations of organisms to adapt to changing in their environments. Indeed, the more genetic variations, the higher chance that there is an organism possess an allele which is proper for new environments (Beebee and Rowe, 2004; Freeland, 2005; Frankham et al., 2010).

Population genetic knowledge leads us to study and determine the genetic diversity. There are many hypotheses and theories concerning genetic diversity, for instant, the neutral theory of evolution and the selection (Beebee and Rowe, 2004). In addition, there are several methods to

examine genetic diversity such as molecular diversity indices, for example, nucleotide diversity (π_n), haplotype diversity ($\hat{H}$) and population comparison F_{st} (Beebee and Rowe, 2004; Freeland, 2005).

Molecular diversity indices comprise number of alleles or haplotypes, nucleotide diversity and haplotype diversity. Number of alleles or haplotype diversity is the total count of alleles or haplotypes in the population. Whereas, nucleotide diversity (π_n) refers to the probability that two randomly chosen homologous nucleotide are different. Nucleotide diversity index can be calculated as:

$$\pi_n = \frac{\sum_{i=1}^{k}\sum_{j<i} p_i p_j \hat{d}_{ij}}{L}$$

where $\hat{d}_{ij}$ is an estimate of the number of mutations having occurred since the divergence of haplotypes *i* and *j*, *k* is the number of haplotypes, p_i is the frequency of haplotype *i*, and p_j is the frequency of haplotype *j*.

Haplotype diversity ($\hat{H}$) refer to the probability that two haplotypes selected by random in sample are different. This index can be calculated as:

$$\hat{H} = \frac{n}{n-1}\left(1 - \sum_{i=1}^{k} p_i^2\right)$$

Where *n* is the number of gene copies in the sample, *k* is the number of haplotypes, and p_i is the sample frequency of the *i* haplotype.

2.5 Mitochondrial DNA and Restriction fragment length polymorphism (RFLP)

DNA is genetic material found in all cells of living organisms and can be recovered. In general, DNA can be classified into two categories, chromosomal (nuclear) DNA and extrachromosomal (organelle) DNA. Nuclear DNA is located in nucleus of eukaryotic cell while organelle DNA is located in mitochondria and chloroplast. Alternatively, it is known as mitochondrial DNA (mtDNA) and chloroplast DNA, respectively. Analysis of polymorphism at DNA level is considered to be a direct approach to investigate interspecific and intraspecific genetic variations.

The in vitro amplification of DNA with the polymerase chain reaction (PCR) enabled for the direct use of nuclear and mitochondrial DNA samples as a powerful tool for investigate population genetic analyses. Mitochondrial DNA (mtDNA) is circular and double stranded DNA located in mitochondria. Each mitochondrion consists about 2 – 10 mtDNA copies (Wiesner et al., 1992). In most animals, the mitochondrial genome is composed of 13 protein coding genes, 2 ribosomal RNAs (rRNAs), 22 transfer RNAs (tRNAs), and non-coding region containing an origin of replication. The size of mtDNA ranges between 14 – 42 kb. In addition, protein coding genes are 3 subunits of cytochrome C oxidase (*COI, COII*, and *COIII*), 7 subunits of NADH dehydrogenase (*ND1-6* and *ND4*L), cytochrome 6, and 2 subunits of ATP synthetase (*ATPase6* and *8*) (Crozier and Crozier, 1993). Unlike nuclear DNA, mtDNA is maternally inherited without recombination (Singh et al., 1995). Basically, mutation rate of mtDNA is much more rapid than that of single-copy nuclear genes and it is not sensitive to environmental selection pressure (Franck et al., 2000). Hence, that makes mtDNA useful and efficient in studying genetic and phylogeographic variations among bee population (Franck et al., 2000; Garnery et al., 1993).

Mitochondrial DNA of *A. mellifera* was first extracted by Moritz and Hawkins (1985). The isolated DNA was digested with restriction enzymes, which revealed a polymorphism in length (Moritz and Hawkins, 1985). After that, Crozier and Crozier (1993) investigated the sequence of mtDNA of *A. mellifera* and showed that the size of mtDNA of *A. mellfera* varies between 16.5 – 17 kb in length

depending on the honeybee subspecies (Figure 4). This size variability is based on the control region and $tRNA^{leu}$ – COII intragenic region (Smith and Brown, 1990). Since then, the information of mtDNA sequences allowed for designing oligonucleotide primer to use in PCR for specific amplification regions of mtDNA. For instance, cytochrome b (Crozier et al., 1991, Collins et al., 2000), the 16S rRNA (Bouga, 2005), and the region, which includes the $tRNA^{leu}$ gene, the $tRNA^{leu}$ – COII intragenic region (Franck et al., 2000; Diniz et al., 2003; Collet et al., 2006; Kozmus et al., 2007; Oxley and Oldroyd, 2006; Suppasat et al., 2007).

Polymorphism of mtDNA have been used to discriminate *A. mellifera* subspecies (Crozier et al., 1991; Hall and Smith, 1991; Moritz et al., 1994) and investigate the pattern of gene flow among introduced European and African honeybees in the New World (Hall and Smith 1991; Moritz and Meusel, 1992). Because of maternal inheritance, honeybee of hybrid origin does not carry a mixture of mtDNA's, but they represent only the pattern of their queen (Smith, 1991; Meusel and Moritz, 1993), making the mtDNA particularly powerful in determine of hybrid origin (Moritz et al., 1994). For example, the $tRNA^{leu}$ – COII intragenic region has been widely used for study DNA polymorphism of honeybee because its restriction site length polymorphism (Moritz et al., 1994; Garnery et al., 1995; Franck et al., 2000, 2001). The RFLP of the $tRNA^{leu}$ – COII intragenic region can be revealed the evolution lineage of *A. meliifera* by endonuclease *Dra* I restriction, resulting from the variability of a specific *P* and *Q* sequence. (Garnery et al., 1993; Franck et al., 2000, 2001; De La Rúa, et al., 2004; Suppasat et al., 2007).

2.6 Microsatellite DNA markers

Among the molecular techniques available, microsatellites have been widely used in studies of the population genetics of various groups of animals including honeybees (Pamilo et al., 1997). Microsatellites are tandem sequence repeats of motifs with 1–6 bases randomly distributed along the euchromatic regions (Arias et al., 2006). Microsatellite loci are considered codominant, selectively neutral, highly polymorphic, and show Mendelian inheritance (Moritz et al., 2003). Due to these

characteristics they have been extremely useful in analyses of relatedness, parentage, intraspecific variation, species hybridization, population dynamics, gene mapping and phylogeographic studies (Arias et al., 2006). This DNA maker have been also used to evaluate the impact of reproductive behavior, social structure, and dispersion in endangered populations (Beaumont and Bruford, 1999). At the population level, the use of multiple highly variable loci brings to the precise analysis of the structure of natural populations including the detection of population growth and decline, bottlenecks in the population history, migration and gene flow. Population sizes are however often difficult to determine and are usually estimated indirectly based on allelic diversity. If the family of structured populations is studied, it is possible to infer the number of families from relatedness estimates among the sampled individuals (Kraus et al., 2005). In haplodiploid social insect species such as honeybees and stingless bees, microsatellites show high alleles difference and high heterozygosity (Brito et al., 2009, 2014). The technique therefore is reliable and precise for determining the genetic structure of honeybee both in inter and intra-specific variation levels (Estoup et al., 1994).

In *A. mellifera*, microsatellites have been used to determine the origin of the species and subspecies (Franck et al., 1998; 2000), to examine the number of patrilines of the colony and matting frequency of the honeybee queens (Estoup et al., 1994; Kraus et al., 2003; Jensen et al., 2005; Rattanawannee et al., 2012), to assess population genetic structure (Sušnik et al., 2004; Kraus et al., 2005; De La Rúa et al., 2006; Bodur et al., 2007; Oleksa et al., 2011; Chahbar et al., 2012, Rattanawannee et al., 2013; Uzunov et al., 2014), and to study gene mapping (Weinstock et al., 2006; Lattorff et al., 2007). Recently, the use of tightly linked microsatellite loci has been demonstrated to be informative to investigate the number of honeybee colonies in particular areas (Moritz et al., 2007, 2008; Jaffé et al., 2010; Hinson et al., 2015).

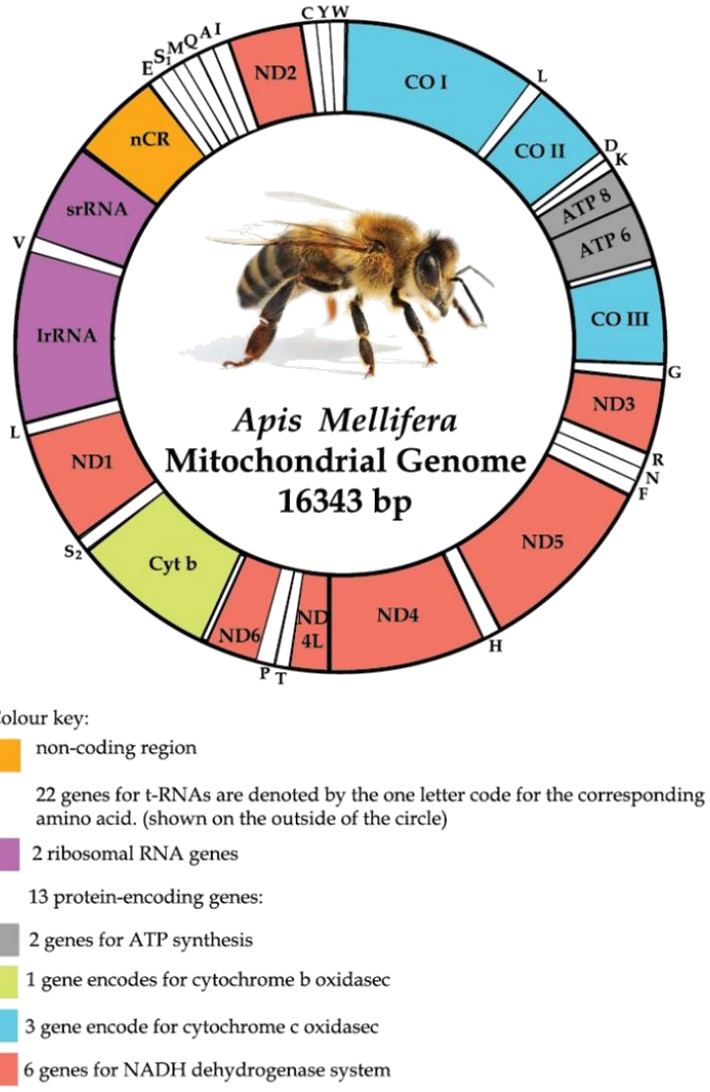

Figure 4 Circular map of *Apis melliefra* mitochondrial DNA (not in scale) showing gene composition (Crozier and Crozier, 1992).

Chapter 3

Material and Methods

3.1 Part I: Genetic characterization of commercial honey bee, *Apis mellifera*, populations in Thailand by using microsatellite and mitochondrial DNA markers

3.1.1 Sampling and DNA Extraction

We sampled a total of 173 managed colonies belonging to 27 beekeepers from 35 commercial apiaries in eight provinces (Fig. 5) during 2017 and 2018. Workers (n = 5 per colony) were collected and preserved in 95% (v/v) ethanol. DNA was extracted from a single worker per colony. For microsatellite markers, genomic DNA was extracted from one right hind leg each bee sample using a 5% Chelex solution (Walsh et al., 1991). For mitochondrial DNA analysis, the thorax was used to extract total DNA for mtCOI analysis using a DNeasy® Blood & Tissue kit (Qiagen, Germantown, MD, US) according to the manufacturer's instructions.

3.1.2 Ethics statement

This study of an exotic invertebrate was welcomed by beekeepers. No specific permits were required. All experimental methods using the animal conformed to the guidelines established by the Animal Experiment Committee, Kasetsart University, Thailand (Approval no. ACKU 01160).

3.1.3 Microsatellite Amplification and Genotyping

The microsatellite target sequences were amplified by using multiplex PCR reaction with fluorescently labeled primers. The microsatellite loci used in this study were A7, A28, A88, A107, and A113 (Estoup et al. 1995). These loci have been shown to be good proxies for determine genetic variation at the population level of honey bees (Delaney et al. 2009). All microsatellite loci were performed using a PCR program of 94 ºC for 10 min, followed by 35 cycles of 94, 54, and 72 ºC for

30 s each, and finally 72 ºC for 10 min. Samples containing no DNA were included as negative controls. The PCR products were sent to Macrogen© (South Korea) for fragment analysis. Resultant data files were subsequently analyzed to examine allele size using GeneMapper software (Applied Biosystems).

3.1.4 Microsatellite Analysis

The number of alleles, number of effective alleles, allele frequencies, and observed (H_o) and expected (H_e) heterozygosity of each locus were estimated using GENEPOP package (Rousset 2008). Exact tests for Hardy–Weinberg equilibrium for each locus and genotypic linkage disequilibrium among loci were calculated in GENEPOP (Rousset 2008). Population structure was examined using a Bayesian model-based clustering method implemented in STRUCTURE 2.3.3 (Pritchard et al. 2000). Analyses were based on the admixture model with correlated allele frequencies with simulations of 100,000 burn-in step and 1,000,000 iterations of MCMC algorithm for each run. Ten runs were computed for each *K* (number of populations) to determine the most likely value of *K*. The Evanno's estimator ΔK (Evanno et al. 2005) implemented in Structure Harvester (Earl and von Holdt 2012) was used to estimate the most likely number of population clusters.

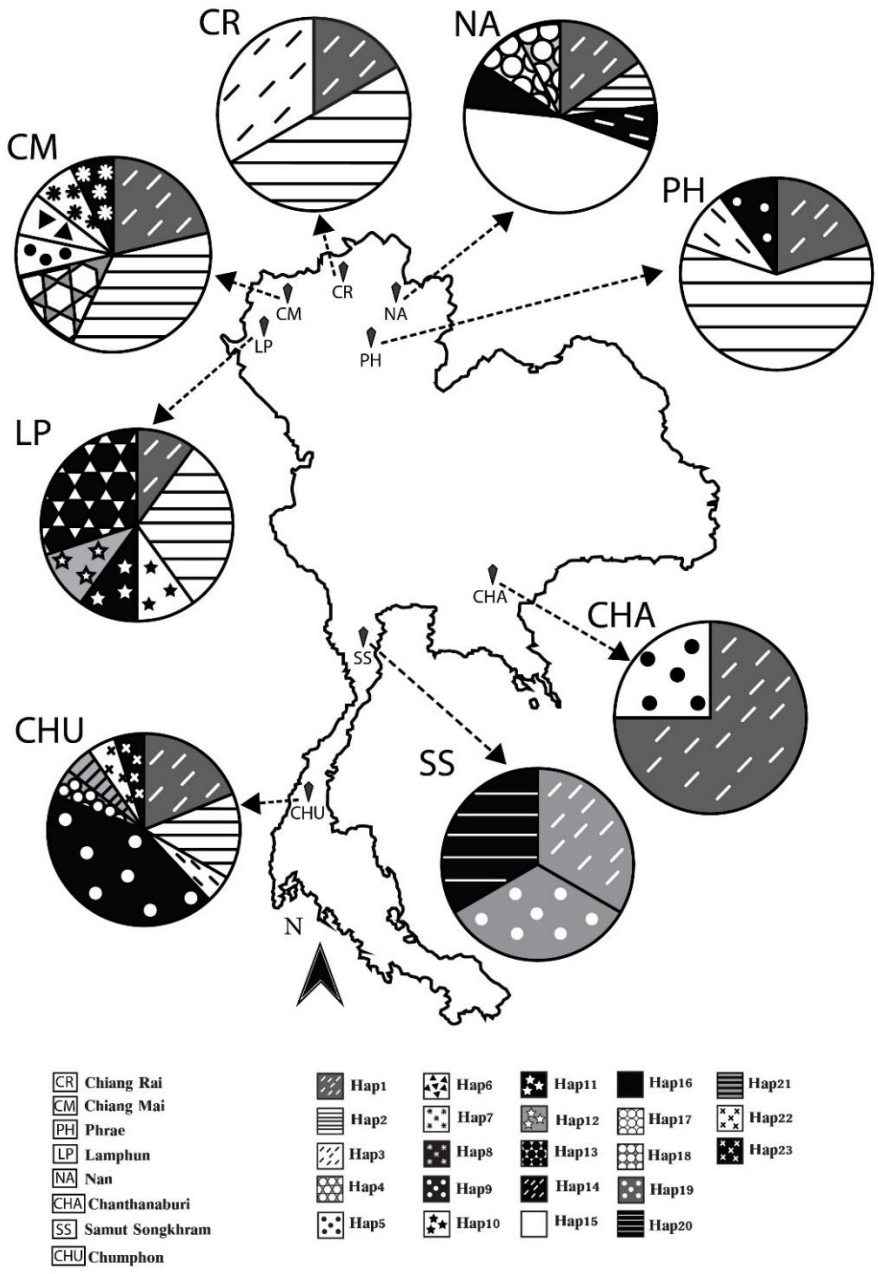

Figure 5 Haplotype distribution map and location of the sample apiaries of *Apis mellifera* from Thailand.

The pairwise genetic distance (F_{ST}) and per-generation migration rate (*Nm*) of commercial *A. mellifera* were estimated the population genetic structure under grouping by province population using ARLEQUIN ver 3.5.2.2 (Excoffier and Lischer 2010). Population pairwise genetic distance was estimated with 1000 bootstraps following Excoffier et al. (1992). Additionally, *F*-statistics were computed overall for all populations and pairwise between populations to assess the degree of genetic differentiation, testing statistical significance with 1000 permutations (α=0.05). The per-generation migration rate was estimated based on the following equilibrium relationship: $Nm = (1–F_{ST})/2\ F_{ST}$ (Szalanski et al. 2016).

