CHAPTER III

MATERIALS AND METHODS

3.1 Instruments

Autoclave (HA-30) by Manufacturing Corporation, Japan.

Automatic micro pipette (Pipetman P20, P100 and P200) by Gilson Medical Electronics S.A., France.

Camera (Pentax super A)

Digital pH meter (PHM 83 Autocal) by Radiometer, Copenhagen, Denmark.

Electrophoresis apparatus : Horizontal gel electrophoresis apparatus for 9x10 cm gels, Power supply (EPS-3200- EPS 5100).

High speed microcentrifuge (MC-15A) by Tomy Seiko, Japan.

Incubator (BM 600) by Memert GmbH, W. Germany.

Magneticstirrer (0188 GMS) by Scientific Instrument Development and Service Center, Faculty of Science, Chulalongkorn University, Bangkok.

Shaking water bath (01PF623) by New Brunswick Scientific Co., Inc., U.S.A.

Standard cassette (8x10 inch) by Okamoto, Japan.

UV Spectrophotometer (Spectronic 2000) by Bausch and Lomb, U.S.A.

UV (302-OR 356-nm wavelength) transilluminator box by UVP San Gabriel, California, U.S.A.

Water bath (A 466) by Charles Hearson & Co.Ltd., England.

3.2 Inventories

Black and white print film (Tri-X pan 400) by Eastman Kodak Company Rochester, New York.

Filter paper (HA 0.45 μm pore size) by Millipore Corporation, U.S.A.

Positively charged nylon membrane (0.45 μm pore size) by Boehringer Mannheim Coporation, Indianapolis, IN., Germany.

Whatman paper 3 MM by Whatman International Ltd., Maidstone, England.

X-ray film (X-Omat XK-1) by Eastman Kodak Company, Rochester, New York.

3.3 Chemicals

Agarose (type II) and Agarose (type VII) Low Gelling Temperature by Sigma Chemical Company, U.S.A.

Bovine serum albumin by Sigma Chemical Company, U.S.A.

Bromphenol blue by Sigma Chemical Company, U.S.A.

Chloroform by BDH Laboratory Supplies, England.

Developer and Fixer by Eastman Kodak Company Rochester, U.S.A.

DIG DNA Labeling and Detection Kit Nonradioactive by Boehringer Mannheim Corporation, Indianapolis, IN., Germany.

Ethidium bromide by Sigma Chemical Company, U.S.A.

Disodium Ethylenediamine tetracetic acid (EDTA) by Fluka Chemika Biochemika, Switzerland.

Isoamyl alcohol by E. Merck, Germany.

Lambda phage DNA by New England Biolabs Company, U.S.A.

Lysozyme chloride by Sigma Chemical Company, U.S.A.

Magnesium chloride by Sigma Chemical Company, U.S.A.

N-Lauryl sarcosine by Sigma Chemical Company, U.S.A.

Phenol by Carlo Erba.

Sodium chloride by E. Merck, Germany.

Sodium dodecyl sulfate (SDS) by Sigma Chemical Company, U.S.A.

Trizma base by Sigma Chemical Company, U.S.A.

3.4 Antibiotics

Ampicillin by Sigma Chemical Company, U.S.A.

Tetracycline by Sigma Chemical Company, U.S.A.

3.5 Enzymes

Alkaline phosphatase by Bethesda Research Laboratories, Inc. (BRL), U.S.A.

Proteinase K by Sigma Chemical Company, U.S.A.

Restriction endonucleases, by Bethesda Research Laboratories, Inc. (BRL), U.S.A. and by New England Biolabs, England.

Ribonuclease A by Sigma Chemical Company, U.S.A.

T4 DNA Ligase by Sigma Chemical Company, U.S.A.

All equipments and reagents are autoclaved or sterilized by passing through a 0.45 μm filter into sterile containers before used.

3.6 Nonradiolabeled *Apis mellifera* probes

Apis mellifera probes number # 24 and # 47, were kindly obtained from Dr. H. A. Sylvester, Honey-Bee Breeding, Genetics, & Physiology Laboratory; United States Department of Agriculture, Agricultural Research Service, Baton Rouge, Louisiana. These probes were cloned in (Lambda) bacteriophage EMBL3 grown in *E. coli* LE 392 and labeled with the Genius® nonradioactive DNA labelling system from Boehringer Mannheim. The probe # 24 has shown variation in *A. mellifera, A. florea* and probably *A. cerana* and the # 47 probe has shown variation in *A. mellifera* and probably *A. cerana.*

3.7 Honey bee specimen collection

3.7.1 *Apis cerana*

Ten to twenty worker pupa (11-13 days) of *Apis cerana* from each colony (Figure 2) were collected from natural colonies and apiaries (Figure 3). About 20 colonies in the following parts of Thailand; 1) the Northern 2) the North-Eastern 3) the Central part 4) the Southern and 5) Samui Island were collected (Figure 4 and Appendix 1-5). For isolation of total DNA, the extraction was immediately in the field whereas the nuclear DNA was extracted in the laboratory.

