CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals

Absolute ethanol (Merck, Germany)

Acrylamide (Merck, Germany)

Activated carbon (Aldrich, USA)

Agarose gel (FMC Bioproducts, USA)

: Seakem LE Agarose

: MetaPhor Agarose

Ammonium persulfate (Merck, Germany)

Ammonium sulfate (Merck, Germany)

Ampicillin (Sigma, USA)

AMV First Strand cDNA Synthesis Kit (Biotechnology Department Bio Basic Inc., Canada)

Aquasorb (Fluka, Switzerland)

Bacto-agar (DIFCO, USA)

100 Base pair DNA ladder (Promaga Coperation, USA)

Basic fuchsin (Biotechnology Department Bio Basic Inc., Canada)

BenchMark™ Protein Ladder (Invitrogen Life Technologies, USA)

Beta-mecaptoethanol (Fluka, Switzerland)

Boric acid (Merck, Germany)

Bovine serum albumin; BSA (Sigma, USA)

Broad pI Calibration kit: pH 3-10 (Amershem Pharmacia Biotech Inc., USA)

5-bromo-4-chloro-3-indoyl-beta-D-galactopyranoside; X-gal (Sigma, USA)

Bromophenoal blue (Merck, Germany)

Catalase (Sigma, USA)

Chloroform (Merck, Germany)

Coomassie brilliant blue R-250 (Sigma, USA)

Copper (II) sulfate (Carlo Erba Reagenti, Italy)

Cytochrome C (Sigma, USA)

DEAE-Cellulose (Sigma, USA)

Deoxynucleotide triphosphate: dNTPs (Promega Corperation, USA)

Diethyl pyrocarbonate (Sigma, USA)

Dithiothreitol; DTT (Sigma, USA)

Ethylene diamine tetra-acetic acid di-sodium; Na_2EDTA (Fluka, Switzerland)

Ferritin (Amershem Pharmacia Biotech Inc., USA)

Ficoll type 400 (Sigma, USA)

Glycine (Sigma, USA)

Hydrochloric acid (Merck, Germany)

Isoamyl alcohol (Merck, Germany)

N, *N*- methylene-bis-acrylamide (Merck, Germany)

N, *N*, *N'*, *N'* Tetramethylethylene diamine (BDH, England)

Ovalbumin (Sigma, USA)

Phenol crytal (BDH, England)

Phenylmethylsulfonyl fluoride: PMSF (UBS, USA)

Q-Sepharose (Amershem Pharmacia Biotech Inc., USA)

QIAquick Gel Eaxtraction kit (QIAGEN, Germany)

QuickPrep® *Micro* mRNA Purification kit (Amershem Pharmacia Biotech Inc., USA)

Sephadex G-200 (Sigma, USA)

Sodium acetate (Merck, Germany)

Sodium chloride (BDH, England)

Sodium dodecyl sulfate: SDS (Sigma, USA)

Sodium metabisulphite (BDH, England)

Sodium hydroxide (Carlo Erba Reagenti, Italy)

SuperScript™ Plasmid System with Gateway™ Technology for cDNA Synthesis and Cloning (Life Technology Inc., USA)

SV total RNA isolation kit (Promega Corperation, USA)

Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech UK Limited, USA)

Tris-(hydroxy methyl)-aminomethane (Fluka, Switzerland)

Tryptone (DIFCO, USA)

Xylene cyanol FF (Sigma, USA)

Yeast extract (DIFCO, USA)

2.2 Equipments

Autoclave: H-88LL (Kokusan Ensinki Co. Ltd., Japan)

Automatic micropipette: pipetman P2, P20, P100 (Gilson Medical Electronics S.A., France)

Camera: Pentax K1000 (Asahi Opt. Co., Japan)

Centrifuge: J2-21 (Beckman Instrument Inc., USA)

Conductivity meter: CDM83 (Radiometer A/S, Denmark)

Densitophotometer: Gs-670 (BioRad Laboratories, USA)

Electronic balance: Alsep EY220A (A&D Co. Ltd., Japan)

Electrophoresis apparatus (BioRad Laboratories, USA)

- Horizontal gel electrophoresis
- Vetrical gel electrophoresis

- 20 °C Freezer (Krungthai Ltd., Thailand)

- 80 °C Freezer (Bara laboratory Co. Ltd., Thailand)

Fraction collector: FRAC-100 (Amershem Pharmacia Biotech Inc., USA)

Ultrasonic: 28H (Ney Dental Inc., USA)

High speed microcentrifuge: MC-15A (Tomy-Seiko Co. Ltd., Japan)

Incubator: BM-600 (Memmert GambH, Germany)

Incubator shaker: GALLENKAMP

Incubator water bath: M20S (Lauda, Germany)

Magnetic stirrer and heater (Fisher Scientific, USA)

Microwave Oven: TRX1500 (Tarbora International Co. Ltd., Korea)

pH meter: PHM 95 (Radiometer, Denmark)

Power supply: POWERPAC 300 (BioRad Laboratories, USA)

Pump: P1 (Pharmacia Biotech Inc., USA)

Spectophotometer: Spectonic2000 (Baush&Lomo, USA)

Thermocycler: GeneAmp PCR system 2400 (Perkin Elmer Cetus, USA)

UV transilluminator: 2001microwave (San Gabriel California, USA)

Vortex: K-550-GE (Scientific Industries, USA)

2.3 Inventory supplies

Black and white print film Tmax-400 (Eastman Kodak Company, USA)

Dialysis tube (Sigma, USA)

Filter paper whatman 3MM (Whatman Internation Ltd., England)

Microcentrifuge tubes 0.5, 1.5 ml (Axygen Hayward, USA)

Pipette tips 10, 20, 100 µl (Axygen Hayward, USA)

PVDF membrane (Amershem Pharmacia Biotech Inc., USA)

Thin-wall microcentrifuge tubes 0.2 ml (Axygen Hayward, USA)

2.4 Enzymes

N-glycosidase F (Boehringer Mannhemn, Germany)

Proteinase K (Life Technologies, Inc., USA)

Restriction endonucleases

: *Dra* I, *Hinf* I (Boehringer Mannhemn, Germany)

: *Ssp* I, *Taq* I (Promega Coperation, USA)

Taq DNA Polymerase (Invitrogen Life Technologies, USA)

2.5 Primers

Oligonucleotides (Life Technologies, Inc., USA or Invitrogen Japan K.K., Japan or QIAGEN Operon, Germany)

2.6 Bactirial Strains

E. coli DH5α competent cells (TAKARA Biomedicals, Japan)

E. coli JM109

2.7 Sample preparations

2.7.1 Honeybee samples

The cavity-nesting honeybee workers, *A. cerana*, were collected from each colony originating from geographically different regions of Thailand (Figure 2.1, N = 225) and used for population genetic studies and identification of population origins.

In addition, bees exhibiting different roles in the colonies were also collected. Newly emerged bees were collected when they bit the brood cell covers. Nurse bees were collected when they were feeding the brood onto the queen cup while forager bees were collected when they returned to the hive after foraging for nectar and pollen. Bees were immediately frozen in liquid nitrogen and stored at –80 °C until further used.

Population origins of bees were initially examined by PCR-RFLP analysis of the large subunit of ribosomal (lr) RNA gene using *Dra* I (TTT/AAA) as described in PART I of experiment (Figure 2.2).

