# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Instruments

- Autoclave, NA-32, Manufacturing corporation, Japan.
- Automatic micropipette, pipetman; $P_2$, $P_{20}$, $P_{100}$, $P_{200}$, $P_{1000}$ Gilson Medical Electrics S.A., France.
- Camera, Pentax Syer A.
- High speed microcentrifuge, Kubota 1300, Kubota corporation, Japan.
- Horizontal gel electrophoresis apparatus (8.5 × 12.5 cm).
- Incubator, BM 600, Memert GmbH, W., Germany.
- Shaker, S03, Stuart Scientific, Great Britain.
- PCR, Gene Amp System 2400, Perkin Elmer, USA.
- PCR workstation, HP-036, C.B. Scientific Co., California.
- pH/ION meter, PHM 95, Radiometer, Copenhagen.
- Power supply, Power PAG 300, Bio-Rad, USA.
- Standard cassette (14 × 17 inch) Okamoto, Japan.
- UV transilluminators: 2011 MA erovue, San Gabrial, USA.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

## 2.2. Chemicals

- Agarose, Seakem LE, FMC Bioproducts, USA.
- Agarose, Metaphore, FMC Bioproducts, USA.
- Boric acid, BDH Laboratory suppliers, England.
- Deoxynucleotide (dATP, dTTP, dCTP, dGTP) Promega coporation, Medison, Wisconsin, USA.
- Chloroform, BDH Laboratory suppliers, England.

- Developer and Fixer, Eastman Kodak company Rochester, USA.
- Ethidium bromide, Sigma chemical company, USA.
- Lamda phage DNA, New England Biology company, USA.
- Oligonucleotide primers, Biosynthesis and Biotechnology Service Unit.
- OmiBase™ DNA Cycle Sequencing System Kit, Promega coporation, Medison, Wisconsin, USA.

2.3 Inventory supplies

- Black and White print film, Tri-Xpan 400, Eastman Kodak company, Rochester, USA.
- Whatman paper 3M, Whatman International Ltd., Maid stone, England.
- X-ray film, X-omat XK-1, Eastman Kodak company Rochester, USA.
- X-ray film, Fuji medical, Fuji photo film Co., LTD, Japan.

2.4 Standard markers

- 10 bp DNA ladder (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 bp), Promega, Madison, USA
- 100 bp DNA ladder (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 and 1500 bp), Promega, Madison, USA
- λ DNA digested with *Dra*I (0.53, 0.69, 1.07, 2.15, 3.03, 3.59, 6.03, 6.82, 7.83, 8.37 and 8.59 kb)
- λ DNA digested with *Hin*dIII (0.6, 2.0, 2.3, 4.3, 6.6, 9.4, and 23.1 kb)

2.5 Enzymes

- Proteinase K, Gibco BRL life Technologies, Inc., USA.
- Restriction endonucleases ;
  - *Ase*I, *Hin*fI, *Hin*dIII, *Ssp*I, *Rsa*I, *Nde*I (Biolabs)
  - *Dra*I (Pramacia)
  - *Alu*I, *Bfr*I, *Eco*RI, *Kpn*I, *Sau*3AI, *Sma*I, *Swa*I (Boehringer Manham).
  - *Taq* DNA polymerase, Perkin Elemer, Cetus, Norwalk, Connecticus.

2.6 Honeybees samples (*A. mellifera*, and *A. cerana*) and tissues preparation.

Samples of adult worker honeybees (*A. mellifera*) were collected from two hives, one from the North/East and the other from Bee Biology Research Unit, Chulalongkorn University. *A. cerana* adult workers were sampled from 177 colonies from 6 different geographic areas in Thailand. Number of sample obtained from the North, North/East, Central, South, Samui Island and Phuket Island was 28, 32, 36, 46, 29 and 6 colonies, respectively. About fifty honeybees from each colonies were immediately preserved in 95% ethanol or kept frozen in liquid nitrogen and were transported to the laboratory for further analysis.

