CHAPTER 2

MATERIALS AND METHODS

2.1 Instruments

Autoclave, HA-32, Manufacturing corporation, Japan

Automatic micropipette, Pipetman; P2, P20, P100, P200, P1000, Gilson Medical Electrics S. A. , France

Camera, Pentax Super A

Gel dryer, model 583, Bio-Rad, USA

High speed microcentrifuge, Kubota 1300, Kubota corporation, Japan

Horizontal gel electrophoresis apparatus for 8.5x12.5 cm gel former

Incubator, BM600, Memert GmbH, W., Germany

Orbital shaker, S03, Stuart scientific, Great Britain

PCR, Gene Amp System 2400, Perkin Elmer, Cetus, Norwalk, Connecticus

PCR workstation, HP-036, C. B. S. scientific Co., California

pH/ ION meter, PHM 95, Radiometer copenhagen

Power supply, Power PAG 300, Bio-Rad, USA

Standard cassette, 14x17 inch, Okamoto, Japan

Univap concentrator centrifuge, Univapo 100H, Uniequip

UV transilluminator

X-ray light box

2.2 Inventory supplies

Black and white print film, Tri-Xpan 400, Eastman Kodak company Rochester, USA

Filter paper, HA 0.45 μM pore size, Millipore corporation, USA

Whatman paper, 3MM, Whatman International Ltd., Maid stone, England

X-ray film, X-Omat XK-1, Eastman Kodak company Rochester, USA

2.3 Chemicals

Agarose,Seakem LE, FMC Bioproducts, USA

Agarose, Metaphore, FMC Bioproducts, USA

Amplitaq DNA polymerase kit, Perkin Elmer, New Jersey, USA

Boric acid, BDH Laboratory suppies, England

100 mM dATP, dCTP, dGTP, dTTP, Promega corporation Medison, Wisconsin

Chloroform, BDH Laboratory supplies, England

Developer and Fixer, Eastman Kodak company Rochester, USA

Ethidium bromide, Sigma chemical company, USA

Disodium ethylenediamine tetraacetic acid (EDTA), Fluka chemica Biochemica, Switzerland

Isoamyl alcohol, E. Merck, Germany

Lamda phage DNA, New England Biology company, USA

Oligonucleotide primers, Bio synthesis

OmniBase™ DNA Cycle Sequencing System kit, Promega, USA

Phenol, Carlo Erba

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Sodium chloride, E. Merck, Germany

Sodium dodecyl sulfate (SDS), Sigma chemical company, USA

Trisma base, Sigma chemical company, USA

2.4 Molecular weight standard

100 bp DNA ladder (1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp)

pBR322 digested with *Msp*I (622, 527, 404, 307, 242/238, 217, 201, 190, 180, 162(2), 147(2), 123, 110, 90, 76, 67, 34(2), 26(2), 15 and 9 bp)

λ DNA digested with *Hin*dIII (23.1, 9.4, 6.6, 4.4, 2.3, 2.1 and 0.6 kp)

2.5 Enzymes

Proteinase K, Gibco BRL life Technologies, Inc., USA

Restriction endonucleases; *Acs*I, *Alu*I, *Bfr*I, *Dra*I, *Eco*RI, *Hin*fI, *Ssp*I, *Swa*I, *Taq*I, *Tru*9I (Boehringer Manham), *Bam*HI, *Bgl*I, *Bgl*II, *Bst*EII, *Bst*NI, *Cla*I, *Hae*III, *Hinc*II, *Kpn*I, *Mlu*NI, *Pvu*II, *Rsa*I, *Sal*I, *Sau*3AI, *Sca*I, *Sma*I, *Tha*I, *Xba*I, *Xho*I (GibcoBRL), *Nde*I, *Pst*I, *Vsp*I (Biolabs)

Taq DNA polymerase, Perkin Elmer, Cetus, Norwalk, Connecticus

All equipments and reagents were autoclaved or alternatively filtered through a 0.45 μm filter into sterile container before used for sterilizing and inactivation of nuclease.

2.6 Biological material

2.6.1 *Apis cerana*

Adult workers honeybee were collected from 206 feral colonies from five different areas in Thailand (appendix 1 and 2) and number of colonies were as follows: The North (36), The North- East (30), The Central region (31), The South (72) and The Samui Island (37). Samples were either frozen in liquid nitrogen for transport to the laboratory where they were stored at - 80°C or immediately preserved in absolute ethanol for later use. Feral colonies were defined as colonies occurring in unmanaged home site (e.g., tree, cave, bridges or buildings) or colonies collected by beekeepers from unmanaged sites and placed in hives without further management, such as queen replacement.