3.1.5 Mitochondrial DNA Amplified and Sequenced

A fragment of the protein-coding mitochondrial COI gene was amplified by polymerase chain reaction (PCR) using primers COI-1908 (5'-TTAAGATCCCCAGGATCATG-3') and COI-2932 (5'-TGCAAATACTGCACCTATTG-3') (Hall and Smith 1991). PCR reactions were amplified in a final volume of 25 µL containing 1x PCR master mix (Fermentas Life Science), 20 µM of each primer and at least 20 ng of DNA template. The thermocycling profiles consisted of an initial denaturation step of 94 ºC for 5 min, followed by 35 cycles of 94 ºC for 1 min; annealing temperature at 55 ºC for 1 min; and extension at 72 ºC for 150 s, with a final extension at 72 ºC for 5 min. The PCR products were visualized by electrophoresis on a 1.0% (w/v) agarose gel, and if a single product was observed, purified using the QIAquick® Gel Extraction kit (Qiagen, Germantown, MD, US). The purified PCR products were direct sequenced by Macrogen© (South Korea). The raw DNA sequences were each checked manually and verified for frame-shifts to avoid pseudogenes (Zhang and Hewitt 1996). Partial DNA sequences were then aligned and edited using MEGA7 version 7.0.26 (Kumar et al. 2016) with default parameters. The sequences obtained were deposited in GenBank under accessions MK450031–MK450111.

3.1.6 Mitochondrial DNA Analysis

Genetic variation was investigated as the number of polymorphic (segregating) sites (*S*), average number of nucleotide differences (*k*), number of haplotypes (*No*), haplotype diversity (*hd*) (Nei 1987), nucleotide diversity (*Pi*) (Nei and Li 1979) by using DNAsp v5.0 program (Librado and Rozas 2009). To assess of possible population expansion as a deviation from the neutrality of commercial *A. mellifera* in Thailand, we calculated Tajima's *D* (Tajima 1989) and Fu's *Fs* (Fu 1997) using ARLEQUIN ver 3.5.2.2 (Excoffier and Lischer 2010).

For phylogenetic analysis, Maximum Likelihood (ML) and Bayesian inference (BI) methods were used to reconstruct the phylogenetic relationships among COI haplotypes and among apiaries. Prior to ML and BI analyses, the program Kakusan4 (Tanabe 2007) was used to determine the best-fit models of nucleotide substitution as judged by the Akaike information criterion (Akaike 1974) implemented for ML and Bayesian information criterion (Schwarz 1978) implemented for BI. The ML analysis was performed on Treefinder program (Jobb et al. 2004), using likelihood-ratchet method with 1000 bootstrap replicates to examine branch confidence values. At least 70% of bootstrap values (bs) were considered as being sufficiently resolved for tree topologies (Huelsenbeck and Hillis 1993). The BI procedure was implemented using MrBayes version 3.2.6 (Ronquist et al. 2012), which recruits a Metropolis-coupled, Markov chain Monte Carlo (MC–MCMC) sampling method. The four-chain MC–MCMC analyses were run twice in parallel (with default heating values) for 1000000 generations, starting with a random tree, and trees were sampled every 1000 generations. The initial 25% of the generations were discarded as "burn-in" samples. The remaining trees were used to investigate the consensus tree topology, branch lengths, and bi-partition posterior probability (Huelsenbeck and Ronquist 2001). A bi-partition posterior probability values with 0.95 or greater were regarded as significant support for the consensus tree topology (Larget and Simon 1999).

We executed a Median-joining network (Bandelt et al. 1999) with the program PopART version 1.7 (Leigh and Bryant 2015) to construct the haplotype network of COI haplotypes. The

median-joining method uses a maximum parsimony approach to explore for the shortest phylogenetic trees based on maximum parsimony approach (Bandelt et al. 1999).

3.2 Part II: The hybrid origin of *Apis mellifera* in Thailand using PCR-RFLP

3.2.1 Sampling

Apis mellifera samples were obtained between 2017 – 2018 from 100 colonies from 4 regions of Thailand (Fig 5). Five adult workers from each colony were randomly selected. All samples were preserved in 95% ethanol and stored at –20 ºC. Then, one individual bee per colony was randomly subjected for genomic DNA extraction.

3.2.2 DNA extraction and PCR amplification

Genomic DNA was extracted from bee thorax using a DNeasy® Blood & Tissue kit (Qiagen, Germantown, MD, US) according to the manufacturer's instructions. Four regions of mitochondrial DNA were amplified by polymerase chain reaction (PCR) amplification: intergenic $tRNA^{leu}$ to the 5' end of COII (including the non-coding between $tRNA^{leu}$ and COII), a fragment of Cytb, Ls rRNA, and COI. Primers used of each region and PCR condition profiles were shown in Table 1. PCRs were performed in a final volume of 40 µL containing 1x Multiplex PCR Master Mix (Biotechrabbit), 20 µmol/L of each primer, and at least 20 ng of genomic DNA template. Amplified PCR products were visualized by electrophoresis on a 1.2 % agarose gel, and those with a single band were purified purified using the QIAquick® Gel Extraction kit (Qiagen, Germantown, MD, US).

Table 1 Primers used and annealing temperature of PCR reactions in this study.

Regions	Primers	Annealing (ºC)	Reference
$tRNA^{leu}$–COII	*Forward* E2: 5'-GGCAGAATAAGTGCATTG-3' *Reverse* H2: 5'-CAATATCATTGATGACC-3'	55	Garnery et al. (1993)
Cytb	*Forward* OLD1: 5'-TATGTACTACCATGAGGACAAATATC-3' *Reverse* OLD2: 5'-ATTACACCTCCTAATTTATTAGGAAT-3'	50	Crozier et al. (1991)
Ls rRNA	*Forward* Ls1: 5'-TTTTGTACCTTTTGTATCAGGGTTG-3' *Reverse* Ls2: 5'-TTTTGTACCTTTTGTATCAGGGTTG-3'	55	Hall and Smith (1991)
COI	*Forward* COI-1908: 5'-TTAAGATCCCCAGGATCATG-3' *Reverse* COI-2932: 5'-TGCAAATACTGCACCTATTG-3'	50	Hall and Smith (1991)

Table 2 Summary of restriction enzyme digested patterns of four mitochondrial lineages of *Apis mellifera*. M = West Europe lineage, A = African lineage, C = East Mediterranean lineage, and O = Middle Eastern lineage.

Regions	**Restriction enzymes**	**Lineages of *Apis mellifera***			
		M	**C**	**A**	**O**
$tRNA^{leu}$–COII	*Hinf* I	+	+	+	+
	Dra I	+	+	+	+
Cytb	*Hinf* I	+	–	+	–
	Bgl II	+	+	–	+
Ls rRNA	*EcoR* I	–	+	–	–
COI	*HinC* II	+	–	–	–

+ indicates restriction site present, – indicates restriction pattern absent

3.2.3 Digestion with restriction enzymes

Each PCR product was digested with the appropriate restriction enzymes (Table 2) in final volume of 20 µL, containing 1X reaction buffer, 17 µL of PCR product and 1 unit of restriction enzyme, with overnight incubation at 37 ºC. The fragment size of restriction enzyme digestion product were visualized by electrophoresis on a 2.0% agarose gel. The size markers used in this study are 100 bp/2.5 kb and 50 bp/1.0 kb.

Chapter 4

Results

4.1 Part I: Genetic characterization of commercial honey bee, *Apis mellifera*, populations in Thailand by using microsatellite and mitochondrial DNA markers

4.1.1 Microsatellite diversity

The number of alleles (N_o), number of effective alleles (N_e), allele frequencies, and observed (H_o) and expected (H_e) heterozygosity of all samples and overall loci are indicated in Table 3.

Bees sampled from different apiaries did not show any evidence sub-structuring as shown by the Bayesian–Markov assignment algorithm implemented in STRUCTURE. The result K=1 was the most strongly supported number of Thai's commercial *A. mellifera* populations [Ln (P)=−2314.54 and Var Ln (P)=11.68] when assuming admixture. We detected significant deviation (P<0.05) from Hardy–Weinberg equilibrium in 10 of 40 population–locus combinations. Only the honey bee samples collected from Phrae and Nan exhibited an excess of heterozygotes for A107 and A07, respectively. Linkage disequilibrium tests showed two significant disequilibria out of 80 locus pair–population combinations. No significant linkage disequilibrium was detected between any microsatellite loci (0.2999< P >0.9897) for the total population.

A measurement of heterozygote deficiency, the F_{is} and F_{it} values were calculated and revealed in Table 4. The local inbreeding coefficient F_{is} was positive values in all populations, except the honey bee from Samut SongKham (F_{is} = –0.045), and significantly greater than zero only in Chanthaburi sample (Table 4).

To assess the genetically differentiated population of commercial *A. mellifera* of Thailand, microsatellite pairwise F_{ST} values were computed among the eight province populations (Table 5). The multilocus F_{ST} values between population pairs ranged from 0.000 (Chiang Rai – Chiang Mai,

Chiang Rai – Samut Songkhram, and Chanthaburi – Chumphon) to 0.0456 (Phrae – Lamphun), and none of them were significantly different from zero (P>0.05). The highest values were between Lamphun and Phrae (0.0456) and Lamphun and Samuth Songkham (0.0341) (Table 5).

Low levels of genetic differentiation (Table 5) most likely arise because of high levels of pairwise per-generation female migration rate (*Nm*) between all populations (raged from 10.465 – Infinite) (Table 5). This suggests that more than 10 queens migrate between all population pairs each generation, a very high rate of gene flow.

Table 3 Number of allele detected (*No*), number of effective allele (*Ne*), observed (H_o) and expected heterozygosity (H_e) at five loci in commercial *Apis mellifera* populations of Thailand. The number of analyzed colony from each province is shown in the brackets.

Locus	*Chiang Rai* (*n = 15)*	*Chiang Mai* (*n = 34)*	*Phrae* (*n = 25)*	*Lamphun* (*n = 29)*	*Nan* (*n = 40)*	*Chanthaburi* (*n = 5)*	*Samut Songkhram* (*n = 6)*	*Chumphon* (*n = 19)*	*All Populations* (*n = 173)*
A07									
N_o	6	9	9	6	8	4	6	8	11
Ne	3.635	3.733	4.921	3.248	4.810	3.333	4.798	4.911	4.396
H_o	0.733	0.676	0.720	0.724	0.600	0.200	0.833	0.895	0.694
H_e	0.725	0.732	0.797	0.692	0.792	0.700	0.792	0.796	0.772
A28									
N_o	5	6	4	6	5	3	4	5	7
Ne	1.931	2.119	1.738	3.020	2.060	2.381	2.97	2.692	2.279
H_o	0.333	0.529	0.440	0.655	0.325	0.600	0.667	0.474	0.474
H_e	0.482	0.528	0.425	0.669	0.514	0.580	0.666	0.628	0.561
A88									
N_o	4	7	7	6	6	3	4	7	9
Ne	1.767	1.672	2.023	1.289	2.199	1.151	3.422	1.687	1.822
H_o	0.467	0.382	0.640	0.241	0.575	0.400	0.833	0.474	0.474
H_e	0.434	0.402	0.506	0.224	0.545	0.340	0.708	0.407	0.451

Table 3 Continue

Locus	*Chiang Rai* (*n = 15*)	*Chiang Mai* (*n = 34*)	*Phrae* (*n = 25*)	*Lamphun* (*n = 29*)	*Nan* (*n = 40*)	*Chanthaburi* (*n = 5*)	*Samut Songkhram* (*n = 6*)	*Chumphon* (*n = 19*)	*All Populations* (*n = 173*)
A107									
N_o	10	15	14	10	12	7	8	11	17
Ne	7.924	8.772	9.470	8.183	6.993	6.250	5.734	5.917	9.033
H_o	0.800	0.853	0.680	0.724	0.750	0.600	0.667	0.684	0.746
H_e	0.874	0.886	0.894	0.878	0.857	0.840	0.826	0.831	0.889
A113									
N_o	8	9	4	7	6	4	4	4	13
Ne	3.607	2.832	1.994	2.799	2.104	2.778	3.126	2.564	2.728
H_o	0.667	0.588	0.360	0.621	0.550	0.600	0.833	0.579	0.566
H_e	0.723	0.647	0.498	0.643	0.525	0.640	0.680	0.610	0.634

Table 4 Multilocus microsatellites variation in the Thai's commercial European honey bee populations. The mean of observed (N_o) and effective (N_e) number of alleles, observed (H_o) and expected heterozygosity (H_e) with standard error (SD), fixation index between individuals and total data set (F_{it}), and fixation index between individuals and the local population (F_{is}).

Province	***n***	N_o	N_e	***Ho***	***He***	F_{it}	F_{is}
Chiang Rai	15	6.60±2.41	3.77±2.48	0.600±0.194	0.648±0.184	0.074	0.074
Chiang Mai	34	9.20±3.49	3.82±2.87	0.606±0.175	0.639±0.186	0.065	0.052
Phrae	25	7.60±4.16	4.03±3.31	0.568±0.158	0.624±0.207	0.123	0.090
Lamphun	29	7.00±1.73	3.71±2.62	0.593±0.202	0.621±0.240	0.085	0.045
Nan	40	7.40±2.79	3.63±2.21	0.560±0.152	0.647±0.164	0.136	0.134
Chanthaburi	5	4.20±1.64	3.18±1.89	0.480±0.179	0.620±0.184	0.259*	0.226*
Samut Songkhram	6	5.20±1.79	4.01±1.20	0.767±0.091	0.734±0.071	–0.184	–0.045
Chumphon	19	7.00±2.73	3.55±1.78	0.621±0.176	0.654±0.170	–0.009	0.050
Mean	21.62±12.69	6.775±1.521	3.712±0.273	0.599±0.080	0.648±0.037	0.124±0.072	0.096±0.065

* $P < 0.05$

Table 5 Genetic distance (F_{ST}) and per-generation migration rate (*Nm*) between pairs of province's apiaries in Thailand based on five microsatellite loci of *Apis mellifera*. Values below the diagonal are the estimates of genetic distance (F_{ST}) and values above the diagonal are the estimates of per-generation migration rate (*Nm*). Inf is infinite.

	Chiang Rai	*Chiang Mai*	*Phrae*	*Lamphun*	*Nan*	*Chanthaburi*	*Samut Songkhram*	*Chumphon*
Chiang Rai	-	Inf	39.822	87.219	134.635	58.324	Inf	55.278
Chiang Mai	0.0000	-	20.333	454.045	73.029	28.071	23.423	155.75
Phrae	0.0124	0.0240	-	10.465	28.740	41.517	23.538	21.921
Lamphun	0.0057	0.0011	0.0456	-	28.071	23.309	14.163	45.371
Nan	0.0037	0.0068	0.0171	0.0175	-	17.748	66.167	53.263
Chanthaburi	0.0085	0.0175	0.0119	0.0210	0.0274	-	55.680	Inf
Samut Songkhram	0.0000	0.0209	0.0208	0.0341	0.0075	0.0089	-	28.071
Chumphon	0.0059	0.0032	0.0223	0.0109	0.0093	0.0000	0.0175	-

4.1.2 Mitochondrial DNA diversity

We obtained high quality DNA sequences comprising 807 base pairs (bp) from 81 bees. Multi-alignment and pair-wise sequence comparisons showed a total of 26 informative single base substitutions, comprising 19 transitions (73.08%) and 7 transversions (26.92%). A summary of molecular diversity indices is given in Table 6. Based on mitochondrial COI gene sequences, the average number of nucleotide differences (*k*), haplotype diversity (*hd*), and nucleotide diversity (*Pi*) of all colony samples were 5.665, 0.875 (±0.022) and 0.007 (±0.0007), respectively. There is high haplotype diversity (ranging from 0.50 to 1.00) but relative low nucleotide diversity (ranging from 0.00062 to 0.00909) (Table 6).

All partial COI sequences of 81 commercial colony samples revealed 23 different haplotypes. Phylogenetic relationships between the 23 haplotypes and the references sequences in GenBank are shown in Figure 6. Haplotypes belong to the C and O lineages. Fifteen haplotypes are of *A. mellifera carnica* (AY114462_1) or *A. m. ligustica* (KX908209_1) origin (the C evolutionary lineage) while the remainder are *A. m. caucasica* (AY114472_1) (the O evolutionary lineage). The generated haplotype network is consistent with the phylogenetic analysis (Fig. 7). The network exhibits a star–like pattern with two common haplotypes (Hap1 and Hap2). Interestingly, Chumphon and Chiang Mai showed the highest haplotype numbers (eight and seven haplotypes, respectively; Fig. 5), suggesting that these two provinces were primary origin commercial *A. mellifera* strains of Thailand.

A summary of neutrality test indices, Tajima's *D* and Fu's *Fs*, were computed and demonstrated in Table 6. The commercial *A. mellifera* populations from Thailand revealed both negative (Fu's *Fs* = –3.749) and positive (Tajima' *D* = 0.2515) values, indicating that balancing selection is occurring. Considering into province population, the allele frequency were not significantly greater than zero (P>0.05; Table 6), demonstrating that no major change in studied population size. These results strongly suggested in a recent managed population in Thai stock are genetic demographically stable.

Table 6 Molecular diversity indices and population expansion test statistics of mitochondrial cytochrome c oxidase I (COI) genes sequence data of commercial *Apis mellifera* population of Thailand. Number of individuals (*N*), number of haplotypes (*No*), number of polymorphic (segregation) sites (*S*), average number of nucleotide differences (*k*), haplotype diversity (*hd*) and nucleotide diversity (P_i) with standard deviation (SD). Tajima's *D*, and Fu's *Fs*.