2.7 Honeybees DNA extraction

Total DNA was individually extracted from each thorace using the modified method of Garnary *et al.*(1993), each thorace was homogenized in 1.5 ml eppendorf tubes containing 400 μl TEN buffer (100 mM NaCl, 100 mM EDTA, 0.1 M Tris-HCl, pH 8.0), then 20% SDS and proteinase K (10 mg/ml) was added. The mixture was incubated

at 55°C for 2 hr. Protein in the mixture was removed by extraction once with phenol/chloroform/isoamyl alcohol (25:24:1) and twice with chloroform/isoamyl alcohol (24:1). After spun at 6,000×g for 10 min, the supernatant (aqeous phase) was transferred to a new eppendrof and the DNA was precipitated by addition of 1/10 sample volumn of 3 M sodium acetate and 2 volumns of 95% ethanol and stored overnight at -20°C. The DNA was pelleted at 10,000×g and washed once with 75% ethanol and resuspened the DNA in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA).

2.8 Measurement of DNA concentration

Measurement of DNA concentration was done according to the principle that the intensity of fluorescence emitted by ethidium bromide molecules which were intercalated into the DNA was directly proportional to the total mass of DNA. The extracted DNA was subjected to 0.7% agarose gel electrophoresis. After finished, the gel was stained in ethidium bromide solution (2.5 μg/ml) and destained in distilled water. The fragments of DNA were visualized under UV light and the gel was photographed through a red filter using Kodax Tri-X-pan 400. The concentration and fragment size were determined by comparing the intensity of the ethidium bromide-DNA complex with those of the known concentration of standard markers.

Approximated 0.7% agarose gel was set up in 1X TBE buffer (89 mM Tri-HCl, pH 8.3; 89 mM boric acid; 2.5 mM EDTA) and solubilized by heating in microwave oven. The solubilizing gel was allowed to cool to 50°C and poured into a gel tray. After the gel was completely set up, the comb was carefully removed. Each DNA sample was mixed with one

fifth by volume of loading dye (0.1% bromphenol blue, 40% ficoll 400 and 0.5% SDS) and loaded into the well. The electrophoresis was performed in 1X TBE buffer at 100 volt for 2-3 hr. until bromphenol blue of tracking dye reached the end of the gel.

## 2.9 PCR amplification of control region

### 2.9.1 Primers

Control region of *A. cerana* mtDNA was amplified by PCR. Twelve oligonucleotides primers were designed by Oligo 4.0 program. Eight primers (H-strand primers) were derived from the $ND_2$ gene region of *A. mellifera* mtDNA sequence (Crozier and Crozier, 1993). Whereas four primers (L-strand primers) were from the region of small ribosomal gene. The designed primer sequences were in Table 2.1. The location of each oligonucleotide primers according to the physical mtDNA map of *A. mellifera* was shown in Figure 2.1. Oligonucleotides primers were synthesized by Bioservice Unit (BSU) of National Science and Technology Development Agency (NSTDA), Bangkok, Thailand.

### 2.9.2 Screening of specific primers for amplification of mtDNA control region.

The different primer combinations (H-strand and L-strand) were used to amplify mtDNA control region of *A.mellifera*. The amplification reaction were performed in 20 µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 0.001%(W/V) gelatin, 200 µM each of dATP, dCTP, dTTP and dGTP, 0.2 µM of each different primers, 50 ng of total DNA and 0.5 unit of

Table 2.1 The oligonucleotide primers used for PCR amplification of mtDNA control region

