CHAPTER 4

DISCUSSION

Classification of related species can be performed based primarily on morphology. Moreover, behaviour and other biological characters (e.g. life history) are also useful for studies of evolutionary relationship at inter- and intraspecific levels. Ruttner (1988) dissociate the *Apis* group to 24 different species using morphometric method. Generally, *A. mellifera* is well studied in several biological and genetical aspects compared to other bee species. Genetic diversity of honeybees based on allozymes has been published. The variation at within and between populations of *A. mellifera* were well documented for discrimination of races (Nunamaker *et al.*, 1984a), for identification of the hybride zone between two races of *A. mellifera* (Sheppard and McPheron, 1986) and for studying of bee behaviour (Robinson and Page, 1988, 1989). Recently, molecular genetic markers from nuclear and mtDNA have been increasingly used because these provide wider information than did the allozymes alone. Introgressive hybridization between European and Africanized *A. mellifera* was found using restriction fragment length polymorphism (RFLP) of nuclear DNA (Hall, 1990). Genetic polymorphism based on RFLP, restriction cleavage maps and length polymorphism of mtDNA in *Apis* has been reported and possibly more popular than that of nuclear DNA. The reason to explain this is maternal transmission of mitochondrial genome resulting in much easier sampling strategy when this extrachromosomal DNA is used in these taxa. Therefore, only one individual can represent the maternal lineage of a colony. In contrast, more number of individuals within a colony need to be investigated when nuclear marker are used.

Biogeography of *A. cerana* in Asia was illustrated by Smith and Hagen, 1996 who sequences the non-coding intergenic region of mtDNA from 110 colonies of *A. cerana* originating from large different geographic regions. Two major forms of *A. cerana*, a western form (India, Sri Lanka and Andaman Islands) and an eastern form (Nepal, Thailand, Malaysia, Indonesia, Philippines, Hong Kong, Korea and Japan). The latter can be further divided to two separated group constituting of the Sundaland found in penisular Malaysia, Borneo, Java, Bali, Lom bok, Timor and Flores and the Philippines group found in Luzon, Mindanao and Sangihe. Both groups of haplotypes were found in the Island of Sulawesi, suggesting that this Island was colonized by both the Philippines and Sundaland (Smith and Hagen, 1996). Additionally, mtDNA polymorphism of *A. cerana* was reported from that originating from Japan, Korea, Taiwan, Vietnam, Thailand, Nepal and the Philippines using conventional RFLP analysis. All geographically investigated samples could be allocated to 6 different groups composing of Japan (1), Nepal, Vietnam and North-to-Central Thailand (2), Korea-Tsushima (3), Taiwan (4), Southern Thailand (5) and Philippines (6) (Deowanish *et al*., 1996). More recently, a large genetic discontinuity between Northern and Southern latitudes of *A. cerana* was reported using PCR-RFLP of mtDNA genes (Sihanantavong, 1997 and Songram, 1997). Both studies could dissociate the Samui sample from the South *A. cerana* based on significant differences in allele distribution frequencies (χ^2 analysis) and population subdivision estimate (Fst) indicating that intraspecific genetic differentiation of *A. cerana* in Thailand did exist and was not resulted from artifacts of sampling errors.

The control region (D-loop) of animal mtDNA is a large non-coding region area containing specific region for initiation of replication and transcription of mitochondrial genome. Generally, base substitutions (transitions and transversions), length polymorphism (insertions and deletions) of this region is greater than those in other regions. Length polymorphism of control region among different individuals within a species has been reported in several species. In insects, the control region is called the AT-rich region because it lacks of any apparent signals for replication and transcription (Desjardins and Morais, 1990; Saccone, Pesole and Sbisa, 1991). Although several mtDNA portion have been used for population genetic and systematic studies of *A. cerana*, the use of the AT-rich region for such purposes is limited. Results for the present study illustrated the possibility to use primer AM8-AM11 originating form *A. mellifera* to amplify the homologous locus in *A. cerana*. Although length polymorphism was observed at intraindividual level (heteroplasmy), interpretation of restriction patterns was not interfered by this phenomenon particularly when three enzymes (*Taq*I, *Rsa*I and *Hin*fI) were carefully chosen based on their simply scorable patterns.