3.7.2 *Apis florea* and *Apis mellifera*

The samples were obtained from the Bee Biology Research Unit (BBRU), Chulalongkorn University. Five to ten worker pupa of a colony were collected and then DNA extraction was performed.

3.8 Honey bee DNA extraction

3.8.1 Total DNA extraction (modified from Devis *et al.*, 1986; Ishii, 1992)

The extraction was performed in the field immediately after collection of worker pupa by throughly homogenizing in 400 µl of a chilled extraction buffer (100 mM Tris; pH 9.0, 100 mM NaCl, 200 mM sucrose, 50 mM EDTA; pH 8.0) in a homemade homogenizer (Figure 5) and continued until they became homogeneous. 10% (w/v) Sodium dodecyl sulfate (SDS) was added to a 1% final concentration, mixed briefly and incubated in a water bath at 65 ^{O}C for at least 30 minutes. The

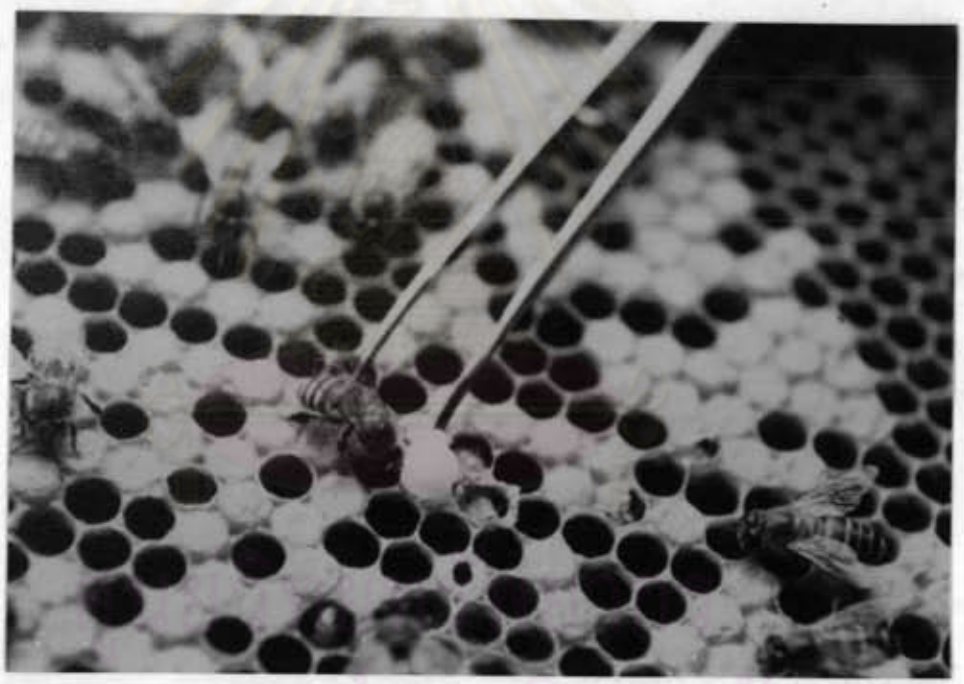

Figure 2 The worker pupae of *A. cerana* used for DNA analysis

Figure 3 The sampling colonies of *A. cerana* located in various places.

(A) Coconut hole (B) Guana hole

(C) The bed

(D) Domestic hive from the North-Eastern

(E), (F) Domestic hive from the Samui Island

(A)	(B)
(C)	(D)
(E)	(F)

Figure 4 Map of Thailand shows Sampling locations of *A. cerana*. Regions are indicated in number;

(1) the Northern
(2) the North-Eastern
(3) the Central part
(4) the Southern
(5) the Samui Island

Figure 5 The homemade homogenizer

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mixture was stored at room temperature for future DNA purification at the laboratory.

