CHAPTER IV

GENETIC DIVERISTY AND POPULATION STRUCTURE OF STINGLESS BEES (*Trigona pagdeni* Schwarz) IN THAILAND DETERMINED BY MITOCHONDRIAL POLYMORPHISMS

INTRODUCTION

The eusocial stingless bees (Hymenoptera, Apidae, Meliponini) exhibiting great abundance in the tropical and subtropical parts of the world, are of high level of diversity in morphology and behavior (Michener, 1974; 1990; 2000). Besides playing an important role in crop pollination of 40-90 % of plant species in both nature and enclosures (Amano *et al.*, 1999; Heard, 1999; Slaa *et al.*, 2000), some species are commercially utilized in honey production (Del Sarto *et al.*, 2005) and in traditional beekeeping (Crane, 1992). In Thailand, a total of 32 stingless bees have been recorded into a genus *Trigona* (Klakasikorn *et al.*, 2005; Michener and Boongird, 2004). Of these, *Trigona pagdeni* is considered to be one of the commonest stingless bee species widely distributed in several regions of Thailand (Sakagami, 1978). The knowledge of genetic variation levels of *T. pagdeni* in Thailand is critical for understanding population structure of this species. The estimation and partitioning of the level of genetic variation within this species can provide fundamental for effective management and efficient conservation.

Mitochondrial DNA (mtDNA) is a small, circular molecule, with a high evolution rate (Gray, 1989). Generally, mtDNA is used for determining population dynamics, systematic, species and subspecies characterization, population structure, and phylogeny (Moritz *et al.*, 1987; Patarnello *et al.*, 1994; Weinlich *et al.*, 2004). In recent years, mtDNA has been commonly used to determine the levels of intraspecific genetic variation and population differentiation of honey bee, *A. cerana* in Thailand. PCR–RFLP of the large (16S rRNA) and small mitochondrial ribosomal subunits (12S rRNA) and the intergenic region of cytochrome c oxidase I (COI) and cytochrome c oxidase II (COII) using *Dra*I digestion revealed non-overlapping distribution of mitotypes between *A.*

cerana from the north-to-central region and peninsular Thailand (Sihanuntavong *et al.*, 1999). Likewise, RFLP patterns of the ATPase(6, 8) gene also revealed strong biogeography between the northern- and southern-latitude bees, especially different mitotype observed in Prachuap Khiri Khan (AAA) and Chumphon (BBB) indicated that the contact zone between bees from north and south of Isthmus of Kra is probably located between Prachuap Khiri Khan and Chumphon (Songram *et al.*, 2006).

In other bees, the portion of the cytochrome b (cyt b) and COI sequences of the mtDNA were used to determine the genetic structure of several *Bombus* species (Estoup *et al.*, 1996; Widmer *et al.*, 1998; Widmer and Schmid-Hempel, 1999). It has been reported that cyt b accumulates nucleotide changes at a rate that is sufficient to resolve phylogenetic relationships among closely related species (Collins *et al.*, 1996).

Additionally, the 16S rRNA gene is usually used in phylogenetic analysis among closely related species and among genus of several stingless bees (Whitfield and Cameron, 1998; Rasmussen and Cameron, 2007 and Costa *et al.*, 2003), because the 16S rRNA gene can give potential information in phylogenetic analysis among closely related species or populations, and among tribes, subfamilies, and families (Whitfield and Cameron, 1998; Rasmussen and Cameron, 2007; Costa *et al.*, 2003).

The goal of this study was to survey genetic diversity and population structure of *T. pagdeni* in Thailand by using polymorphisms of the cyt b, 16S rRNA and ATPase(6, 8) genes of *T. pagdeni*. Here, single strand conformational polymorphism (SSCP) analysis was used, to investigate the polymorphism because it is relatively sensitive method for determining genetic variation, convenient, rapid and inexpensive (Weder *et al.*, 2001).

MATERIAL AND METHODS

Sampling

Trigona workers were collected from each locality in Thailand (Figure A.1 and Table A.1, APPENDIX A). Specimens were stored in 95% ethanol and maintained at 4°C until required. Taxonomic identification was performed according to Sakagami (1978) and Sakagami *et al.* (1983), and kindly verified by Professor Charles D. Michener (University of Kansas).

DNA extraction

Genomic DNA of *T. pagdeni* was extracted from one entire bee per nest according to the procedure of Smith and Hagen (1996) with a few modifications. DNA concentration was semiquantitated on 1.0% agarose gel and assessed by comparison with a known quantity of digested lambda DNA (Sambrook *et al.*, 2001).

PCR and development primers

The cyt b gene sequences (432 bp) was known from GenBank (accession no. AY575080), whereas the portions for the 16S rRNA and ATPase(6, 8)+COIII genes of *T. pagdeni* were amplified using the specific primers (Table A.6; APPENDIX A). The 16S rRNA and ATPase(6, 8)+COIII gene fragments (550 bp and 1700 bp, respectively) were amplified by PCR conditions described in Table A.7; APPENDIX A. The PCR products were then purified (QIAGEN) and cloned into pGEM® -T easy vector (Hoelzel and Green, 1992). The insert sizes were verified by colony PCR. Plasmid DNA was extracted from recombinant clones and sequenced for both directions. The known sequences of the cyt b and 16S rRNA genes were used to design the primers (Fast PCR program version 5.2.21; Kalenda, 2007) used for genome walking technique (Topic 5.2 and 5.3; APPENDIX A). After the flanking, regions of known sequence are known from genome working, then the specific primer pairs of the cyt b, 16S rRNA and ATPase(6, 8) genes were designed from each known fragment. The expected sizes of PCR products of the cyt b, 16S rRNA and ATPase(6, 8) genes were 600, 500 and 500 bp, respectively.

