CHAPTER IV

DISCUSSION

Geographic distribution of the honeybee, *A. cerana* in Thailand based on morphometric approach was studies by Limbipichai (1990) using Canonical and Cluster analyses. The former divided *A. cerana* to two different groups; the Northern and Southern latitudes whereas the latter could further dissociate the Samui sample from the Southern group. In addition, genetic diversity of *A. cerana* in Thailand has been studies by several molecular techniques.

Pramual (1994) demonstrated that *A. cerana* gene pool was not homogeneous but fragmented to several groups using RFLP analysis with *Bgl*II, *Cla*I, *Eco*RI, *Hae*II, and *Nde*I. The *Eco*RI-restricted DNA probed with a probed no.3035 clearly illustrated the genetic differences between *A. cerana* originating from the Samui Island and other geographic locations.

Uthaisang (1994) investigated genetic variation in this species using the same approach as did Pramul (1994). Six different RFLP patterns were observed when *Hae*III digested total DNA of *A. cerana* was probed with the DNA probe no. 99 (repetitive sequence). Besides the nuclear DNA markers, level of genetic variability in *A. cerana* was also examined using mtDNA polymorphism. Several mtDNA genes were amplified by PCR and digested with informative restriction endonucleases (hereafter called PCR-RFLP). A total of 16 single haplotype was found from digestion of sRNA gene (3), lrRNA gene (5) and the intergenic COI-COII region (8) with *Dra*I (TTT↓AAA) (Sihanuntavong, 1997). The additional analysis of genetic structure in *A. cerana* by PCR-RFLP of the amplified ATPase6-ATPase8 with *Taq*I (T↓CGA), *Ssp*I (AAT↓ATT) and *Vsp*I (AT↓TAAT) generated 2, 5 and 6 restriction patterns, respectively (Songram, 1997). The results from both experiments allocated five investigated

samples (North, North-East, Central, South and Samui Island) to be three heterogeneous populations.

There have been no reports concerning genetic diversity in *A. cerana* using nuclear DNA markers. Although sampling strategies and laboratory techniques are relatively simple when mtDNA is employed, contribution of the male component on population genetic sense cannot be inferred from mtDNA. As a result, it is necessary to investigate an agreement of population genetic information of two different sources of DNA markers. The ITS region and microsatellite DNA from nuclear genome of *A. cerana* were then chosen. Based on the fact that, these markers inherit in the co-dominant fashion, further applications for breeding programmes of *A. cerana* using these markers are promising.

Ribosomal DNA has been widely used for phylogenetic studies in various taxa. The polymorphism within the internal transcribed spacer (ITS) of the nuclear ribosomal RNA (rRNA) genes has been used to differentiate closely related organisms (Hillis and Davis, 1986; Mindel and Honeycutt, 1990; Hillis and Dixon, 1991). For examples, the RFLP analysis of PCR amplified ribosomal DNA of three *Trichogramma* species ; *T. minutum, T. brassicae* and *T. sibirium*, were reported (Sappal *et al.*, 1995). Length polymorphism in the ITS region was clearly observed in theses three species. Population specific patterns were observed from RFLP analysis. Tang *et al.*(1996) sequences the ITS of the West - African black fly species complex (*Simulium damnosum* s.l.) and demonstrated extensive intra-individual (length heteroplasmy) and intra-specific (length polymorphism) polymorphism. The polymorphic subregion in the ITS was primarily due to the ITS1 domain. An analysis of the ITS1 sequences of *S. damnosum* indicated differences in both length and sequence composition of this subregion. Some individuals gave up to four ITS bands after PCR amplification.

The ITS region of *A. cerana* in Thailand also showed length heteroplasmy. This indicate the existence of both inter- and intraindividual polymorphism of the ITS in *A. cerana.* Amplification of the ITS from the five geographic samples of *A. cerana*

through PCR yielded DNA products ranging in size from approximately 500-800 bp. The length of these amplified fragments was comparable to that of grasses, 588-603 bp, (Hsiao *et al.*, 1994) and *Tropilaelaps clareae*, about 620 bp (Tangjingjai, 1998). In the present study, a 580 bp product was the most common allele which were available in most of investigated specimens. This band were then selected for further characterizations by direct sequencing.