Approximately seventy-one percent of all investigated colonies were successfully amplified by the polymerase chain reaction (PCR). The main amplification product from *A. cerana* was 2750 bp in length. This fragment was coexisted with a slightly smaller PCR product after gel fractionation in all investigated individuals. The homologous product from *A. mellifera* and unidentified *Apis* species (S60) showed the same size of PCR product implying their closed relationship. It is interesting to directly compare the whole amplification sequence of

A. cerana, A. mellifera and S60 with other local *Apis* species in Thailand for a better conclusion of the systematic status of the unidentified S60.

Sequencing of the most common amplification fragment of *A.cerana* was carried out. Based on the fact that the inner primer (5′-AAA ATA AAT AAA GCA GTG GTA-3′) was designed from mitochondrial ND_2 gene of *A. mellifera* , high similarity (>90%) between this and previously deposited ND_2 sequences in the GenBank comfirmed that the amplification product was the actual target fragment composing of the expected control region.

Additionally, digestion of this PCR product amplified from *A. mellifera* in the present study with various restriction enzymes exhibit expected restriction patterns and fragments inferred from a restriction map of *A. mellifera ligustica* reported by Crozier et al (1993). All evidences confirmed that the amplification fragment was the control region of *A. cerana* mitochondrial genome.

Fifteen restriction endonucleases (*Eco*RI, *Kpn*I, *Sma*I, *Sau*3AI, *Bfr*I, *Dra*I, *Ssp*I, *Ase*I, *Hin*fI, *Hin*dIII, *Nde*I, *Swa*I, *Alu*I, *Taq*I and *Rsa*I) could digest the PCR product. Three restriction enzyme (*Taq*I, *Rsa*I and *Hin*fI) were used to analysis the control region. Basically, their enzymes produced with discrete a coexisted digestion band and a fainter fragment with smaller molecular length. Presumably, the fainter band was originated from heteroplasmic product. Nevertheless, scoring of restriction patterns and fragments resulted from these three enzymes were not interfered by length heteroplasmy. Conversely, results from *Ssp*I, *Dra*I and *Ase*I did not include in analysis of genetic polymorphism. The

reason for this was that the sum of restriction fragments resulted from these enzymes was significantly smaller than the size of undigested fragments indication that several smaller DNA fragments may move out of the gels. Moreover, different restriction patterns could not be interrelated by simple loss and gain of restriction site. Therefore, it was not possible to eliminate effects of length heteroplasmy that interfere scoring of band sharing between compared specimens.

Phylogenetic relationship based on genetic distance (the number of nucleotide substitutions per site) indicated two different clades of composite haplotypes. While clade B was observed only in the South, Samui and Phuket samples, clade A genotypes were possessed by specimens from the North, North/East and Central *A. cerana*. No overlapping haplotype between the Northern and Southern latitudes of *A. cerana* was observed indicating large genetic difference between these two groups of *A. cerana* in Thailand. Moreover, clearly differentiation of Thai *A. cerana* should be resulted from restricted gene flow between these populations. Similarity, a UPGMA dendrogram based on nucleotide divergence between pairs of samples well allocated 6 investigated samples to two separated groups, Northern latitude and Southern latitude of *A. cerana*. This supported the results inferred from phylogeny of composite haplotypes as described earlier. The results in the present study were in agreement with those of Sihanantavong (1997) and Songram (1997).