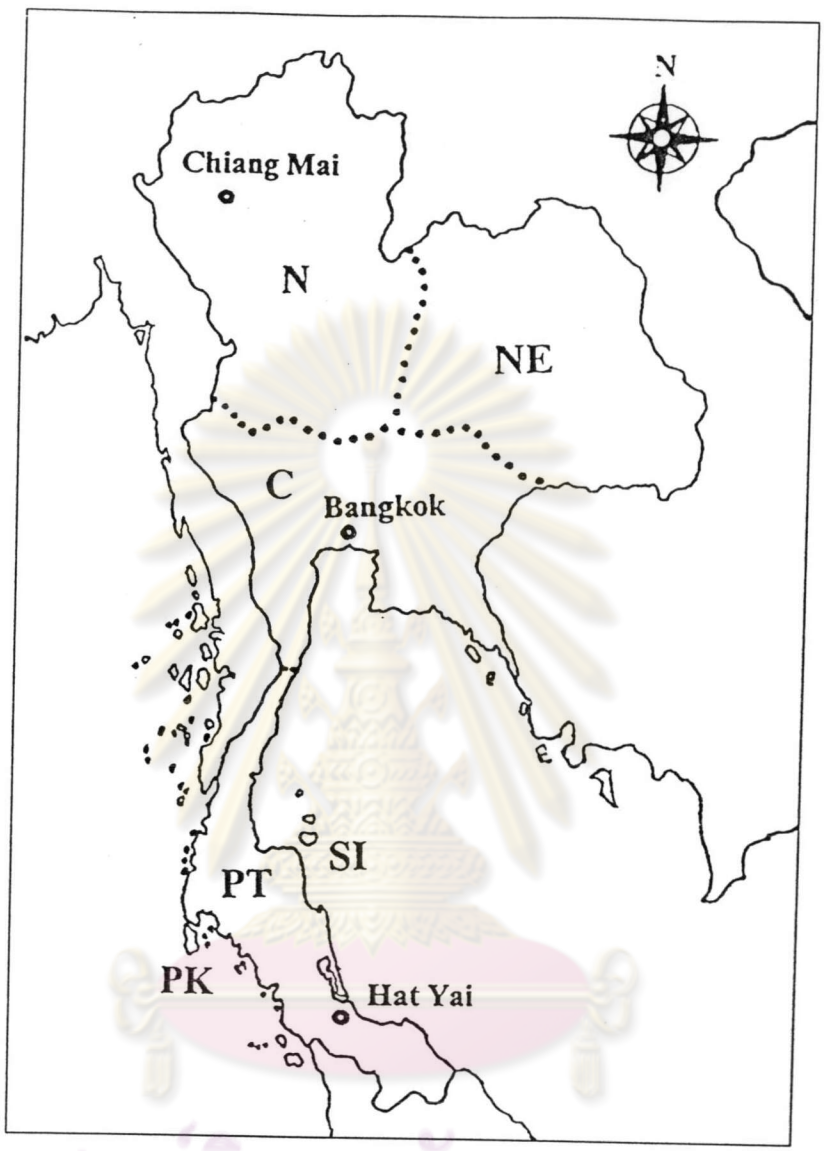

Figure 2.1 Map of Thailand showing sample collection sites of *A. cerana* used in this study. Specimens were collected covering a major part of each location. Abbreviation: C = the central region, N = north, NE = north-east, PT = peninsular Thailand, PK = Phuket Island and SI = Samui Island

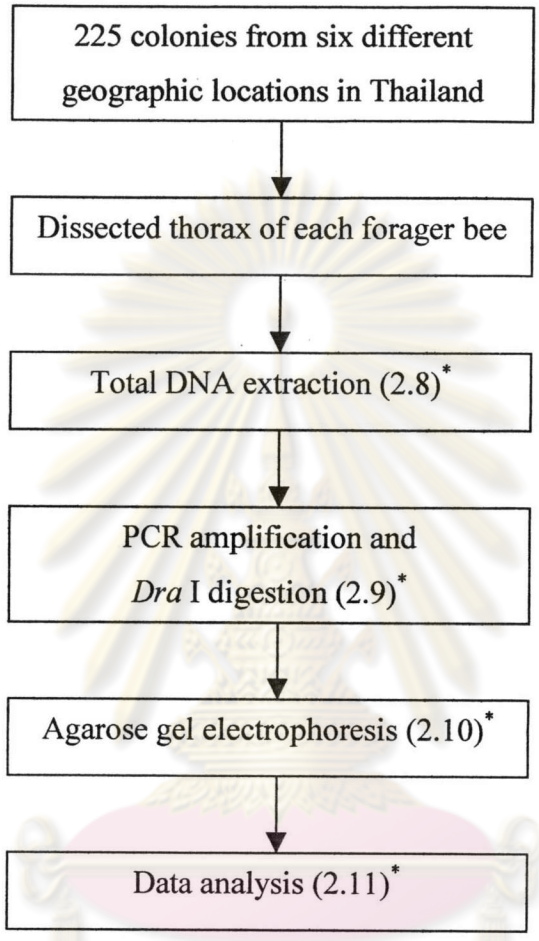

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Figure 2.2 A workflow for the PART I experiment on identification population origins of *A. cerana*.

2.7.2 Royal jelly (RJ) samples

RJ was produced using a modification of the method of queen rearing described by Kavinseksan, 1994. Six healthy colonies of *A. cerana* were chosen. These were composed of two colonies from Samutt Songkhrom and Chuntaburi provinces, which represent the northern bee population, three colonies from Chumporn provinces, which represent peninsular bee population and one colony from Samui Island located in Surajthani province, which represents the non-mainland population of *A. cerana* in Thailand (Table 2.1). RJ was directly collected from queen cells in the natural field.

Table 2.1 Sampling locations of *A. cerana* colonies used for the production of royal jelly used for purification and characterization

Colony no.	Sampling area	Population origin
1	Lungsuan, Chumporn	Peninsular Thailand
2	Lungsuan, Chumporn	Peninsular Thailand
3	Sawee, Chumporn	Peninsular Thailand
4	Muang, Samutt Songkrom	North to central Thailand
5	Makham, Chuntaburi	North to central Thailand
6	Tham Bon Maenam, Samui, Surat Thani	Samui Island

A bee colony was firstly stimulated by removing the queen outside. Forty to sixty wax queen cups were prepared from the wood stick template and adhered onto the queen frame. After that, the frame was incubated in the hive for 4-6 hours without the availability of the queen. This technique greatly enhanced the new queen cup acceptance. Then, newly emerged worker larvae (one-day-old larvae) were transferred