Province	***N***	***No***	***S***	***k***	***hd (±SD)***	***P_i (±SD)***	***Tajima'D***	***Fu's Fs***
Chiang Rai	6	3	12	6.467	0.733(0.155)	0.0080(0.0024)	1.4020	3.610
Chiang Mai	14	7	12	2.890	0.846(0.074)	0.0036(0.0008)	–0.9323	–0.992
Phrae	10	4	12	4.378	0.644(0.152)	0.0054(0.0022)	0.1448	2.582
Lamphun	9	6	8	2.444	0.889(0.091)	0.0030(0.0007)	–0.7695	–1.659
Nan	13	7	14	3.923	0.795(0.109)	0.0049(0.0015)	–0.5412	–0.381
Chanthaburi	4	2	1	0.500	0.500(0.265)	0.0006(0.0003)	–0.6124	0.172
Samut Songkhram	3	3	11	7.333	1.000(0.272)	0.0091(0.0036)	/	/
Chumphon	22	9	19	7.052	0.805(0.070)	0.0087(0.0008)	1.3056	1.452
All samples	***81***	***23***	***26***	***5.665***	***0.875(0.022)***	***0.0070(0.0007)***	***0.2515***	***–3.749***

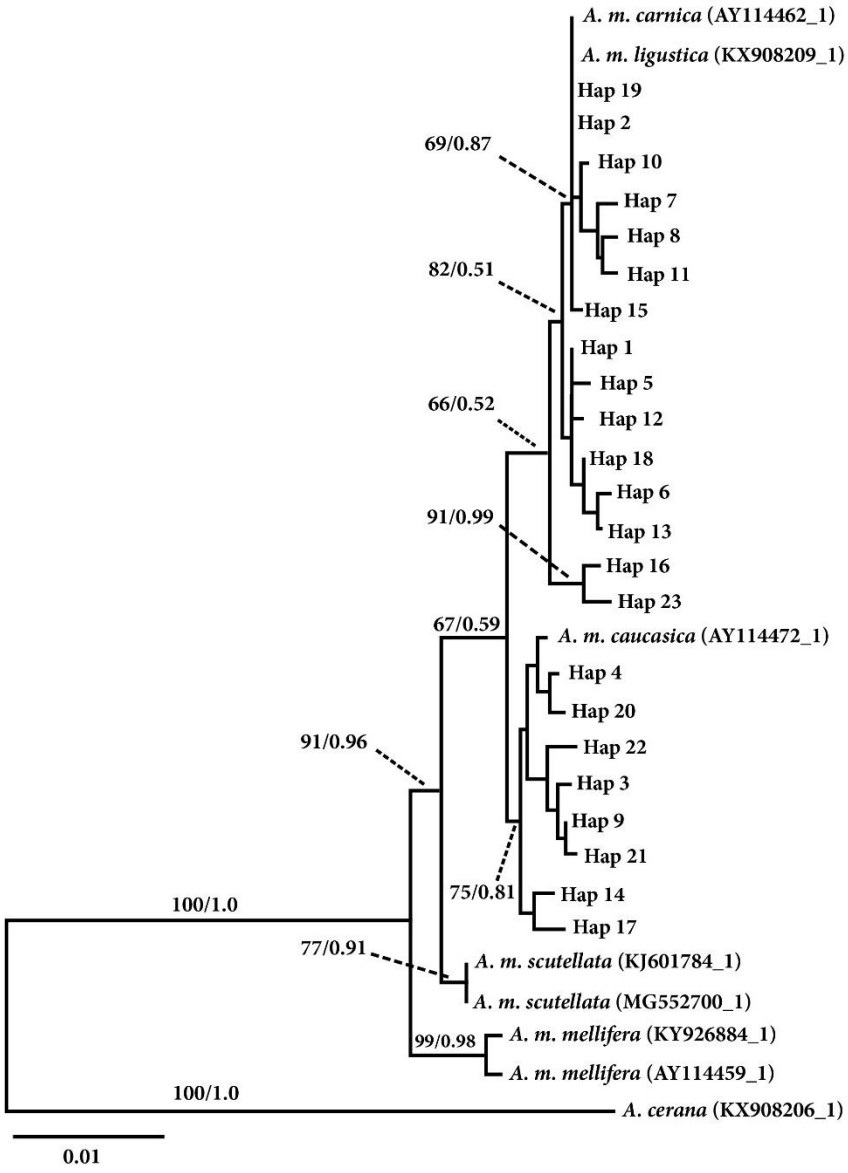

Figure 6 Phylogenetic relationships among cytochrome c oxidase I (COI) haplotypes of commercial *Apis mellifera* in Thailand based on maximum likelihood (ML). Two Asian honey bee species, *Apis cerana* (KX908206_1) and Apis nuluensis (NC036235_1) were included as outgroup. Node supports inferred from Bayesian posterior probability and bootstrap value for ML.

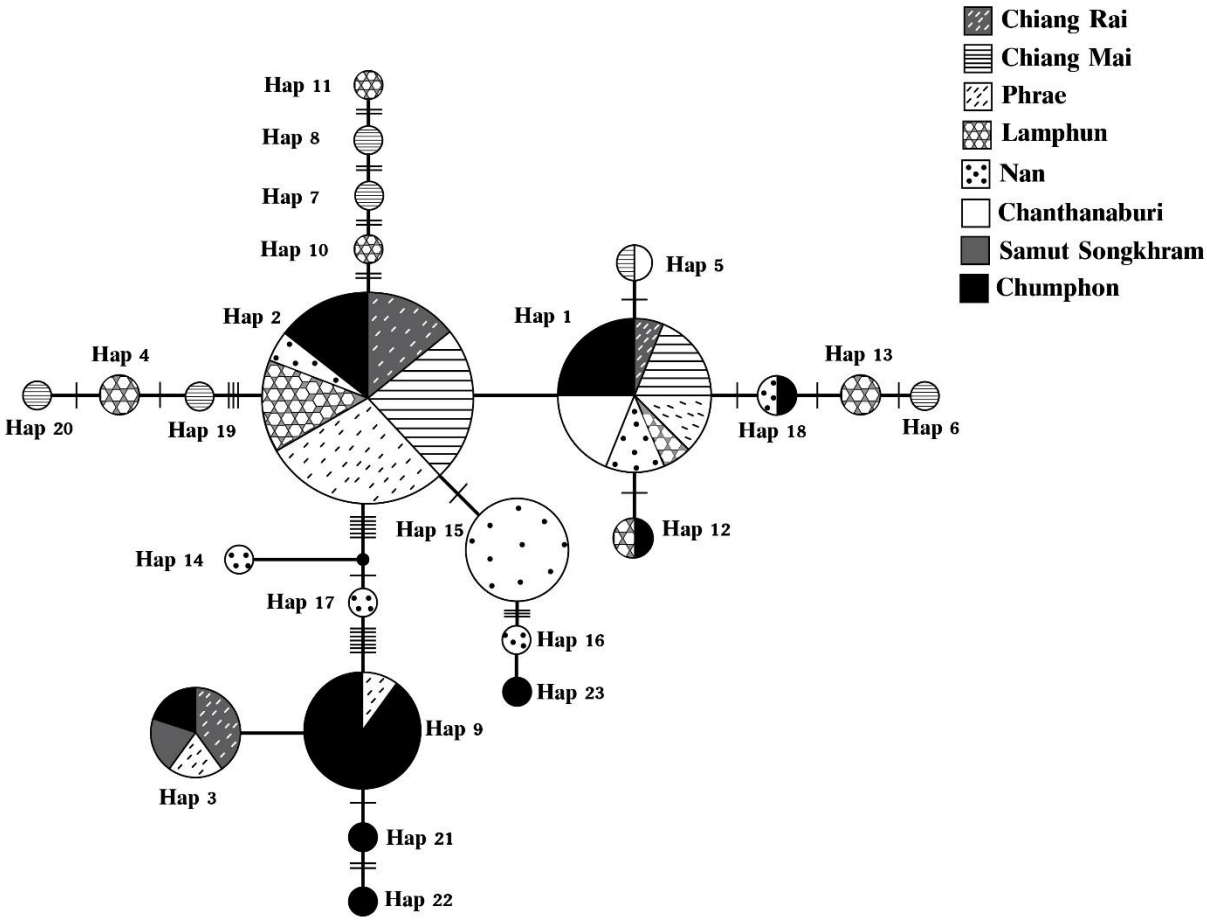

Figure 7 Median-joining haplotype networks of *Apis mellifera* for mitochondrial COI gene. Each circle represents one unique haplotype and the size of the haplotype circle corresponds to the abundance of individual samples. Each pattern indicate a sampling location. Dark spots and crossbars demonstrate missing haplotype and one mutational step, respectively.

4.2 Part II: The hybrid origin of *Apis mellifera* in Thailand using PCR-RFLP

4.2.1 PCR amplification of mitochondrial DNA of Apis mellifera

All four gene fragments were successfully amplified for all honeybee samples, including COI, intergenic $tRNA^{leu}$–COII region, Ls rRNA, and Cytb of mitochondrial DNA. The PCR product size of COI, intergenic $tRNA^{leu}$–COII region, Ls rRNA, and Cytb are ca. 1000, 850, 800, and 500 bp respectively (Fig. 8). These product sizes are in close agreement with those from previous reported (Crozier et al., 1991; Hall and Smith, 1991; Gernery et al., 1993; Suppasat et al., 2007; Wu et al., 2017).

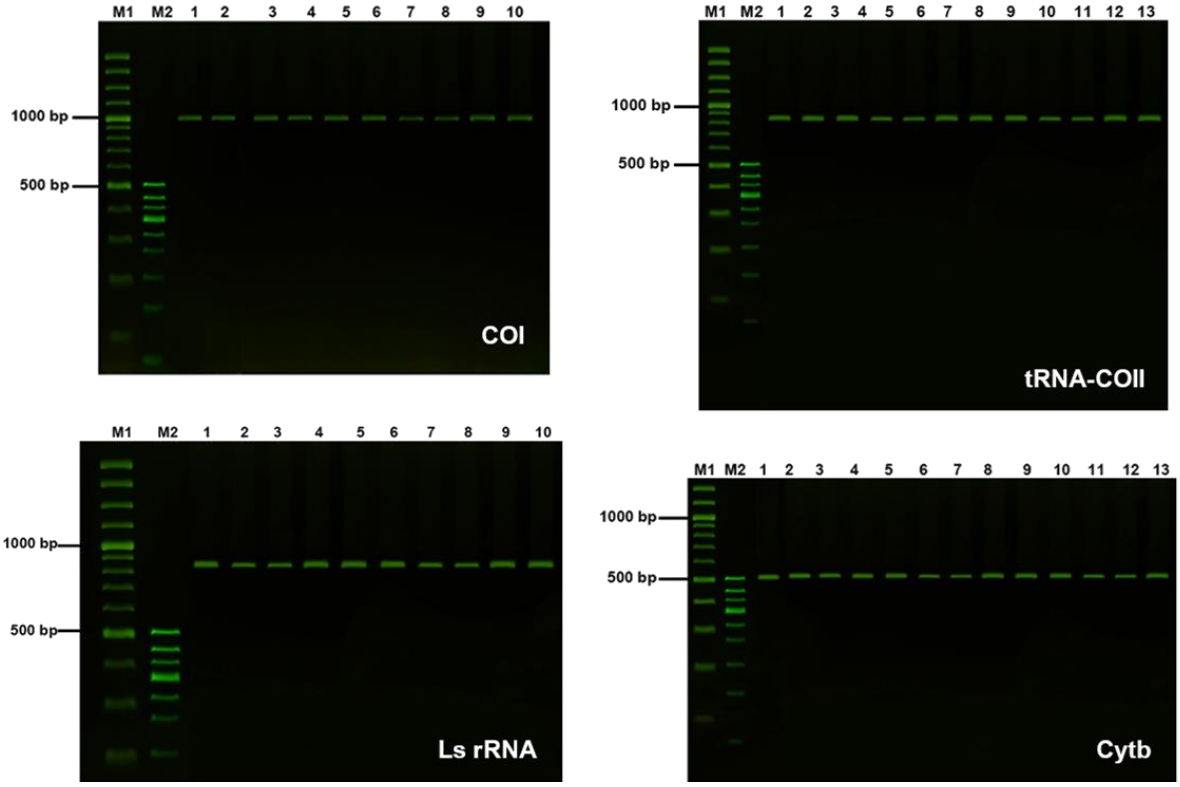

Figure 8 PCR amplification of four mitochondrial gene fragment of *Apis mellifera*. Lane M1: 100 bp DNA maker; Lane M2: 50 bp DNA marker; Lane 1 – 13: PCR product of *Apis mellifera* samples.

4.2.2 PCR–RFLP of Apis mellifera

The patterns of restriction enzyme digests of four regions of mitochondrial DNA used in the characterization of evolution lineages of *A. mellifera* were summarized in Table 2 and Figure 9. All Thai commercial *A. mellifera* samples absence a *Hinc* II site in the 5' end of COI fragment (Fig. 9A), indicating that none of them belong to the West European or M evolution lineage. Additionally, the presence of a *Bgl* II restriction site in Cytb fragment in all Thai's *A. mellifera* samples excludes the African or A evolution lineage (Fig. 9B).

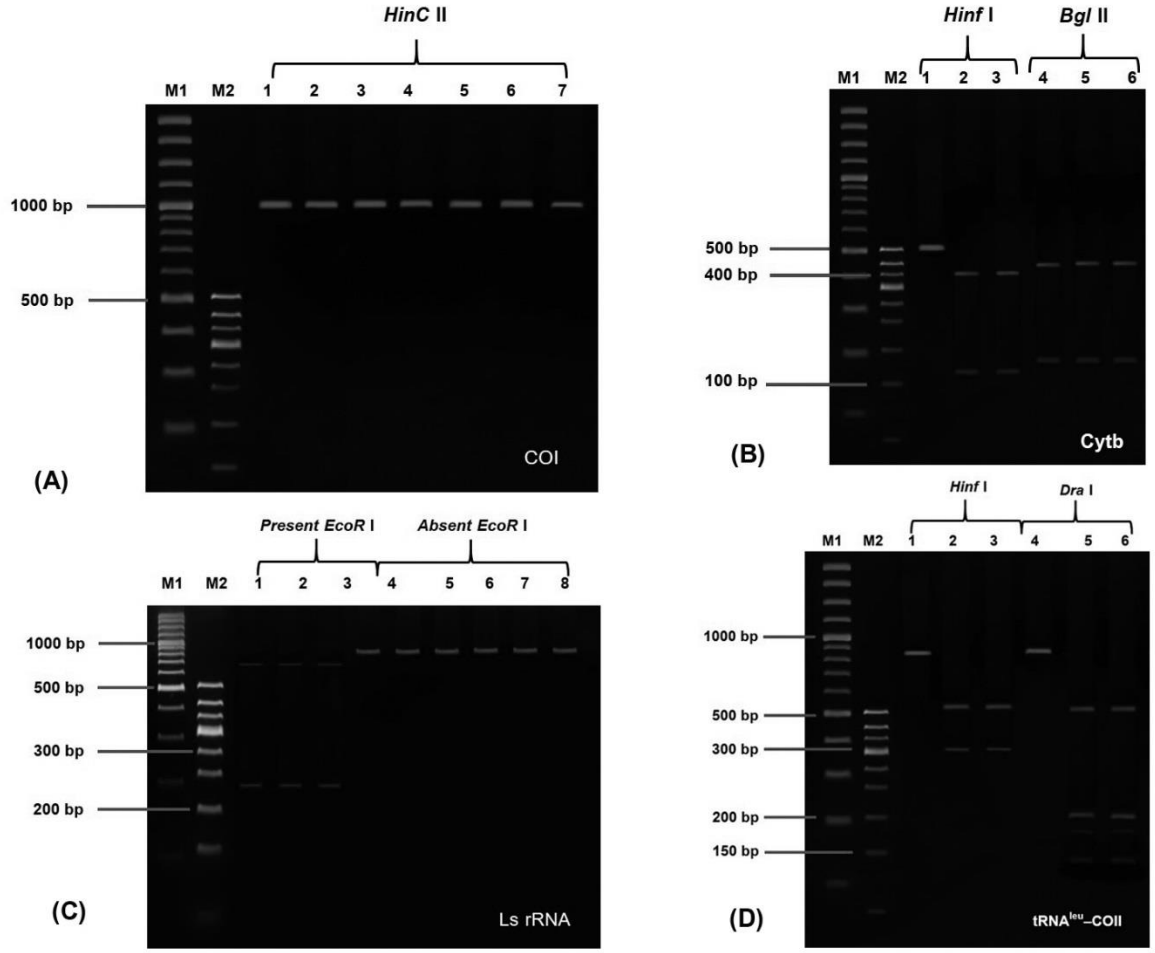

Figure 9 Restriction enzyme digested patterns of four mitochondrial gene fragments of Thai commercial *Apis mellifera* stocks. (A) COI digested by *HinC* II; (B) Cytb digested with *Hinf* I and *Bgl* II; (C) Ls rRNA digested with *EcoR* I; and (D) intergenic $tRNA^{leu}$–COII region digested with *Hinf* I and *Dra* I.

Amplification of the intergenic tRNA^{leu}–COII region of Thai *A. mellifera* demonstrated only one fragment size (about 850 bp). The digestion of these fragments with *Dra* I produced single pattern but absent of restriction site of *Hinf* I identical to that found in *A. melliefra ligustica* and *A. m. carnica* (C evolution lineage) (Suppasat et al., 2007). Whereas, the single pattern *Hinf* I digestion but absent restriction site of *Dra* I similar to that from O evolution lineage or Middle Eastern (Suppasat et al., 2007) (Fig. 10).