| Primer designation | Primer sequence | Location on mtDNA of *A. mellifera* |
|---|---|---|
| **H-strand primer** | | |
| AM-1 | 5'-CAG GGT ATG AAC CTG TTA GCT TTA TTT AG-3' | 260-231 |
| AM-2 | 5'-CAG GGT ATG AAC CTG TTA GC-3' | 260-241 |
| AM-3 | 5'-AAA ATA AAT AAA TCA GTG GTA-3' | 543-523 |
| AM-4 | 5'-AAT CTA ATT CTA ATG ATT GTA CCA AAT TC-3' | 635-607 |
| AM-5 | 5'-ATC TTG GGG TTT TAT TTG TGG-3' | 670-650 |
| AM-6 | 5'-TAT TCA AAA ATG AAA GGG GAA AGT TCC-3' | 831-805 |
| AM-7 | 5'-GAA TGA ATT AAA AAT TGT TGA ACA TGC TAG-3' | 1044-1016 |
| AM-8 | 5'-CTA TGA TAT ATA TAT TTA TTA TCT TTA TC-3' | 1439-1411 |
| **L-strand primer** | | |
| AM-9 | 5'-TTA TAA GTC AAG TTT AAC CGC TAT TGC-3' | 15309-15335 |
| AM-10 | 5'-CCG CTA TTG CTG GCG ACT CAT-3' | 15326-15346 |
| AM-11 | 5'-ACA ATT AAT CTA AAA AAC TAC AAC ATG-3' | 15369-15495 |
| AM-12 | 5'-ACT TTC ATT CAT TGT TTC AGA-3' | 15380-15400 |

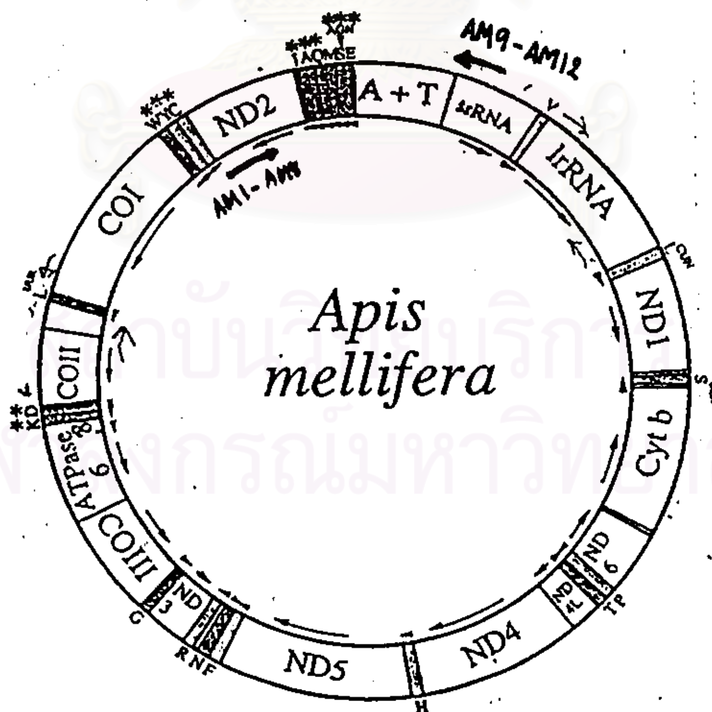


Figure 2.1 Location of oligonucleotide primers in *A. mellifera*. Descriptions of the primers are as in Table 2.1

Ampli *Taq* polymerase. Amplification were performed in a Gene Amp System 2,400 (Perkin Elmer) for pre-denaturation 2 min at 95°C, and then 35 cycles of denaturation at 94°C for 30 sec, annealing at 45°C for 30 sec, extension at 72°C for 1 min 50 sec to 2 min and the final extension was carried out at 72°C for 10 min. The amplification products were then electrophoretically analyzed by agarose gel electrophoresis.

2.9.3 PCR products analysis by agarose gel electrophoresis

The appearance of size of PCR products and their size were determined by 1.5% agarose gel electrophoresis with standard markers (λ/*Hin*dIII and 100 bp DNA marker). Primer pairs which gave single expected band of PCR product at the size were be selected for further amplification of the mtDNA control region of *A. cerana* DNA samples.

2.10 Amplification of control region of *A. cerana* DNA samples

The selected primer pairs (from 2.9.3) were used to amplify mtDNA control region of *A. cerana* DNA samples using the same PCR condition as in 2.9.2. The primer pair which could give PCR product were selected and then optimization of DNA template, $MgCl_2$ concentration and primer concentration were performed.