The homogenate was treated with 100 mg/ml Proteinase K and 50 mg/ml Dnase-free Rnase (pre-boiled for 10 minutes and cooled on ice), mixed gently and incubated at 37 ^{o}C at least 3 hours. Five molar potassium acetate was then added to a 1% final concentration, let cool in a microcentrifuge tube on ice for 45 minutes. This was centrifuged at 5,000xg for 10 minutes and the supernatant transfered to a new 1.5 ml microcentrifuge tube. This step may be repeated if the denatured protein in the interphase layer is incompletely removed. After the final extraction, the upper layer was transferred and a half volume of 7.5 M ammonium acetate was first mixed and 2 volumes of cold absolute ethanol were added later. After the tube had been inverted several time, it was placed at -20 ^{o}C at least 2 hours and then centrifuged at 5,000xg for 2 minutes. The supernatant was discarded and the pellet was washed with 70% cold ethanol and allowed to air-dry. The DNA was finally dissolved in an appropriate volume of TE buffer (10 mM Tris-HCl; pH 8.0, 1 mM EDTA) and stored at 4 ^{o}C until used. The resultant DNA solution was then able to have the quantity and quality determined.

3.8.2 Nuclear DNA extraction

An individual honey bee pupae was placed in a 1.5 ml centrifuge tube and homogenized in 700 µl of cold HomI buffer (100 mM Tris; pH 7.4, 250 mM sucrose, 1 mM EDTA, 2.5 mM $CaCl_2$, 5 mM $MgCl_2$). The tube was centrifuged at 5,000xg for 5 minutes and the supernatant was discarded. The pellet was homogenized with 850 µl of HomII buffer

(100 mM Tris; pH 7.4, 250 mM sucrose, 10 mM EDTA, 2.5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1% (v/v) Triton X-100) and spun at 5,000xg for 10 minutes and the supernatant was discarded. The pellet was mixed with lysis buffer (10 mM Tris; pH 8.0, 10 mM EDTA, 500 mM NaOAc, 1% SDS) plus w/10 µl Proteinase K (50 mg/ml), and incubated at 42 °C for at least 2 hours. Two hundred microlitres of phenol/chloroform : isoamyl alcohol (24:1) were added and mixed gently, spun at 6,000xg for 2 minutes. The supernatant was transferred to a new microcentrifuge tube and this step was repeated 2-3 times. Two volumes of cold absolute ethanol were added to the supernatant and placed at-20 °C for 30 minutes. Centrifuged at 8,000xg for 5 minutes, the supernatant was discarded, the pellet was washed with 70% cold ethanol then centrifuged again and the supernatant was discarded. The pellet was allowed to air dry and redissolved with a suitable volume of TE buffer and then stored at 4 °C.

3.8.3 Quantification of honey bee DNA

Measurement of DNA concentration was performed by using spectrophotometric analysis. 25 µl of DNA solution was added to 475 µl of TE buffer and its absorbance at 260 nm was recorded. The absorbance optical density (OD) at 260 nm of a 50 µg/ml solution of double-standed DNA is equal to 1. The ratio of $OD_{260/280}$ is used to, estimate the purity of a sample and it should fall between 1.65 and 1.85.

The semi-quantitation of DNA sample can be performed by comparison with the intensity ethidium bromide fluorescent DNA standards (*Hind*III digestion of 0.5 mg λ phage DNA). After staining with ethidium bromide, the intensity of orange-red fluorescence of the DNA bands was

observed under ultra-violet light. The fluorescent intensity of the standard DNA fragments of 23.13, 9.42, 6.56, 4.36, 2.32, 2.03 and 0.57 Kb λ/*Hind* III fragments) corresponded to 240, 98, 67, 45, 24, 21 and 6 ng DNA, respectively when 500 ng of DNA (48 kb) was digested with *Hind*III.

3.9 Cloning of *A. cerana* chromosomal DNA

3.9.1 Chromosomal DNA preparation (Modified from Maniatis, 1982)

The DNA sample purified according to section 3.8.1 was digested with restriction endonuclease *Bgl*II (1 mg DNA/ 5 U *Bgl*II) and the fragments were separated by electrophoresis on 0.8% (w/v) of low melting agarose (Type VII). The DNA fragments at 2 to 4 and 5 to 7 kb on agarose were excised and placed in individual 1.5 ml microcentrifuge tubes. The agarose gels were mixed with 2 volumes of TE buffer, and then heated in a water bath at 65 ^{o}C for approximately10 minutes (until the gel was melted). After that, they were left to cool down at room temperature. The solution was purified with phenol/chloroform and precipitated with cold absolute ethanol as described in section 3.8.1. The dried-pellet was dissolved in a suitable volume of TE buffer. The concentration DNA solution was estimated (as mentioned in section 3.8.3) and stored at 4 ^{o}C until used.