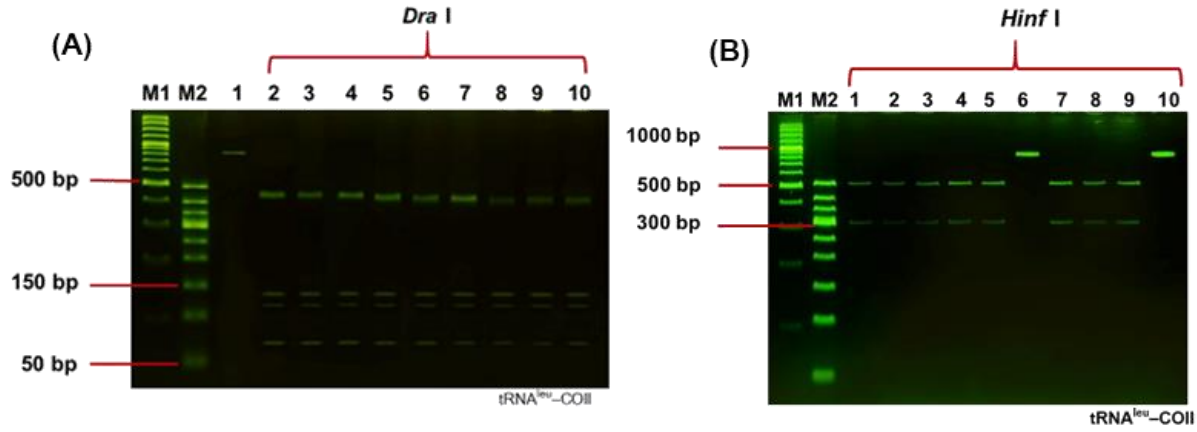

Figure 10 Restriction enzyme digested patterns of the intergenic tRNA^{leu}–COII region of Thai *Apis mellifera* (A) tRNA^{leu}–COII fragments digested with *Dra* I; (B) tRNA^{leu}–COII fragments digested with *Hinf* I.

The results of the gene fragment/digestion combinations (*Hinf* I and *Dra* I digest of tRNA^{leu}–COII, *Hinf* I digests of COI, *Dra* I digests of Ls rRNA, and *Hinf* I digests of Cytb) are shown in Table 7 and Figure 11. The digested patterns demonstrated by five restriction enzyme digests were combined into composite haplotype. Nineteen composite haplotypes were found among 100 colonies of Thai *A. mellifera* stock. The three highest frequency of composite haplotype of Thai *A. mellifera* were haplotype 10 (babab), haplotype 5 (aabab) and haplotype 6 (aabaa), which were 0.28, 0.17, and 0.13 respectively (Table 7).

Table 7 Geographic distribution of 19 haplotypes from 100 Thai *Apis mellifera* colonies. The five letters of the composite haplotype indicate restriction fragment patterns for five gene/enzyme combinations ($tRNA^{leu}$ – COII/*Hin*f I, $tRNA^{leu}$ – COII/*Dra* I, COI/Hinf I, Ls rRNA/*Dra* I, and Cytb/*Hinf* I, respectively).

Province	**Haplotype**																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
	aabbb	abbab	aaaab	abaab	aabab	aabaa	bbaba	ababa	baaaa	babab	babaa	babbb	baaab	ababb	baabb	baaba	abbaa	babba	bbbab
Chiang Rai	1	1	1	1	1	1													
Chiang Mai		1			3					5	2					1			2
Phrae			1		2	5	1	1											
Lamphun		1			2					6			2						1
Nan		1			6	1				5					1				
Chanthaburi						1				2	1		1						
Samut Songkhram						3			2				1						
Chumphon		1	2	2	3	2			3	8		4	4	2			1	1	
All samples	***1***	***5***	***4***	***3***	***17***	***13***	***1***	***1***	***5***	***26***	***3***	***4***	***8***	***2***	***1***	***1***	***1***	***1***	***3***
(frequency)	***(0.01)***	***(0.05)***	***(0.04)***	***(0.03)***	***(0.17)***	***(0.13)***	***(0.01)***	***(0.01)***	***(0.05)***	***(0.26)***	***(0.03)***	***(0.04)***	***(0.08)***	***(0.02)***	***(0.01)***	***(0.01)***	***(0.01)***	***(0.01)***	***(0.03)***

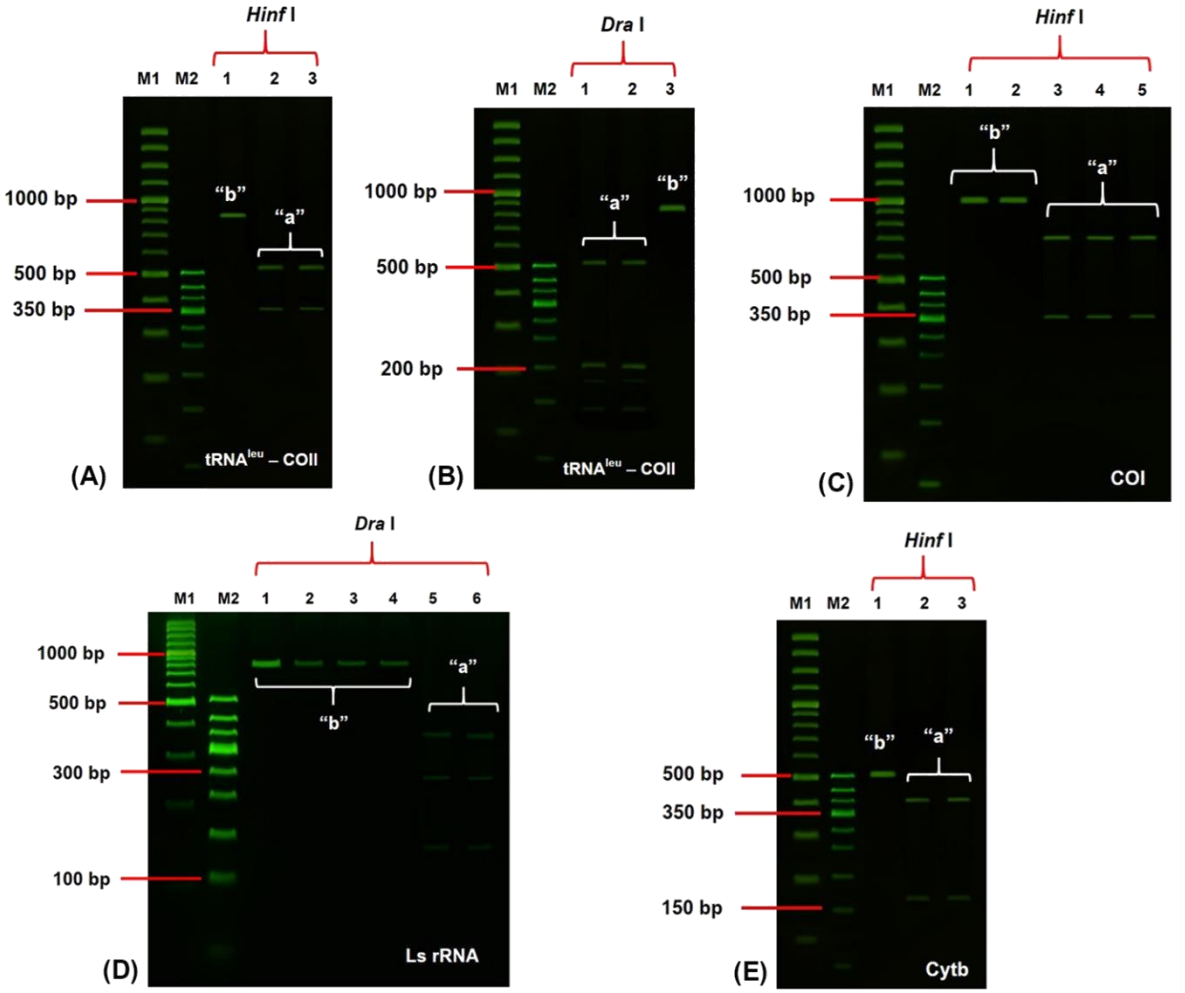

Figure 11 Restriction patterns of five gene/enzyme combinations of Thai *Apis mellifera*. (A) tRNA^{leu} – COII digested by *Hinf* I; (B) tRNA^{leu} – COII digested by *Dra* I; (C) COI digested by *Hinf* I; (D) Ls rRNA digested by *Dra* I; (E) Cytb digested by *Hinf* I. The symbol "a" and "b" represent restriction pattern found in the analyzed samples.

Chapter 5

Discussion

The reduction of genetic diversity is a familiar character of domesticated plant and animal populations (Bruford et al. 2003). Low level of genetic variation has been suggested as a source of colony declines in honey bees (Oldroyd 2007). In this study, we observe moderate low genetic diversity of the managed *A. mellifera* populations. Our microsatellite data show a sign of inbred. Most of observed heterozygosity values were lower than expected heterozygosity under Hardy–Weinberg equilibrium. However, we detect no significant evidence of heterozygote deficiency among apiaries within province and total commercial population (F_{is} = –0.045 – 0.226; P>0.05 for all test, except for Chanthaburi), with an average inbreeding coefficient of F_{is} = 0.096 within populations (Table 4). Our results were not consistent to the study of Harpur et al. (2012), which shows the managed *A. mellifera* populations have higher genetic diversity than that their progenitors in East and West Europe. They suggested that beekeeper management practices are the major divers of increased genetic diversity via genetic admixture in managed populations (Harpur et al. 2012). High genetic diversity was also detected in Australian commercial *A. mellifera* that reported by Chapman et al. (2008). They revealed no evidence of inbreeding and the commercial breeding stocks maintain considerable genetic diversity by the Western Australia Bee Breeding Program for 25 years. Delaney et al. (2009) also demonstrated that genetic diversity of both western and southern commercial breeding population in the US did not change for 10 years. The microsatellite analysis showed there loss of some alleles in both population, but not significant due to spontaneous receive new alleles into these populations. Likewise, low genetic diversity of Thai managed *A. mellifera* populations detected here is probably because we found no evidence of recent changes in effective population size. The all frequency spectra were not significantly greater than zero in all provinces (Tajima's D = –0.93 – 0.14, P > 0.05; Fu's Fs = –1.66 – 3.61, P > 0.05) or when considering the honey bee samples as a single population (Tajima's D = –0.25, P > 0.05; Fu's Fs = –3.75, P > 0.05), suggesting no recent major change in effective population size (Fay and Wu 1999, Garrigan and Hammer 2006).

Using mitochondrial COI sequence data and PCR-RFLP of four mitochondrial gene fragments, the managed *A. mellifera* population in Thailand derived from the Eastern Mediterranean (C) and the Middle Eastern Europe (O) evolutionary lineages (Fig. 6 and 11). We did not detected either the African (A) and/or the Western Europe (M) evolutionary lineages. These observation were close agreement with Suppasat et al. (2007). The authors reported that researchers and beekeepers have imported *A. mellifera* queens from Australia, Russia, the USA and Europe, which may have included hybrids with Middle Eastern Europe ancestry. Thus, our results confirmed that there is no inflow of new alleles from Africanize honey bees into Thai's commercial breeding lines more than 10 years.

The STRUCTURE program revealed a single population of managed *A. mellifera* in Thailand, indicating panmictic of managed *A. mellifera* in Thailand. The result was supported by low level of F_{ST} values but high value of *Nm* between pair of provinces (Table 5). Additionally, haplotype network analysis shows a high interconnected and clearly exhibited a star–like pattern with two common haplotypes (Hap1 and Hap2), suggesting that no haplotype or haplotype group had diverged (Fig 7). These showed opposite pattern when compared to other native bee species in Thailand. For instance, *A. cerana* shows high level of genetic differentiation between north and south of a biogeographic transition in the Kra of Isthmus (Smith and Hagen 1996, Warrit et al. 2006). The same population pattern was detected in Thai's stingless bee (*Tetragonilla collina*). Mitochondrial COI sequences combined with geometric morphometric analysis revealed two separate population of *T. collina* in Thailand, roughly divided into the population northeast of the Thai-Malay Peninsula and the western and Thai-Malay Peninsula population (Rattanawannee et al. 2017). A panmictic of commercial *A. mellifera* stocks in Thailand observed in our results suggest that no geographic structure to the distribution of the species due to the colonies transportation throughout the country by beekeepers. Also, the queens may interchange among beekeepers and/or between beekeeper and Apicultural Extension Centers.

For most agricultural plants and animals, genetic variation is essential not only in a commercial breeding environment but also for survival in a changing natural environment

(Delaney et al. 2009). Increased overall genetic health and longevity of an organism are corresponded increased levels of genetic diversity. For instance, honey bee workers live at high densities and face to numerous pathogens and diseases. To be effective, innate immune system and behavioral defenses require a high level of genetic diversity within colonies (Oldroyd 2007, Oldroyd and Fewell 2007). Thus, the honey bee queens mate on flight with up to 30 drones (Palmer and Oldroyd 2000), leading to increase colonial genetic variation. Interestingly, only a few subspecies of *A. mellifera* were imported for commercial stock. Additionally, no feral *A. mellifera* population was found in Thailand caused by *Tropilaelaps* and *Varroa* mites. Therefore, commercial *A. mellifera* are more likely to mate with close relatives, potentially leading to reduce genetic diversity. This study suggests that the queen daughter production from current queen mother stocks might effect on genetic diversity of Thai's commercial *A. mellifera*. Moreover, without the added of new alleles from other import breeding lines there was a decline in the genetic variation of country stocks. Therefore, the maintenance of adequate genetic diversity in Thai's commercial honey bee population will probably depend on the inflow of new alleles in the future.

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Appendices

Appendix 1: Manuscript entitle "*Genetic Characterization of Exotic Commercial Honey Bee (Hymenoptera: Apidae) Populations in Thailand Reveals High Genetic Diversity and Low Population Substructure*" was submitted to **Journal of Economic Entomology** (Q1: Impact factor = 1.936).

Appendix 2: Original article – **Rattanawannee, A.**, Jeratthitikul, E., Duangpakdee, O. and Oldroyd, B. P. 2017. Mitochondrial sequencing and geometric morphometrics suggest two clades in the *Tetragonilla collina* (Apidae: Meliponini) population of Thailand. **Apidologie**, 48(6): 719–731 (Q1: Impact factor = 2.856) acknowledged the Thailand Research Fund (**grant no. MRG6080272**).

Appendix 1

Appendix 1: Manuscript entitle "*Genetic Characterization of Exotic Commercial Honey Bee (Hymenoptera: Apidae) Populations in Thailand Reveals High Genetic Diversity and Low Population Substructure*" was submitted to **Journal of Economic Entomology** (Q1: Impact factor = 1.936).

Genetic Characterization of Exotic Commercial Honey Bee (Hymenoptera: Apidae) Populations in Thailand Reveals High Genetic Diversity and Low Population Substructure

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Abstract

Domestication is of animal species is often associated with a reduction in genetic diversity. The honey bee, *Apis mellifera*, has been managed by beekeepers for millennia for both honey and wax production and for crop pollination. Here we use both microsatellite markers and sequence data from the mitochondrial COI gene to evaluate genetic variation of managed *A. mellifera* in Thailand, where the species is introduced. Microsatellite analysis revealed high average genetic diversity with expected heterozygosities, ranging from 0.620±0.184 to 0.734±0.071 per locus per province. Observed heterozygosities were generally lower than those expected under Hardy–Weinberg equilibrium, both locally and across the population as a whole. Mitochondrial sequencing revealed that the frequency of two evolutionary linages (C - Eastern European and O – Middle Eastern) are similar to those observed in a previous survey 10 years ago. Our results suggest that Thai beekeepers are managing their *A. mellifera* in ways that retain overall genetic diversity, but reduce genetic diversity between apiaries.

Keywords: Honey bee, genetic variation, microsatellites, mitochondrial COI

Introduction

Animal domestication often results in dramatic morphological and behavioral modifications over short time scales (Mignon-Grasteau et al. 2005, Trut et al. 2009), and a reduction in genetic diversity relative to their wild progenitors (Bruford et al. 2003). Loss of genetic diversity probably arises as a result of a small initial population sizes and intense artificial selection for economic traits (Wang et al. 2014). The former effect, also known as the 'domestication bottleneck', is often evidenced by genome-wide loss of genetic diversity during the founding of a new domestic lineage. Both the magnitude and duration of the bottleneck affects levels of genetic variation in domesticated plants and animals (Wright et al. 2005). For example, chickens have 50% less genetic variation than their wild progenitors (Muira et al. 2008). Surprisingly, however, most other domesticated animal species retain significant genetic variation (Bruford et al. 2003), though variation within breeds and subpopulations can be limited.

The Western honeybee, *Apis mellifera* L., is managed by beekeepers for honey and wax production and crop pollination. This species is native to Africa, Europe and the Middle East (Seeley 1985) but has been spread around the world by humans (Ruttner 1988). Based on morphology, ecological and behavioral traits, *A. mellifera* has been grouped into 4 main evolutionary lineages (Ruttner 1988): an African lineage (A), an Eastern Europe lineage (C), a Western Europe lineage (M), and a Middle Eastern lineage (O). Within these broad groupings there are at least 26 named subspecies (Whitfield et al. 2006, Wallberg et al. 2014). Ruttner's classification has generally been supported by molecular genetic analysis of mitochondrial (Estoup et al. 1995, Franck et al. 2000) and nuclear (Whitfield et al. 2006, Wallberg et al. 2014) DNAs.

Selective breeding is believed to have caused reductions in genetic variation in many populations of commercial *A. mellifera* (Delaney et al. 2009, Meixner et al. 2010; but see Harpur et al. 2012). Indeed, several authors have speculated that unusually high rates of mortality among commercial colonies over the last decade are due in part to reduction in genetic diversity within commercial populations (Oldroyd 2007, van Engelsdorp and Meixner 2010, Sheppard 2012). Honey bee queens are highly polyandrous leading to high levels of intra-colonial genetic diversity (Palmer and Oldroyd 2000, Tarpy and Seeley 2006, Tarpy et al. 2013). Genetic diversity is important to colony fitness and productivity because it increases nest homeostasis (Oldroyd and Fewell 2007), disease resistance (Tarpy 2003) and honey production

(Oldroyd et al. 1992, Mattila and Seeley 2007). Therefore, maintenance of genetic diversity within commercial populations is essential for ongoing productivity.

Outside Asia, *A. mellifera* is generally regarded as a 'partially' domesticated animal. While beekeepers provide hives for bees to live in, propagate queens and manage diseases among other management practices, there is little control of mating, and regular exchange of genetic material between the domestic and wild (or feral) populations (Chapman et al. 2008). However in Asian countries like Thailand, feral *A. mellifera* cannot establish due to competition with native *Apis* species and parasitism from mites such as *Varroa* spp and *Tropilaelaps* spp that are extant on the native *Apis* (Oldroyd and Nanork 2009). Thus Asian *A. mellifera* qualify as a truly domesticated species, being wholly dependent on human management for survival.