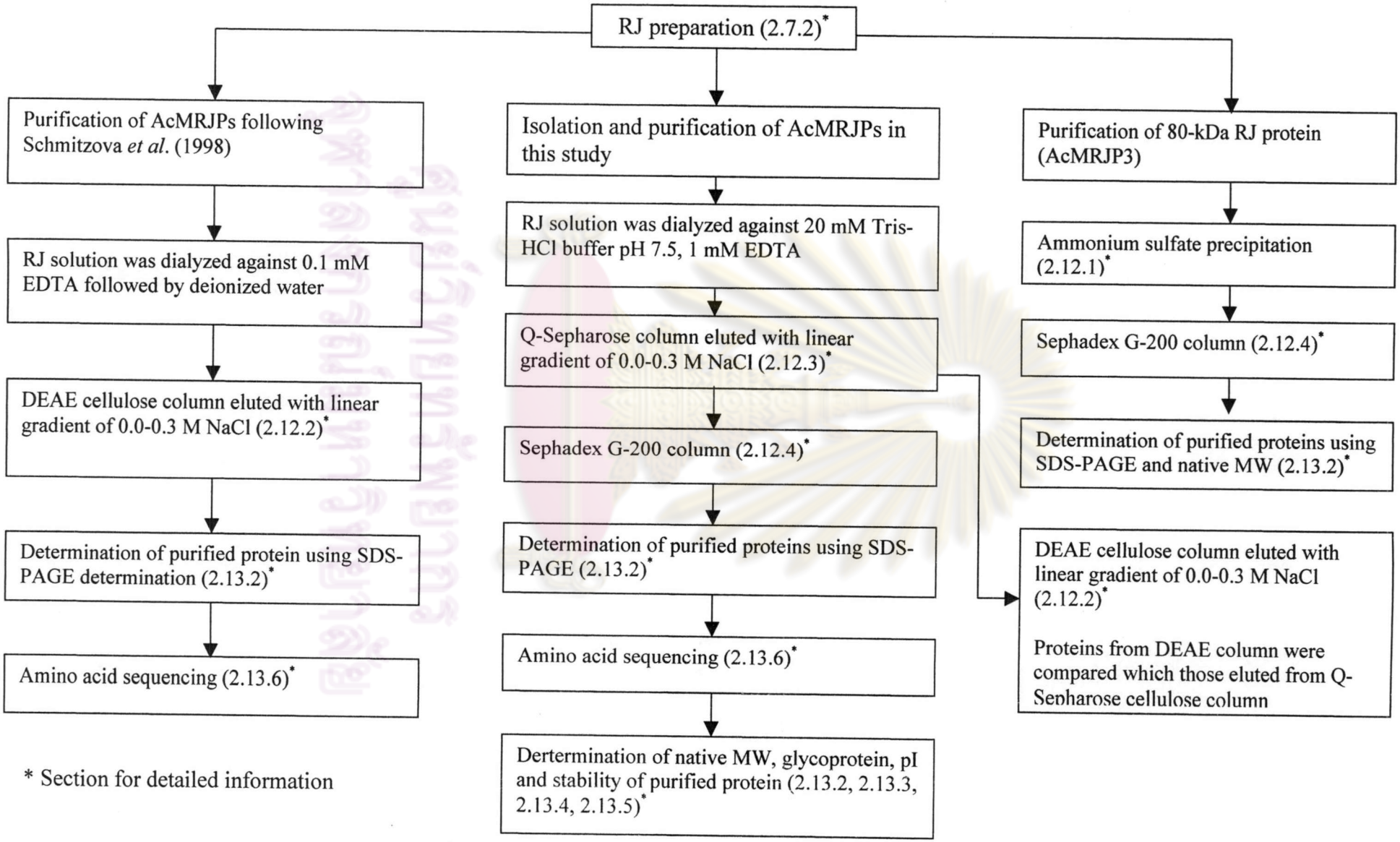
into the queen cups with the grafting tool and the queen frame was then returned to the original position in the hive. The larvae were left in the queen cups for 2-3 days. Finally, RJ was harvested from each queen cell, mixed and quickly frozen in liquid nitrogen and stored at –80 °C until used.

Approximately 0.3 g of frozen RJ was dissolved in 10 ml of 50 mM phosphate buffer (NaH_2PO_4/Na_2HPO_4; pH 7.5, 10 mM NaCl and 20 mM EDTA), gently mixed and centrifuged at 10,000xg for 20 minutes at 4 °C. The supernatant was then collected and used as crude RJ solution. The workflow of the PART II experiment (isolation, purification and characterization of ***A. cerana*** **M**ajor **R**oyal **J**elly Proteins, **AcMRJPs**)) is shown in Figure 2.3.

2.7.3 Hypopharyngeal gland samples

A hypopharyngeal gland was dissected out from the head of each worker under a binocular microscope. A knife was used to cut through the wall of the mask, across the vertex, round the margins of the compound eyes, and round the edges of the mask. The mask was then taken off. A hypopharyngeal gland located in front of the brain was removed and placed in a tube containing pre-chilled buffer constituting of guanidium thiocyanate and *N*-lauroyl sarcosine and stored in liquid nitrogen.

Fifty hypopharyngeal glands were used for direct mRNA extraction. Construction of a cDNA library, characterization of EST clones and RT-PCR are shown in the workflow of the PART III experiment (Figure 2.4) while twenty hypopharyngeal glands were used for extraction of total RNA. Quantification of expression levels of AcMRJPs using semi-quantitative PCR is shown in the work flow of the PART IV experiment.



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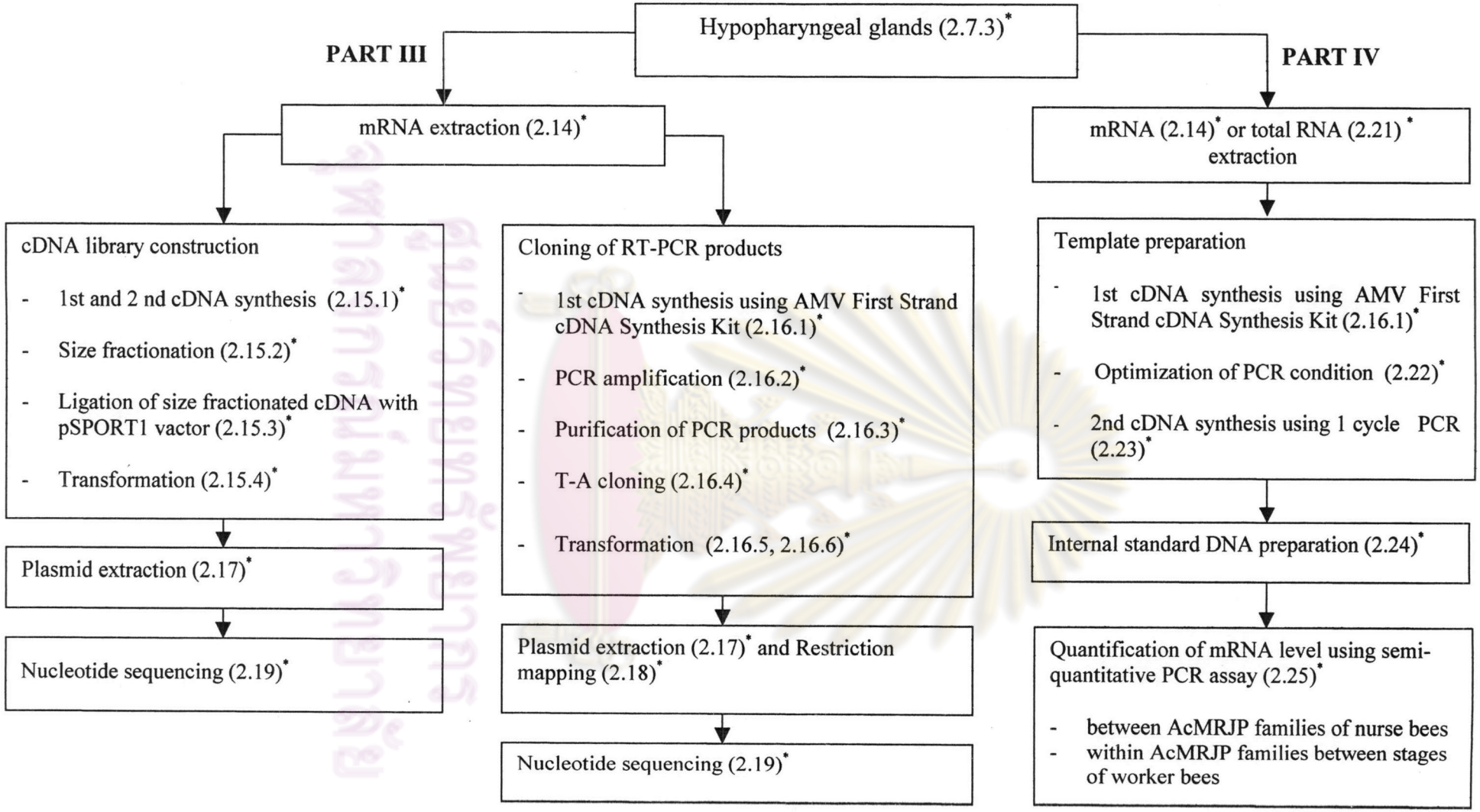

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PART I: POPULATION GENETIC STUDIES AND IDENTIFICATION OF POPULATION ORIGINS OF THAI HONEY BEES, *A. cerana*