3.9.2 Plasmid DNA extraction (Modified from Maniatis, 1982)

Plasmid pBR322 was used as the vector DNA (restriction map is shown in Appendix 6) and prepared by using the minipreparation method,

starting from an *E. coli* strain HB101 colony containing plasmid pBR322. *E. Coli* was aerobically grown at 37 ^{o}C for overnight in 50 ml of Luria Bertani broth medium (LB medium) (appendix 7) including 25 μg/ml ampicillin and 12.5 g/ml tetracycline, in a 250 ml flask with shaking. Cells were collected by centrifugation at 4,000xg for 10 minutes, and washed with TE buffer. One ml of solution I (25 mM Tris-HCl; pH 8.0, 10 mM EDTA, 50 mM Glucose) plus 2 mg/ml of lysozyme (freshly prepared) was then used to suspend the pelleted cells. The tube was vortexed and left on ice for 10-15 minutes. 2 ml of solution II (10 mM NaOH, 10% (w/v) SDS) was added, and gently mixed by inversion until a clear solution appeared, left on ice for 10 minutes, 1.5 ml of solution III (3 mM NaOAc; pH 4.8) was then added, gently mixed, and returned to ice for 30-60 minutes. After that, the tube was centrifuged at 4,000xg for 10 minutes. The upper aqueous phase was then transfered to a new tube. An equal volume of phenol/chloroform was added to the transfered phase, vortexed and centrifuged. The supernatant was removed to a new tube, ensuring that none of the organic layer (interphase) was transfered. The supernatant was subsequently precipitated 2 volumes of absolute ethanol at -20 ^{o}C for at least 1 hour, afterward the salt concentration of the supernatant should be adjusted to greater than 100 mM by adding of 5 M NaCl. Then the tube was centrifuged and the supernatant decanted. The DNA pellet was washed with 70% (v/v) ethanol and allowed to dry at room temperature. Finally, the plasmid DNA was dissolved in a suitable volume of TE buffer, and the concentration was determinded and stored at 4 ^{o}C until used.

3.9.3 Plasmid DNA digestion and dephosphorylation (Maniatis, 1982)

About 1 μg plasmid DNA (from section 3.9.2) was completely digested with 5 U of *Bam*HI in 20 μl of the reaction mixture (as described in section 3.10). The reaction mixture was extracted with phenol/chloroform and the DNA precipitated with absolute ethanol as outlined in the previous procedures. Then, the linearized plasmid DNA was dephosphorylated by adding 3.5 μl (0.06 U) of Calf intestine phosphatase (CIP), 0.5 μl (1 U) of Alkaline phosphatase, and 1 μl of sterile distilled water in 35 μl reaction mixture. After that, the mixture was then incubated at 37 ^{O}C for one and a half hours. and One-tenth volumes of 3M NaOAc were added. After extracted with phenol/chloroform the ethanol precipitation was performed. The pellet was resolved in TE buffer and determined its concentration.

3.9.4 Recombinant plasmid preparation (modified from Maniatis, 1982)

DNA fragments of *A. cerana* after digestion with *Bgl*II and dephosphorylated plasmid DNA (from section 3.9.3) were mixed in a 3:1 (mole/mole) ratio in 40 μl of reaction mixture (20 μl (750 ng) of DNA fragments and 8 μl (250 ng) of dephosphorylated plasmid DNA). The mixture was incubated at 50 ^{O}C for 10 minutes. Then, 8 μl of T_4 ligation 5x buffer (with ATP), 0.5 μl (200U) of T_4 DNA ligase and 3.5 μl of sterile distilled water were added. The solutions were mixed and frozen

for 5 minutes, then prior incubated at 15 ^{O}C for 15 hours. After phenol/chloroform extraction, ethanol precipitation was performed. Finally, the DNA pellet was dissolved in a suitable volume of TE buffer. This recombinant plasmid DNA solution was now ready for transfromation.

3.9.5 Transformation of competent *E. coli* (modified from Mandel and Higa, 1970)

E. coli strain HB101 was cultured in 200 µl of LB broth medium; pH 7.4 at 37 ^{O}C for 2.5-3 hours or until the cells were in the log phase stage (OD_{260}=0.6). After that, the cells were chilled on ice for 5 minutes, then spun down by centrifugation at 4,000xg for 10 minutes. The pellet was resuspended in 500 µl of cold 100 mM $CaCl_2$ and then, let stand on ice at least 1 hour for future use.