A. mellifera was first imported to Thailand on a large scale from Taiwan in the 1970s (Wongsiri et al. 1995). Apiaries were established in the northern provinces of Chiang Mai and Lamphun (Wongsiri et al 1995). In later years *A. mellifera* is thought to have been imported from Russia, Europe and Australia (Kavinseksan et al. 2004) though the frequency and success of these latter imports is unknown. *A. mellifera* beekeeping is now practiced throughout Thailand (Thapa and Wongsiri 1997) with more than 300,000 managed colonies (Chantawannakul 2018). However no feral population has established. The ancestry and genetic diversity of the commercial population is unclear. There are beekeeper concerns that increasing homogenization both within and between Thai honeybee populations may be impacting production and disease resistance, leading to calls for further imports of genetic material. In this study we assess genetic variation and population structure of commercial *A. mellifera* stocks of Thailand using microsatellites and mitochondrial cytochrome oxidase subunit I (mtCOI) sequences. Our goal is to assess whether there is sufficient genetic variation to prevent the effects of inbreeding or whether importation of additional genetic material is warranted.

Materials and Methods

Sampling and DNA Extraction

We sampled 173 colonies belonging to 27 beekeepers from 35 commercial apiaries in eight provinces (Fig. 1) during 2017 and 2018. Workers (n = 5 per colony) were collected and preserved under ethanol. Genomic DNA was extracted from a single worker per colony. For microsatellite markers, genomic DNA was extracted from the right hind leg using a 5% Chelex

solution (Walsh et al. 1991). For mitochondria, used the DNeasy® Blood & Tissue kit (Qiagen, Germantown, MD, US) to extract DNA from the thorax.

Ethics statement

This study of an exotic invertebrate was welcomed by beekeepers. No specific permits were required. All experimental methods conformed to the guidelines established by the Animal Experiment Committee, Kasetsart University, Thailand (Approval no. ACKU 01160).

Microsatellite Amplification and Genotyping

We genotyped one worker per colony using microsatellite loci A7, A28, A88, A107, and A113 (Estoup et al. 1995). PCR products were sent to Macrogen (South Korea) for fragment analysis. Resultant data files were checked manually to determine allele sizes using GeneMapper software (Applied Biosystems).

Microsatellite Analysis

We used option 5 of GENEPOP package (Rousset 2008) to compute the number of alleles (*N*), the number of effective alleles (*Ne*), and observed (H_o) and expected (H_e) heterozygosities of each population and each locus. To assess departures from Hardy–Weinberg equilibrium, we conducted genotypic linkage disequilibrium tests and exact tests for Hardy–Weinberg equilibrium among populations using GENEPOP (Rousset 2008). A Bayesian model-based clustering method implemented in STRUCTURE 2.3.3 (Pritchard et al. 2000) was used to assess the sub-structure of population. Analyses were based on an admixture model with correlated allele frequencies with a 100,000 step burn-in and 1,000,000 iterations of the MCMC algorithm for each run. Ten runs were computed for each *K* (number of populations) to estimate the most likely *K* value. We used Evanno's estimator ΔK (Evanno et al. 2005) implemented in Structure Harvester (Earl and von Holdt 2012) to estimate the most likely number of population clusters.

We calculated the pairwise genetic distance (F_{ST}) and gene flow (*Nm*) between provinces using ARLEQUIN ver 3.5.2.2 (Excoffier and Lischer 2010). Pairwise genetic distances were calculated between provinces with 1000 bootstraps (Excoffier et al. 1992). Additionally, *F*-statistics were computed overall for all populations and pairwise between populations to assess the degree of genetic differentiation, testing statistical significance with 1000 permutations

(α=0.05). The per-generation migration rate was estimated based on the following equilibrium relationship: $Nm = (1–F_{ST})/2\ F_{ST}$ (Szalanski et al. 2016).

Mitochondrial DNA Amplified and Sequenced

A fragment of the protein-coding mitochondrial COI gene was amplified by polymerase chain reaction (PCR) using primers COI-1908 (5'-TTAAGATCCCCAGGATCATG-3') and COI-2932 (5'-TGCAAATACTGCACCTATTG-3') (Hall and Smith 1991). PCR products were visualized by electrophoresis on a 1.0% (w/v) agarose gel, and if a single product was observed, purified using the QIAquick® Gel Extraction kit (Qiagen, Germantown, MD, US). Purified PCR products were direct-sequenced by Macrogen© (South Korea). Partial mtDNA COI sequences alignment and editing were performed using MEGA7 version 7.0.26 (Kumar et al. 2016) with default parameters. The sequences obtained are deposited in GenBank under accessions MK450031–MK450111.

Mitochondrial DNA Analysis

We used DNAsp v5.0 (Librado and Rozas 2009) to calculate genetic variation indices, including number of segregating sites (*S*), average number of nucleotide differences (*k*), number of haplotypes (*No*), haplotype diversity (*hd*), and nucleotide diversity (*Pi*).

Bayesian inference (BI) under Bayesian information criterion (BIC) (Schwarz 1978) and Maximum Likelihood (ML) under Akaike information criterion (AIC) (Akaike 1974) were used to determine phylogenetic relationships among mitochondrial haplotypes and among apiaries. Prior to ML and BI analyses, the program Kakusan4 (Tanabe 2007) was used to estimate the best-fit evolutionary models of nucleotide substitution. The best-fit model for BI and ML analyses were HKY85_Gamma and GTR_Gamma Invariant, respectively. The BI analysis was generated on MrBayes version 3.2.6 (Ronquist et al. 2012), which uses a Metropolis-coupled, Markov chain Monte Carlo sampling approach. The program parameters were set according to Panyamang et al. (2018). The first 25% of generations were eliminated as “burn-in” samples. Bi-partition posterior probability values of 0.95 or greater were regarded as significantly verifying for the consensus tree topology (Larget and Simon 1999). The ML tree was reconstructed on Treefinder (Jobb et al. 2004) with 1000 replicates. The ML tree topologies with bootstrap values greater than 69% were considered as adequately resolved (Janique et al. 2017).

We executed a Median-joining network (Bandelt et al. 1999) with the program PopART version 1.7 (Leigh and Bryant 2015) to construct the haplotype network of COI haplotypes based on maximum parsimony.

Results

Microsatellite diversity

The number of alleles (N_o), number of effective alleles (N_e), allele frequencies, and observed (H_o) and expected (H_e) heterozygosity of all samples and overall loci are indicated in Table 1.

Bees sampled from different provinces did not show any evidence sub-structuring as shown by the Bayesian–Markov assignment algorithm implemented in STRUCTURE. The result K=1 was the most strongly supported number of populations [Ln (P) = −2314.54 and Var Ln (P)=11.68] when assuming admixture. We detected significant deviation ($P < 0.05$) from Hardy–Weinberg equilibrium in 10 of 40 population–locus combinations. Only the samples collected from Phrae and Nan provinces exhibited an excess of heterozygotes for A107 and A07, respectively. Linkage disequilibrium tests showed two significant disequilibria out of 80 locus pair–population combinations. There was no evidence of significant linkage disequilibrium between any microsatellite locus pairs ($0.2999 < P > 0.9897$) for the total population.

The local inbreeding coefficient, F_{IS}, was positive in all populations, except Samut SongKham (F_{IS} = –0.045), and significantly greater than zero in the Chanthaburi subpopulation only (Table 2).

The multilocus F_{ST} values between population pairs ranged from 0.000 (Chiang Rai – Chiang Mai, Chiang Rai – Samut Songkhram, and Chanthaburi – Chumphon) to 0.0456 (Phrae – Lamphun), and none of them were significantly different from zero ($P>0.05$). The highest values were between Lamphun and Phrae (0.0456) and Lamphun and Samuth Songkham (0.0341) (Table 3).

Low levels of genetic differentiation (Table 3) most likely arise because of high levels of migration (Nm) between all population pairs raged from 10.465 – Infinite) (Table 3). This suggests that more than 10 queens are exchanged between all population pairs each generation, a very high rate of gene flow.

Mitochondrial DNA diversity

We obtained high quality DNA sequences comprising 807 base pairs (bp) from 81 bees. Multi-alignment and pair-wise sequence comparisons showed 26 informative single base

substitutions, comprising 19 transitions (73.08%) and 7 transversions (26.92%) (Table 4). Based on mitochondrial COI gene sequences, the average number of nucleotide differences (*k*), haplotype diversity (*hd*), and nucleotide diversity (*Pi*) of all colony samples were 5.665, 0.875 (±0.022) and 0.007 (±0.0007), respectively. There is high haplotype diversity (ranging from 0.50 to 1.00) but relatively low nucleotide diversity (ranging from 0.00062 to 0.00909) (Table 4).

COI sequences of revealed 23 haplotypes. Phylogenetic relationships between haplotypes and reference sequences in GenBank are shown in Figure 2. The reference sequences represented the four evolutionary lineages of *A. mellifera* proposed by Ruttner et al. (1978): M (West European: KY926884_1 and AY114459_1), A (African: KJ601784_1 and MG552700_1), O (Middle Eastern: AY114472_1) and C (East Mediterranean: AY114462_1 and KX908209_1).Based on these reference sequences, 15 of the Thai haplotypes are of *A. mellifera carnica* (AY114462_1) or *A. m. ligustica* (KX908209_1) origin (the C lineage) while the remainder are *A. m. caucasica* (AY114472_1) (the O lineage). The haplotype network is congruent with the phylogenetic analysis (Fig. 3). The network shows a star–like pattern with two common haplotypes (Hap1 and Hap2). Interestingly, Chumphon and Chiang Mai showed the highest haplotype diversity (eight and seven haplotypes, respectively; Fig. 1), suggesting that these provinces are the primary origins of the commercial *A. mellifera* strains of Thailand.

Discussion

Reduced genetic diversity is a familiar characteristic of domestic plant and animal populations (Bruford et al. 2003), particularly those that are genetically isolated from wild populations. In this study, we observed low genetic heterogeneity of managed *A. mellifera* among the provinces of Thailand, a population that was established 49 years ago from a narrow genetic base, with few if any successful subsequent imports. We detected moderate levels of inbreeding within provinces with an average inbreeding coefficient of F_{is} = 0.096 (Table 2).

Our results contrast with those of Harpur et al. (2012), who showed high levels of genetic admixture in the managed *A. mellifera* populations in North America. They suggested that beekeeper management practices are the major drivers of increased genetic diversity relative to progenitor populations in Europe and Africa (Harpur et al. 2012). High genetic diversity was also detected in Australian (Chapman et al. 2008) and US (Delaney et al. 2009) commercial *A. mellifera* stocks. The situation in Thailand is different because of infrequent

imports, the lack of a feral population, and high intra-country exchange of genetic material between beekeeping enterprises.

Based on mitochondrial COI sequence data, the *A. mellifera* population of Thailand is derived from the Eastern Mediterranean (C) and the Middle Eastern Europe (O) evolutionary lineages (Fig. 2). We did not detect African (A) or West European (M) haplotypes in our samples. The high level of haplotype diversity suggests that there have been numerous independent successful introductions of *A. mellifera* queens into Thailand.

This study suggests that although overall genetic diversity and heterozygosity is high in Thai *A. mellifera*, there is local inbreeding. Likely this is because beekeepers mate queens within their own breeding lines. This suggests that Thai beekeepers should pay more attention to providing large numbers of unrelated males when mating queens. However, there is no evidence that further imports are required. Country-wide genetic diversity appears to be high, and introduction of new genetic material risks introducing new parasites and pathogens. Thailand is home to four indigenous *Apis* species (Oldroyd and Wongsiri 2006). Therefore biosecurity needs to be a high priority to protect these native species (Oldroyd and Nanork 2009).

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Figure Legends

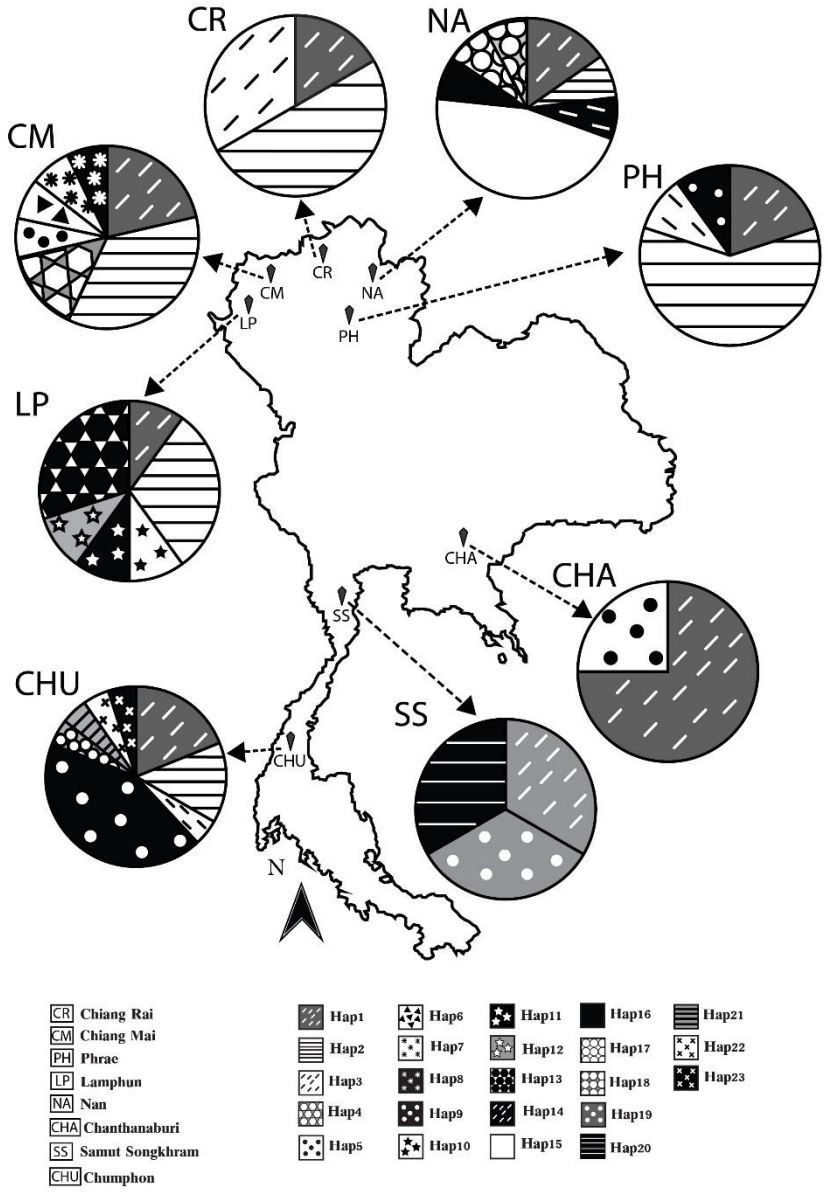

Fig. 1 Haplotype distribution map and location of the sampled apiaries

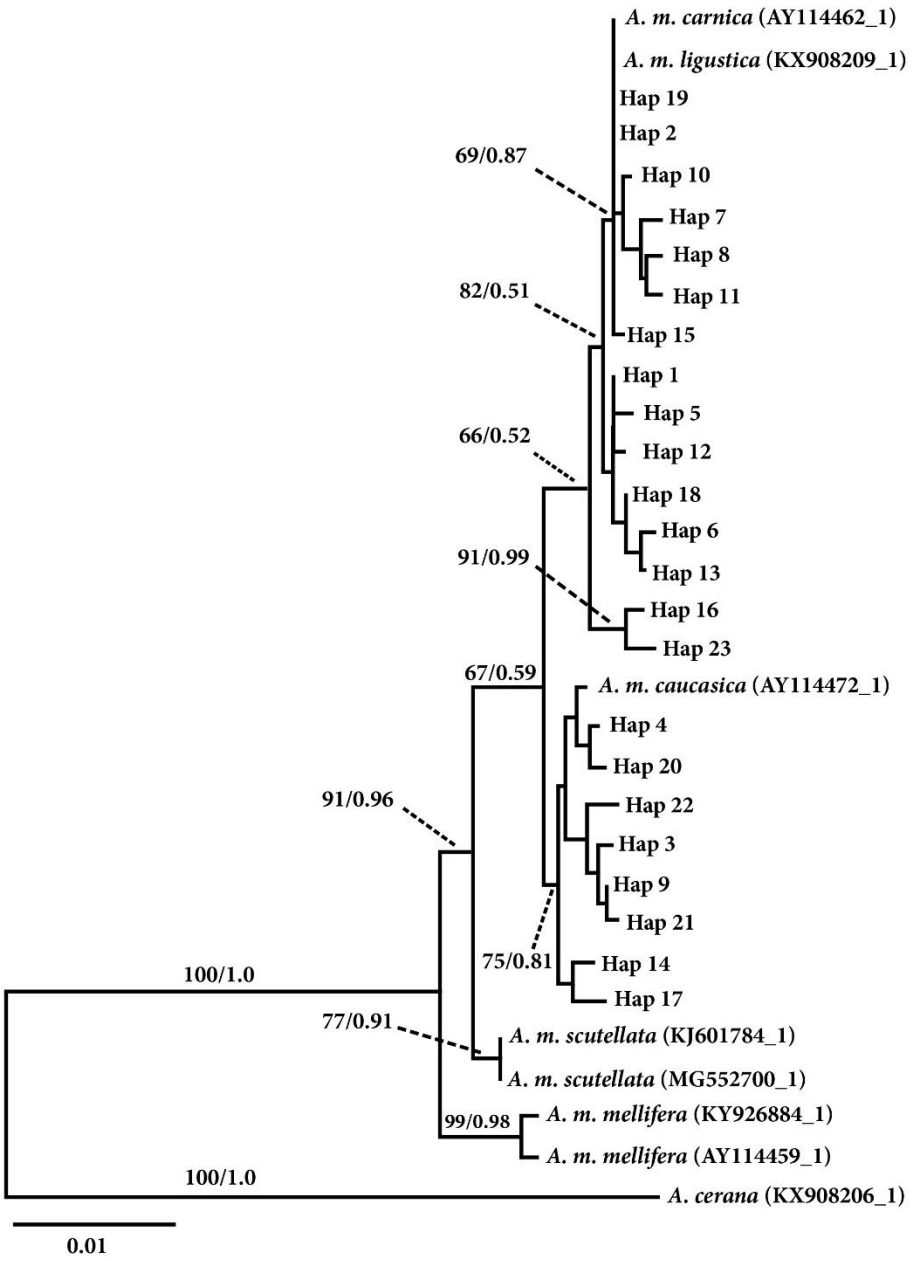

Fig.2 Phylogenetic relationships among cytochrome c oxidase I (COI) haplotypes of based on maximum likelihood (ML). The Asian honey bee species, *Apis cerana* (KX908206_1), was included as outgroups. Node support is inferred from Bayesian posterior probability and the bootstrap value for ML.