2.8 Total DNA extraction

Total DNA was extracted from the thorax of each forager bee using a modification of the method of Smith and Hagen (1997). Briefly, a thorax was homogenized in 500 µl of STE buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 mM EDTA). After that, 20 % SDS and 10 mg/ml proteinase K were added to final concentrations of 1 % SDS and 500 µg/ml proteinase K, respectively, The homogenate was incubated at 37 °C for 3 hours. The mixture was centrifuged at 8,000xg for 5 minutes art room temperature. The supernatant was removed to a new microcentrifuge tube and extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The mixture was gently mixed for 15 minutes and centrifugation at 8,000xg for 5 minutes at room temperature. The upper aqueous phase was collected after and transferred to a new sterile tube. This phenol/chloroform extraction was repeated. The supernatant from the 2nd phenol/chloroform extraction was further extracted once with an equal volume of chloroform/isoamyl alcohol (24:1) as above. One-tenth volume of 3 M sodium acetate pH 7.5 was added. DNA was precipitated by the addition of 2 volume of cold absolute ethanol. The mixture was kept at –20°C overnight. The DNA pellet was recovered by centrifugation at 12,000xg for 15 minutes at room temperature and briefly washed with 1 ml of 70 % ethanol. The DNA pellet was air dried and dissolved in the appropriate volume of TE buffer (10 mM Tris-HCl pH 7.5 and 1 mM EDTA). DNA concentration was roughly estimated by comparing the intensity with that of a λ/*Hin*d III DNA marker after electrophoresed through a 0.7 % agarose gel stained with ethidium bromide. The DNA concentration was finally adjusted to a final concentration of 25 ng/µl and kept at 4 °C until further used.

2.9 Polymerase Chain Reaction (PCR) – Restriction Fragment Length Polymorphism (RFLP) analysis

Previously, nucleotide sequences of *A. cerana* mitochondrial lrRNA gene was reported (Sihanuntavong, 1997). Aligned sequences of lrRNA gene of representative

A. cerana individuals from different geographic origins indicated that *Dra* I (recognition site TTT/AAA) could differentiate *A. cerana* originating from different regions accurately. Analysis of population origins was then simplified to restriction analysis of the lrRNA gene with the informative restriction enzyme *Dra* I.

The mitochondrial lrRNA gene segment of each bee was amplified by PCR using forward (5'-CTA TAG GGT CTT ATC GTC CC–3') and reverse (5'-TTT TGT ACC TTT TGT ATC AGG GTT G-3") primers designed from *A. mellifera* sequence at the position 13708th and 14447th, respectively (Hall and Smith, 1991; Crozier and Crozier, 1993). PCR was carried out in a 25 µl reaction volume containing 50 ng DNA template, 20 mM Tris-HCL pH 8.4, 50 mM KCl, 3.0 mM $MgCl_2$, 100 µM of each dNTP (dATP, dCTP, dGTP and dTTP), 0.1 µM of each primer and 0.6 units of Ampli*Taq* DNA polymerase (Perkin Elmer, USA). Amplification conditions were predenatured at 94 °C for 1 minute followed by 35 cycles of a denaturation step at 92 °C for 1 minute, an annealing step at 53 °C for 1 minute and an extension step at 72 ° C for 2 minutes. The final extension was performed at the same temperature for 10 minutes.

Five microlitres of the PCR product from each individual was analyzed on 0.7% agarose gels to determine whether the amplification was successful. Eight microlitres of the PCR product was subsequently digested with 2 -3 units of *Dra* I (Boehringer Manheim) The reactions were incubated at 37 °C for 4 – 5 hours. The digests were then by electrophoretically analyzed through 3 % Metaphor agarose gels. Digestion patterns were visualized under a transilliminator after ethidium bromide staining (Maniatis et al., 1982).

2.10 Agarose gel electrophoresis

Agarose gel electrophoresis was used to size-fractionate total DNA, the amplification product and *Dra* I-digested lrRNA gene segment. Different concentrations of agarose gel were prepared depending on sizes of DNA fragments. Generally, 0.7-1.0 % agarose gels were used for general purposes whereas 2.5-3.0 % Metaphor agarose gels were used to determine digestion patterns of the lrRNA gene segment. An appropriate amount of agarose was weighted out and dissolved in the appropriate volume of 1X TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5

mM EDTA pH 8.0). The gel slurry was heated until completed solubilization. The agarose gel solution was incubated at 65°C and further left to 50°C before poured into the electrophoretic gel mould. The comb was inserted. For Metaphor agarose, solidified gel are required to pre-chilled at 4°C for 30-60 minutes to achieve the sieving ability of the gels. When needed, the comb was carefully removed. The gel was placed in the chamber. An enough volume of 1X TBE was poured to cover the gel for 2-3 cm. One-fifth volume of loading dye (0.25 % bromophenol blue, 0.25 % xylene cyanol FF and 15 % Ficoll 400) was added into the sample and loaded into the gel. Electrophoresis was usually operated at 100 volts for 0.7-1.5% gel or 130 volts for 2.5-3.0 % Metaphhor agarose gel until bromophenol blue reached approximately 1 cm from the bottom of gel. The gel was stained with a 2.5 μg/ml ethidium bromide solution for 5 minutes and destained in deionized water for 30 minutes. The DNA was visualized under a long wavelength UV light and photographed through a red filter using a Kodax Tri -Xpan 400 film.

2.11 Data analysis

Nucleotide sequences of mitochondrial lrRNA gene of *A. cerana* from different geographic origins (accession numbers 140508-140511) were retrieved from the GenBank and aligned using Clustal X. Restriction sites were searched using Genetyx 3.1.

Restriction patterns of the amplified lrRNA gene digested with *Dra* I were assigned alphabetically (A, B, C and D) in order of discovery. Sequences of the lrRNA gene of *A. cerana* were used to facilitate the mapping of restriction sites. The percent nucleotide divergences between geographic samples (Nei, 1987) were calculated usind REAP 4.0 (McEroy *et al.*, 1991) and used to construct a neighbor-joining tree (Saitou and Nei, 1987) using Neighbor in PHYLIP 3.56c (Felsenstein, 1993). Geographic heterogeneity among different geographic samples was analyzed using a Monte Carlo simulation (Roff and Bentzen, 1989) routine in REAP 4.0 (McEroy *et al.*, 1991). An analysis of molecular variance (AMOVA) and *F*-statistics were calculated using Arlequin 1.1 (Schneider *et al.*, 1997).

PART II: ISOLATION, PURIFICATION AND CHARACTERIZATION OF AcMRJPs

2.12 Isolation and Purification of AcMRJPs

2.12.1 Ammonium sulfate precipitation

Crude RJ proteins were precipitated with different concentrations of ammonium sulfate (0-20, 20-30, 30-40, 40-50, 50-60, 60-70 and 70-80 % saturation, respectively). Solid ammonium sulfate was slowly added to the RJ solution to 20 % saturation with continuous stirring for 1 hour on ice. The precipitate was collected by centrifugation at 10,000xg for 25 minutes at 4 °C. Subsequently, the supernatant was sequentially brought to 30 %, 40 %, 50%, 60% 70 % and 80% saturation. The precipitate of each concentration was collected by centrifugation at 10,000xg at 4 °C for 25 minutes and dissolved in 50 – 1200 µl of 20 mM Tris-HCl, pH 7.5 and 10 mM EDTA. Five microlitres of the precipitate were electrophoretically analyzed by 10 % SDS-PAGE. The precipitate from 50-60 % saturation fraction contained high quantity of 80 kDa AcMRJPs was selected to further purify by a Sephadex G-200 column chromatography. The purified protein (characterized as AcMRJP3 afterwards) obtained after size-fractionated was electrophoresed through 10% SDS-polyacrylamide gel for determination of denatured molecular weight of this AcMRJP.

2.12.2 Ion-exchanged chromatography using DEAE-cellulose

DEAE-cellulose, supplied as a dry powder, was hydrated with deionized water for approximately 12 hours. The hydrated cellulose was washed several times with deionized water to remove residual fine particles. The slurry matrix was then activated by washed with 0.5 N HCl for 30 minutes followed by several washes with deionized water until the pH of the solution reached to 7.0. The slurry was then washed with 0.5 N NaOH for 30 minutes followed by several washes with deionized water until the pH of the solution was lowered to 7.0. Finally, it was resuspended with the buffer containing 20 mM Tris-HCl pH 7.5 and 1 mM EDTA and packed into a 2.5 ∅ x 12 cm column. The packed column was equilibrated with 3-5 column volume of the same buffer at the constant flow rate of 0.5 ml/min.