PCR-SSCP analysis

The polymerase chain reaction (PCR) was used to amplify a 600 bp segment of the cytb gene (n = 122) using the primers cytb-F (5'-TTCACTATATTATAAAAGAT GTAAGTTC-3') and cytb-R (5'-GGCAAAAAGAAAATATCATTCAGG-3'). A 500 bp segment of the ATPase(6, 8) (n = 98) and 16S rRNA gene (n = 129) was also amplified using the primer pairs ATPS6-F (5'-AAGATATATGGAAATAAGCT-3')/tRNA-ASP-R (5'-ATAAAATAACGTCAAAATGTCA-3') and LR-F (5'-TGGCTGCAGTATAACTG ACTGTACAAAGG-3') / LR-R (5'-GAAACCAATCTGACTTACGTCGATTTGA-3'), respectively. PCR was performed on a Perkin Elmer 9700 thermocycler in a reaction mixture containing10mM Tris-HCl (pH 8.8), 50 mM KCl and 0.1%Triton X-100,2.0 mM $MgCl_2$, 100 µM dNTPs, 1 unit of DyNAzyme™ DNA polymerase (Finnzymes, Finland), 0.1 µM of each primer. After an initial denaturation at 95°C for 3 minutes and 5 cycles of low stringent amplification (94°C for 30 seconds, 42°C for 30 second; and 72 °C for 50 seconds or 60 seconds) was performed, followed by 35 cycles of higher stringent conditions (94°C for 30 seconds; 58-62°C for 30 seconds; and 72°C for 50 seconds- 60 seconds) and a final extension at 72°C for 7 minutes. PCR products were analyzed by 1.0% agarose gel electrophoresis.

For SSCP analysis, 5 µl of the PCR product of each individual was added with 4 volumes of the loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH), denatured at 95°C for 3 minutes, immediately placed on ice for 2 minutes, then loaded in 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 (Klinbunga *et al.*, 2006).

Data analysis

SSCP bands were scored as present (1) or absent (0) for each sample. The binary data matrix was input into GenAlEx program (Peakall and Smouse, 2006). The following indices were used to quantify the amount of genetic diversity within each population (North, Northeast, Central, Prachapkhirikhan, Chumphon and Peninsular)

examined: the percentage of polymorphic loci (P) and the mean expected heterozygosity (H_e) (Lynch and Milligan, 1994). Bands that were unique to a population, or to a region, were treated as private fragments. To compute the degree of population differentiation of *T. pagdeni*, an analysis molecular variance (AMOVA) was conducted (Excoffier *et al.*, 1992). The population genetic structure of *Trigona pagdeni* was estimated using Analysis of Molecular Variance (AMOVA; Excoffier *et al.*, 1992) implemented in GenAlEx (Peakall and Smouse, 2006). The significance of covariance components was tested using a random permutation test (999 permutations). The Phi (Φ) statistics are analogous to traditional Wright's F-statistics for co-dominant data (Wright, 1951; 1965). Increasing positive Φ_{PT} values (between 0-1) suggest increasing genetic differentiation among populations.

Additionally, genetic distances (Nei, 1978) between pairs of populations were calculated using GenAlEx V6 (Peakall and Smouse, 2006). A neighbor-joining tree based on genetic distances was constructed using Neighbor-Joining method (Saitou and Nei, 1987) in MEGA 4 (Tamura *et al.*, 2007).

For geographic pattern, *T. pagdeni* samples were divided into six populations (North, Northeast, Central, Prachuap Khiri Khan, Chumphon and Peninsular populations). Samples from North, Northeast, Central and Prachuap Khiri Khan populations were allocated to north of the Isthmus of Kra or the Asian mainland group (ranged from 20 °N to 11 °N), whereas samples from Chumphon and Peninsular populations (ranged from 11 °N to 5 °N) were allocated to south of the Isthmus of Kra or the Sundaland group. Analysis of genetic differentiation among six populations and between north and south of Isthmus of Kra were performed using significance testing by comparing observed statistics to the distributions generated by 999 random permutations of the data into populations of the same size.

RESULTS

The three mtDNA gene segments (cyt b, ATPase(6, 8) and 16S rRNA) were chosen for genetic diversity and population structure studies of *T. pagdeni* in different geographic localities in Thailand. The specific primer pairs of the cyt b, ATPase (6, 8) and 16S rRNA genes were successfully amplified when genomic DNA was used as the template. The PCR products of each individual of *T. pagdeni* were analyzed by SSCP analysis to examine mtDNA polymorphisms (Figure 4.1 – 4.3; APPENDIX H). The results of each mtDNA gene indicated high variation of SSCP banding patterns in each individual of *T. pagdeni*. These mtDNA genes were scored for the presence (1) or absence (0) of SSCP bands as the dominant marker (homozygotes and heterozygotes can not be discriminated). Since inconsistent amplifications in each mtDNA gene were found, the PCR products obtained from each *T. pagdeni* mtDNA gene amplification were: cyt b gene:122/129 accounting for 95% of investigated individuals, 16S rRNA gene: 129/129, 100% and ATPase(6, 8) gene: 98/129, 76%. Therefore, the results were separately analyzed for each mtDNA gene. The SSCP banding patterns for the cytb, 16S rRNA and ATPase(6, 8) genes could be unambiguously scored. The results of mtDNA genes showed that no monomorphic fragments were observed for all populations of *T. pagdeni*.