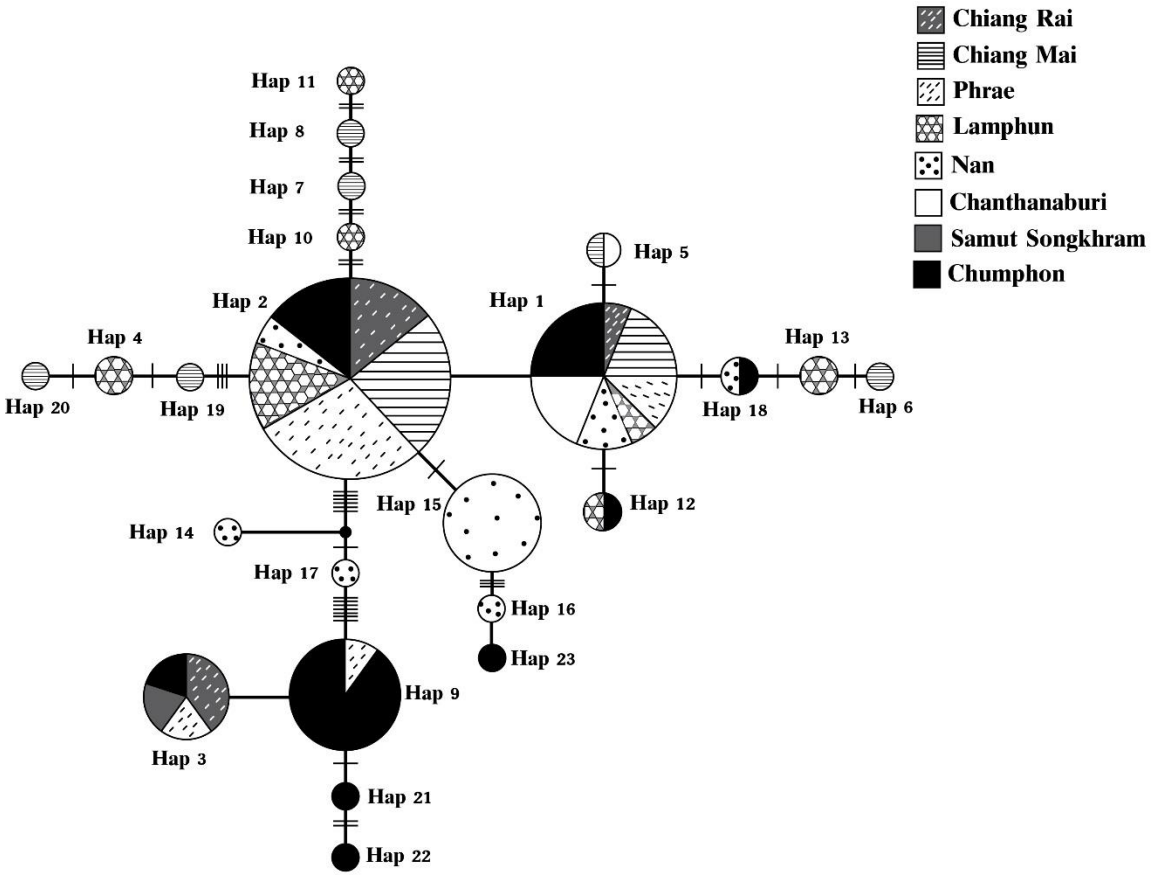

Fig. 3 Median-joining haplotype networks of *Apis mellifera* for mitochondrial COI gene. Each circle represents one unique haplotype and the size of the haplotype circle corresponds to the abundance of individual samples. Each pattern indicate a sampling location. Dark spots and crossbars demonstrate missing haplotype and one mutational step, respectively.

Table 1 Number of alleles detected (*N*), number of effective alleles (*Ne*), observed (H_o) and expected heterozygosity (H_e) at five microsatellite loci in commercial *Apis mellifera* populations of Thailand. The number of analyzed colonies from each province is shown in the brackets.

Locus	*Chiang Rai (n = 15)*	*Chiang Mai (n = 34)*	*Phrae (n = 25)*	*Lamphun (n = 29)*	*Nan (n = 40)*	*Chanthaburi (n = 5)*	*Samut Songkhram (n = 6)*	*Chumphon (n = 19)*	*All Populations (n = 173)*
A07									
N	6	9	9	6	8	4	6	8	11
Ne	3.635	3.733	4.921	3.248	4.810	3.333	4.798	4.911	4.396
H_o	0.733	0.676	0.720	0.724	0.600	0.200	0.833	0.895	0.694
H_e	0.725	0.732	0.797	0.692	0.792	0.700	0.792	0.796	0.772
A28									
N	5	6	4	6	5	3	4	5	7
Ne	1.931	2.119	1.738	3.020	2.060	2.381	2.97	2.692	2.279
H_o	0.333	0.529	0.440	0.655	0.325	0.600	0.667	0.474	0.474
H_e	0.482	0.528	0.425	0.669	0.514	0.580	0.666	0.628	0.561
A88									
N	4	7	7	6	6	3	4	7	9
Ne	1.767	1.672	2.023	1.289	2.199	1.151	3.422	1.687	1.822
H_o	0.467	0.382	0.640	0.241	0.575	0.400	0.833	0.474	0.474
H_e	0.434	0.402	0.506	0.224	0.545	0.340	0.708	0.407	0.451

Table 1 Continue

Locus	*Chiang Rai* *(n = 15)*	*Chiang Mai* *(n = 34)*	*Phrae* *(n = 25)*	*Lamphun* *(n = 29)*	*Nan* *(n = 40)*	*Chanthaburi* *(n = 5)*	*Samut Songkhram* *(n = 6)*	*Chumphon* *(n = 19)*	*All Populations* *(n = 173)*
A107									
N	10	15	14	10	12	7	8	11	17
Ne	7.924	8.772	9.470	8.183	6.993	6.250	5.734	5.917	9.033
H_o	0.800	0.853	0.680	0.724	0.750	0.600	0.667	0.684	0.746
H_e	0.874	0.886	0.894	0.878	0.857	0.840	0.826	0.831	0.889
A113									
N	8	9	4	7	6	4	4	4	13
Ne	3.607	2.832	1.994	2.799	2.104	2.778	3.126	2.564	2.728
H_o	0.667	0.588	0.360	0.621	0.550	0.600	0.833	0.579	0.566
H_e	0.723	0.647	0.498	0.643	0.525	0.640	0.680	0.610	0.634

Table 2 Multilocus microsatellite variation in the Thailand's commercial European honey bee populations.

Province	*n*	N_o	N_e	*Ho*	*He*	F_{it}	F_{is}
Chiang Rai	15	6.60±2.41	3.77±2.48	0.600±0.194	0.648±0.184	0.074	0.074
Chiang Mai	34	9.20±3.49	3.82±2.87	0.606±0.175	0.639±0.186	0.065	0.052
Phrae	25	7.60±4.16	4.03±3.31	0.568±0.158	0.624±0.207	0.123	0.090
Lamphun	29	7.00±1.73	3.71±2.62	0.593±0.202	0.621±0.240	0.085	0.045
Nan	40	7.40±2.79	3.63±2.21	0.560±0.152	0.647±0.164	0.136	0.134
Chanthaburi	5	4.20±1.64	3.18±1.89	0.480±0.179	0.620±0.184	0.259*	0.226*
Samut Songkhram	6	5.20±1.79	4.01±1.20	0.767±0.091	0.734±0.071	–0.184	–0.045
Chumphon	19	7.00±2.73	3.55±1.78	0.621±0.176	0.654±0.170	–0.009	0.050
Mean	21.62±12.69	6.775±1.521	3.712±0.273	0.599±0.080	0.648±0.037	0.124±0.072	0.096±0.065

The mean of observed (N_o) and effective (N_e) number of alleles, observed (H_o) and expected heterozygosity (H_e) with standard error (SD), fixation index between individuals and total data set (F_{it}), and fixation index between individuals and the local population (F_{is}). * $P < 0.05$

Table 3 Genetic distance (F_{ST}) and per-generation migration rate (*Nm*) between pairs of province's apiaries in Thailand based on five microsatellite loci of *Apis mellifera*. Values below the diagonal are the estimates of genetic distance (F_{ST}) and values above the diagonal are the estimates of per-generation migration rate (*Nm*). Inf is infinite.

	Chiang Rai	*Chiang Mai*	*Phrae*	*Lamphun*	*Nan*	*Chanthaburi*	*Samut Songkhram*	*Chumphon*
Chiang Rai	-	Inf	39.822	87.219	134.635	58.324	Inf	55.278
Chiang Mai	0.0000	-	20.333	454.045	73.029	28.071	23.423	155.75
Phrae	0.0124	0.0240	-	10.465	28.740	41.517	23.538	21.921
Lamphun	0.0057	0.0011	0.0456	-	28.071	23.309	14.163	45.371
Nan	0.0037	0.0068	0.0171	0.0175	-	17.748	66.167	53.263
Chanthaburi	0.0085	0.0175	0.0119	0.0210	0.0274	-	55.680	Inf
Samut Songkhram	0.0000	0.0209	0.0208	0.0341	0.0075	0.0089	-	28.071
Chumphon	0.0059	0.0032	0.0223	0.0109	0.0093	0.0000	0.0175	-

Table 4 Molecular diversity indices and population expansion test statistics of mitochondrial cytochrome c oxidase I (COI) genes sequence data of commercial *Apis mellifera* population of Thailand. Number of individuals (*N*), number of haplotypes (*No*), number of polymorphic (segregation) sites (*S*), average number of nucleotide differences (*k*), haplotype diversity (*hd*) and nucleotide diversity (P_i) with standard deviation (SD).

Province	*N*	*No*	*S*	*k*	*hd (±SD)*	P_i *(±SD)*
Chiang Rai	6	3	12	6.467	0.733(0.155)	0.0080(0.0024)
Chiang Mai	14	7	12	2.890	0.846(0.074)	0.0036(0.0008)
Phrae	10	4	12	4.378	0.644(0.152)	0.0054(0.0022)
Lamphun	9	6	8	2.444	0.889(0.091)	0.0030(0.0007)
Nan	13	7	14	3.923	0.795(0.109)	0.0049(0.0015)
Chanthaburi	4	2	1	0.500	0.500(0.265)	0.0006(0.0003)
Samut Songkhram	3	3	11	7.333	1.000(0.272)	0.0091(0.0036)
Chumphon	22	9	19	7.052	0.805(0.070)	0.0087(0.0008)
All samples	***81***	***23***	***26***	***5.665***	***0.875(0.022)***	***0.0070(0.0007)***

Appendix 2

Appendix 2: Output – **Rattanawannee, A.**, Jeratthitikul, E., Duangpakdee, O. and Oldroyd, B. P. 2017. Mitochondrial sequencing and geometric morphometrics suggest two clades in the *Tetragonilla collina* (Apidae: Meliponini) population of Thailand. **Apidologie**, 48(6): 719–731 (Q1: Impact factor = 2.856) acknowledged the Thailand Research Fund (**grant no. MRG6080272**).

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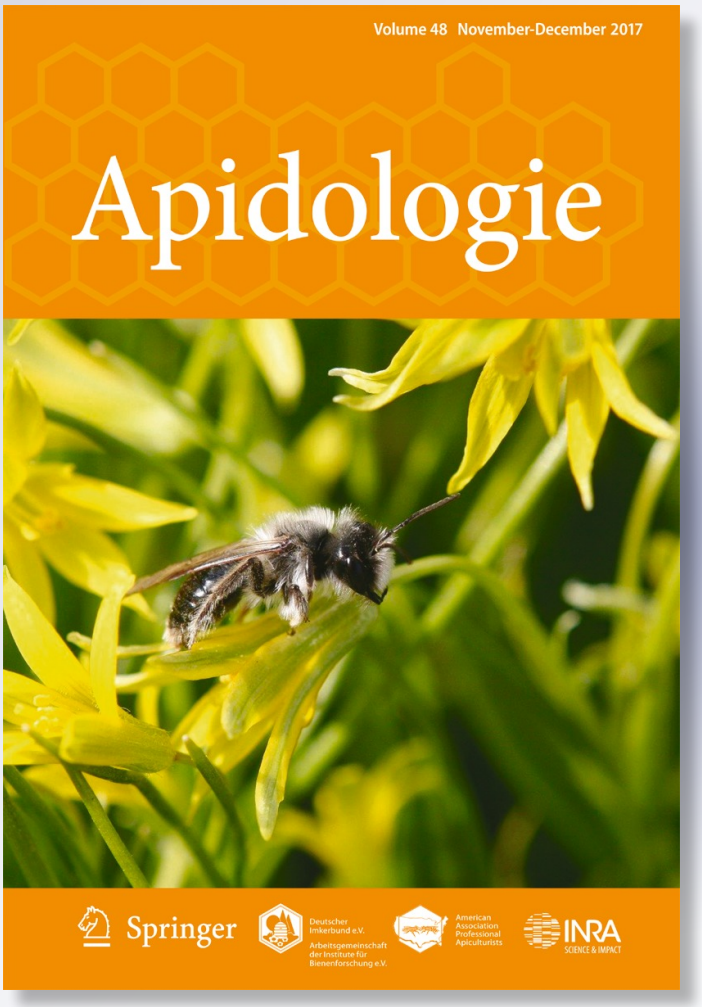

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Original Article

Mitochondrial sequencing and geometric morphometrics suggest two clades in the *Tetragonilla collina* (Apidae: Meliponini) population of Thailand

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Abstract – The stingless bee *Tetragonilla collina* Smith, 1857, is broadly distributed across Indochina. In this study, we use a combination of molecular and geometric morphometric analyses to quantify the genetic structure and diversity of the *T. collina* population of Thailand. We found striking regional differences in both mitochondrial haplotype frequencies and morphology. A Bayesian analyses of molecular diversity of the mitochondrial *COI* region revealed two clades, roughly divided into the population northeast of the Thai-Malay Peninsula (clade A) and the western and Thai-Malay Peninsula population (clade B). In addition, morphometric analysis showed that bees in clade A have significantly larger wings than bees from clade B. These results suggest that the *T. collina* population of Thailand is divided into two distinct populations. The spatial distributions seem to reflect contemporary ecological features such as annual flooding (bees of clade B are absent from areas subject to inundation), rather than past biogeography. Thus, *T. collina* differs from the honey bees *Apis dorsata* and *A. cerana* that show genetic differentiation north and south of the Isthmus of Kra, perhaps reflective of past separation during the Pleistocene when sea levels were much higher.

stingless bees / *Tetragonilla collina* / genetic variation / geometric morphometrics / biogeography / Isthmus of Kra

1. INTRODUCTION

Geographical features in a landscape are often associated with abrupt transitions in species or subspecies distribution. These may be legacies of historical barriers to gene flow, caused by the geographical feature, or by a change in contemporary ecological processes that affect species distribution. The Isthmus of Kra on the Malay peninsula, between 10° 34′ N and 11° 24′ N, is a transition zone between seasonal rainforest and mixed moist deciduous forest (Hughes et al. 2003), and between Indochinese and Sundaic biota (Woodruff 2003). During the early Pliocene (5.5–4.5 million years ago), many plant and animal populations north of the Kra Isthmus were repeatedly separated from populations to the south by changing sea levels, leading to the creation of new species and subspecies (Woodruff 2003).

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Contemporary distributions often reflect these historical events. For example, three shrew species show a transition zone just south of Isthmus of Kra near Satun to Sai Suri (Roberts 2011). Additionally, the honey bees *Apis cerana* (Deowanish et al. 1996; Smith and Hagen 1996; Sihanuntavong et al. 1999; Sittipraneed et al. 2001; Warrit et al. 2006) and *A. dorsata* (Insuan et al. 2007) show significant genetic variation among geographical regions of Thailand, particularly between populations north and south of the Kra Isthmus (Sihanuntavong et al. 1999; Warrit et al. 2006). Interestingly, *Varroa jacobsoni*, a parasitic mite of *A. cerana*, also shows different haplotypes north and south of the Kra ecotone (Warrit et al. 2006).

The stingless bees are a large group of tropical eusocial bees of the tribe Meliponini (Kerr and Maule 1964). In contrast to honey bees, which reproduce and migrate by swarms that can travel many tens of kilometers from the natal nest (Koeniger and Koeniger 1980; Nakamura et al. 1991; Dyer and Seeley 1994; Itioka et al. 2001; Paar et al. 2004), stingless bee colonies reproduce by a gradual process of budding, which restricts the distance of daughter colonies from their natal nest to 100 m or so (Michener 1979; Inoue et al. 1984; van Veen and Sommeijer 2000; Roubik 2006; Francisco and Arias 2010). This reproductive behavior means that stingless bee populations tend to show much greater structure than honey bee populations (Francisco et al. 2008, 2014; Francisco and Arias 2010; Brito et al. 2014) and might be expected to reflect the vestiges of past biogeography even more strongly than honey bee populations. *Tetragonilla collina* Smith, 1857, is one of the most common and broadly distributed stingless bee species in the Indochina region (Sakagami and Khoo 1987) and is found throughout Thailand (Theeraapisakkun et al. 2010). It is therefore an ideal species to examine the hypothesis that the Isthmus of Kra is a transition zone for stingless bee subpopulations that were formerly separated north and south of the Isthmus during the Pleistocene.