One hundred microlitres of the competent cells were mixed with 20 µl of recombinant plasmid DNA (from section 3.9.4) and then placed on ice for 30 minutes. The mixture was incubated at 42 ^{O}C for 5 minutes and 500 µl of LB broth medium was added, then incubated at 37 ^{O}C for 15 minute. After that, 60 µl of the solution was spread onto an LB-agar plate (see Appendix 7) containing 50 µg/ml of ampicillin. The plate was incubated overnight at 37 ^{O}C and the transformant colonies were counted and selected.

Using sterile tooth picked, each colony from the master LB-agar plate was stabed onto an LB-agar plate containing 25 µg/ml ampicillin (the first plate) and an LB-agar plate containing both ampicillin and

tetracycline (the second plate) at 25 and 12.5 μg/ml, respectively. Both the first and second plates should have the colony in the same position as the master colonies (replica plating method). The plates were incubated overnight at 37 ^{O}C and then the colonies from the first plate; which did not grow in the second plate ($Ap^{r}Tc^{r}$), were selected and amplified by culturing on ampicillin LB agar plates. The transformant cells were proved again by dot hybridization.

The cells were preparatively regrown on ampicillin LB-agar plates and the recombinant plasmid DNA was isolated as described in a previous section (3.9.2). The plasmid DNA was digested with 5-10 U of restriction endonuclease *Pst*I (the linearized form). After electrophoresis 0.7% agarose, the molecular weights of DNA fragments were compared with the plasmid vector (pBR322; 4.361 kb). Only the higher fragments (>4.361 kb) were selected and used for dot hybridization with *A. cerana* DNA nonradioactive labeled probes (see section 3.10.2). The clones which showed an intense signal after dot hybridization were selected and further used as DNA probes.

3.10 Analysis of honey bee DNA by restriction pattern

DNA samples were digested with various restriction endonucleases (Table 1) in 20 μl of reaction mixture which contained approximately 800 ng of DNA, 2.5-5 units (U) of restriction endonuclease, 2μl of 10x restriction endonuclease buffer appropriate to the enzyme

Table 1 Some of restriction endonucleases used for restriction pattern analysis.

Restriction endonuclease	**Recognition sequence**	**Buffer used**	**Temperature (^{O}C)**
*BamH*I	5'-G/GATCC-3'	High	37
*Bgl*II	5'-A/GATCT-3'	High	37
*Cla*I	5'-AT/CGAT-3'	Low	37
*EcoR*I	5'-G/AATTC-3'	High	37
*Hae*III	5'-GG/CC-3'	Low	37
*Hind*III	5'-A/AGCTT-3'	Low	37
*Sau*3AI	5'-/GATC-3'	Specific	37
*Sca*I	5'-AGT/ACT-3'	Medium	37
*Sma*I	5'-CCC/GGG-3'	Specific	30
*Nde*I	5'-/CATAGA-3'	Medium	37

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used as recommend by the suppliers (appendix 3), 2 μl of 10x BSA (final concentration of 100 μg/ml) and sterilized distilled water. The reactions were incubated in a microcentrifuge tube at suitable digestion temperature for at least 2 hours. One-fifth by volume of loading dye (50% (v/v) glycerol, 0.1% (w/v) bromphenol blue, 0.1% (w/v) Xylene cyanol FF) was added to stop the reaction and then subjected to agarose gel electrophoresis.

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, λ/*Hind*III (λ-DNA digested with restriction endonuclease *Hind* III) (Appendix 8)

The horizontal gel was prepared by weighing out 0.7-1.0% (w/v) agarose, dissolving it in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM Na_2EDTA; pH 8.3) and heating to completion. The agarose solution was left to cool down to about 60 ^{o}C and poured into the chamber set with a comb of a desired thickness. One-fifth by volumes of loading dye was added to the DNA samples together with the size marker and then loaded into the preformed sample well of the gel. Electrophoresis proceeded until the bromphenol blue dye reached 1 cm from the bottom of the gel, taking at 80 V about 3 hours. After electrophoresis was stopped, the gel was stained by using 2.5 μg/ml

ethidium bromide and removed out the excess by destaining with distilled water. Fluorescence under ultra violet light was photographed and the agarose gel was ready for restriction pattern analysis, Southern transfer or fragment preparation.