Forty-seven unique SSCP phenotypes were obtained from the cyt b amplification (APPENDIX G). Exsample of banding patterns were shown in Figure 4.1. No common bands were presented in all 122 *T. pagdeni*. Sixteen scored fragments were considered polymorphic. Thirteen of these bands (81%) had inferred allele frequencies in the range of 5% to 95%. The mean expected heterozygosity, H_e, was 0.182 (st. error 0.100).

For the ATPase(6, 8) gene, forty-one unique SSCP patterns were observed (APPENDIX G). Esample of banding patterns were shown in Figure 4.2. No common bands were presented in all 98 *T. pagdeni*. The 21 SSCP bands surveyed were considered polymorphic. Nine of these bands (43%) had inferred allele frequencies in the range of 5% to 95%. The mean expected heterozygosity, H_e, was 0.102 (st. error 0.074).

Additionally, thirty-nine unique SSCP patterns obtained from the 16S rRNA amplification were observed (APPENDIX G) and some banding patterns are shown in Figure 4.3. No common bands were presented in all 129 *T. pagdeni*. Seventeen SSCP scored fragments were considered polymorphic. Nine of these bands (53%) had inferred allele frequencies in the range of 5% to 95%. The mean expected heterozygosity, H_e, was 0.124 (st. error 0.088). The genetic diversity for the cytb gene, one private band was presented in only north population. The genetic diversity quantified by the percentages of polymorphic loci and the expected heterozygosity were high in Central, Prachuap Khiri Khan and Northeast population (P = 81%; H_e = 0.148; P = 69%, H_e = 0.139 and P = 69%; H_e = 0.134, respectively), and lower in Peninsular, North and Chumphon population (P = 50%, H_e = 0.161, P = 50 %, H_e = 0.146 and P = 25 %, H_e = 0.060, respectively). For the ATPase (6, 8) gene, the number of private fragments observed in Northeast, Central and Prachuap Khiri Khan population were 2, 1 and 1, respectively. We found high levels of genetic diversity in Central, Prachuap Khiri Khan and Northeast population (P = 67%; H_e = 0.092, P = 43%; H_e = 0.085 and P = 33%, H_e = 0.085, respectively) and lower in North, Chumphon and Peninsular population (P = 29%; H_e = 0.078 and P = 29%, H_e = 0.061 and P = 14%; H_e = 0.042, respectively) (Table 4.2). For the 16S rRNA gene, one private band in Central population was detected, and another one was specific for Peninsular population. The polymorphic loci and the mean expected heterozygosity for each population were given in Table 4.3. The both values exhibited a similar trend. Among the six populations, Central, Northeast and Prachuap Khiri Khan populations were of high level of variability (P = 88%; H_e = 0.112, P = 59%; H_e = 0.107; and P = 59%; H_e = 0.107, respectively), while lower diversity was observed in the remaining geographic samples; Peninsular, North and Chumphon (P = 35%, 35% and 18%, H_e = 0.096, 0.098 and 0.033, respectively).

When statistics of genetic diversity were compared among the cyt b, ATPase(6, 8) and 16S rRNA genes, the results of each mtDNA gene revealed a similar trend but the cyt b gene provided the highest value of genetic diversity in each population than those of 16S rRNA and ATPase(6, 8) genes.

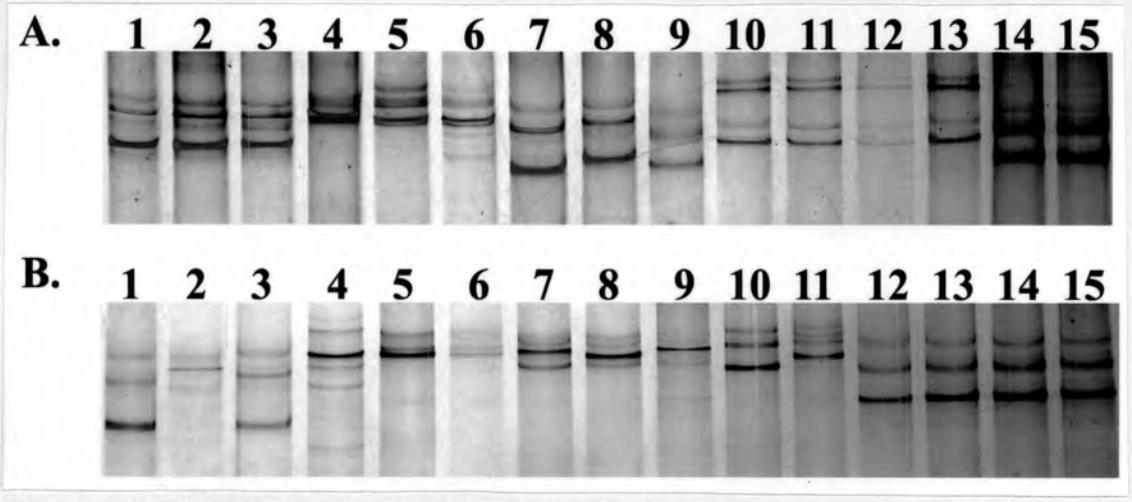

Figure 4.1 The SSCP pattern of the amplified cytb gene of *T. pagdeni* from different geographic regions in Thailand; North population (lanes 1-3, A), Northeast population (lanes 4A and 7-11, B), Central population (lanes 5-8, A and 3-6, B), Peninsular population (lanes 9-15, A), Chumphon population (lanes 12-15, B), and Prachuap Khiri Khan population (lanes 1-2, B).

