CHAPTER V

DISCUSSION

In this study, honey bee DNA was isolated from individual worker pupae (11-13 days old) beacuse of firstly; the soft tissue of pupae facilitated homogenization, secondly; ensuring that the sample was originated from the collected colony (whereas adults can drift among colonies) (Hall and Smith, 1991; Seveson *et al.*, 1988) and thirdly; the pupae provides consistant amounts of DNA from each individual (Seveson *et al.*, 1988). The total DNA was used for restriction pattern and RFLP analysis instead of nuclear DNA since nuclear DNA extraction is highly appropriate only for bee samples growing at the laboratory, but it is the inappropriate method for bee samples collected from natural places. The nuclear DNA extraction of the pupae which was collected on ice during transport to laboratory was alway unsuccessful because of the autodigestion of the chromosomal DNA. The total DNA extraction, was simple and had to be pre-extracted immediately in the field by homogenized the sample in lysis buffer after warm at 65 ^{O}C then the pre-extracted solution could keep at room temperature during transport. However, the total DNA extraction alway contaminate with mitochondrial DNA, but our previous experiments demonstrated that with this stage pupae (11-13 days old) only small amount of the mitochondrial DNA (less than 50 ng) was contaminated

which was only 1% comparing to nuclear DNA. Futhermore, the results of total DNA extraction method and nuclear DNA extraction method were clearly presented in the similar result of 1) purify of the isolated honey bee DNAs (range of OD_{260}/OD_{280} were from 1.65 to 1.85), 2) high molecular weight of DNA; after agarose gel eletrophoresis, only one band was presented the same pattern which is larger than 23.1 kb marker (Figure 7) 3) which was not the band represent the mitochondrial DNA, total and nuclear honey bee DNAs digested with restriction endonucleases *Bgl*II, *Cla*I, *Eco*RI, *Hae*III and *Nde*I generated the same patterns (Figure 11).

The restriction pattern analysis is performed by the restriction endonucleases digested DNA. The DNA fragments are separated through agarose gel under electrophoretic-field and distinct patterns appear from different restriction endonucleases. Some of restriction endonucleases which simply generated discrete bands, were selected for consequents analysis. From the experiments of this study, restriction endonucleases *Bgl*II, *Cla*I, *Eco*RI, *Hae*III and *Nde*I were selected (Figure 8). The restriction pattern analysis should be ensuring that the high molecular weight of honey bee DNA was completely digested with restriction endonucleases. The complete digestion relies on the appropriate amount of DNAs, the incubation time, and the amount of restriction endonucleases. The experiment in Figure 10 showed that, 5 U of each restriction endonucleases from *Bgl*II, *Cla*I, *Eco*RI, *Hae*III and *Nde*I were completely digested for in at least 1 hour. In order to compared the restriction patterns among DNA samples obtained from various regions of

Thailand, total DNA of the same colony could be previously tested to ensure that within a colony one honey bee represented the similar pattern. The result showed (Figure 13) that an individual of twenty total DNA given the same restriction pattern when completely digested with *Bgl*II, *Cla*I, *Eco*RI, *Hae*III and *Nde*I. Since the queen honey bee is the multiple insermination, it may be the important part to explain that 1) sperms of the same or different males are used nonrandomly 2) sperms are in complete mixed and stored in queen's spermatheca (Seeley, 1985).

The results of restriction pattern analysis from three species of different bees, *A. florea*, *A. mellifera* and *A. cerana*, were shown in distinct patterns when completely digested with restriction endonuclease *Eco*RI (Figure 12). The results of this study agree with the research presented by Dary in 1991.

The results of restriction patterns analysis using *Bgl*II, *Cla*I, *Eco*RI, *Hae*III and *Nde*I (Table 9) showed that *A. cerana* populations from different regions of Thailand are highly diversified. The results of restriction from the Northern showed only one groups of B1, C1 and N1 when DNA samples digested with *Bgl*II, *Cal*I and *Nde*I respectively. But restriction patterns, showed that *A. cerana* could be categorized into three groups; E1, E2 and E3 if digested with *Eco*RI and also presented as two groups; H1 and H2 when digested with *Hae*III. More details were shown in appendix 11.