Combinations of morphometric and molecular techniques are often used to quantify genetic diversity and to determine intra-generic boundaries of bee subpopulations (Sittipraneed et al. 2001; Arias et al. 2006; Mendes et al. 2007; Tofilski 2008; May-Itzá et al. 2012; Rattanawannee et al. 2012; Wappler et al. 2012). For instance, Francoy et al. (2006) showed that a single wing cell, the radial cell, carries sufficient information to correctly classify three groups of *Apis mellifera* (Africanized, Italian, and Carniolan) with a fidelity of nearly 99%. Morphometric analysis of wing venation of the Brazilian stingless bee *Plebeia remota* revealed cryptic species within the population (Francisco et al. 2008). Francoy et al. (2011) proposed that geometric morphometric analysis of wing shape could be used as a first step for assigning genetic lineages and geographic origins samples of the stingless bee *Melipona beecheii*.

Here we use a phylogenetic analysis of the *COI* region of the mitochondria in combination with geometric morphometrics of wing venation to determine whether the *T. collina* population of Thailand shows a pattern of differentiation about the Kra ecotone that parallels that of *A. cerana*. If so, then this would reinforce the idea that there is a sharp biological division north and south of the ecotone that acts as a barrier to gene flow and enhances differentiation of bee populations. If there is no population subdivision in *T. collina*, then it may be inferred that the sharp boundary observed in *A. cerana* arises from a hybrid zone brought about by the reunification of the Sunderland and Indo-Chinese subpopulations of *A. cerana* in this area after the Pleistocene.

2. MATERIALS AND METHODS

2.1. Sample collection

T. collina workers were sampled from 71 colonies from 25 locations throughout Thailand. Sampling locations were grouped into seven geographical subpopulations (Table I, Figure 1). The bees were collected directly at the nest entrance tube of each colony. For morphometric analysis, at least 15 workers were collected, immediately killed with ethyl acetate, and then stored in 70% (*v*/*v*) ethanol. For molecular analysis, bees were immediately preserved in 95% (*v*/*v*) ethanol and then kept at −20°C until analysis.

Table I. Sampling sites of *Tetragonilla collina*

No.	Population	Locality name	Coordinates	Specimen examined	
				Morphology (individuals)	DNA (GenBank ID)
1	North	Maerim, Chiang Mai	18° 53.27 N 98° 51.45 E	20	CMO1 (KU934113)
2	North	Mueang, Chiang Mai	18° 48.11 N 98° 56.08 E	10	CMO3 (KU934119)
3	North	Hangchad, Lumpang	18° 25.38 N 99° 12.25 E	20	LPANG1 (KU934117), LPANG2 (KU934126)
4	North	Lee, Lumpoon	17° 56.53 N 98° 53.18 E	30	LPON1 (KU934118), LPON2 (KU934127)
5	Central	Wangthong, Phitsanulok	16° 52.46 N 100° 39.38 E	10	-
6	Central	Bang Rakhum, Phitsanulok	16° 44.49 N 100° 07.38 E	20	PLOK (KU934128)
7	Central	Lan Krabbue, Kamphaeng Phet	16° 35.43 N 99° 54.02 E	30	KPANG (KU934111)
8	Central	Mueang, Phichit	16° 26.49 N 100° 20.38 E	20	–
9	Central	Banphot Phisai, Nakhon Sawan	16° 02.26 N 99° 54.10 E	60	NSAWAN (KU934114)
10	Central	Chaloem Phra Kiat, Saraburi	14° 40.21 N 100° 53.26 E	40	LOP2 (KU934112)
11	West	Nong Ya Plong, Phetchaburi	13° 13.14 N 99° 42.40 E	30	PHET1 (KU934129), PHET2 (KU934130)
12	West	Sangkhla Buri, Kanchanaburi	15° 09.12 N 98° 27.31 E	20	SUNGKLA1 (KU934116), SUNGKLA2 (KU934144)
13	West	Suan Phueng, Ratchaburi	13° 35.38 N 99° 30.42 E	10	RATRY (KU934132)
14	Northeast	Mueang, Sakon Nakhon	17° 10.07 N 104° 7.04 E	30	SAKON (KU934123)
15	Northeast	Kuchinarai, Kalasin	16° 35.39 N 104° 7.45 E	50	KASIN2 (KU934120)
16	Northeast	Khao Wong, Kalasin	16° 38.35 N 104° 7.59 E	10	KASIN6 (KU934121)
17	Northeast	Kantharawichai, Maha Sarakham	16° 14.49 N 103° 15.12 E	20	SAKAM (KU934122)
18	East	Mueang, Chonburi	13° 19.15 N 100° 55.47 E	10	CHON (KU934138)
19	East	Mueang, Rayong	12° 41.44 N 101° 14.24 E	20	RY (KU934139)
20	East	Khlung, Chanthaburi	12° 30.40 N 102° 10.33 E	50	JANTHA2 (KU934115)
21	Kra	Mueang, Prachuap Khiri Khan	11° 40.35 N 99° 41.31 E	40	PAJOB1 (KU934135), PAJOB4 (KU934131)
22	Kra	Tha Sae, Chumphon	10° 57.03 N 99° 10.52 E	30	TASAE2 (KU934140), TASAE3 (KU934143)
23	Kra	Sawi, Chumphon	10° 20.01 N 99° 05.27 E	40	SAWEE2 (KU934136), SAWEE4 (KU934133)
24	South	Kanchanadit, Surat Thani	09° 06.55 N 99° 38.20 E	40	SURAT2 (KU934137), SURAT3 (KU934141), SURAT4 (KU934142)
25	South	Sichon, Nakhon Si Thammarat	09° 01.36 N 99° 46.20 E	50	NKONSI2 (KU934134), NKONSI3 (KU934124), NKONSI4 (KU934125)
Total				710	34

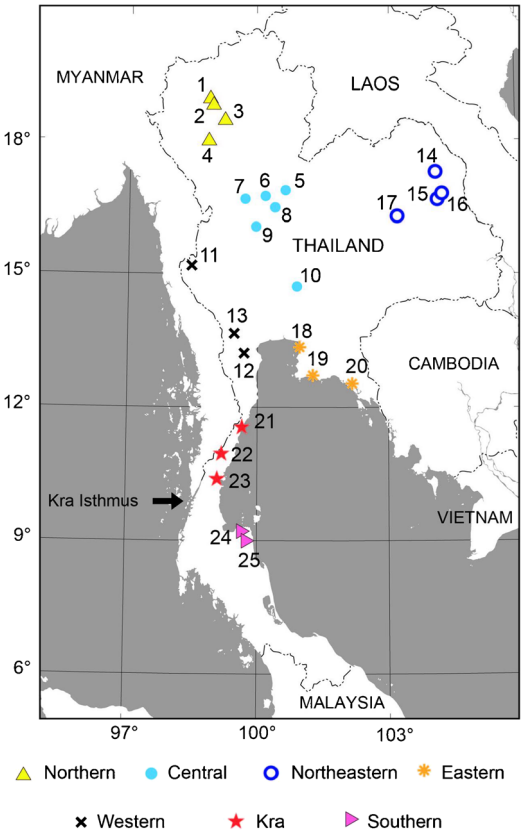

Figure 1. *Tetragonilla collina* collection sites in Thailand. The numbers correspond to those in Table I.

2.2. DNA extraction, amplification, sequencing, and alignment

Genomic DNA was extracted from thoracic muscle of one worker bee per colony (34 colonies) using the DNeasy® Blood & Tissue Kit (Qiagen, Germantown, MD) following the manufacturer's instructions. We amplified a fragment of the mitochondrial cytochrome oxidase subunit I (*COI*) in a 50-μL final reaction volume containing 1× PCR master mix (Catalog#K0171, Fermentas Life Science), 200 nmol of forward (LCO1490: 5′-GGTCAACAAATCATAAAGATATTGG-3′) and reverse (HCO2198: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) primers (Folmer et al. 1994), and at least 200 ng of DNA template. Thermal profiles consisted of an initial denaturation step of 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 48 °C for 1 min, and 72 °C for 150 s, with a final extension step of 72 °C for 5 min.

Amplified PCR products were purified using QIAquick® Gel Extraction Kit (Qiagen, Germantown, MD) and directly sequenced by AITbiotech Pty Ltd. (The Rutherford Science Park 1, Singapore). Partial DNA sequences were aligned and edited using MEGA6 v6.06 (Tamura et al. 2013). Sequences have been deposited in GenBank under accessions KU934111–KU934146 (Table I). To obtain outgroups for phylogenetic analysis, we sequenced the same COI fragment from three additional stingless bee species [*Tetragonula pagdeni* Schwarz, 1939 (GenBank ID: KU934145), *Homotrigona fimbriata* Smith, 1857 (GenBank ID: KU934146), and *Tetrigona apicalis* Smith, 1857 (GenBank ID: KU934147)], all collected in Thailand.

2.3. Molecular diversity indices analysis and phylogenetic reconstruction

Measures of genetic diversity including the average number of nucleotide differences (k), number of polymorphic sites (S), haplotype diversity (h) (Nei 1987), and nucleotide diversity (π) (Nei and Li 1979) were obtained using DnaSP v5.0 (Librado and Rozas 2009).

Maximum likelihood (ML) and Bayesian inference (BI) methods were used to reconstruct the phylogenetic relationships among *COI* haplotypes. The program Kakusan4 (Tanabe 2007), with maximum likelihoods calculated in TREEFINDER (Jobb et al. 2004), was used to estimate the best-fit models of nucleotide substitution as determined by the Akaike information criterion, AIC, (Akaike 1974) implemented for ML and the Bayesian information criterion, BIC, (Schwarz 1978) for BI. The ML analysis was performed using the likelihood-ratchet method in TREEFINDER (Jobb et al. 2004), with 1000 bootstrap replicates to estimate branch confidence values. Tree topologies with bootstrap values 70% or greater were regarded as being sufficiently resolved (Huelsenbeck and Hillis 1993). The BI analysis was performed with MrBayes v3.1 (Huelsenbeck and Ronquis 2001), which employs a Metropolis-coupled, Markov chain Monte Carlo

(MC-MCMC) sampling approach. A four-chain MC-MCMC analysis was run twice in parallel (with default heating values) for one million generations starting with a random tree, and trees were collected every 100 generations. The log-likelihood values of the sample points were plotted against the generation time, and 25% of the generations were discarded as "burn-in" samples. The remaining trees were used to estimate consensus tree topology, bipartition posterior probability (bpp), and branch length (Huelsenbeck and Ronquis 2001). A bi-partition posterior probability of 0.95 or greater was regarded as significant support for the consensus tree (Larget and Simon 1999).

2.4. Geometric morphometrics

Ten workers were randomly selected from each of 71 colonies for dissection, giving a total of 710 bees analyzed. The right forewing of each bee was dissected and slide-mounted. Wings were photographed with a digital camera attached on a stereomicroscope (Olympus SZX16) under ×25 magnification with the same camera setting and by the same person (AR). The wing images were randomly ordered using tpsUtil v.1.49 (Rohlf 2012) before bi-dimensional coordination of landmarks. A set of 13 homologous was digitized using the tpsDig2 v.2.16 software (Rohlf 2010) by the same person (AR). Landmarks are shown in Figure 2.

All specimens were digitized twice by the same person (AR). Procrustes ANOVA (Klingenberg and McIntyre 1998) was then performed using MorphoJ v.1.05c (Klingenberg 2011) to ensure that the observed variation was attributable to biological variation and not to measurement error. Repeatability (R), the proportion of variance due to true variation among individuals in relation to the total variance, was calculated according to Arnqvist and Mårtensson (1988). To reduce the effects of measurement error, repeated measurements were averaged in MorphoJ and used in subsequent analyses.

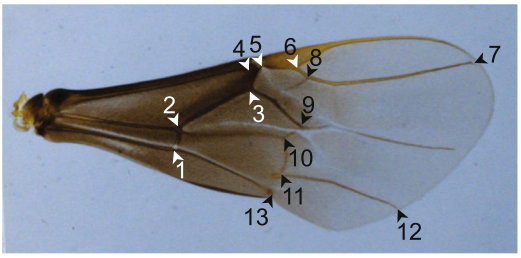

Figure 2. Right forewing of a *Tetragonilla collina* worker. The *arrows* indicate the respective position of each of the plotted landmarks.

Samples were grouped into seven *prior groups* according to geographical area (Table I) and into two groups based on the molecular analysis (see Sect. 3). Centroid size (CS) of the landmarks were calculated as an indicator of overall size of the wing (Zelditch et al. 2004) and used to assess whether bees of different subpopulations differed in size. We compared CS of the landmarks between two of the clades suggested by the *COI* analysis using an independent sample *t* test.

For variation of wing shape, the two-dimensional landmark data were first subjected to the Procrustes superimposition, which removes variation (i.e., scale, position, and orientation) that is not attributable to wing shape variation (Dryden and Mardia 1998). Canonical variate analysis (CVA) was then performed to examine the relative difference in wing shape among populations using MorphoJ. Mahalanobis distances and Procrustes distances between pairwise populations were determined and the significance of differences assessed by a permutation test (10,000 iterations). In order to infer phenotypic relationships of wing-shape variation among populations, a neighbor-joining tree (NJ) (Saitou and Nei 1987) based on Mahalanobis distances between the centroids of each population derived from CVA was constructed using MEGA6 v6.06.

3. RESULTS

3.1. Molecular data analysis

We obtained DNA sequence comprising 519 base pairs (bp) from 34 individuals. The nucleotide composition showed high A+T content (average 71.48%). Multi-alignment and pair-wise sequence comparisons showed a total of 119 variable sites (89 informative), with 131 single base substitution sites comprising 93 transitions (70.99%) and 38 transversions (29.01%).

Molecular diversity indices are shown in Table II. In total, 19 different haplotypes were detected from the 34 *T. collina* individuals examined. The estimates of haplotype diversity (h) were high overall (0.95), ranging from 0.67 to 1.00 per subpopulation (Table II). Overall nucleotide diversity (π) was 0.073, and ranged from 0.011–0.070 per population. Populations from the east had the highest levels of nucleotide diversity, while those from the south had the lowest (Table II). When the bee samples were divided into two groups based on the two major clades suggested by the phylogenetic analysis of mitochondrial *COI* sequences (see below), molecular diversity indices of clade A were higher than those of clade B (Table II).

3.2. Phylogenetic analysis

The best-fit evolutionary model for a ML tree under the AIC was J2+G+I and that for a BI tree under BIC was HKY85+G. The topology of the Bayesian tree suggests that Thai populations of *T. collina* are monophyletic but divided into two major clades—clade A and clade B. Clade A comprises bees from northern, central, northeastern, eastern, and western1 (PHET1 and PHET2) populations, with a ML bootstrap value of 90.7% and a Bayesian posterior probability of 0.55. Clade B includes populations from western2 (SUNGKLA1, SUNGKLA2, and RATRY), Kra, and southern populations with high support values (ML bootstrap value of 89.9% and a Bayesian posterior probability of 1.0) (Figure 3 and Table I).

3.3. Geometric morphometric analysis

Procrustes ANOVA (Klingenberg and McIntyre 1998) showed that the measurement error was low relative to overall shape variation. The between-individual mean square significantly exceeded the mean square attributable to measurement error ($F_{15,598} = 52.76$; $P < 0.0001$). In addition, the repeatability of landmark acquisition was high ($R = 0.96$).

The bees from seven geographic populations differed significantly in wing centroid size (ANOVA: $F_{6, 703} = 114.8$; $P < 0.0001$). When the bee samples were divided into two groups based on the two major clades suggested by the phylogenetic analysis of mitochondrial *COI* sequences (Figure 3), bees of clade A (mean wing CS = 772.64 ± 26.82) had significantly ($P < 0.001$ two-tailed t test) larger wings than bees from clade B (734.78 ± 21.98) (Figure 4).