2.6.2 *Apis mellifera*

Adult workers honeybee were collected from two colonies from the North- East and one colony from the Central region of Thailand and preserved in absolute ethanol and stored at -20°C.

2.7 DNA isolation

Total DNA was extracted from individual thorax, which was homogenized with a plastic pestle in 500 µl of STE (100 mM NaCl; 50 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 8.0), then proteinase K (10 mg/ml) and 20% SDS were added. The mixture was incubated at 55°C for 2 hr and the suspension was centrifuged at 8000 rpm for 10 min (high speed microcentrifuge, Kubota 1300). The supernatant was extracted

once with an equal volume of phenol/chloroform/isoamyl alcohol(25:24:1) and then twice with chloroform/isoamyl alcohol (24:1). After each extraction, phases were separated by centrifugation at 8000 rpm for 10 min. Subsequently, 3 M sodium acetate were added to the final concentration of 0.3 M. DNA was precipitated with two volumes ethanol at -20°C overnight, centrifuged at 12000 rpm for 10 min and washed with 70% ethanol (v/v). The DNA pellet was air-dried and dissolved in TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0)

2.8 Measurement of DNA concentration

The electrophoresis was the standard method used to separate DNA fragments under the influence of an applied electrical field on the basis of their molecular weight. The rate of movement in the gel is inversely proportional to the log of the molecular weight. The size of the fragments generated can be estimated by comparison with a standard curve of the distance migrated through the gel versus log molecular weight of a known DNA fragment molecule.

The extracted total DNA was running on 0.7% agarose gel in 1xTBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM Na_2EDTA; pH 8.3). The gel was prepared by adding 0.35 g of agarose to 50 ml of 1xTBE buffer. Agarose was solubilized by heating in a microwave oven and then allowed to cool to 50 to 60°C before pouring gel into plastic gel (8.5x12.5cm) former with preset locations for the well forming combs for casting the gel. Subsequently, electrophoreses at about 10 volts/cm gel length. When electrophoresis is complete, and then gel was stained with ethidium bromide. DNA concentration was estimated from fluorescent

band after gel staining and then comparison to the intensity of red fluorescent bands of standard DNA (500 ng lamda phage DNA digested with *Hin*dIII). The orange fluorescent intensity of standard DNA bands of 23.1, 9.4, 6.6, 4.4, 2.3, 2.1 and 0.6 kb corresponding to 240, 98, 67, 45, 24, 21 and 6 ng DNA, respectively.

2.9 Amplification of a DNA segment using the polymerase chain reaction (PCR)

Each PCR amplification should be conducted as an experiment, complete with control reactions to test the purity and viability of reagent. Two major types of controls are important; firstly a no template control to test for the absence of contamination in the reagents, secondary a positive control on a known template to test the performance of the buffers, enzyme, and the temperature cycle parameters.

2.9.1 Standard amplification condition

ATPase6-ATPase8 gene of *A. cerana* mtDNA was amplified via PCR, with a set of primers based specifically on sequences from ATPase6-ATPase8 gene of *A. mellifera* mtDNA. Oligonucleotides primers were designed by Oligo4.0 program and synthesized by Bioservice Unit (BSU) of National Science and Technology Development Agency (NSTDA), Bangkok, Thailand. The primers were at the start (5′end) and the end (3′end) of ATPase6-ATPase8 gene of *A. mellifera* mtDNA (Figure2.1) that enabled amplification of honeybees mtDNA through the PCR.

4440 5244

5′TTTAATTCCTCAAATAATAC3′..............3′TAAAGAGACTTAGTTTAATT5′

Figure2.1 Showing the primers and their orientation (5′→3′) used in PCR for ATPase6-ATPase8 gene of honeybees mtDNA amplification. The numbered position above the sequence correspond to those in the original sequence data (Crozier and Crozier, 1993).

Amplification reactions were performed in 50 μl volumes containing 10 mM Tris-HCl (pH8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.001% (w/v) gellatin, 200 μM each of dATP, dCTP, dGTP, and dTTP, 0.2 μM of each primer, 25 ng of total DNA, and 0.5 unit of Ampli *Taq* polymerase; the desired volume was restored by addition of a suitable amount of steriled double distilled water. Amplifications were performed in a Gene Amp System 2400 (Perkin-Elmer) for 2 min at 95°C, and then 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 1 min and extension at 72°C for 2 min, the last extension at 72°C for 10 min.