These events indicated that, different restriction endonucleases gave the DNA samples into distinct groups. Because of each of restriction

Table 9 The restriction pattern classification of total DNA digested with different restriction endonucleases

The sampling location	Restriction endonucleases				
	***Bgl*II**	***Cla*I**	***Eco*RI**	***Hae*III**	***Nde*I**
The Northern	B1	C1	E1, E2, E3	H1, H2	N1
The North-Eastern	B1	C1	E1	H1, H2	N1
The Central part	B1	C1	E1	H3	N1
The Southern	B1	C1, C2	E1, E4	H1, H2	N1, N2
The Samui Island	B1, B2	C1	E1, E2, E5	H1, H3	N1, N2

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endonucleases are recognized and cleavaged in different bases sequence. Therefore in the same DNA sample could be classified as different groups when digested with different enzymes. Furturemore, the restriction pattern of *Eco*RI could be identicated that *A. cerana* from the Northern of Thailand have the DNA diversity at least three groups.

The results of restriction patterns from the North-Eastern could be divided into at least two different groups when DNA samples digested with *Hae*III. The DNA samples from the Central part could not be classified into subgroups when digested with these 5 enzymes. The other restriction patterns from the Southern could be divided into at least two different groups when digested with *Cla*I, *Eco*RI, *Hae*III and *Nde*I. The restriction pattern from the Samui Island could be divided into at least three groups when digested with *Eco*RI (Appendix 11-15).

The study of diversity of *A. cerana* was also performed using RFLP analysis. The DNA-DNA hybridization was performed between restriction fragments of *A. cerana* DNA bounded on nylon membrane and the labeled DNA profied. The steps of DNA-DNA hybridization were performed by the following; firstly, prehybridization step that the proteinous blocking reagent hybridized to non-specific hybridization on the membrane surface secondly, hybridization step allowing probe hybridized to homologous sequence immobilized on the membrane surface and finally, non-bound is removed from the membrane, refered to post hybridization (washing) step.

The hybridiaztion was firstly performed using *A. mellifera* probes (# 24 and # 47) with *A. cerana* total DNA, the result of dot blot hybridization clearly showed that probe from *A. mellifera* could hybridized with *A. cerana* DNA target from different locations (Figure 19). However, the Southern hybridized of *Eco*RI digested *A. cerana* DNA with probe # 47, this result showed only weak signal and also no signal appeared by using probe # 24 (Figure 20B and 20A). The results demonsteaded the low homology between *A. mellifera* probes and *A. cerana* genomes. Morover, dot hybridization is the procedure to enable rapid screening by passing electrophoresis and DNA fragments transfer. The results clearly indicated on DNA-DNA hybridization by using probe # 24 that no signal appeared. Whereas in the same condition, λ phage DNA digested with *Hind*III (hybridized with λ/*Hind*III probe) was intensely presented. The result of probe # 47 which showed unclear bands seems to show a few homology between *A. mellifera* probe and *A. cerana* target DNA including unappropriate condition influency. Moreover this study has been tested by increasing the rate of hybridization at low temperature (42 °C, when 50% formamide is used) (Maniatis *et al.*, 1982). The result is also given the same as the first result. Moreover, much work remains to be studied.

Because of failure of the hybridization using DNA probe from *A. mellifera*, therefore the DNA probe from *A. cerana* was prepared by DNA cloning. From the results of restriction pattern, *Bgl*II usually generated conservation DNA fragments at about 3 and 5 kb (Figure 14, 21). But the only results from Samui Island were rather different when digested with *Bgl*II, which were found at the range of 2 to 4 kb. For this

reason, the DNA fragments of Samui Island were used to prepare the DNA probe. To prepare the appropriate DNA probes, the DNA fragments at the range of 2 to 4 kb and 5 to 7 kb were cut and purified to use as the source of DNA probes.

Plasmid pBR322 is the widely used in molecular cloning because of its properties as follows; 1) small size plasmid (4.361 kb) 2) relaxed plasmid, the copy numbers can increased to 1,000-3,000 by adding chloramphenical to the culture medium (Calwell, 1972), 3) carring two drug resistance markers, tetracycline (Tc) and ampicillin (Ap) resistance genes which easily to handle 4) the insertion sites (restrictionsites) located in these markers (the sensitive results of the success cloning). Therefore, plasmid pBR322 was selected to use as plasmid vector for propragation of these probes.