A crude RJ solution was prepared by dialyzed against 0.1 M EDTA at 4 °C until the ratio of A280/A260 reached 1.8. Dialysis was further carried out against deionized water at 4 °C for 24 hours. The pH of crude RJ solution was adjusted with 1 M Tris-HCl pH 7.5 and 0.5 M EDTA to the final concentration of 20 mM Tris-HCl pH 7.5 and 1 mM EDTA, respectively. Dialyzed RJ was loaded onto the DEAE column and washed with at least 3 column volumes of a 20 mM Tris-HCl pH 7.5 and 1 mM EDTA solution to remove unbound proteins. Bound proteins were eluted out with a linear gradient of 0.0 - 0.3 M NaCl in a total volume of 400 ml at the flow rate of 0.5 ml/min. Two-milliliter fractions were collected. The protein elution profile was monitored using the absorbance at 280 nm. Conductivity of each fraction was also measured and converted to the NaCl concentration. Fractions representing the same protein peak were pooled. The protein concentration was determined by a Lowry *et al* (1951) method. Five micrograms of the purified protein in each peak were analyzed by 10 % SDS-PAGE. Two nanomoles of each protein were further used for N-terminal and internal peptide sequencing.

2.12.3 Ion-exchaged chromatography using Q-Sepharose

Purification of AcMRJPs using a DEAE column chromatography was not successful and was replaced by Q-Sepharose. Q-Sepharose was supplied as a pre-swollen form in 20 % ethanol. Ethanol was decanted and replaced with an enough volume of a 20 mM Tris-HCl pH 7.5 and 1 mM EDTA solution. The matrix solution was degassed *in vacuo* and poured into a column (1.25 ∅ X 20 cm) in a single continuous motion and equilibrated with 3-5 column volume of the same buffer at the constant flow rate of 0.5 ml/min.

A crude RJ solution was dialyzed twice against 20 mM Tris-HCl pH 7.5 and 1 mM EDTA for at least 3 hours each and applied onto the Q-Sepharose column. The column was washed with 20 mM Tris-HCl pH 7.5 and 1 mM EDTA for at least 3 column volumes to remove unbound proteins. A linear gradient of 0.0 - 0.3 M NaCl in the same buffer was used to elute bound proteins. Two milliliter fractions were collected and protein elution profiles were monitored using the absorbance at 280 nm. The concentration of NaCl in each fraction was converted from conductivity. Fractions representing the same protein peak were pooled. The protein concentration was determined by a Lowry *et al* (1951) method. An aliquot of 5 μg protein from each

peaked protein was analyzed by 10 % SDS-PAGE. The remaining protein of each peak was then filled in the dialysis tube and covered with aquasorb at 4 °C until the volume reduced to approximately 1 ml. This solution was further purified using Sephadex-G200 column chromatography.

2.12.4 Size-exclusion chromatography using Sephadex-G200

Sephadex G-200 was added to an enough volume of deionized water and left overnight at 50 °C. The equilibrated gel was then degassed and packed into a column (2.5 ∅ x 80 cm.) using a peristaltic pump at the constant flow rate of 20 ml/hr. The Sephadex G-200 column was then equilibrated with 20 mM Tris-HCl pH 7.5, 1 mM EDTA and 0.1 M NaCl for 5 column volumes at the constant flow rate of 12 ml/hr to allow column stability. Blue dextran 2000 and potassium dichromate were used to determine the void and the total volumes by measuring the absorbance at 630 nm and 410 nm, respectively.

Concentrated proteins derived from a Q-Sepharose column chromatography were loaded onto the Sephadex-G200 column. Proteions were eluted out with the buffer containing 20 mM Tris-HCl pH 7.5, 1 mM EDTA and 0.1 M NaCl at the constant flow rate of 12 ml/hr. Two milliliter fractions were collected. Protein elution profiles were monitored using absorbance at 280 nm. Protein concentration was determined by the Lowry *et al* (1951) method. Purified protein of each fraction was dialyzed against 20 mM Tris-HCl pH 7.5 and store at –20 °C until further characterization. Native molecular weight of each protein was estimated by comparing with a standard protein maker. Denatured molecular weight of each protein (5 μg) was determined by 10 % SDS-PAGE as described in the section 2.13.2.

2.13 Characterization of AcMRJPs

2.13.1 Protein concentrations

The concentration of each protein sample was determined according to a Lowry *et al* (1951) method (Appendix A). A 0.5-ml sample solution was mixed with 2.5 ml of solution C (1 ml of 0.5 % $CuSO_4$ and 1 % sodium citrate into 50 ml of 2 % Na_2CO_3 in 0.1 M NaOH) and left at room temperature for 5-10 minutes. Two hundred and fifty microlitres of solution D (two fold dilution of Folin-Ciocalteau reagent) was

added to the mixture, mixed and left at room temperature for 20-30 minutes. The A_{750} of each sample was measured. Protein concentrations of samples were estimated from a standard curve of BSA (Appendix B).

2.13.2 Molecular weight determination

Native molecular weight of purified proteins in RJ was determined by Sephadex G-200 column chromatography. The elution volume (V_e) of protein was compared with those of standard protein markers; ferritin (440 kDa), catalase (233 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa) and chymotrypsinogen (23 kDa). Blue dextran 2000 and potassium dichromate were used to determine the position of void (V_o) and total volumes (V_t), respectively. The relative mobility, K_{av}, derived from $(V_e - V_o) / (V_t - V_o)$, was plotted against log molecular weight of each standard protein to make a calibration curve. K_{av} of protein samples was then calculated and used to estimate their native molecular weight.

The molecular weight of denatured proteins was determined by SDS-PAGE (Laemmli, 1970; Appendix C). This method separated proteins based primarily on their sizes. A discontinuous slab gel (10 x 10 x 0.75 cm) composed a 10 % separating and 5 % stacking gels in the presence of 0.1 % SDS (w/v) was prepared and placed in the eletrophoretic tank. The electrode buffer (25 mM Tris, 192 mM glycine; pH 8.3 and 0.1 % SDS) was filled in the tank. One-fifth volume of the sample buffer (60 mM Tris-Hcl (pH 6.8), 25 % glycerol, 2 % SDS, 14.4 mM 2-mercaptoethanol and 0.1 % bromophenol blue) was added to each sample. The mixture was boiled for 10 min before applying to the gel. Electrophoresis was performed at 20 mA constant current per slab gel. After electrophoresis, the gel was stained with a Coomassie brialliant blue R-250 solution (0.1 % Coomassie brialliant blue R-250 in 45 % methanol and 10 % acetic acid) for 15 minutes and destained with a solution containing 10 % methanol and 10 % acetic acid overnight on slow rocking shaker. A BenchMark™ Protein Ladder was used as the standard molecular weight marker.

2.13.3 Determination of carbohydrate side chain

2.13.3.1 Qualitative analysis using a Periodic Acid-Stiff (PAS) staining method (Segrest and Jackson, 1972)

Purified proteins were firstly electrophoresed on 10 % SDS-PAGE as described in section 2.13.2. The gel was then fixed in the fixative (7.5 % acetic acid), left at room temperature for 1 hour and carefully rinsed with deionized water. The gel was then immersed in a 0.2 % periodic acid solution and incubated at 4 °C for 45 minutes. The gel was immediately placed in the Schiff's reagent and chilled at 4 °C for 45 minutes and destained at room temperature with two or three changes of 10 % acetic acid. For a faster destaining, the gel was soaked in a solution containing 0.05 N HCl and 0.5% potassium metabisulphite for 15 minutes (see Appendix D for preparation of PAS staining solutions).