2.10 Optimization in the Amplification conditions

2.10.1 Optimization of the amount of DNA Template

Different amounts of *A. cerana* total DNA, 12.5, 25, 50, and 100 ng, were amplified under standard conditions with primer in figure 2.1.

2.10.2 Optimization of the magnesium ion concentration

ATPase6-ATPase8 gene of *A. cerana* mtDNA was amplified with the primer in figure 2.1 under standard condition and the total $MgCl_2$ concentration varied from 1.0 mM to 4.0 mM.

2.10.3 Optimization of the primers concentration

ATPase6-ATPase8 gene of *A. cerana* mtDNA was amplified under standard conditions, except that the $MgCl_2$ concentration was 2.5 mM and the primer concentration varied from 0.1 μM to 0.5 μM.

2.11 Characterization of the amplification product

It is essential to assess the purity of the amplification product before continuing with more detailed characterization of the amplified DNA. Contamination of reagents by foreign DNA is only one cause of inappropriate products. Annealing of primers to alternative sites in the template DNA also can produce a variety of unwanted DNA fragments. Finally, it is important to quantify the amplification achieved before further characterizing the fragment.

2.11.1 Gel electrophoresis of the amplification products

The homogeneity of the amplified DNA is most conveniently assessed by electrophoresis through an agarose gel. Because of the small size of the chain reaction products, an agarose specifically formulated to separation of small DNA fragments should be used.

Five μl of each of the amplification product and molecular weight DNA size maker (100 bp DNA ladder) were loaded into 1.5%

agarose gels (8.5x12.5 cm). Electrophoresis was performed in 1xTBE buffer at constant voltage of 10 volt/cm until the faster migration dye (bromophenol blue) has traveled at least 10 cm and then stained with ethidium bromide. The DNA fragments on agarose gel was visualized and photographed under UV light (long-wave UV transilluminator).

2.12 Analysis of the amplification product by restriction fragment length polymorphism (RFLP)

2.12.1 Restriction endonuclease digestion

An amplification products were digested with a panel of 31 restriction endonucleases (Appendix 4). The reaction mixture was carried out in 20 µl which consisting of 10 µl aliquots of amplification product usually containing 500 ng of amplified ATPase6-ATPase8 gene, 1x the recommended buffer for the respective enzymes, 5 units of enzyme and sterilized distilled water. The reaction was incubated in a microcentrifuge tube at suitable digestion temperature for at least 3 hours. The reaction was stoped by the addition of one fifth by volume of loading dye (0.25% bromophenol blue, 0.25% xylene cyanols, 15% (w/v) Ficoll type 400, 1% SDS, 0.1M Na_2EDTA, pH8.0).

2.12.2 Metaphore gel electrophoresis

Digestion of an amplified ATPase6-ATPase8 with restriction endonucleases resulted in small DNA fragments which their resolution in metaphor agarose gel was higher than in agarose gel. Therefore, metaphor agarose gel is more appropriate than agarcse gel because high resolution are required in this study.

The horizontal gel was prepared by weighting out an enough amount of metaphor agarose to prepared 3-4% (w/v) gel, dissolving in 1xTBE buffer and heating to boiling in a microwave. The metaphor solution was left at 80°C and poured into the chamber set (8.5x12.5 cm). The total digestion volume was loaded into the gel. Electrophoresis using 1xTBE buffer at 10 volt/cm. The gels were stained with ethidium bromide and photographed under UV light.

2.13 DNA sequencing

The amplified ATPase6-ATPase8 gene of some *A. cerana* samples were sequenced by using OmniBase™DNA Cycle Sequencing System and Omnibase™ Sequencing Enzyme Mix.

The sequence of a deoxyribonucleic acid molecule can be elucidated using enzymatic method of sequencing is based on the ability of a DNA polymerase to extend a primer, which 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 reactions, each of which contains all four deoxyribonucleotide triphosphate (dNTPs) supplemented with a limiting amount of a different dideoxyribonucleotide triphosphate (ddNTP) per reaction. Because ddNTPs lack the 3′OH group necessary for chain elongation, the growing oligonucleotide is terminated selectively at G, A, T or C, depending on the respective dideoxy analog in the reaction. 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.

