CHAPTER VI
DISCUSSION AND CONCLUSION

Trigona pagdeni which belongs to the taxonomically difficult subgenus *Tretragonula* of *Trigona*, is one of the most common stingless bees in Thailand (Sakagami, 1978). Species diagnostic marker for identification of the stingless bees (*Trigona pagdeni* Schwarz) was successfully developed. Initially, amplified fragment length polymorphism (AFLP) analysis was carried out across representatives of 12 stingless bee species using 64 primer combinations. A 284 bp band restrictively found in *T. pagdeni* was cloned and sequenced. A primer pair (CUTPTP 1-F/R) was designed and tested for specificity. In total, 100% (129/129 individuals) of *T. pagdeni* provided a 163 bp expected product. Nevertheless, the amplified CUTPTP was also found in *T. fimbriata* (1/3, 33.3%), *T. collina* (11/112, 9.8%), *T. laeviceps* (1/12, 8.3%) and *T. fuscobalteata* (15/15, 100%) but not the remaining species. SSCP analysis of the CUTPTP fragment could successfully differentiate *T. fuscobalteata* and *T. collina* from *T. pagdeni*, but no successful discrimination of *T. laeviceps* and *T. fimbriata* from *T. pagdeni*. Nevertheless, *T. pagdeni*, *T. laeviceps*, *T. collina* and *T. fimbriata* can be easily differentiated by using their morphology. The AFLP and SSCP analysis could be applied to establish the species-specific marker to support the taxonomic classification of *T. pagdeni* based on morphological characters. It may be useful to discriminate *T. pagdeni* from other *Trigona* species in the iridipennis group or in the subgenus *Tetragonula* which may play very similarity in morphology. The molecular marker at genetic level which was obtained could be used to verify their taxa of *T. pagdeni* for further studies to avoid sacrifice of specimens. Furthermore, molecular population genetic techniques can provide nesscesery informations to be fundamental for establishing management efficiency of resources for conservation of this native species. However, genetic diversity and population structure of stingless bees *T. pagdeni* in Thailand have not been studied by using nuclear DNA markers and mtDNA markers.

Samples of the stingless bee *Trigona pagdeni* Schwarz were collected from North, Northeast, Central and peninsular Thailand. Genetic variation and population structure were investigated using a DNA fingerprinting technique, TE-AFLP, and Analysis of Molecular Variance (AMOVA). TE-AFLP methods followed those of van der Wurff *et al.* (2000) using two primer pairs: a ^{32}P-labeled *Bam*HI primer with the arbitrary extension "C", paired with an *Xba*I primer with arbitrary extensions -CC or -AC. Amplified fragments were separated by electrophoresis for 5-6 hours at 500 V on 8% denaturing polyacrylamide gels. Dried gels were used to expose X-ray film. Presence of a TE-AFLP band was scored as 1, absence as 0; thus each bee was characterized by a multi-locus phenotype. A band was considered polymorphic if our samples showed any variation for presence or absence. Individuals were grouped into four populations based on collection site: North, Northeast, Central and Peninsular (Figure 3.1, Table 3.1). Percentage of polymorphic loci, expected heterozygosity, genetic distance among populations, and population structure statistics were calculated using Genetic Analysis in Excel (GenAlExV6) (Peakall and Smouse, 2006). We used AMOVA (Analysis of Molecular Variance; Excoffier *et al.*, 1992) to examine differentiation among populations. Because *Apis cerana* as well as other taxa show genetic differentiation between populations north and south of 11° N in the Isthmus of Kra (Hughes *et al.*, 2003; Woodruff, 2003; Warrit *et al.*, 2006), we tested for this pattern in our *T. pagdeni* samples. Based on genetic distances among populations, we also tested for differentiation between the bees from the Northeast vs. all other samples. Table 3.2 compares genetic diversity in the four populations of *T. pagdeni*. Expected heterozygosity and percentage of polymorphic loci were highest in the Northeast. Table 3.3 presents pairwise genetic distances among the four *T. pagdeni* populations; pairwise genetic distances were greater between the Northeast and other populations than between other pairs of populations. AMOVA results are presented in Tables 3.4 -3.5. These showed differentiation among all four populations ($\Phi_{PT} = 0.18$, $p = 0.001$), and between bees north and south of the Kra ecotone ($\Phi_{PT} = 0.13$, $p = 0.001$), but it was the contrast between Northeast bees and all other samples that accounted for the greatest proportion of observed genetic variation

(Φ_{PT} = 0.21, p = 0.001). This differs from the pattern observed for mitochondrial sequences of honey bees, *A. cerana*. Therefore, TE-AFLP method can be applied to the study of population structure in *T. pagdeni* and other stingless bees, and may provide a useful tool for management and conservation of this species.

