CHAPTER IV

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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