2.10.1 Optimization of the $MgCl_2$ concentration

*A. cerana* mtDNA was amplified using selected primer pair for control region. The amplified condition was as described in 2.9.2 excepted the magnesium ion concentration which was varied from 1.0 mM to 6.0 mM (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mM).

2.10.2 Optimization of DNA template concentration

*A. cerana* mtDNA was amplified using selected primer pair for control region. The amplified condition was as described in 2.9.2 the magnesium ion concentration from 2.10.1 excepted the DNA concentration which serial dilution (12.5, 25, 50 and 100 ng).

2.10.3 Optimization of the primer concentration

*A. cerana* mtDNA was amplified using selected primer pair for control region. The amplified condition was as described in 2.9.2 the magnesium ion concentration from 2.10.1 and DNA concentration as in 2.10.2 excepted the primer concentration which varied from 0.1 μM to 0.5 μM (0.1, 0.2, 0.3, 0.4 and 0.5 μM).

2.11 Characterization of PCR product by sequencing.

PCR products (control region) of *A. mellifera* and *A. cerana* were sequenced using inner primer (5′-AAA ATA AAT AAA TCA GTG GTA-3′) which designed from $ND_2$ gene of *A. mellifera ligustica* (Crozier and Crozier, 1992). The sequence of a deoxyribonucleic acid molecule of mtDNA $ND_2$ gene can be elucidated using enzymatic methods. This method is based on the ability of a DNA polymerase to extend a primer which 5′-end-labeled using $T_4$ polynucleotide kinase with [γ- $^{32}$P] ATP or [γ- $^{33}$P] ATP by one-step extension/termination reaction. The primer is hybridized to the template that is to be sequenced, until a chain terminating nucleotide is incorporated. Sequence determination is carried out as a set of four separate reaction, each of which contains all four deoxyribonucleotide triphosphates (dNTPs) supplemented with limiting amount of a different dideoxyribonucleotide triphosphate (ddNTPs) per reaction. Because ddNTPs lack the 3′OH

หอสมุดกลาง สถาบันวิทยบริการ จุฬาลงกรณ์มหาวิทยาลัย



group necessary for chain reaction, the growing oligonucleotide is terminated selectively at G, A, T or C, depending on the respective dideoxy analog in the reaction. The relative concentrations of each of the dNTPs and ddNTPs can be adjusted to give a nested set of terminate chains over several hundred to a few thousand bases in length. The resulting fragments, each with a common origin but ending in a different nucleotide are separated according to size by high resolution denaturing polyacrylamide gel electrophoresis.

2.11.1 Template preparation

The PCR product for characterization was purified by QIAEX II agarose gel extraction. The PCR-product was electrophoresed in 1.5% agarose gel and the desired DNA band was excise from the agarose gel with a clean, sharp scalpel. The gel slice was weighted in 1.5 ml eppendrof and added 3 vol of buffer QX I to 1 volume of DNA fragment gel. The QIAEX II was resuspened by vortexing for 30 sec after addition of 10 µl QIAEX II to the sample. After , incubated at 50°C for 10 min to solubilize the agarose and bind the DNA. Mixed by vortexing every 2 min to keep QIAEX II in suspension. The sample was centrifuged for 30 sec and carefully removed supernatant with a pipet and washed a pellet with 500 µl of buffer QX I. The pellet was resuspended by vortexing, the sample was centrifuge for 30 sec and carefully removed all trace of supernatant with a pipet and washed the pellet twice with 500 µl of buffer PE. The pellet was collected by centrifuge and the supernatant was removed with a pipet. After air-dried the pellet until it became white the DNA would be eluted by ultrapure water. Following, incubate at room temperature for 5 min and centrifuged for 30 sec. The supernatant was carefully transfered into a

clean tube. The supernatant containing the eluted DNA was collected for sequencing.