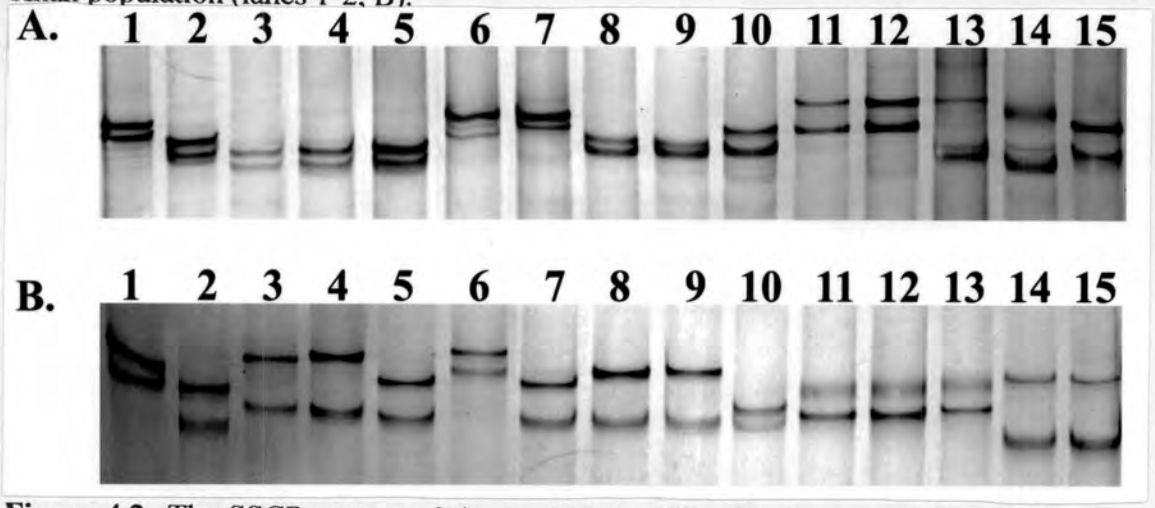

Figure 4.2 The SSCP pattern of the amplified ATPase(6, 8) gene of *T. pagdeni* from different geographic regions in Thailand; North population (lanes 1A and 1-2, B), Northeast population (lanes 2-7,A and 8-10, B), Central population (lanes 3-7, B), Peninsular population (lanes 14-15, B), Chumphon population (lanes 11-13, B), and Prachuap Khiri Khan population (lanes 8-15, B).

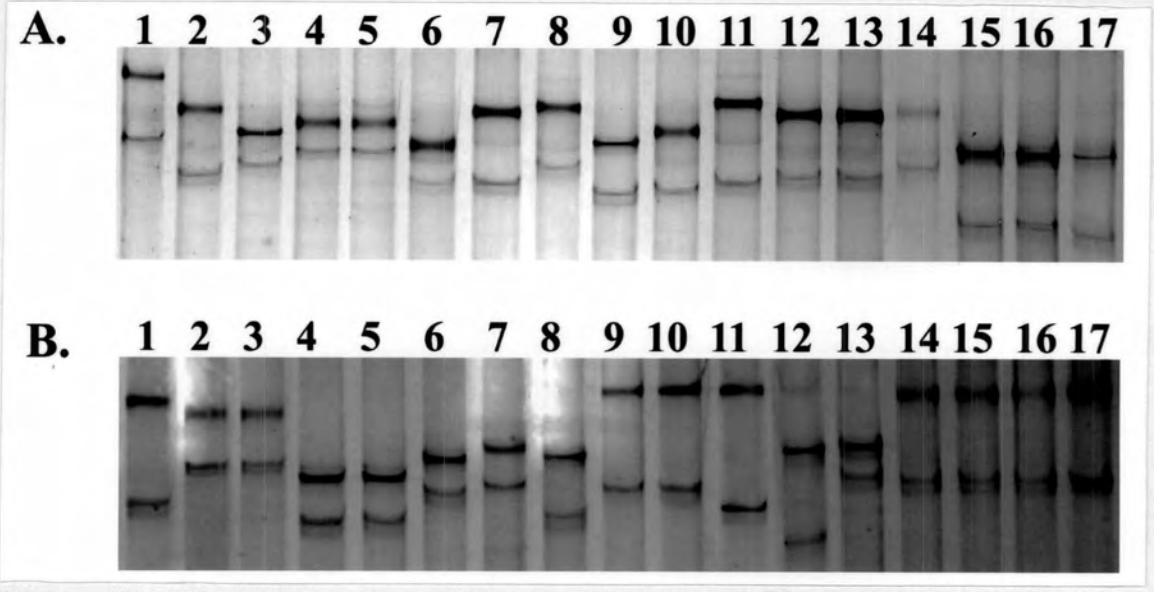

Figure 4.3 The SSCP pattern of the amplified 16S rRNA gene of *T. pagdeni* from different geographic regions in Thailand; North population (lanes 1-2, A and 1B), Northeast population (lanes 9-13, A and 8-11, B), Central population (lanes 3-8, A and 2-5, B), Prachuap Khiri Khan population (lanes 6-7, B), Peninsular population (lanes 15-17, A and 12-14, B), and Chumphon population (lanes 14A and 15-17, B).

Table 4.1 Summary of comparison of genetic diversity in six populations of the cyt b gene (600 bp) in *T. pagdeni* in Thailand computed from the SSCP binary data.