2.13.1 Template preparation

PCR products were purified by recovered from agarose gel after electrophoresis, by using QIAquick Gel Extraction Kit. After electrophoresis, DNA fragment was excised from the 2% agarose gel with a clean, sharp scalpel and the gel slice was weighed in a microcentrifuge tube. Then 3 volumes of buffer QG was added to the gel slices. The sample was incubated at 50°C for 10 min. During the incubation, the sample was mixed by vortexing every 2-3 minutes. After the gel slice was dissolved completely, the color of the mixture would be yellow. The sample was then applied to the QIAquick column, and centrifuged for 1 min at 10,000 rpm (high speed microcentrifuge, Kubota 1300). The flow-through was discarded and 0.5 ml of buffer QG was added to QIAquick column and centrifuged for 1 minute. The QIAquick column was washed with 0.75 ml of buffer PE and centrifuged for 1 min. The flow-through was discarded and centrifuged again for additional 1 minute. Subsequently the QIAquick column was placed into a clean 1.5 ml microfuge tube. DNA was eluted by addition of 50 μl of buffer EB (10 mM Tris-HCl, pH 8.5) to the center of the QIAquick column and centrifuged for 1 min at maximum speed.

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2.13.2 End-labeled primer with the Omnibase™ DNA cycle sequencing system

2.13.2.1 Primer radiolabeling reaction

The following procedure was designed to label enough primer with [γ- ^{32}P]ATP, a specific activity of 3,000 Ci/mmol, for 6 sets of double-stranded sequencing reaction, and can be scaled

proportionately according to the number of reactions to be performed. The amount of [γ- ^{32}P]ATP in the reaction should be doubled if the isotope has decayed by one half- life (approximately 14 days).

The labelling reaction was prepared by combined the following in a 0.5 ml microcentrifuge tube: 4-10 pmol of primer, 10 pmol of [γ-^{32}P]ATP , 1 μl of T4 polynucleotide Kinase 10xbuffer, 5 units of T4 polynucleotide Kinase (5-10 U/μl) and nuclease-free water was added to final volume of 10 μl. The reaction mixture was incubated at 37°C for 10 min and then inactivated the Kinase at 90°C for 2 min. Briefly centrifuged the reactions in a microcentrifuge to collect any condensation. The end- labeled primers may be stored at -20°C for as long as one mount and still generate clear sequence data. The primer was used directly without further purification.

2.13.2.2 Extension/Termination reaction

For each set of sequencing reactions, four 0.5 ml microcentrifuge tubes for G, A, T and C were prepared. Then 2 μl of the appropriate d/ddNTP nucleotide mix was added to each tube. The tubes were capped and stored on ice or at 4°C until needed. Mixture of template DNA and [γ- ^{32}P]ATP labeled primer were prepared in the other microcentrifuge tube, each tube containing 4-40 fmol of template DNA, 5 μl of DNA sequencing 5x buffer, 1.5 μl [γ- ^{32}P]ATP labeled primer and nuclease-free water to final volume of 16 μl. After that 1 μl of OmniBase™ 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 to the inside wall of each tube

which containing d/ddNTP mix. The reaction tubes were placed in a thermal cycles for 30 cycles of 30 seconds at 95°C, 30 seconds at 42°C and 1 minute at 70°C, then 4°C. After the cycling program has been completed, 3 µl of DNA sequencing stop solution was added to the inside wall of each tube. Briefly centrifuged in a microcentrifuge to mix the stop solution with the reactants and terminated the reaction. The reactions were heated at 70°C for 2 minutes immediately before loading on a sequencing gel. Load 2.5-3.0 µl of each reaction on the gel.

2.13.3 Sequencing gel preparation and electrophoresis

The DNA products of sequencing reactions were separated in denaturing polyacrylamide gels as a function of the log of their molecular weight. As a result, the distance between smaller fragments was greater than that of larger fragments. The rate of migration of DNA fragments in the gel is a function primarily of the voltage gradient (volts/cm of gel length). Thus, longer gels require a greater voltage to achieve a given speed of separation.

2.13.3.1 Preparation of the gel

The glass plates were cleaned thoroughly in warm water. It was advisable not to used abrasive or detergents to clean the plates, and this should not be necessary if the plates were washed immediately after used. Wipe a few millilitres of silanizing solution over the surface of the shorted plate with Kimwipes, on the side which will contact the gel and leave to air dry. Both plates were wiped with 96%(v/v) ethanol, and polish thoroughly, taking care to remove dust from

the plates surfaces. Assemble the gel would with the two spacers, and used gel-sealing tape to seal around the bottom and sides of the mould. Ensure that sealing was completed. Subsequently, allow sufficient 6% denaturing acrylamide gel mix: 460 g urea, 150 ml 40%(w/v) acrylamide, 100 ml 10x TBE made up to 1 litre with deionized water, to warm up to room temperature. For a 43 cm x 0.35 mm x 33 cm gel would used 60 ml and then added 300 µl 10%(w/v) ammonium persulphates and 42 µl TEMED. Mix and then poured into the gel would using a 50 ml disposable syringe. Start pouring the gel with the mould at about 45°C to the horizontal, and gradually decrease this angle as the mould fills up with gel mix. Try to keep the flow of gel mix between the plates continuous, as this will minimize the risk of air bubbles. The gel comb was inserted to a depth of about 0.5 cm, the plate was clamped with fold back clips directly over the spacers and the gel was left to polymerize for at least 30 min.