2.11.2 End-label the sequencing primer

The primer can be labelled at 5′end by using either [γ- $^{32}$P] ATP or [γ- $^{33}$P] ATP. In this studied [γ- $^{32}$P] ATP with specific activity of 3,000 ci/mmol was used. The labelling reaction mixture was performed in 0.5 eppendrof which contained 20 ng of sequencing primer, 10 pmol of γ- labeled ATP, 1 μl of 10X buffer (500 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 50 mM DTT and 1.0 mM spermidine), $T_4$ polynucleotide kinase 5 unit and nuclease-free water to final volume of 10 μl. The mixture was incubated at 37°C for 10 min and then the kinase was inactivated at 90°C for 2 min. This volume (10 μl) is appropriate to label enough primer for 6 sets of double-stranded sequencing reactions. These end-labeled primers could be stored at -20°C as long as one month and still generate clear sequence data.

2.11.3 Extension and termination reactions

For each set of sequencing reactions, four 0.5 ml eppendrof was labeled (G, A, T, C) and 2 μl of the appropriate d/ddNTPs was added. After mixing each tube was capped and stored on ice or at 4 °C until need. Primer template mix consisted of template DNA 50 ng, 5 μl of DNA sequencing 5X buffer (250 mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$), 1.5 μl of labeled primer and nuclease-free water to final volume of 16 μl. One microliter of OmmiBase™ Sequencing Enzyme Mix (10 U/μl) was added to the primer/template mix. After mixed briefly by pipetting, 4 μl of the enzyme/primer/template mix was added into the d/ddNTPs mix

tube and placed the reaction tube in a Perkin Elmer Cetus DNA Thermal Cycler (Model 2400). The extension are performed for 95 °C for 2 min, 30 cycles at 95°C for 30 sec, 45°C for 30 sec, 70°C for 1 min and final extension at 70°C for 7 min then stored at 4°C. After the cycling program had been completed, 3 µl of DNA sequencing stop solution (10mM NaOH, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol) was added each tube. Immediately before loading on a sequencing gel, the reactions was heated at 70 °C for 2 min.

2.11.4 Preparation of the polyacrylamide gel

The polyacrylamide gel was set between two glass plates, the shorter glass plate and the longer glass plate. The shorter glass plate should be siliclonized to available water repellent and both plates were cleaned with water followed by 95% ethanol. The spacer was placed on both side of the longer plate and then cover with the shorter plate with the siliconized side facing down. Both sides and the bottom were sealed with sealing tape. The plates were optionally clamped on both sides with clips and ready for gel setting. Six percent denaturing acrylamide gel (460 g urea, 150 ml 40% (w/v) acrylamide, 100 ml 10X TBE and made up to 1 lite with deionized water) was prepared and could be stored in the refrigerator for a few week. For sequencing, gel mix (6%) of 60 ml was transfered to breaker and add immediately 150 µl and 42 µl of 10% ammonium persulfate and TEMED, respectively. Quickly mixed and poured the gel plates, avoid air bubble, let the solution flow continuously down along one side of the plate. After full the plate with gel mixed, comb and clamp were inserted at both sides with clips. The gel was usually polymerized after let stand for another 15 min.

2.11.5 Polyacrylamide gel electrophoresis

The comb and the tape were removed from the bottom and both sides from the polymerize gel. Then the inside well was clean with water for a few times. After the gel plate was fitted with a sequencing gel box and 1X TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM $Na_2EDTA$; pH 8.3) was added into the chamber. Before loading the samples, the residual gel mix and urea was removed from the well using syring with needle. The samples was loaded and subjected the gel with constant 40 watts. After electrophoresis was completed the glass plates were separated and the gel was exposed to X-ray film for about 10 hr, at -80°C. The X-ray film was developed and fixed using the method recommended by the supplier.