2.13.3.2 Qualitative analysis by N-glycosidase F digestion

Five micrograms of each purified RJ protein was treated with 1 unit of N-glycosidase F and incubated at 37 °C for 3 hours. The digested products were then electrophoretically analyzed to determine whether the molecular weight was shifted on 8-10 % SDS-PAGE. Human ferritin and haemoglobin were used as positive and negative, respectively.

2.13.4 Isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE)

2.13.4.1 Preparation of gel supporting film

A small volume of deionized water was dropped on the glass plate. The hydrophobic side of the gel supporting film was then coated against the glass plate and smoothly rolled with a test tube to remove any excess water and air bubble. It was then placed on the casting tray by the gel support film side facing down.

2.13.4.2 Preparation of the IEF gel

The gel solution composed of 30 % acrylamide, 1 % bis-acrylamide, 240 μl of ampholyte pH 3-10, 50 % sucrose, 10 % ammonium persulfate and TEMED was

gently mixed and carefully pipetted to the space between the gel support film coated glass plate and the casting tray (Appendix E). The gel was then left at room temperature about 45 minutes for polymerization and fixing on the gel support film. After complete polymerization, the gel was lifted from the casting tray using of spatula.

2.13.4.3 Sample preparation and IEF electrophoresis

Purified protein derived from Sephadex G-200 column was dialyzed against deinoized water for 2 hours and concentrated by a speed vacuum. Samples were then loaded on a small piece of filter paper placed on the gel and allowed its diffusion into the gel for 5 minutes. The filter paper was carefully removed from the gel. The gel with adsorbed samples was turned upside-down and directly placed on the top of the electrodes. The focusing was carried out under constant voltage condition in a stepwise procedure. The focusing gel was initially run at 100 V for 15 minutes. The voltage was then increased to 200 V for 15 minutes. Finally, the voltage was increased to 450 V and further operated for additional 60 minutes. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (0.1 % W/V Coomassie brialliant blue R-250 in 40 % methanol and 1 % acetic acid). Standard proteins with known pI of 3-10 were also electrophoretically analyzed in parallel. The pI values of proteins were determined by comparing their mobility with the standard calibration curve (a plot between a pI value of each protein marker versus their distances from the cathode).

2.13.5 Effect of temperatures on AcMRJPs stability

Stability of each AcMRJP family at different temperatures was studied. Five micrograms of purified proteins were incubated at –20 °C, 4 °C and 37 °C for 1 to 15 days. At the end point of incubation, one-fifth volume of sample buffer (60 mM Tris-Hcl; pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue) was added and boiled for 10 minutes. The mixtures were electrophoretically analyzed through 10 % SDS-PAGE.

2.13.6 *N*-terminal amino acid sequencing

N-terminal amino acid sequences of purified protein were analyzed. The proteins purified from Q-Sepharose followed by Sephadex G-200 column chromtography (peak A1 and B1) were electrophoresed through 10 % SDS-PAGE and transferred onto a piece of PVDF membrane (Towbin *et al.*, 1979).

The gel was soaked in the electroblotting buffer (25 mM Tris, 192 mM glycine and 10 % methanol) for 15-30 minutes. A piece of PVDF membrane cut to the dimensions of the gel and pre-wetted with methanol was transferred to the electroblotting buffer and left for 15-30 minutes. Transferring of proteins on the gel to the PVDF membrane was carried out using a Mini Trans-Blot® Electrophoretic Transfer Cell (BioRad Laboratories, USA) and operated at 50 V for 90 minutes. After that, the membrane was washed with deionized water, saturated with 100 % methanol for a few seconds and stained with 0.1 % Coomassie Brilliant Blue R-250 in 1 % acetic acid and 40 % methanol. Finally, the membrane was destained in 50 % methanol, rinsed with deionized water and air-dried. The *N*-terminal amino acid sequences were analyzed by automated amino acid sequencer at the Department of Biological Sciences, National University of Singapore, Singapore.

Two nanomoles of purified proteins derived from a DEAE cellulose column chromatography (peak A2 and C1) were analyzed by internal peptide sequencing at the Department of Bioresources Science, Kochi University, Japan. Each protein was evaporated and dissolved in 20 µl of 8 M urea and incubated at 37 °C for 1 hour prior to the addition of 60 µl of 0.3 M Tris-HCl, pH 9.0 and 10 µl (33 pmol) of lysyl endopeptidase. The solution was mixed and incubated at 37 °C for 16 hours before applying to a Shimadzu HPLC System (C18 column). Peptide peaks were collected for further sequencing analysis.

2.13.7 Data analysis

For homology searching, *N*-terminal amino acid sequences of RJ proteins were blasted against amino acid sequences that were previously submitted to GenBank (http://www.ncib.nlm.nih.gov/).

PART III: MOLECULAR CLONING AND CHARACTERIZATION OF AcMRJPs cDNAs

2.14 mRNA extraction

The mRNA was extracted from 50 hypopharyngeal glands of nurse bees using QuickPrep® *Micro* mRNA Purification kit (Amershem Pharmacia Biotech Inc., USA). Hypopharyngeal glands were homogenized in 0.4 ml of extraction buffer containing guanidinium thiocyanate and *N*-lauroyl sarcosine. The homogenate was diluted with 0.8 ml of the elution buffer (10 mM Tris-HCl pH 7.5 and 1 mM EDTA), mixed and centrifuged at 10,000xg for 1 minute. This can be done in parallel with centrifugation of 1 ml Oligo(dT)-cellulose. Cleared homogenate was obtained and transferred to the tube containing Oligo(dT)-cellulose pellet and mixed for 3 minutes and centrifuged at 10,000xg for 1 minute at room temperature.

The pellet was washed 5 times with 1 ml of the high-salt buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA and 0.5 M NaCl) and 2 times with 1 ml of the low-salt buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA and 0.1 M NaCl). In each step, Oligo (dT)-cellulose was mixed and centrifuged at 10,000xg for 1 minute. After washing, Oligo(dT)-cellulose was resuspended in 0.3 ml of the low-salt buffer and transferred to MicroSpin column which was placed in a microcentrifuge tube. The column was then centrifuged at 10,000xg for 1 minute. Three additional times of of low-salt washing were performed. The column was placed in a new microcentrifuge tube and 0.2 ml of the pre-warmed at 65 °C elution buffer was added on the top of the resin bed. The mRNA was collected by centrifugation at 10,000xg for 1 minute. The second 0.2 ml of pre-warmed at 65 °C elution buffer was added to recover the residual mRNA. To concentrate mRNA, 10 µl of 10 mg/ml glycogen and 40 µl of 2.5 M potassium acetate solutions pH 5.0 were added followed by two volume of cold absolute ethanol. The resulting solution was kept in –20 °C for a minimum of 30 minutes. Precipitated mRNA was collected by centrifugation at 10,000 g for 10 minutes at room temperature, air dried and redissolved in DEPC-treated water. Two microliters of mRNA solution was electrophoretically analyzed by 1.0 % agarose gel. Quality of mRNA was then spectrophometrically determined. The concentration of mRNA was calculated following the formula: [mRNA] = A260 x 40 x dilution factor

μg/ml. Aliquots of mRNA was also electrophoretically analyzed on 0.7 % agarose gel.

2.15 Construction of a cDNA library from hypopharyngeal mRNA using a SuperScript™ Plasmid System with Gateway™ Technology for cDNA Synthesis and Cloning Kit

2.15.1 First and secondary stranded cDNA preparation

Three micrograms of mRNA (10 μl) were heated at 70 °C for 10 minutes and quickly chilled on ice for 5 minutes. The contents of the tube was collected by brief centrifugation and then 4 μl of 5X of the first strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl and 12 mM $MgCl_2$), 2 μl of 0.1 M DTT and 1 μl of 10 mM dNTPs mixture were added, mixed and incubated at 37 °C for 2 minutes prior to the addition of 3 μl of 200 units/μl of SuperScript II Reverse Transcriptase, briefly mixed and incubated at 37 °C for 1 hour. At the end of incubation period, the mixture was placed on ice to terminate the reaction. Two microliters of the first stranded product were removed for electrophoretically analyzed.