The mtDNA diversity within *A. cerana* samples showed higher haplotype diversity of the Northern sample (0.6088-0.6629) than did the Southern *A. cerana* (0.0000-0.5032). Patterns of nucleotide diversity

within geographic samples also showed the same trend. Approximately equal distributions in frequencies of AAC, AAB and AAD in each of the Northern latitude *A. cerana* caused high genetic diversity within each geographic sample. On the other hand, the most common haplotype BBA was contributed by 91.9%, 68.2% and 100% of *A. cerana* originating from the South, Samui and Phuket, respectively. This reflected lower genetic polymorphism in the Southern latitude of *A.cerana*. It should be noted that only 3 samples from the Phuket Island was investigated and the sample size of this geographic location was unfortunately too small to draw several conclusions in this sample. Therefore, increasing of the sample size of *A. cerana* from the Phuket Island is necessarily required.

The average nucleotide divergence in this study was 4.647% which was greater than that previously reported by Sihanantavong (1997) and Songram (1997) at approximately 2 %. The contradictory results between these results should be primarily responded by the limited number of restriction endonucleases used in this and other experiments.

Considering levels of nucleotide divergence within each group (Northern and Southern), the divergence was lower than 0.2% whereas such levels were larger than 7% when comparing a geographic sample in one region to each of the other. This evidenced strong genetic differentiation of North/South *A. cerana* in Thailand. Nevertheless, it was not possible to compare this circumstance with data from previous publications of *A. cerana* from different locations. This due to lack of standard parameters indicating level of distance and differentiation (e.g. Fst and divergence) in these previously reported.

Genetic heterogeneity analysis using psudo Chi-square indicated significant different in allele distribution frequencies of *A. cerana* in Thailand ($P<0.0028$). Therefore, population subdivision was existance in this species. The results from all possible pairwise comparisons showed the same conclusions drawn from phylogenetic studies with the exception that *A. cerana* from the South had significant genetic heterogeneity compared to that from the Samui Island ($P=0.0014$). Therefore, the Samui *A. cerana* should be considered as different population. It should be noted that, the level of confidence in statistical analysis was further adjusted by the Sequential Bonferroni technique [0.05/(no. of population × no. of restriction enzyme)], for the presence of the significant level of genetic heterogeneity and subsequently, population differentiation analysis.

Likewise,Fst statistics showed overview comparable results with those from Chi-square analysis confirming genetic differentiation of *A. cerana* in Thailand. Restricted gene flow levels were observed between pair of geographic samples from different regions ($Fst>0.2$) whereas high gene flow levels were observed when compared pairs of samples within the same region. Nevertheless, the data on gene flow in *A. cerana* might have been underestimated as transferring of *A. cerana* from different location has been promoted by beekeepers.

Based on all analysis carried out by the present study, all investigated *A. cerana* could be differentiated into 3 different population composing of A (North, North/East and Central *A.cerana*), B (South and Phuket) and C (Samui). It is extremely interesting to test the performance in some economically important traits of *A. cerana* carrying BBA with

those carrying AAD, AAB and AAC haplotypes. The information obtained will be significantly useful for selective breeding programes of *A. cerana* in Thailand. Moreover, patterns of genetic differentiation in other local *Apis* species should not be overlooked and actually need to be classified by molecular genetic approaches.

The most important disadvantage to use the control region (D-loop) for population genetic and systematic studies was possibly due to the extensive length heteroplasmy found in this region producing difficulties for analysis of the results. Although, this phenomenon was previously found in the amplified ATPase6-ATPase8 region of *A. cerana* in Thailand. It was restricted to specimens originating from the South and Samui Island (Songram, 1997). In contrast, heteroplasmy in the control region was observed in all investigated specimens and did not relate to geographic origin of the experimental *A. cerana*. Moreover, the approach used for interspecific analysis among *Apis* species needs to be altered from RFLP to restriction cleavage maps because it was not possible to use band sharing method to compare the amplified products exhibiting length polymorphism between different species.

The results from this study indicated that mtDNA polymorphism analyzed by PCR-RFLP provided useful genetic information in *A.cerana*. The large genetic discontinuity in the continuously distributed species like *A. cerana* indicated that *A. cerana* in the Northern and Southern latitudes experienced restricted gene flow level. This basic information requests for a requirement of further study to test whether *A. cerana* from different populations exhibit different level of performance on

economically important phenotypes (e. g. disease resistance, more honey production).

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