2.12 Analysis mtDNA control region polymorphism

The amplified mtDNA control region from each individual workers of *A.cerana* was digested with a panel of 15 restriction enzymes which include 10 six-base cutters (*Ase*I, *Ssp*I, *Nde*I, *Hin*dIII, *Dra*I, *Bfr*I, *Eco*RI, *Kpn*I, *Sma*I and *Swa*I), 1 five-base cutter (*Hin*fI) and 4 four-base cutters (*Rsa*I, *Alu*I, *Sau*3AI and *Taq*I). The reaction mixture of 20 µl containing about 500 ng of DNA, 10 units of each restriction endonucleases, 2 µl of 10X reaction buffer and adjusted the volumn to 20 µl by sterile deionized water. Then the reaction mixture was incubated at 37 °C for 2-3 hr and the reaction was stop by addition of one-fifth volume of a loading dye. The fragments of DNA were separated by electrophoresed in 1.5% agarose gel or 2-5% metaphor gel and stained in ethidium bromide. The DNA fragments on agarose and metaphor gel were visualized and photographed under UV light.

## 2.13 Data analysis

In statistical analysis, two input files was prepared and analyzed in Restriction Enzyme Analysis Package, REAP version 4.0. First, the composite haplotype file consists largely of a rectangular data matrix, with alphanumeric characters corresponding to alternative restriction phenotyped across OTUs for restriction enzymes; the corresponding enzyme profile input file is a tabular matrix (by enzyme) of the binary representations of those restriction phenotypes specified in the haplotype file. Second, the enzyme profile was generated from restriction pattern of each restriction enzyme with digested control region. The fragments size in restriction pattern were recorded in binary matrix for each haplotype. The character state '1' denoting the presence of a particular fragment and '0' the absence of a fragment.

### 2.13.1 Genetic distance

The nucleotide substitution per site between composite haplotype was calculated and showed in genetic distance (d-value) which using the formula.

$$d = -(2/r) \ln G$$

Where r is the number of recognized sequences of each restriction endonuclease

G is $[F(3-2GI)]^{1/4}$ and repeatly calculated G = GI then $GI = F^{1/4}$ is the recommended to initial trial value

F is the similarity index between haplotypes was estimated by

$$F = 2n_{xy}/(n_x + n_y)$$

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Where $n_{xy}$ is the number of fragments shared by individuals x and y and $n_x$ and $n_y$ are the number of fragments scored for each individual.

2.13.2 Haplotype and nucleotide diversity

The haplotype and nucleotide diversity within population were calculated from combined haplotype frequency distributions for each population and d values among haplotypes

The haplotype diversity was calculated by

$$h = n (1- \Sigma x_i^2)/ (n-1)$$

Where n is the number of individuals investigated and $x_i$ is the frequency of the $i^{th}$ haplotype.

The nucleotide diversity (d) is an average number of nucleotide differences per site between two sequences which could be calculated using

$$d = (n/n-1) \Sigma x_i x_j d_{ij}$$

Where n is the number of individuals investigated and $x_i$ and $x_j$ are the frequencies of i and j genotype and $d_{ij}$ is the number of nucleotide difference per site between the $i^{th}$ and $j^{th}$ haplotype.

The nucleotide diversity is more appropriate than of the haplotype diversity when compare the same DNA markers with difference in length because the haplotype diversity value vary enormously with the length if investigated genome.

2.13.3 Nucleotide divergence

The nucleotide divergence was estimated to nucleotide diversity between haplotypes in population X and Y ($d_x$ and $d_y$) as

equation 2.13.2. The average nucleotide diversity between haplotypes from population x and y ($d_{xy}$) was estimated by

$$d_{xy} = \sum x_i x_j d_{ij}$$

Where $d_{ij}$ is nucleotide substitution between the $i^{th}$ and $j^{th}$ haplotype from population x and y. Then the nucleotide divergence between two populations ($d_a$) is calculated from

$$d_a = d_{xy} - (d_x + d_y)/2$$

2.13.4 Chi-square analysis

The Chi-square ($\chi^2$) analysis based on Monte carlo simulation which performed on composite haplotype frequencies to as certain the difference between population.

2.13.5 Fst analysis

The genotypic differentiation among population was analyzed by Fst value using GENEPOP( version 2)

2.13.6 Dendrograms

The genetic distance (d-value) among composite haplotypes and the nucleotide divergence between populations was used to construction phenograme using UPGMA in phylip version 3.57c.