For the second stranded cDNA synthesis, 93 μl of DEPC-treated water was added to the first stranded cDNA reaction followed by 30 μl of 5X second strand buffer (100 mM Tris-HCl pH 6.9, 450 mM KCl, 23 mM $MgCl_2$, 0.75 NAD^+ and 50 mM $(NH_4)_2SO_4$), 3 μl of 10 mM dNTPs mix, 10 units of *E. coli* DNA ligase, 40 units of *E. coli* DNA polymerase I and 2 units of *E. coli* RNase H. The mixture was gently mixed and incubated at 16 °C for 2 hours. At the end of the incubation period, 10 units of T4 DNA polymerase was added and further incubated at 16 °C for 5 minutes. The reaction mixture was placed on ice and 10 μl of 0.5 M EDTA was added to stop the reaction.

An equal volume (150 μl) of phenol-chloroform-isoamyl alcohol (25:24:1) was added, thoroughly mixed by vortexing and centrifuged at 14,000xg for 5 min. The upper aqueous phase (140 μl) was carefully removed to a new microcentrifuge tube. A half volume of ammonium acetate and 0.5 ml of cold absolute ethanol were added and mixed. The mixture was immediately centrifuged at 14,000 g for 20 minutes. The supernatant was discarded. The pellet was washed with 0.5 ml of 70%

ethanol. After centrifugation at 14,000xg for 2 minutes, the supernatant was removed. The pellet was air-dried at 37 °C for 10 minutes.

For adapter ligation, the reagent composed of 25 µl of DEPC-treated water, 10 µl of 5X T4 DNA ligase buffer (250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT and 25 % PEG8000), 10 µl of *Sal* I adapter and 5 units of T4 DNA ligase was added to cDNA pellet, gently mixed and incubated at 16 °C for 16 hours. The reaction was then extracted using phenol-chloroform method as described above. The cDNA was recovered using ethanol precipitation.

To produce *Not* I overhang, 41 µl of DPEC-treated water was added to the ligated cDNA followed by 5 µl of REACT 3 buffer (500 mM Tris-HCl pH 80, 100 mM $MgCl_2$ and 1 M NaCl) and 4 µl of 15 units/µl of *Not* I. The reaction was gently mixed and incubated at 37 °C for 2 hours before extracted with phenol-chloroform. The cDNA was recovered with ethanol precipitation. The cDNA was dissolved in 100 µl of TEN buffer (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA and 25 mM NaCl) and further used for size-fractionated by column chromatography.

2.15.2 Size fractionation of cDNA using column chromatography

The cDNA derived from section 2.15.1 was size fractionated using a pre-packed column chromatography. The top and bottom caps were removed from the pre-packed column. The column was washed 4 times with 0.8 ml of TEN buffer (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA and 25 mM NaCl) by pipetting the buffer onto the upper frit and allowed to completely drain out (approximately 15 minutes per wash).

A set of twenty microcentrifuge tubes was prepared and labeled. The cDNA solution was applied into the center of the top of the frit. The column was completely drained and the effluent was collected. Additionally, 100 µl TEN buffer was sequentially added to the column and the effluent was fully collected in the second tube. This step was continually repeated by adding 100 µl of TEN buffer. A single drop (approximated 35 µl) was individually collected into the tube no 3 trough no 20 while additional 100 µl of TEN buffer was continually added after the column

stopped dripping. Aliquot of 5 µl from each fraction was electrophoretically analyzed by a 1 % agarose gel.

2.15.3 Ligation of cDNA using *Sal* I-*Not* I-cut pSPORT I vector

Four microliters of fractions 9 and 10 and five microliters of fractions 11 and 12 were pooled. Five microliters of 5X T4 DNA ligase buffer (250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT and 25 % PEG8000), 50 ng of *Sal* I-*Not* I-cut pSPORT1 and 1 unit of T4 DNA ligase were added, mixed by pipetting and incubated at 4 °C overnight.

2.15.4 Transformation of cDNA to host cells

Three microliters of the ligation product were added to a microcentrifuge tube placed on ice. Commercially purchased *E. coli* DH5α (TAKARA Biomedicals, Japan) was removed from a -80 °C freezer and placed on ice bath until the competent cells were just thawed. One hundred microliters of *E. coli* DH5α competent cells were transferred to the tube containing the ligation product and gently mixed. The mixture was left on ice for 20 minutes. The cells were heat-shocked at 42 °C for exact 45 seconds. The tube was immediately placed to ice for 2-3 minutes. One milliliter of SOC medium prewarmed to room temperature was added to the tube and incubated with shaking at 37 °C for 1.5 hours containing 50 µg/ml ampicillin, 0.5 mM IPTG and 80 µg/ml X-Gal. The plates were incubated overnight at 37 °C.

2.15.5 Blue-White colony screening for recombinant plasmid

The selective plate (1% tryptone, 0.5% yeast extract, 1.0 % NaCl and 1.5 % Bacto-agar) containing 50 µg /ml ampicillin and coating with 4 µl of 20 % ITPG, 40 µl of 20 mg/ml X-gal was prepared. Aliquots of the cultured cell (100, 10, 1, and 0.1 µl) were plated to LB agar plates and incubated at 37 °C for 16-18 hours. White colonies were selected for further analysis.

2.15.6 Colony PCR for inserted size screening

Colony PCR was carried out using primers pUC1 (5'-CCG GCT CGT ATG TTG TGT GGA-3') and pUC2 (5'-GTG GTG CAA GGC GAT TAA GTT GG –3')

to examine sizes of inserts. Positive colonies were randomly picked up and added to a 10 µl reaction volume containing (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.001% gelatin, 2 mM $MgCl_2$, 100 µM of each dNTP, 0.1 µM of each prim and 0.5 U of *Taq* DNA polymerase. The amplification reaction was carried out for 35 cycles consisting of denaturation at 94 °C for 30 seconds, annealing at 53 °C for 45 seconds and extension at 72 °C for 90 seconds. The final extension was carried out at the same temperature for 7 minutes. PCR products were electrophoretically analyzed on 1.0 % agarose gel. Sixty-eight positive clones showing insert sizes greater than 500 bp were selected for sequencing.

2.16 Reverse transcription-polymerase chain reaction (RT-PCR)

2.16.1 First stranded cDNA synthesis

The first stranded cDNA was synthesized using an AMV First Strand cDNA Synthesis Kit (Biotechnology Department Bio Basic Inc., Canada). Approximately 0.6 µg of mRNA and 0.5 µg of $Oligo(dT)_{18}$ primer in the reaction volume of 12 µl was gently mixed and incubated at 70 °C for 5 min. The mixture was quickly chilled on ice for at least 5 minutes and spun down. Subsequently, 20 units of ribonuclease inhibitor, 2 µl of 10 mM each of dNTP mixture and 4 µl of 5X reaction buffer were added, mixed and incubated at 42 °C for 5 minutes. Finally, 20 units of AMV reverse transcriptase was added in the reaction, mixed and further incubated at 42 °C for 60 minutes. At the end of the incubation period, the reaction was terminated by heating at 70 °C for 10 minutes before chilled on ice for 2-3 minutes.

2.16.2 Polymerase chain reaction (PCR) amplification

Specific primers for amplification of the full length AcMRJP3 are designed from AmMRJP sequences (Albert *et al.*, 1999b). A pair of primers for AcMRJP3 were 5'-GTC AAT TGG AAA ATA TCT GTA TTA T-3' and 5'-TTT TAA TTG ATA ATT GAT TGA TTT AAT G-3'. Amplification reaction was carried out in a 25 µl reaction volume containing 1 µl of the 1st stranded cDNA, 1X PCR buffer (20mM Tris-HCl pH 8.4, 50 mM KCl), 100 mM of each dNTP, 1.5 mM $MgCl_2$, 0.2 µM of each primer and 1 unit of *Taq* DNA polymerase. The reaction was predenatured at 94 °C for 5 minutes following by 30 cycles of denaturing at 94 °C for 50 seconds,

annealing at 53 °C for 50 seconds and extension at 72 °C for 90 seconds. The final extension was performed at 72°C for 10 minutes. After amplification, 5 µl of reaction product was electrophoretically analyzed using a 0.7 % agarose gel to determine whether the amplification was successful.