3.11 Analysis of honey bee DNA by DNA-DNA hybridization

3.11.1 Preparation of nonradioactive probed labeling (Boehringer Mannheim Biochemica, 1993)

The nonradioactive DIG-DNA Labeling and Detection Kit of Boehringer Mannheim Biochemica was employed in this research. The DNA was labeled with Digoxigenin-11 UTP using the random primed method and the hybridized-probe detected with the Genius® nonradioactive detection system.

In a microcentrifuge tube, 500 ng of probe DNA was labeled. The DNA was denatured in 1.5 µl of distilled water by boiling for 10 minutes and quickly chilled on ice. Then 2 µl of hexanucleotide mixture, 2 µl of a dNTP labeling mixture and 1 µl (2 U) of Klenow enzyme were added. The reaction mixture was incubated at 37 ^{o}C for 20 hours. In order to stop the reaction, 2 µl of 200 mM EDTA solution; pH 8.0 were added. The labeled DNA was precipitated with 2.5 µl of 4 M LiCl and 7.5 µl of cold absolute ethanol and then, kept for 2 hours at -20 ^{o}C or 30 minutes at -70 ^{o}C. After centrifugation at 12,000xg for 10 minutes, the pellet was washed briefly with 70% ethanol and allowed to air dry. The pellet was dissolved in 50 µl of TE buffer and stored at -20 ^{o}C for future use.

The yield of labeled DNA can be quantified by directly comparing with digoxigenin-labeled control DNA provided with the Kit. Each one μl of a 10-fold dilution series of control labeled DNA and labeled DNA (Table 2) were spotted in parallel on the nylon membrane and air-dried. After fixing by ultra violet light for 3 minutes. The nylon membrane was readily detected (as described in section 3.10.3).

3.11.2 DNA dot blot

Usually to 250 ng of DNA (1 μl) sample was added to one-tenth volume of denaturing solution (4 M NaOH, 100 mM EDTA). The mixture was incubated at room temperature for 10 minutes. One μl of each sample was dispensed onto the nylon membrane, then, the spots were allowed to dry at room temperature and fixed by UV crosslinking for 3 minutes. After that, the membrane was ready for future hybridization. The hybridization and detection procedures used were performed as on Southern blotting, described in section 3.3.5.1.

3.11.3 Southern hybridization (modified from Maniatis, 1982; Carter, 1991)

After 1.6 μg of DNA was digested with restriction endonuclease and subjected to 0.7% agarose gel electrophoresis, the agarose gel was treated by placing it in a tray and flooding it with 250 mM HCl for 12-15 minutes. The gel was rinsed with distilled water prior to flooding in the denaturing solution (0.5 N NaOH, 1.5 N NaCl) with gentle shaking for 45 minutes. Then, the denaturing solution was replaced with transfer solution (0.25 N NaOH, 1.5 M NaCl) for 15 minutes. The blotting apparatus was

Table 2 The dilution series for DNA labelling quantification

Labeled control DNA concentration		Total dilution in
Starting concentration	Final concentration	DNA dilution buffer
100 µg/ml	20 ng/µl	1:5
20 ng/ml	1 ng/µl	1:20
1 ng/ml	100 pg/µl	1:200
100 pg/ml	10 pg/µl	1:2,000
10 pg/ml	1 pg/µl	1:20,000
1 pg/ml	0.1 pg/µl	1:200,000
0.1 pg/ml	0.01 pg/µl	1:2,000,000
0.01 pg/ml	0.001 pg/µl	1:20,000,000

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then set up, the reservior filled with transfer solution and the Whatman 3 MM filter paper prewetted with transfer solution to act eventually as a wick. The wick was placed over a glass plate with the ends hanging down into the transfer solution and all air bubbles pressed out. The gel was then placed onto the wick and the nylon membrane (cut about 0.5 cm larger than the gel) put onto the gel, again ensuring that all air bubbles trapped between the gel and the membrane were removed. The Whatman 3 MM filter papers were put over the nylon membrane, paper towers were placed on the 3 MM filters and the blotting covered with a Glad® wrap. About 0.75-1.0 kg of weights were put on the top to allow the capillary transfer process to proceed for 12-16 hours (see Figure 6). The nylon membrane was removed, rinsed in 5xSSC (750 mM NaCl, 75 mM sodium citrate; pH 7.0) and dried at room temperature. The DNA on the nylon membrane was fixed by irradiating with UV light for 3 minutes and kept between 3 MM filter papers at room temperature until used for Southern hybridization.