Bands	North (13)	Northeast (24)	Central (33)	Prachuap Khiri Khan (19)	Chumphon (16)	Peninsular (17)
Number scored	8	11	13	11	5	8
Number fixed	1	0	0	0	0	0
Number absent	7	5	3	5	11	8
No. of polymerphic loci	8	11	13	11	4	8
% polymorphic	100% of 8 (50% of 16)	100% of 11 (69% of 16)	100% of 13 (81% of 16)	100% of 11 (69% of 16)	80% of 5 (25% of 16)	100% of 8 (50% of 16)
Loci						
# loci with inferred allele frequencies 5-95%	7	7	10	9	2	6
% with inferred allele frequencies 5-95%	88% of 8 (44% of 16)	64% of 11 (44% of 16)	77% of 13 (62% of 16)	82% of 11 (56% of 16)	40% of 5 (12% of 16)	75% of 8 (38% of 16)
mean *He* (std. error)	0.146 (0.044)	0.134 (0.038)	0.148 (0.031)	0.139 (0.041)	0.060 (0.035)	0.161 (0.051)

Table 4.2 Summary of comparison of genetic diversity in six populations of the ATPase(6, 8) gene (500 bp) in *T. pagdeni* in Thailand computed from the SSCP binary data.

Bands	North (9)	Northeast (23)	Central (29)	Prachuap Khiri Khan (18)	Chumphon (14)	Peninsular (5)
Number scored	6	7	14	9	6	4
Number fixed	0	2	1	1	0	0
Number absent	15	12	6	11	15	18
No. of polymerphic loci	6	7	14	9	6	3
% polymorphic	100% of 6 (29% of 21)	100% of 7 (33% of 21)	100% of 14 (67% of 21)	100% of 9 (43% of 21)	100% of 6 (29% of 21)	75% of 4 (14% of 21)
Loci						
# loci with inferred allele frequencies 5-95%	6	5	9	9	3	3
% with inferred allele frequencies 5-95%	100% of 6 (28% of 21)	71% of 7 (24% of 21)	64% of 14 (43% of 21)	100% of 9 (43% of 21)	50% of 6 (14% of 21)	75% of 4 (14% of 21)
mean *He* (std. error)	0.078 (0.030)	0.085 (0.031)	0.092 (0.022)	0.085 (0.028)	0.061 (0.029)	0.042 (0.024)

Table 4.3 Summary of comparison of genetic diversity in six populations of the 16S rRNA gene (500 bp) in *T. pagdeni* in Thailand computed from the SSCP binary data.

Bands	North (13)	Northeast (24)	Central (32)	Prachuap Khiri Khan (20)	Chumphon (17)	Peninsular (23)
Number scored	6	10	15	10	4	6
Number fixed	0	0	1	0	0	1
Number absent	11	7	1	7	14	10
No. of polymerphic loci	6	10	15	10	3	6
% polymorphic	100% of 6 (35% of 17)	100% of 10 (59% of 17)	100% of 15 (88% of 17)	100% of 10 (59% of 17)	75% of 4 (18% of 17)	100% of 6 (35% of 17)
Loci						
# loci with inferred allele frequencies 5-95%	5	7	8	9	1	5
% with inferred allele frequencies 5-95%	83% of 6 (29% of 17)	70% of 17 (41% of 17)	53% of 15 (47% of 17)	45% of 20 (53% of 17)	25% of 17 (6% of 17)	83% of 6 (29% of 17)
mean *He* (std. error)	0.098 (0.039)	0.107 (0.032)	0.112 (0.020)	0.107 (0.029)	0.033 (0.026)	0.096 (0.041)

AMOVA results were analyzed separately for the cyt b, ATPase(6, 8) and 16S rRNA genes in *T. pagdeni*. AMOVA analysis of polymorphisms of the cytb, ATP(6, 8) and 16S rRNA genes in each individual of *T. pagdeni* from 6 population groupings (North, Northeast, Central, Prachuap Khiri Khan, Chumphon and Peninsular) revealed high molecular variability which was attributed to differences among populations (35%, Φ_{PT} = 0.35, P = 0.001; 27%, Φ_{PT} = 0.27, P = 0.001 and 28%, Φ_{PT} = 0.28, P = 0.001, respectively), and 65%, 73% and 72% of observed variation occurred among individuals within populations of *T. pagdeni* (Table 4.4).

When samples of *T. pagdeni* were grouped into two populations: South (same as Peninsular population) and North of Isthmus of Kra, high genetic differences could be detected between bees from north and south of the Isthmus of Kra by analyzing polymorphisms of the cyt b, ATPase(6, 8) and 16S rRNA genes; 15% (Φ_{PT} = 0.15, P =0.001), 20% (Φ_{PT} = 0.20, P = 0.001) and 18% (Φ_{PT} = 0.18, P = 0.001), respectively (Table 4.5).