Homology among investigated ITS sequenced was analyzed. Only 4 point mutations including 1 transversion (G ↔ C) and 3 transitions (T ↔ C) were observed indicating extremely low sequence divergence of the ITS region. No genetic polymorphism within each geographic sample was found. Although this sequence information was not sufficient to represent patterns of genetic variation and differentiation in Thai *A. cerana*, dissociation of geographic origin of *A. cerana* from the Northern, Central and North-East), the Southern (South) and the Samui Island were consistently observed in the sequencing data set. Based on the fact that, DNA sequencing is tedious and time-consuming, the number specimens investigated was limited. Considering level of polymorphism obtained, sequencing of a 580 bp amplified ITS is not appropriate for determination of genetic differentiation in this species. No genetic variation between the ITS sequences of 11 lake trout (*Salvelinus namaycush*) individuals from five different location was reported (Zhuo *et al.*, 1994). Analysis of 20 *Tropilaelaps Clareae* individuals (Tangjingjai, 1998) by sequencing of the amplified ITS did not show any sequence divergence at this region. Within Apidae, interspecific polymorphisms between *Anthophora abrupta*, *Apis mellifera*, *Euglossa imperialis*, *Bombus terricola* and *Trigona capitata* were reported. A total of 7 phylogenetically informative sites were available at the homologous region of that in this study (Sheppard and McPheron, 1991). Disregarding the small sample sizes of investigated *A. cerana*, the information on fixed differences in nucleotides between different geographic samples, allow developing of population specific primers. The primer could be designed for selective amplification of different product sizes. The existence of length

heteroplasmy in the ITS made the approach more complicated. A 580 bp band need to be eluted from an electrophoresed gel and served as the template for selective amplification second round of *A. cerana* from different geographic origin.

The same *A. cerana* individuals used for DNA sequencing analysis in this study was also genetically analyzed by PCR-RFLP (Sihanuntawong, 1997 and Songram, 1997). Several haplotypes were observed from digestion of sRNA gene, lrRNA, the intergenic COI-COII and the ATPase6-ATPase8 with selected restriction enzymes. Moreover, the PCR-RFLP approach is much more convenient than DNA sequencing and, in this case, provide better results. Therefore the other molecular technique should be used to replace the advantages of DNA sequencing using the amplified ITS region.

Microsatellite marker was additional chosen for this study because it previously showed high level of polymorphism in *A. mellifera*. Estoup *et al.* (1993) reported that primers developed from flanking sequences of microsatellite loci in *A. mellifera* are similar enough to allow PCR amplification in other *Apis* species as well as members of its related group from the genus *Bombus*. Microsatellites is potentially useful for not only population structure and genetic differentiation analysis (Estoup *et al.*, 1995 and Estoup *et al.*, 1996) but also determination of polyandry (multiple queen mating) (Mortz *et al.*, 1995 and Oldroyd *et al.*, 1997) in the social insect like honeybees. Microsatellite polymorphism was used to confirm that *A. mellifera* evolved in three distinct and deeply differentiated lineage previously detected by morphological and mtDNA studies. Large genctic differences between European and Africanized *A. mellitera* were detected by 7 microsatellite loci (each exhibit 7-30 alleles per locus). The Africanized subspecies showed higher heterozygosity and the number of alleles per locus than did the European subspecies (H = 0.748-0.829 for the former and H = 0.082-0.503 for the latter). Oldroyd *et al.* (1995) studied levels of polyandry and intracolonial genetic relationships in *A. florea* using 5 microsatellite primer successfully sets amplify those in *A. mellifera*.

In this study, 13 sets of microsatellite primers originally developed from *A. mellifera* were examined in *A. cerana* using the PCR condition further improved from

that for *A. mellifera*. Five sets of microsatellite primers were not successfully amplified the homologous loci in *A. cerana* even though PCR conditions were carefully optimized. Presumably, these microsatellite loci are not sufficiently conserved for other species. Eight microsatellite loci were successfully amplified in *A. cerana* but only one (A14, A81 and A88) and two alleles (A8) were found when analyzed with at least 40-50 individuals of *A. cerana*. Two microsatellite loci exhibited 3 alleles (A24 and A113). While 121 of 151 individuals from 5 geographic samples possessed a 96 bp allele when analyzed at locus A24 interpopulation polymorphism was observed at locus A113. The A28 and A107 loci exhibited greater number of alleles than any others.

Therefore, three microsatellite loci (A28, A107 and A113) were chosen for determination of genetic differentiation within five geographic samples of *A. cerana* in Thailand. All of which are compound microsatellites showing lower level of polymorphism in *A. cerana* than in *A. mellifera* expect at the locus A28 where level of polymorphism was converse.