The *Bgl*II sites on *A. cerana* fragments were used to represent on *Bam*HI site of plasmid pBR322, located in Tc resistance gene. Therefore, both sites of *Bgl*II and *Bam*HI were used to generate the DNA probes. Then the ligation was performed after dephosphorylated of linearized plasmid to prevent the self-ligated and increase the efficiency of ligation.

After *A. cerana* fragments were ligated with vector pBR322. This recombinant DNA was transformed into host cell (*E. coli*) in order to propagate the number of recombinant DNA. The result of transformation showed that the DNA fragments at the range of 2 to 4 kb gave the higher efficiency than the DNA fragments at the range of 5 to 7 kb (Figure 23). Afterward the clones of transformation were identified by dot hybridizing with *A. cerana* chromosomal DNA labeled probe. The intense signal from

dot hybridization indicated that DNA probe also contained the repetitive DNA. In addition, the ligation success confirmed by size of recombinant DNA (> 4.36).

Actually, the efficiency of hybridization relies on sizes of probes. The shorter probes give higher sensitivity than the longer probes. Accroding to the efficiency of hybridization all probes should be reduced their sizes before labeling. From the actual procedure the source of DNA probes in this research were prepared from *Bgl*II and *Bam*HI sites of *A. cerana* DNA and plasmid pBR322 vector, respectively, so that these sites of recombinant DNA were defaulted. Moreover, the other restriction sites of vector were used to reduce the size of DNA probe and this sites might locate within *A. cerana* DNA fragment or generated the inserted fragment into the same, nearly equivalent sizes both of foreign DNA and vector DNA. Therefore the elution of these fragment were difficult. So DNA probes carrying vector were not yet labeled after ensuring that vector were not hybridized with *A. cerana* target DNA (Figure 24).

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The great sensitivity of the radioactive labeling method is well established, but unfortunately short lasting, hazardous and requires special laboratories (Mathews and Kricka, 1988). For these reasons, the nonradioactive probe is more advantage. Therefore the better technique is applied for labeling DNA probes in this study.

The labeling probe was prepared by using DIG-11-dUTP of random primed labeling from the DIG DNA labeling system. This reaction was characterized by the ELISA principle (Enzyme linked immunosorbent assay (Kassler *et al.*, 1992; Lion and Haas, 1990; Martin *et al.*, 1987) and

catalyzed by Klenow polymerase. The steroid hapten digoxigenin is linked with uracil base at position C_5 of deoxyribonucleotide by 11-atom spacer. The denatured single stranded templates are annealed to the random mixture of hexanucleotides. The synthesis is started by adding Klenow polymerase and all deoxynucleotides (one is the hepten modified substrated (DIG-dUTP)). During polymerization reaction, Klenow polymerase incoporates not only the nonmodified deoxynucleotide but also DIG-dUTP. The primers are able to bind all posible target sequences (both of two single strands may act as templates). However, this small amount of DNA can be used and gives the specific activity (Boehringer, 1993). Due to detection of nucleic acid-bound with a cardenoid-steroid digoxigenin, this research was done by using two methods. The method colorimetric detection; the hybrids are detected with 5-bromo-4-chloro-3-indolyphosphate (BCIP), the substrate of alkaline phosphatase-linked digoxigenin antibody. The oxidation of dye nitrobluetetrazolium (NBT) is produced insoluble colored-product signals located on nylon membrane. In order to reuse of membrane for rehybridization, the DNA probe and the color precipitate must be removed out of the membrane. The color precipitate is difficult to remove and the poisonous chemical; Dimethylformamide, is used in this step. The second method, which is called Chemiluminescent, generally uses the emission of light that occured by alkaline phosphatase (linked dioxigenin antibody) using lumigen PPD as a substrate. Chemiluminescent detection can be considered as a better method than colorimetric detection and also detected small amount of target DNA (Carlson *et al.*, 1990).