Table 4.4 Analysis of Molecular Variance (AMOVA) of polymorphisms of the cyt b , ATPase(6,8) and 16S rRNA gene in each individual of *T. pagdeni* from 6 population groupings (North, Northeast, Central, Prachapkhirikhan, Chumphon and Peninsular). df = degrees of freedom, SS = sums of squares, MS = mean squares, Est. Var. = estimated variance within and among populations, % = percentage of observed variance within or among populations, Φ_{PT} = correlation of individuals within populations relative to the collection as a whole. Significance testing was carried out by comparison of observed values to those generated by 999 permutations in which individuals were randomly assigned to four populations of the same size as the four study populations. Prob. = the probability of obtaining a value of Φ_{PT} as large or larger when individuals are randomly assigned to groups.

		df	SS	MS	Est. Var.	%	Φ_{PT} (p)
Cytb	**Among Populations**	5	89.234	17.847	0.821	35%	0.35 (P = 0.001)
	Within Populations	116	173.717	1.498	1.498	65%	
ATPase (6, 8)	**Among Populations**	5	45.017	9.003	0.494	27%	0.27 (P = 0.001)
	Within Populations	92	123.370	1.341	1.341	73%	
16S rRNA	**Among Populations**	5	57.972	11.594	0.487	28%	0.28 (P = 0.001)
	Within Populations	123	156.849	1.275	1.275	72%	

Table 4.5 Regional differentiation: AMOVA of polymorphisms of the cytb, ATPase (6,8) and 16S rRNA gene in each individual of *T. pagdeni.* Samples of *T. pagdeni* were grouped into two populations: South (same as Peninsular population) and North of the Kra ecotone. Significance testing by comparison of observed values to those generated by 999 random permutations of individuals into populations of the same size. Other abbreviations are as in Table 4.4.

		df	SS	MS	Est. Var.	%	Φ_{PT} (p)
Cytb	**Among Populations**	1	19.701	19.701	0.367	15%	0.15 (P =0.001)
	Within Populations	120	243.250	2.027	2.027	85%	
ATPase (6,8)	**Among Populations**	1	13.728	0.396	13.728	20%	0.20 (P =0.001)
	Within Populations	96	1.611	1.611	154.660	80%	
16S rRNA	**Among Populations**	1	20.335	20.335	0.341	18%	0.18 (P =0.001)
	Within Populations	127	194.487	1.531	1.531	82%	

When pairwise population Φ_{PT} values were estimated from the binary matrix of each mtDNA gene, the result of the cytb gene showed high differentiation between paired populations. For the results of ATPase(6, 8) and 16S rRNA genes indicated that low genetic divergence was found between pairs of populations within the northern and southern samples but larger divergence was found between samples from different regions (Table 4.6). Furthermore, pair-wise genetic distance values of the cytb gene were calculated. Genetic distance was observed between pairs of populations ranged from 0.041 (between Central and Prachuap Khiri Khan) to 0.303 (between North and Chumphon). The pair-wise genetic distance comparisons among populations analyzed by the ATPase(6, 8) gene were ranged from 0.025 (between Northeast and Central) to 0.131 (between Chumphon and Peninsular). For the 16S rRNA gene, the pair-wise genetic distance comparisons among populations were ranged from 0.021 (between Northeast and Central) to 0.162 (between Chumphon and Northeast) (Table 4.7).

Using Nei's genetic distances from the three mtDNA genes and the neighbor joining method of clustering, the neighbor-joining trees of relationships among six populations based on cytb gene (Figure 4.4) revealed two different lineages in it. The Prachuap Khiri Khan population had position within the six populations, near the Chumphon population.

Nevertheless, the neighbor-joining tree analysis of ATPase(6, 8) gene in Figure 4.5; the Chumphon and Peninsular populations were grouped together. The North, Northeast, Central and Prachuap Khiri Khan had unique branch. Likewise, the neighbor-joining tree analysis of 16S rRNA gene based on genetic distance values between pairs of samples (Figure 4.6) also revealed phylogeographic differentiation between bees north and south of the Isthmus of Kra.

Table 4.6 Φ_{PT} values and Nm (number of migrants) analysis between paired geographic samples of *T. pagdeni*

Pairwise comparison	Cyt b gene			ATPase (6, 8) gene			16S rRNA gene		
	Φ_{PT}	Nm	P-value	Φ_{PT}	Nm	P-value	Φ_{PT}	Nm	P-value
North & Northeast	0.398	0.378	0.001	0.327	0.514	0.001	0.238	0.802	0.001
North & Central	0.280	0.644	0.001	0.215	0.915	0.001	0.139	1.544	0.001
Northeast & Central	0.160	1.312	0.001	0.096	2.350	0.002	0.051	4.611	0.016
North & Prachuap Khiri Khan	0.407	0.364	0.001	0.123	1.778	0.021	0.267	0.685	0.001
Northest & Prachuap Khiri Khan	0.299	0.587	0.001	0.262	0.703	0.001	0.165	1.264	0.001
Central & Prachuap Khiri Khan	0.094	2.423	0.001	0.127	1.716	0.003	0.062	3.760	0.005
North & Chumphon	0.670	0.123	0.001	0.477	0.274	0.001	0.582	0.180	0.001
Northest & Chumphon	0.599	0.167	0.001	0.455	0.300	0.001	0.536	0.216	0.001
Central & Chumphon	0.388	0.395	0.001	0.305	0.570	0.001	0.390	0.390	0.001
Prachup Khiri khan & Chumphon	0.321	0.528	0.001	0.322	0.526	0.001	0.297	0.591	0.001
North & Peninsular	0.311	0.555	0.001	0.472	0.279	0.002	0.412	0.357	0.001
Northest & Peninsular	0.406	0.366	0.001	0.410	0.360	0.001	0.351	0.461	0.001
Central & Peninsular	0.290	0.612	0.001	0.284	0.630	0.001	0.232	0.826	0.001
Prachuap Khiri Khan & Peninsular	0.418	0.348	0.001	0.383	0.403	0.001	0.242	0.785	0.001
Chumphon & Peninsular	0.635	0.143	0.001	0.601	0.166	0.002	0.559	0.197	0.001

Table 4.7 Pair-wise genetic distance of the cytb, ATPase(6, 8) and 16S rRNA genes. SSCP variance among six populations of *Trigona pagdeni* in Thailand, computed in GenAlEx6 (Peakall and Smouse, 2006).