The fixed DNA-nylon membrane (8x10 cm) was prehybridized at 65 ^{o}C at least 3 hours in a sealed plastic bag containing at least 20 ml of standard pehybridization solution (5xSSC, 1% (w/v) blocking reagent 0.1% N-lauroyl sarcosine, 0.02% SDS) per 100 cm^2 of membrane. After that, it was replaced by 3 ml of standard hybridization solution (DNA-labeled denatured probe diluted in standard prehybridization solution in a ratio of 5-10 ng/ml) and hybridized overnight at 65 ^{o}C. Then the membrane was washed twice with 2xSSC washing solution (2xSSC, 0.1% SDS) for 5 minutes each at room temperature and washed twice with 0.5xSSC

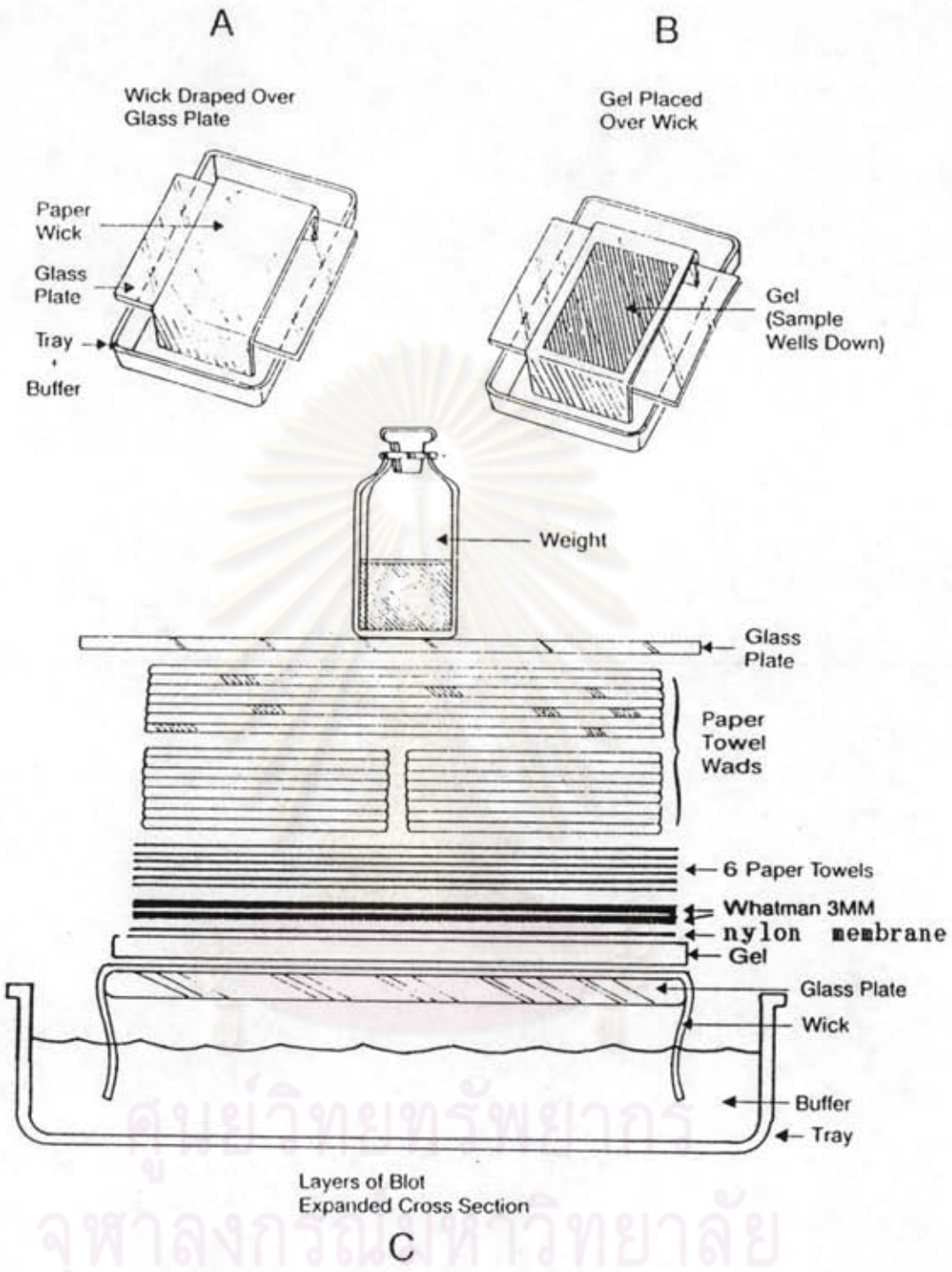

Figure 6 Preparation of Southern transfer of DNA fragment from an agarose gel onto nylon membrane.