The allelic size differences by a single nucleotide were found at A28 and A107 loci. These may be the effects of single base mutation in the flanking region of the repeated motifs or, in a rave case, from mutations within the cluster of repetitive sequences. In addition, slippage during DNA replication *in vivo* may result a single nucleotide spacing of amplified microsatellite products. This circumstance was evident in several species such as *A. mellifera* (Estoup *et al.*, 1995) and *A. cerana* (Oldroyd *et al.*, 1998). At locus A113, three polymorphic alleles were found and not suffered from single changes. Although, overall loci (A28, A107 and A113) can be used to determine level of genetic variation in *A. cerana*, effects of homoplasy at a given locus should be determined by sequencing of suspected alleles. This is necessary for population genetic studies but it can be overlooked if the three microsatellite loci are analyzed for analysis of individuality and parentage.

At locus A28, 24 alleles (108-132 bp in size) were observed in *A. cerana*. Five of these (122, 127, 129, 130 and 132 bp) were also observed in *A. mellfera* from previous

publication (Estoup *et al.*, 1994 and Estoup *et al.*, 1995). Only three alleles (182,186 and 196 bp) were observed in Thai *A. cerana* whereas higher number of alleles (19 alleles, 200-238 bp) was reported in *A. mellifera*. No shared allelic size was found between these species at this locus. Likewise, low number of alleles in *A. cerana* (10) in compared with *A. mellifera* (22) was observed at the locus A107. Again, only 3 alleles (161, 167 and169 bp alleles) were overlapping distributed between species.

Nine alleles were found across all investigated *A. cerana* samples (7 alleles (114, 118, 119, 120, 121, 125 and 126 bp) for A28, a 167 bp allele for A107 and a 196 bp allele for A113). At locus A28, three common alleles (118, 119 and 120 bp) were found in all investigated samples. These genotype were possessed by at least 30% of each samples. The most common genotype at locus A107 was a 167 bp allele carrying a high frequencies in all mainland samples. This allele were fixed in the Samui implying limited genetic diversity of this *A. cerana* sample. At locus A113, a 196 bp allele was nearly fixed in the Samui Island. This frequencies of this allele in other geographic samples were 2-4 times less than that in Samui. The observed heterozygosity was relatively high in all mainland samples for all loci. The Samui *A. cerana* showed high heterozygosity at locus A28 but lack of diversity was observed at locus A107 (*Ho* = 0.000). Limited heterozygosity was observed at locus A113. Considering the number of alleles per locus and levels of heterozygosity, the low level of genetic diversity of the Samui *A cerana* interred from microsatellite loci were in contrast with results, from mtDNA-RFLP reported by Sihanuntavong (1997) and Songram (1997).

Large genetic differences were observed between *A. cerana* from the Northern and Southern latitudes. The neighbor-joining based on Cavalli-Sforza and Edwards chord distance allocated five geographic samples into 3 groups : 1) North, Central and North-East, 2) South and 3) Samui Island.

Geographic homogeneity was observed between North and Central geographic samples at all loci while North and North-East were not significantly different

genetically at locus A107 and A113, Central and North-East did not show the difference in genotype frequency at locus A107 (P = 0.691). These were supported by negative *Fst* value between such comparisons. The results could be explained by high gene flow level occurred within the Northern latitude *A. cerana* but restricted gene flow was found between Northern and Southern groups. Geographic heterogeneity between the South and Samui samples may be resulted from genetic drift in the latter as it was suffered from gene flow. The reason to explain this phenomenon is that Samui Island are isolated from the South by the geographical barrier of water gap (18 km from the closest of the Malaysian Peninsula (Nakamura *et al.*, 1991)).

The *Fst* of all *A. cerana* samples were significantly larger than zero indicating that *A. cerana* in Thailand are genetically differentiated to several gene pools. On the basis of this estimate five different geographic samples of *A. cerana* were allocated into three genetic populations, 1) North, Central and North-East, 2) South and 3) Samui Island.

Lower number of allele and observed heterozygosity in the Samui sample indicated limited genetic diversity of this population. Although, these results was contradictory to those previously reported by Sihanuntavong (1997) and Songram (1997), were in consonant with those of Pootong (1999). Sampling errors may be one of the most important reason to explain this circumstance.

Although, the ITS sequence may not be appropriate for determination of genetic differentiation in *A. cerana*, it proved to be useful for identification of geographic origin of suspected *A. cerana*. Additionally, microsatellite loci are useful for analysis of genetic variation and population structure of *A. cerana* in Thailand. This results from this marker provided a large genetic discontinuity between the Northern and Southern *A. cerana* in Thailand as previously clarified by mtDNA polymorphism.