		North	Northeast	Central	Prachuap Khiri Khan	Chum Phon	Peninsular
cytb	North (n = 13)	*					
	Northeast (n = 24)	0.187	*				
	Central(n = 33)	0.138	0.064	*			
	Prachuap Khiri Khan (n = 19)	0.200	0.116	0.041	*		
	Chumphon(n = 16)	0.303	0.248	0.142	0.072	*	
	Peninsular(n = 17)	0.139	0.191	0.140	0.208	0.285	*
ATPase(6,8)	North (n = 9)	*					
	Northeast (n = 23)	0.083	*				
	Central(n = 29)	0.061	0.025	*			
	Prachuap Khiri Khan (n = 18)	0.033	0.063	0.034	*		
	Chumphon(n = 14)	0.099	0.110	0.074	0.065	*	
	Peninsular (n = 5)	0.125	0.114	0.089	0.108	0.131	*
16S rRNA	North (n = 13)	*					
	Northeast (n = 24)	0.073	*				
	Central(n = 32)	0.047	0.021	*			
	Prachuap Khiri Khan (n = 20)	0.085	0.053	0.025	*		
	Chumphon(n = 17)	0.124	0.162	0.110	0.056	*	
	Peninsular (n = 23)	0.120	0.108	0.070	0.066	0.127	*

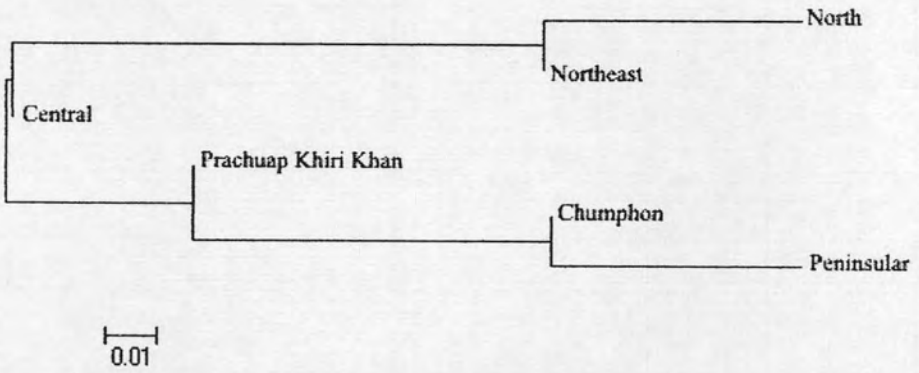

Figure 4.4 A neighbor-joining tree summarizing genetic relationships between each population of *T. pagdeni* in Thailand analyzed by polymorphisms of the cytb gene.

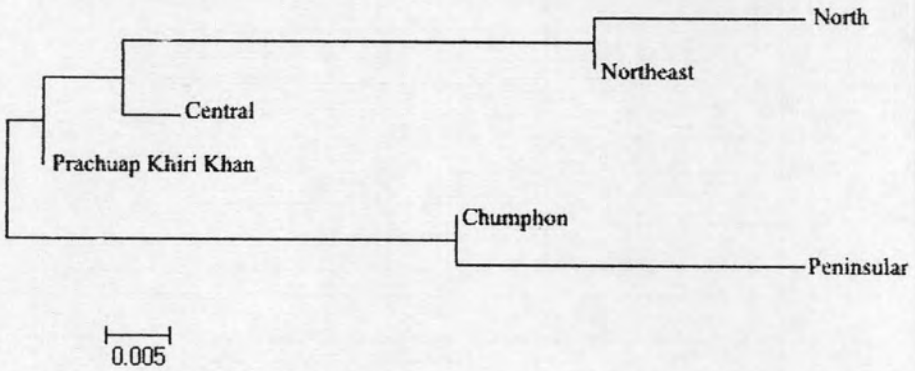

Figure 4.5 A neighbor-joining tree summarizing genetic relationships between each population of *T. pagdeni* in Thailand analyzed by polymorphisms of the ATP(6, 8) gene.

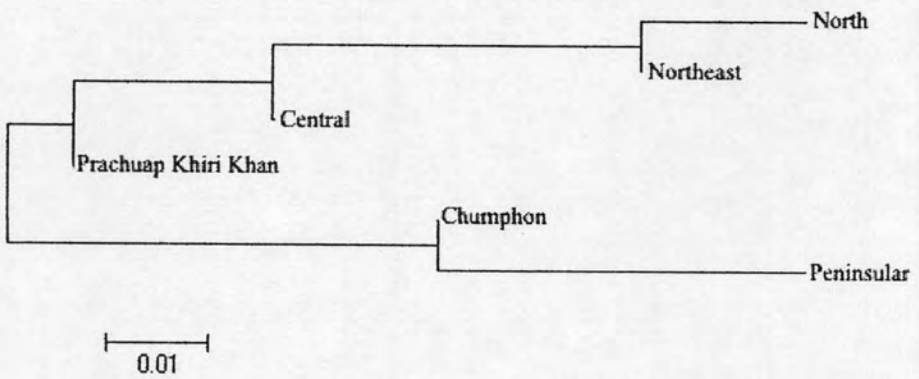

Figure 4.6 A neighbor-joining tree summarizing genetic relationships between each population of *T. pagdeni* in Thailand analyzed by polymorphisms of the 16S rRNA gene.