(A) Position of wick over glassplate. (B) Gel is placed on wick.

(C) Schematic illustration of complete Southern bloting set-up. (Devis *et al.*, 1986)

washing solution (0.5xSSC, 0.1% SDS) for 20 minutes each at hybridization temperature. The membrane can be used directly for the detection.

3.11.4 Detection (Boehringer Mannheim Biochemica, 1993)

3.11.4.1 Colorimetric detection (NBT and X-phosphatemethod)

The membrane was washed briefly in buffer I (100 mM Tris-HCl, 150 mM NaCl; pH 7.5) followed by 1 hour in buffer II (1% (w/v) blocking reagent dissolved in buffer I). The anti-digoxigenin antibody conjugate (dilute 1: 20,000 to final concentration of 50 mU/ml in buffer I) was added for 30 minutes. The membrane was washed twice with buffer I for 15 minutes each and equlibrated in buffer III (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5). The membrane was ready for enzyme reaction, then incubated in the color solution (45 µl of NBT solution and 35 µl of x-phosphate solution in 10 ml of buffer III). A colour precipitate was formed within 30 minutes to 12 hours in the dark. The color reaction was stopped by buffer IV (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and the membrane was stored in the stopped buffer and photographed.

3.11.4.2 Chemiluminescent detection (Lumigen ™ PPD method)

The procedure was the same as described in the colorimetric detection method until the membrane was incubated with buffer III. After that, the membrane was incubated with Lumigen PPD dilution solution (100 µl; 2.35 mM Lumigen PPD in 20 ml of buffer III) for a few minutes. Excess liquid was allowed to drip off the membrane and it was covered with a plastic sheet. This was rolled to remove air bubbles over the

membrane, incubated and stored in the dark at 37 ^{o}C for 2 hours. After that, X-ray film exposure was done in a cassette with two tungsten intensifying screens for 30 minutes at room temperature. The signal intensity can be adjusted by decreasing or increasing the exposure time. The results were visualized by developing the film with developer and fixer solution.

3.11.5 Removal of DNA probe

3.11.5.1 Removal of color-detected (NBT/X-phosphate) membranes

After stopping the color reaction and photographing the results, the membranes were stripped immediately in a glass tray containing heated (50-60 ^{o}C) 100 % dimethylformamide (DMF) until the color was removed (frequently changing this solution increases the speed of decolorization). Then, the membranes were rinsed throughly in distilled water and incubated with probe stripping solution (50% (v/v) DMF, 1% (w/v) SDS, 50 mM Tris-HCl; pH 8.0) at 68 ^{o}C for 1 hour. The membranes were rinsed with distilled water and stored in 2xSSC or immediately hybridized again.

3.11.5.2 Removal of Chemiluminescence detection (Lumigen PPD) membranes

The membranes were washed with distilled water for 1 minute and then incubated four times with 0.2 N NaOH, 0.1% (w/v) SDS solution at 37 ^{o}C for 30 minutes each. After that, the membranes were briefly rinsed with 2xSSC and used for rehybridization.

CHAPTER IV

RESULTS

4.1 Total DNA and nuclear DNA extraction

In this study both of total DNA and nuclear DNA of *A. cerana* were extracted from individual worker pupae. The total DNA was pre-extracted immediatly at the collection-location and transported for future purification at the laboratory, whereas, all steps of nuclear DNA extraction were done at the laboratory. The initial preparation of DNA was previously tested for achievement of future analysis, so that the absorption spectrum of extracted DNA was measured from 200 to 400 nm (Appendix 5). The purity and concentration of DNA was estimated where the absorbance at 260 nm equal to 1 as equivalent to 50 μg/ml of double-standed DNA and the ratio of $OD_{260/280}$ between 1.65 and 1.85 showing the purity of DNA. Usually, about 3.9 and 2.9 μg were obtained single worker pupae total DNA extraction and nuclear DNA extractions, respectively. The extracted DNA was always dissolved in 40 μl of TE buffer. The $OD_{260/280}$ ratios of total and nuclear DNA ranged from 1.70 to 1.95. In addition, agarose gel electrophoresis of the undigested total and nuclear DNA migrated as the high molecular weight, larger than the 23.1 kb marker, and sheared fragments were minimal (Figure 7). Those results demonstrated that total DNA extraction and nuclear DNA extraction were suitable for subsequent experiments.