2.13.3.2 Gel loading and running

The gel-sealing along the bottom of the cost gel was removed or sliced with a razor blade. The gel mould was placed in the gel running tanks, and clamped securely into place. It may be necessary to seal around the upper rubber seal of the apparatus with silicone wax to prevent buffer leaking. 1x TBE buffer was poured in to the upper and lower buffers chambers and then remove the gel comb, and immediately flush out the gel wells with a pasture pipette and the buffer in the upper chamber. The sample should be heat-denatured by heating at 80°C for 2 min. Immediately prior to loading, flush out the sample wells

again to remove any residual urea. The end of the tip was placed close to the bottom of the well and do not attempt to load all of the sample, as this will probably introduce air bubbles. When gel loading was complete, placed the cover over the gel and turned the power supply on. The gel was run at constant power for a 50 cm-long gel run at 35-40 watts for about 2.5 hours. By this time, the bromophenol blue marker dye should reach the end of the gel.

2.13.3.3 Autoradiography

When electrophoresis was completed, removed the shorted plate, and then laid a piece of Whatman 3MM paper, cut to a suitable size, over the gel, and slowly peel back. The gel should stick to the paper. The gel and paper were coverd with saran wrap and dried the gel under vacuum at 80°C for 2 hours. Then the gel was placed in a cassette next to the X-ray film and exposed for 10 hours, at -80°C. The X-ray film was developed using the method recommended by the supplier.

2.14 Data analysis

2.14.1 Restriction fragment data analysis

All statistical analyses were carried out in Restriction Enzyme Analysis Package, REAP version 4.0 (McElroy, 1991) which two input files are required. The composite haplotype file consists largely of a rectangular data matrix, with alpha numeric characters corresponding to alternative restriction phenotype across OTUs for a series of 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.

The restriction fragment patterns of each restriction endonuclease digested ATPase6-ATPase8 gene of *A. cerana* mtDNA were assigned a molecular weight and recorded in a binary matrix for each haplotype with character state '1' denoting the presence of a particular fragment and '0' the absence of a fragment. The different haplotype were designated in a single alphanumeric character (A, B, C, ...) in order to their frequencies which have been observed.

2.14.1.1 Genetic distance

Genetic distance (*d* values) were showing the relationship between composite haplotype that could be computed as the number of nucleotide substitution per site using the formula :

$$d = -(2/r) lnG$$

where r is the number of recognized sequences of each restriction endonuclease and G is $[F(3-2GI)]^{1/4}$ and repeatly calculated $G = GI$ then $GI = F^{1/4}$ is recommended to initial trial value.

F is the similarity index between haplotypes was estimated by

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

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.

2.14.1.2 Haplotype and nucleotide diversity

Haplotype frequency distributions for each population and the associated d values among haplotypes are used to

estimate haplotype and nucleotide diversity of mitochondrial DNA within population. 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 ith haplotype. Nucleotide diversity (π) is an average number of nucleotide differences per site between two sequences which could be calculated using

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

when 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 differences per site between the ith and j^{th} haplotype. This value is more appropriate than of the haplotype diversity for comparisons of the same DNA markers with difference in length because the haplotype diversity value varies enormously with the length if investigated genome.

2.14.1.3 Nucleotide divergence

For nucleotide divergence, total nucleotide diversity between two populations was estimated, and the component of this diversity not explained by within population polymorphism was extracted. An average nucleotide diversity between haplotypes from population x and y (d_{xy}) was estimated by

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

where d_{ij} is nucleotide substitution between the ith and j^{th} haplotype from population x and y. Then the nucleotide divergence between two populations (d_A) is calculated from

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

2.14.1.4 Chi-square analysis

A model of restriction fragment pattern was referred to a haplotype which was generated from ATPase6-ATPase8 gene of *A. cerana* mtDNA was digested with three restriction endonuclease. Chi-square (χ^2) test, a Monte carlo simulation was also performed on composite haplotype frequencies to as certain the difference between population.

2.14.2 Dendrograms

The genetic distance (*d* values) between composite haplotype and the nucleotide divergence between population (d_A) were subjected to cluster analysis using the UPGMA in Phylip version 3.57c.

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