DISCUSSION

Accurate estimation and partitions of genetic diversity in any species are very useful for conserving and managing natural genetic resources (Avise, 1994). In the present study, the detection of high levels of polymorphisms for the cytb, ATPase(6, 8) and 16S rRNA gene (P: 25 to 81%; H_e: 0.060 to 0.148; P: 14.29 to 67%; H_e: 0.042 to 0.092; P: 17.65 to 88%; H_e: 0.107 to 0.112 respectively) maked SSCP analysis a powerful tool for assessing genetic diversity in *T. pagdeni*. Additionally, the number of haplotypes analyzed from polymorphisms of the cyt b gene was 47 patterns, corresponding to the results of the ATPase(6, 8) (41 patterns) and 16S rRNA genes (39 patterns) that *T. pagdeni* species revealed high levels of genetic diversity.

However, using SSCP analysis, the statistic values for the cytb gene analysis were higher than those of the ATPase(6, 8) and 16S rRNA genes. Although comparing these three genes could be problematic due to differences in sampling sizes used to examine the data, the same sample set was used.

Earlier findings, Franck and coworkers (2004) studied genetic diversity of the *T. cabonaria* species group (*Trigona carbonaria*, *T. hockingsi* and *T. davenporti*) from eastern Australia using 13 microsatellite loci. In addition, *T. laeviceps* (Chiang Mai, northern Thailand and Chantraburi, eastern Thailand; 41 colonies), *T. collina* (Chantraburi, eastern Thailand; 35 colonies) and *T. pagdeni* (Chantraburi, eastern Thailand; 11 colonies) were also collected and genotyped. The results suggested that proportions of polymorphic loci at the 95% and 99%, the allele and gene diversities were particularly high in *T. pagdeni*
(83%, 92%, 3.58 and 0.42, respectively).

AMOVA analysis for the cytb, ATPase(6, 8) and 16S rRNA genes of *T. pagdeni* samples revealed genetic differentiation among six populations (the Peninsular, Chumphon, Prachuap Khiri Khan, Central, North and Northeast populations). Partition of genetic variance of the cyt b, ATPase(6, 8) and 16S rRNA genes indicated significant genetic differentiation between samples from north and south of the isthmus of Kra. Among the results of the cyt b, ATPase(6, 8) and 16S rRNA genes, the ATPase(6, 8)

gene showed the highest genetic differentiation between samples from north and south of the isthmus of Kra.

Φ_{PT} values and Nei's genetic distances for pair-wise comparisons analyzed by polymorphisms of the cyt b, ATPase(6, 8) and 16S rRNA genes of geographic samples of *T. pagdeni* showed a similar trend, especially ATPase(6, 8) and 16S rRNA genes showed greater values when those were comparing the northern (North, Northeast, Central and Prachuap Khiri Khan) to southern regions (Chumphon and Peninsular).

A neighbor joining tree based on genetic distances of the ATPase(6, 8) and 16S rRNA genes revealed two major clusters corresponding to geographical regions within the distribution of *T. pagdeni*. The two clusters included north of Isthmus of Kra group (North, Northeast, Central and Prachuap Khiri Khan), and south of Isthmus of Kra group (Chumphon and Peninsular). The neighbor joining tree indicated a clearly split between north of Isthmus of Kra and south of Isthmus of Kra.

Smith *et al.* (2000) examined biogeography of *Asian* honey bees, *A. cerana* using sequences of a noncoding region between tRNA-Leu and COII of the mitochondrial genome. The results indicated four groups of mitochondrial haplotypes, including an Asian mainland group (Japan, Korea, China, Nepal, northern and southern Vietnam, northern Thailand, and some of the bees from India), a Sundaland group (peninsular Thailand and Malaysia, Samui and Phuket islands, Borneo, Java, Bali, Lombok, Timor and Flores, and Sulawesi), a Palawan group, and a Luzon-Mindanao group. It appears that changing in the sea level during Pleistocene glaciations can influence to the geographic distribution of mtDNA haplotypes.

Thus, research on mtDNA polymorphism 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 in the Isthmus of Kra. However, the exact area for the shift from the Asian mainland to the Sundaland mitotypes of honey bees, *A. cerana* in Thailand has not been studied.

Strong and significant differences had been also reported in honey bees, *Apis cerana* in Thailand between north and south of isthmus of Kra based on mtDNA (Sihanuntavong *et al.*, 1999; Smith *et al.*, 1999; 2005; Sittipraneed *et al.*, 2001; Warrit *et al.*, 2006) and microsatellites (Sittipraneed *et al.* 2001). Hughes *et al.* (2003) suggested that the Isthmus of Kra is the region corresponded to climate changes between seasonal evergreen or seasonal rainforest and mixed moist deciduous or monsoon forest.

In present study, the analysis of population structure within *T. pagdeni* samples showed differentiation among six populations (Peninsular, Chumphon, Prachuap Khiri Khan, Central, North and Northeast). There was significant differentiation between bees from north and south of Isthmus of Kra. This pattern was similar to those of *A. cerana* and *T. pagdeni* previously reported. However, the greatest proportion of genetic differentiation between Northeast bees and all other samples combined was not observed by using polymorphisms of mtDNA. This differred from the pattern obtained from the TE-AFLP result of *T. pagdeni*. The SSCP analysis was successfully used to examine intraspecific genetic variability and population subdivision of *T. pagdeni*. This may give useful information for decisions in conservation programs of this native species, whose natural habitat is declined because of human activities (see, for example, Araujo *et al.*, 2004). Nowadays, *T. pagdeni* is introduced to domesticate in boxes. Thus human activities may have influence on mixing of genetically divergent populations.