The first two canonical variates explained 77.640% of total variance (Table III). Based on permutation tests for Mahalanobis and Procrustes distances, most subpopulation pairs showed significant differences in forewing shape (Table IV). In addition, individuals from clade A were

Table II. Summary of molecular diversity indices of Thai *Tetragonilla collina* populations based on the mitochondrial *COI* gene

Population	*N*	*S*	*No.*	*h* (s.d.)	π (s.d.)	*k*
North	6	22	3	0.80 (0.12)	0.023 (0.01)	12.000
Central	4	22	3	0.83 (0.22)	0.021 (0.004)	11.000
Northeast	4	12	4	1.00 (0.18)	0.012 (0.003)	6.000
East	3	54	3	1.00 (0.27)	0.070 (0.024)	36.333
West	5	52	3	0.80 (0.31)	0.058 (0.015)	30.200
Kra Isthmus	6	19	4	0.86 (0.12)	0.019 (0.004)	9.733
South	6	0	1	–	–	–
Clade A	19	87	12	0.95 (0.03)	0.049 (0.004)	25.63
Clade B	15	38	7	0.83 (0.08)	0.024 (0.002)	12.69
Total	34	119	19	0.95 (0.02)	0.073 (0.003)	37.90

N number of individuals, *S* number of polymorphic (segregation) sites, *No.* number of haplotypes, *h* haplotype diversity, π nucleotide diversity, *k* average number of nucleotide differences

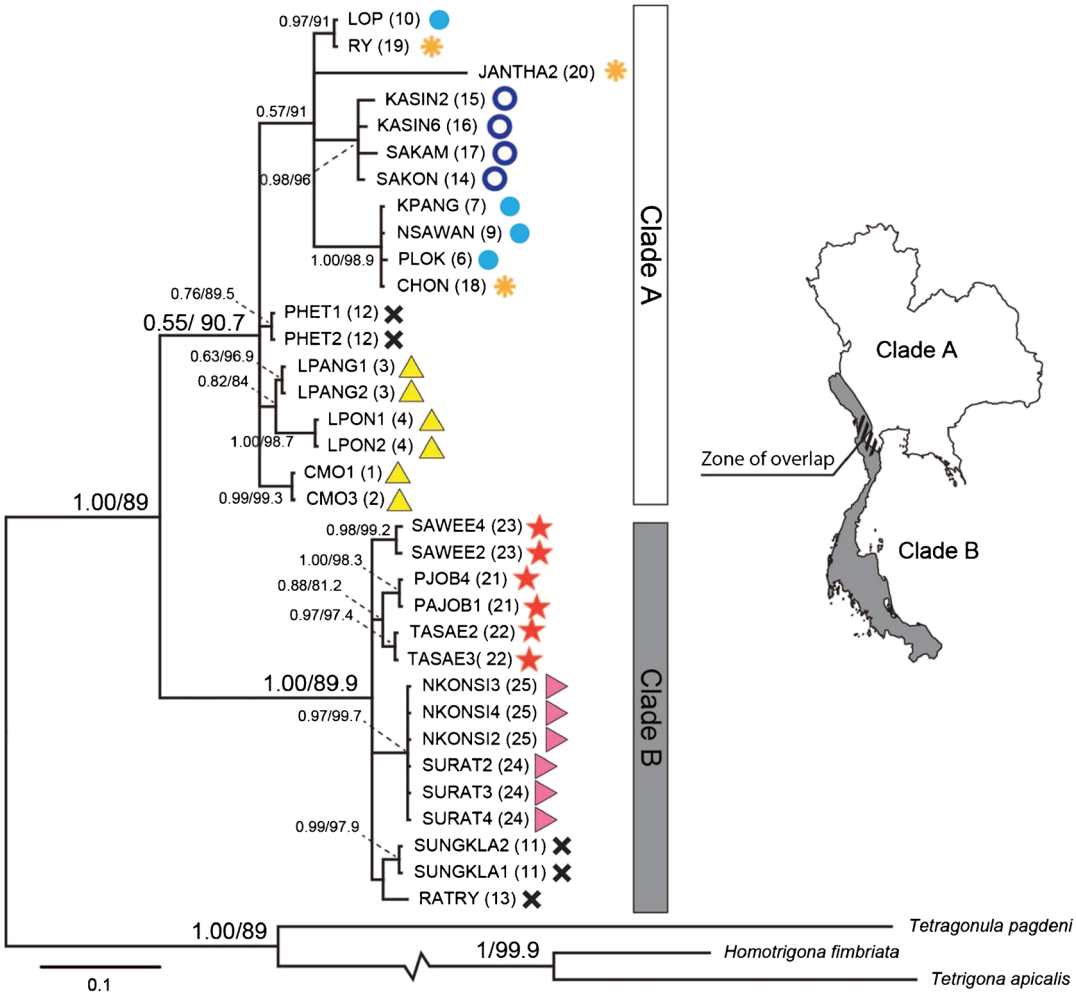

Figure 3. Phylogeny relationships of *Tetragonilla collina* populations in Thailand and outgroups based on Bayesian inference analysis (BI) of mitochondrial *COI* gene. Node supports inferred from Bayesian posterior probability and bootstrap value for ML.

generally well separated from those of clade B (Figure 5).

Deviations in shape from the consensus configuration along the first two CV axes of the CVA plot, as represented by wireframe graphs in Figure 5, showed that individuals located in the positive dimension of CV1 had an extended cross vein between the cubitus and vannal vein (cu-v) (landmarks 1 and 2) compared to individuals in the negative dimension. Further, the first branch of cubitus (Cu1) (landmarks 11 and 12) was constricted in the positive group relative to the negative group. Wing shape change along CV2 arose from the distal shift of landmarks 7 and 8 (radial sector) in the positive group when compared to the negative group.

A neighbor-joining tree constructed using Mahalanobis distances between population centroids revealed two distinct groups that are similar to the A and B clades revealed by the mitochondrial phylogenetic analysis (Figure 6).

4. DISCUSSION

Both genetic and morphological data strongly suggest that the *T. collina* population of Thailand

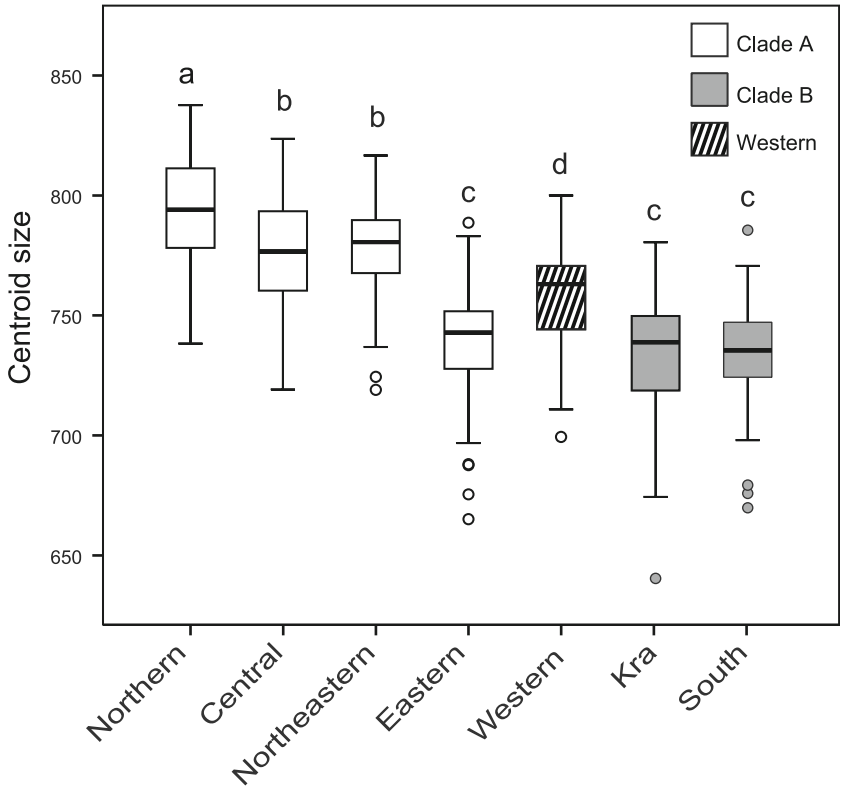

Figure 4. Box-and-whisker plot showing forewing centroid size variation of *Tetragonilla collina* populations in Thailand. *Boxes* exhibit the median, *whiskers* exhibit the minimum and maximum observation, and *circles* exhibit the outliers of the data sets.

is divided into two distinct clades. One clade (B), which is extant on the Thai-Malay Peninsula, also extends north of the Peninsula along the Myanmar border (Figure 3). This Thai-Malay clade is significantly smaller in wing size than the second clade (A) that occupies all other areas. There is a small area of overlap between clades A and B well north of the Kra Isthmus (Figure 3).

Table III. Eigenvalues and percentages of variance explained by six canonical variates produced from canonical variate analysis (CVA) of *Tetragonilla collina* wing venation pattern

CV	Eigenvalues	% Variance	Cumulative %
1	3.823	55.884	55.884
2	1.488	21.756	77.640
3	0.712	10.404	88.043
4	0.418	6.111	94.155
5	0.300	4.391	98.546
6	0.099	1.454	100

Various studies of population genetic structure of Thai honey bees have found a biogeographical transition zone between mainland and peninsula populations focused on a sharp boundary at the Kra ecotone (at Bang Saphan, Prachuap Khiri Khan: 11° 24′ N, 99° 31′ E; and Tup Sa Kae, Prachuap Khiri Khan: 11° 31′ N, 99° 35′ E) (Limbipichai 1990; Deowanish et al. 1996; Smith and Hagen 1999; Warrit et al. 2006). Woodruff (2003) suggests that sea level rises submerged sections of the Thai-Malay Peninsula on at least two occasions during the early/middle Miocene (24–13 mya) and in the early Pliocene (5.5–4.5 mya). These historical inundations most likely led to the contemporary subdivisions in various honey bee populations, but what about stingless bees?

Thummajitsakul et al. (2008) examined genetic variation and population structure of the arboreal

Table IV. Mahalanobis distances and Procrustes distances of *Tetragonilla collina* populations in Thailand derived from CVA of the worker bee's forewings with p values calculated by 10,000 random permutations per test to examine statistical significant differences between pair of stingless bee populations

Population	North	Central	NEast	East	West	Kra	South
North	–	2.3792 $P < 0.0001$	2.9194 $P < 0.0001$	4.1679 $P < 0.0001$	2.2854 $P < 0.0001$	4.0312 $P < 0.0001$	4.1403 $P < 0.0001$
Central	0.0159 $P = 0.0003$	–	2.7574 $P < 0.0001$	4.2265 $P < 0.0001$	2.9509 $P < 0.0001$	4.2138 $P < 0.0001$	4.6529 $P < 0.0001$
NEast	0.0128 $P = 0.0105$	0.0158 $P = 0.0001$	–	2.9926 $P < 0.0001$	3.6002 $P < 0.0001$	5.3063 $P < 0.0001$	5.3436 $P < 0.0001$
East	0.0210 $P = 0.0009$	0.0168 $P = 0.0001$	0.0211 $P < 0.0001$	–	4.3476 $P < 0.0001$	5.5186 $P < 0.0001$	5.5495 $P < 0.0001$
West	0.0170 $P = 0.0048$	0.0221 $P < 0.0001$	0.0216 $P < 0.0001$	0.0251 $P = 0.0001$	–	2.8094 $P < 0.0001$	3.9051 $P < 0.0001$
Kra	0.0199 $P = 0.0005$	0.0257 $P < 0.0001$	0.0277 $P < 0.0001$	0.0278 $P < 0.0001$	0.0137 $P = 0.0267$	–	2.8180 $P < 0.0001$
South	0.0194 $P < 0.0001$	0.0264 $P < 0.0001$	0.0262 $P < 0.0001$	0.0271 $P < 0.0001$	0.0164 $P = 0.0005$	0.0092 $P = 0.077$	–

Above diagonal is Mahalanobis distances and below diagonal is Procrustes distances

stingless bee, *Trigona pagdeni* Schwarz, based on molecular markers. They detected differentiation between samples collected north and south of the Isthmus of Kra, but a much stronger differentiation between populations in the northeast with respect to all other populations. This differentiation parallels that seen here in *T. collina*, and suggests that stingless bees and honey bees differ strongly in their biogeography in Thailand.

In stingless bees, gene flow via females is restricted by their reproductive biology. Stingless bee colonies propagate by establishing a new nest nearby the parent nest (Roubik 2006). Food and other resources are transferred from the parent colony to the daughter nest over several weeks or months, greatly restricting the dispersal distance (Michener 1979; Inoue et al. 1984; van Veen and Sommeijer 2000) and the extent to which mitochondrial haplotypes can spread per generation via queens (Francisco and Arias 2010; de J. May-Itzá et al. 2012; Nogueira et al. 2014). Males probably disperse further than queens (Paxton 2000; Cameron et al. 2004; Kraus et al. 2008; Mueller et al. 2012), but nonetheless the flight distance of *Melipona scutellaris* males, for example, is only 0.8–1 km (Carvalho-Zilse and Kerr 2004) and less than 10 km for *Scaptotrigona mexicana* (Kraus et al. 2008). Therefore, high population subdifferentiation is expected (Francisco et al. 2014) and usually observed (e.g., Franck et al. 2004; Quezada-Euán et al. 2007; Tavares et al. 2007; Thummajitsakul et al. 2008, 2010; de J. May-Itzá et al. 2012; Brito et al. 2014) in stingless bee populations worldwide. Geographical barriers such as rivers, oceans, and mountain ranges can further restrict the dispersal of stingless bees (Brito et al. 2014). Despite limited dispersal, the clade B of *T. collina* appears to have expanded its range into northwest Thailand, where it apparently out-competes the mainland clade. This may be because the forest types of western Thailand have more in common with the Malay Peninsula than with central and northern Thailand (Maxwell 2001, 2004; Wikramanayake et al. 2002).

More important than forest type may be a combination of altitude and climate. The transition zone between clades A and B appears to be the Central Plane of Thailand (Figure 3). This area is

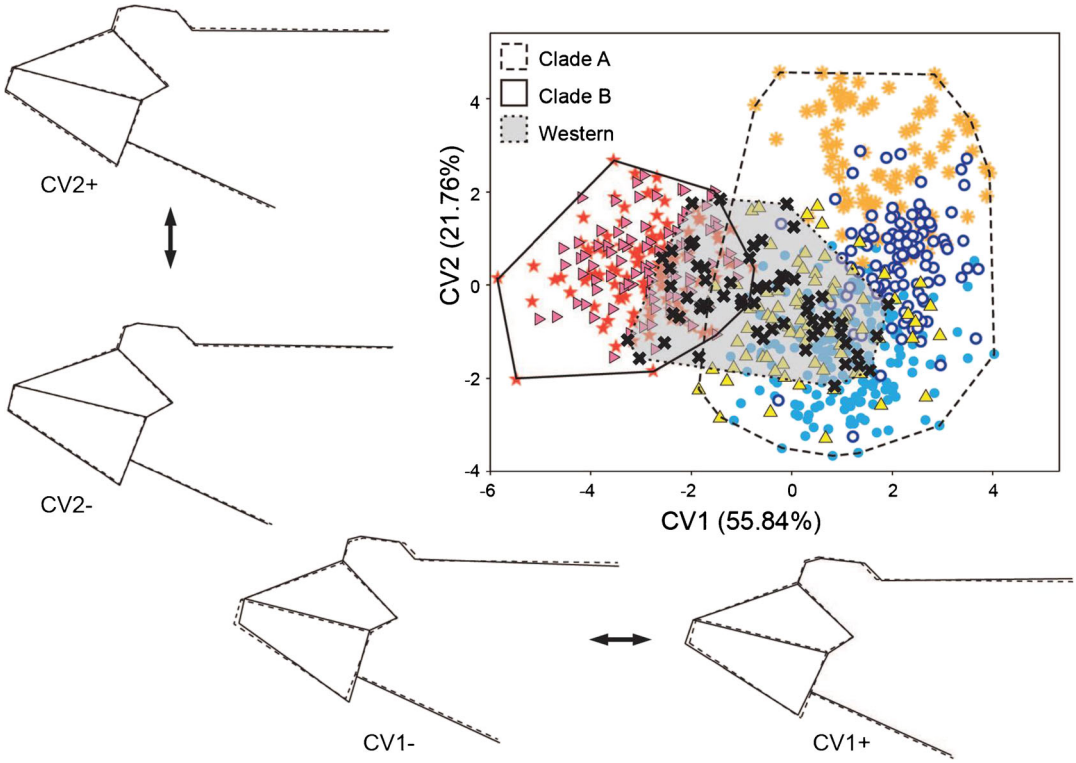

Figure 5. Scatterplot of individual scores for the first two canonical variates derived from the canonical variant analysis (CVA) of landmarks. Wireframes representing the shape change (*solid line*) from the consensus configuration of landmarks (*dash line*) to each extreme negative and positive CV scores. The *symbols* correspond to those in Fig. 3.

very dry in the dry season but endures prolonged periods of inundation during the wet season. As *T. collina* nest in cavities, either underground (typically beneath a tree), or in a termite mound (Jongjitvimol and Wattanachaiyingcharoen 2007), topology and climate combine to make the Central Plane an inhospitable environment for ground-nesting *T. collina*, and the area probably acts as

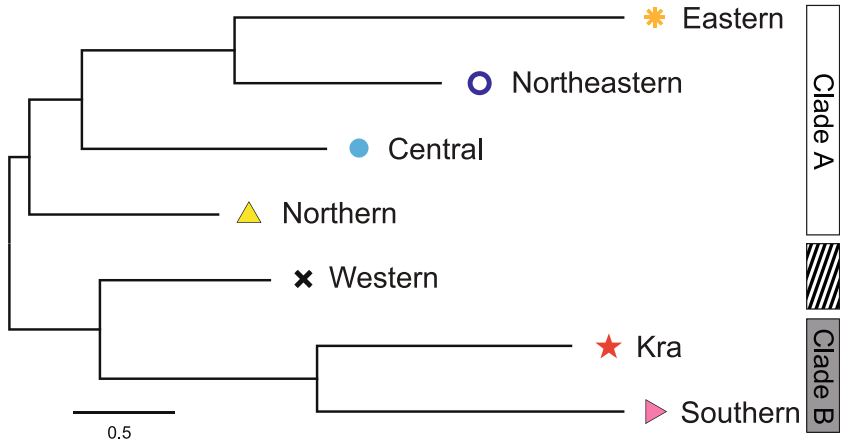

Figure 6. Neighbor-joining tree based on Mahalanobis distances between population centroids derived from canonical variate analysis.

a strong impediment to gene flow between clades A and B. We therefore speculate that this area acts as a natural barrier to gene flow between the two clades, and maintains their integrity. Thus, we propose that it is ecological factors rather than historical biogeography that drive the contemporary distribution of the clades of *T. collina*. In contrast, species like *A. cerana* that nest in tree cavities are able to survive prolonged periods of flooding. Thus, the central plane does not affect gene flow within honey bee species. Nonetheless, the structure of the *T. pagdeni* population of Thailand is also separated by the central plane (Thummajitsakul et al. 2008), yet this species also nests in trees above ground, and is therefore typically unaffected by seasonal flooding.

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AUTHORS' CONTRIBUTIONS

AR and OD conceived the study and collected the data. AR and EJ analyzed the data. AR and BPO wrote the paper. All authors approved the final manuscript.

Le séquençage mitochondrial et la morphométrie géométrique suggèrent l'existence de 2 taxons monophylétiques dans la population de *Tetragonilla collina* (Apidae : Meloponini) de Thaïlande

Abeille sans aiguillon / variation génétique / biogéographie / Isthme de Kra

Mitochondriale Sequenzdaten und geometrische Morphometrie deuten auf zwei monophyletische Gruppen in der Population von *Tetragonilla collina* (Apidae: Meliponini) in Thailand hin

Stachellose Bienen / genetische Variation / geometrische Morphometrie / Biogeographie / Isthmus von Kra

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