After *A. cerana* total DNA labeled-probe investigated dot hybridization of recombinant plasmids, the positive signals were then selected (Figure 22). Finally the suitable probe, # 3035 was chosen for subsequent performing on Southern hybridization of total DNA of *A. cerana* from different regions. The results did not agree with the highest signal intensity of dot. This may be attributable that, probe # 3035 contains with low repetitive segment of DNA (fragment). But given the high homology of specific sequences which disperse throughout the genome. When the *Eco*RI cleavaged sites within the repeated DNA of *A. cerana*. The sequence interspresed through the varying lengths. If DNA sample from different locations are presence or absence from *Eco*RI recognition site, the length polymorphisms will occure.

The investigation on *A. cerana* samples within a colony showed exactly the same intense band in every one of 20 honey bees per colony when target DNA hybridized with probe # 3035 and # 3018. These results represented on restriction pattern analysis.

Eventhough *A. cerana* are from the total DNA, the mitochondrial DNA does not interfere because the mitochondrial DNA are found very little (< 50 ng which could not be detected by restriction pattern observation). However, the hybridization of *Bgl*II digested total DNA of *A. cerana* with *A. cerana* # 3035 probe, the intense band appear at 20 kb and 3 kb which were the size of # 3035 DNA probe (Figure 26). These results indicated that DNA probe # 3035 is not the mitochondrial DNA fragment since the size of intacted mitochondrial DNA was about 10 kb.

DNA-DNA hybridization could classify *A. cerana* from various regions of Thailand by using major and minor intense bands. The classification based on the various sizes of the major intense bands which are clearly shown at 2.1 and 3.1 kb (Table 8) distinguishes DNA samples of Samui Island from the samples of the other locations. The minor bands can classify the population of *A. cerana* into nine groups. Three groups of them were the population on Samui Island. The other, six groups were the mainland bees (Table 10).

The diversity of Samui Island bees has suggested that the disjunct population are isolated by the geographical of water gaps (18 km from the closest of the Malay Penisula (Nakamura *et al.*, 1991)). The pure native line are mated within their pupulation for generations. The bees population of Samui Island remain highly conserved and slowly evolution of genes.

While the diversity of bees from the mainland are classified into six groups, five of them present in the Northern and the other represent all each regions of the mainland. The result has suggested that the swarm habit of *A. cerana* occured for serveral times. For the fact of unlimited area, they can sprease and mate among population. So these factors tend to allow the population remain slowly conserved and fast evolutions of gene in the mainland bees.

The highly diversity of honey bee from the Northern also supported with the investigation of Ruttner (1988) which classified the Eastern honey bees collections in Chiang-Mai as *A. c. himalayama*, and the lowerland (vast area) of Thailand classified as *A. c. indica*. Therefore,

Table 10 The Southern hybridization classification of *Eco*RI digested total DNA hybridized with probe # 3035

The sampling location	Group of classification based on intense bands
The Northern	I, II, III, IV, V
The North-Eastern	I
The Central part	I, II, VI
The Southern	I
The Samui Island	VII, VIII, IX

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the long distance between the Northern and the other regions tended to cause higher diversity of honey bee than the other regions.

The DNA-DNA hybridization showed higher diversity than the morphometrical methods. The morphometrics are measured on the phenotypes which are yields of the structural gene with non-coding region excised. In contrast, the DNA-DNA hybridization is measured on the structural genes where consist of either coding region and non-coding region. Therefore the variation which occure in the non-coding region cannot be detected by the morphometric method.

From the experiment of Southern hybridization some errors might occure, for example some of Southern hybridization pictures showed DNA form and evidentiary curve (⌒) after chemiluminescent detection. But these results corresponded to ethidium bromide staining pattern after agarose gel electrophoresis. Therefore, the evidentary curved (⌒) of intense bands are assumed that they are the same sizes. These results could be influenced by many possible factors; 1) ethidium bromide staining the gel 2) excess voltage during electrophoresis, gel over heating 3) ethanol in the DNA sample or 5) well overloaded with DNA (Kirby, 1992). The future experiments using various restriction endonucleases, two or more probes mixed, and also the longer of agarose gel (long distance of agarose gel electrophoresis) to test the DNA samples should give more detailed evidence.