Polymorphisms of the cyt b, ATPase(6,8) and 16S rRNA genes were also used to estimate genetic diversity and population structure of *T. pagdeni*. PCR-SSCP analyses of the cyt b, ATPase(6, 8) and 16S rRNA gene segments were done by using the primer pairs; cytb-F/cytb-R, ATPS6-F/tRNA-ASP-R and LR-F/LR-R, respectively. The expected PCR products were 600, 500 and 500 bp, respectively. Amplified fragments were separated on 11% non-denaturing polyacrylamide gels (75:1 crosslink) and run in a vertical electrophoresis at 12.5 V/cm at 4°C for 16 hours. SSCP variants were directly visualized in the gel by silver staining. Individuals were grouped into six populations (North, Northeast, Central, Prachuap Khiri Khan, Chumphon and Peninsular). *T. pagdeni* from North, Northeast, Central and Prachuap Khiri Khan populations were allocated to north of Isthmus of Kra, whereas samples from Chumphon and Peninsular populations were allocated to south of Isthmus of Kra. The proportion of polymorphic loci and the expected heterozygosity obtained from estimation of each mtDNA gene showed the greatest genetic diversity in Central population (Tables 4.1-4.3). AMOVA results of each mtDNA gene are presented in Tables 4.4-4.5. Strong population differentiation and restricted female gene flow (cyt b; Φ_{PT} = 0.35, P = 0.001, ATPase(6, 8); Φ_{PT} = 0.27, P = 0.001 and 16S rRNA; Φ_{PT} = 0.28, P= 0.001), were observed between different populations of Thai *T. pagdeni*. Siginificant genetic differentiation was also observed between samples from north and south of Isthmus of Kra (cyt b; Φ_{PT} = 0.15, ATPase(6, 8); Φ_{PT} = 0.20 and 16S rRNA; Φ_{PT} = 0.18, P =0.001, respectively). This resembled the pattern observed for the TE-AFLP result of *T. pagdeni*, but provided greater differentiation between bees from north and south of Isthmus of Kra than those of TE-AFLP analysis. The Isthmus of Kra was known as the Kra ecotone, where corresponds to the transition between seasonal evergreen or seasonal rainforest and mixed moist deciduous or monsoon forest, and between the Indochinese and Sundaic biotas (Hughes

et al., 2003). Shifts from one species or subspecies to another north and south of this ecotone have been documented in many animal taxa as well (e.g., birds,). For honey bees, *Apis cerana,* in Thailand using mtDNA (Smith and Hagen, 1996; Sihanuntavong *et al.*, 1999; Smith and Hagen, 1999 and Warrit *et al.*, 2006) and microsatellites analysis (Sittipraneed *et al.*, 2001) showed strong genetic differentiation among geographic regions within Thailand, particularly between populations from north and south of 11°N in the Isthmus of Kra (Warrit *et al.*, 2006). However, the greatest proportion of genetic differentiation between *T. pagdeni* samples from Northeast population and all other samples was observed by using TE-AFLP analysis but this pattern was not observed by mtDNA analysis. It may be explaned by the effect of the male component. The phylogeography of *T. pagdeni* in Thailand was also observed from a neighbor-joining tree constructed from genetic distances between pairs of geographic regions. This further supported the biogeographic differentiation of Thai *T. pagdeni* from mtDNA and TE-AFLP analysis. The ability to identify population differentiation within *T .pagdeni* is important for estabishing natural management of resources and conservation programs for this native species. The information suggests that *T. pagdeni* from north and south of the Isthmus of Kra of Thailand should be treated and genetically managed separately. Although the mtDNA and TE-AFLP analysis showed useful results for levels of genetic variation and differentiation in *T. pagdeni*, more mtDNA genes and nuclear DNA markers should be further studied for understanding genetic relationships of this species accurately and the exact area for biogeographic boundary in Thai *T. pagdeni* should be studied further.

However, the region of mtDNA gene proved in honey bees is a non-coding region between cytochrome c oxidase I and cytochrome c oxidase II genes. This region was known as hypervariable region (Cournuet *et al.*, 1991). A non coding region between COI and COII gene is not appeared in stingless bee at least 16 *Meliponini* species (Arias *et al.*, 2006; Silvestre *et al.*, 2008). For the *Trigona* genus, the information of mtDNA sequence is still limited. Therefore, much more information of mtDNA sequence of *Trigona* genus may provide application in other *Trigona* species. We

designed three sets of primer pairs (LR12647-R+COI2494, LR12677+cytb10729 and COIII9821+cytb5031, respectively) on known sequences (COI, 16S rRNA, cyt b and ATPase(6, 8)+COIII), which were used to amplify the three long PCR products (5855, 5089 and 4879 bp, respectively) (Figure 5.1). The three PCR products were purified and cloned into pGEM® T-easy vectors which were sequenced by primer walking. The known sequences of each fragment were then overlapped and analyzed. Ten internal primer pairs (Figure 5.3) were used in each long PCR reaction to verify the gene order on the overlapped fragment. The expected sizes of the PCR products were obtained (Figure 5.4). The partial mtDNA sequence was obtained with size 12,802 bp containing 12 protein coding genes (11 complete gene sequences and a COI partial sequence), both rRNA genes (12S and 16S rRNA) and 12 tRNA genes. The AT bias in codon usage of *T. pagdeni* revealed by the ratio of "G+C" (Pro, Ala, Arg and Gly) to "A+T" rich codons (Phe, Ile, Met, Tyr, Asn and Lys) was 0.20. The A+T content was very high in *T. pagdeni* mtDNA, similar those of *M. bicolor* and *A. mellifera*. Eleven protein coding genes of mtDNA were analyzed and translated into amino acid sequence. The standard insect mitochondrial genetic codes were used. Protein coding genes were started with ATA, ATG, ATC and ATT, and ended with TAA or TAG. Most of these mitochondrial genes were similar in length to their counterpart genes in either *M. bicolor* and *A. melliferra*. In addition, fourteen non-coding regions were observed with total intergenic region 419 bp. The non-coding region between COI and COII genes was absent in *T. pagdeni*. Four tRNA genes (tRNA-Glu, tRNA-Gln, tRNA-Thr and tRNA-Pro) of *T. pagdeni* are on different positions when compared with *M. bicolor*. Likewise, the rearrangement of 12S rRNA, 16S rRNA, ND1, cyt b and ND6 genes of *T. pagdeni* mtDNA was different from those of *M. bicolor*. This knowledge may provide critical information on biology, ecology and evolution at intra and inter-specific levels, and be useful for future mtDNA analyses of other stingless bees.