2.16.3 Purification of PCR product

The remaining of AcMRJP3 amplified product in 2.16.2 was electrophoresed analyzed through a 0.7 % agarose gel. After electrophoresis was complete, the marker lane (λ/*Hind* III) was cut off and stained with ethidium bromide (2.5 µg /ml) for 5 minutes. The position of desired DNA fragment was located and excised from the gel and placed into the pre-weight microcentrifuge tube. Three volumes of GQ buffer (w/v) were added. The gel mixture was then incubated at 50 °C for 10 minutes or until the gel slice was completely dissolved. The color of the gel mixture was yellow.

The gel mixture was applied in a QIAquick spin column, which was placed into a provided 2-ml collection tube and centrifuged at 10,000xg for 1 minute at room temperature. The effluent was discarded. Optionally, 0.5 ml of QG buffer was added to remove all traces of agarose from the column and centrifuged at 10,000xg for 1 minute. After that, 0.75 ml of the PE buffer was added, left for 2-5 min and centrifuged at 10,000xg for 1 minute. The column was placed into a new microcentrifuge tube. Finally, DNA was eluted by adding 50 µl of the EB buffer (10 mM Tris-HCl pH8.5) and centrifuged at 10,000xg for 1 minute.

2.16.4 Ligation of RT-PCR product using pGEM®-T easy vector system

The pGEM®-T easy vector was used for cloning of PCR product. The ligation reaction was performed in the total volume of 10 µl containing 250 ng of purified RT-PCR product from 2.15.2, 50 ng pGEM®-T easy vector, 5 µl of 2X rapid ligation buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2mM ATP and 10 % PEG 8000), 3 units of T4 DNA ligase. The ligation mixture was mixed by pipetting and incubated at 4 °C overnight. The ligation product was electro-transformed to *E. coli* JM109.

2.16.5 Preparation of host cells for electro-transformation

A 5 ml overnight culture of *E. coli* JM109 was inoculated to 500 ml of LB broth (1% tryptone, 0.5% yeast extract and 1.0 % NaCl). The cell was incubated at 37 °C with shaking at 250 rpm for 2-3 hours until the optical density at 600 nm of culture reached 0.5-0.7. The culture was chilled on ice for 20-30 minutes and harvested by centrifugation at 8,000xg for 15 minutes at 4 °C. The supernatant was carefully decanted. The cell pellet was washed three times with 500 ml, 250 ml and 20 ml of ice-cold 10% glycerol, respectively. The cells were collected by centrifugation at 8,000xg for 15 minutes at 4 °C. Finally, the concentrated cells were resuspended in a total volume of 1.0 ml of ice-cold 10% glycerol, divided into 40 µl aliquots and stored at –80 °C until used.

2.16.6 Electro-transformation

An aliquot of 40 µl of concentrated cell in 2.16.5 was thawed on ice and mixed with 1 µl of the ligation product. The mixture was transferred into the narrow gap of cold electroporation cuvette (0.2 cm) and tapped to the bottom. The cuvette was then placed in the chamber slide, pushed into the chamber until the cuvette was seated between the contacts in the base of the chamber and pulsed once. The condition of electrophoration was set as follows; 25µF, 200Ω and 2.50 kV of the pulse controller unit.

After one pulse was applied, 1 ml of the LB broth (1.0 % trptone, 0.5 % yeast extract and 1.0 % NaCl) was immediately added to the cuvette and the cells were immediately resuspended with a Pasteur pipette. The cell suspension was transferred to the tube and incubated at 37 °C for 1 hour with shaking at 250 rpm before aliquopts of the cells were spread on the selective plates and incubated for 16 hours as described in 2.15.5. Positive clones were selected and cultured in LB broth (1% tryptone, 0.5% yeast extract, 1.0 % NaCl) containing 50 µg/ml of ampicillin. The recombinant plasmid from RT-PCR was extracted using an alkaline lysis method as describe in 2.17. The extracted plasmid was digested with *Eco* RI and electrphoretically analyzed through 0.7% agarose gel electrophoresis.

2.17 Plasmid extraction for nucleotide sequencing

A recombinant clone was inoculated into 3 ml of LB broth (1% tryptone, 0.5% yeast extract, 1.0 % NaCl) containing 50 μg/ml of ampicillin and incubated at 37 °C with constant shaking at 250 rpm overnight. The culture was transferred into 1.5-ml microcentrifuge tube and centrifuged at 12,000xg for 1 min. The cell pellet was collected and resuspended with 100 μl of solution I (25 mM Tris-HCL, pH 8.0, 10 mM EDTA and 50 mM glucose). The mixture was completely dispersed by vortexing and placed on ice for 15 min. The mixture was then treated with 200 μl of freshly prepared solution II (0.2 N NaOH and 1% SDS), gently mixed and placed on ice for 10 min. Additionally, 150 μl of solution III (3 M sodium acetate, pH 4.8) was added, gently mixed and placed on ice for 30 minutes. To separate the cell debris, the mixture was centrifuged at 12,000xg for 10 minutes. The supernatant was transferred into a new microcentrifuge tube and extracted with an equal volume of phenol : chloroform : isoamly alcohol (25:24:1). The mixture was centrifuged at 8,000xg for 5 minutes. The supernatant were removed to a new microcentrifuge tube. Plasmid DNA was precipitated with the addition of 2 volumes of absolute ethanol for 30 minutes and recovered by centrifugation at 10,000xg for 10 minutes at room temperature. The DNA pellet was washed with 70% ethanol, centrifuged at 10,000xg for 10 minutes, air died and dissolved in 50 μl TE buffer (10 mM Tris-HCl pH 7.5 and 1 mM EDTA) containing 20 μg/ml RNase A. To purify plasmid DNA for nucleotide sequencing, 32 μl of 20 % PEG6000 in 2.5 M NaCl was added. The mixture was mixed, placed on ice for 1 hours and centrifuged at 10,000xg for 10 minutes. The DNA pellet was washed with 70 % ethanol, centrifuged at 10,000xg for 10 minutes, air-dried and resuspended in 50 ml TE buffer (10 mM Tris-HCl pH 7.5 and 1 mM EDTA).

2.18 Restriction mapping for RT-PCR clones

The recombinant plasmid of the positive clone was separately digested with *Ssp* I (AAT/ATT), *Sal* I (G/TCGAC) and *Cla* I (AT/CGAT). The reaction was carried out in 20 μl containing approximately 500 ng of recombinant plasmid, 1 unit of each restriction enzyme, 1X reaction buffer, 1X BSA. The reaction mixture was incubated at 37 ° C for 3 hours. The digested product was electrophoretically analyzed through 0.7 % agarose gel.

2.19 Nucleotide sequencing and data analysis

Sixty-eight clones of the hypopharyngeal gland cDNA library of nurse bees were unidirectional sequenced using the thermo sequenase cycle sequencing with 7-deaza-dGTP and fluorescent 1 dye primer system kit (Amersham Pharmacia Biotech UK Limited, England) on an automated sequencer (Licor 4100). The M13 reverse primer was used as the sequencing primer, Briefly, 0.5-5 μg of double stranded DNA was required. A set of nucleotide base A, C, G and T reaction was prepared by combining 0.6 – 1.0 μl of fluorescent primer and DNA template and thoroughly mixed. Three microliters of the reaction were aliquoted to four separate reactions and 1 μl of A, C, G or T reagent mixes were added into each reaction. The sequencing reaction mixtures were then subjected to amplification cycle consisting of a pre-denaturing at 95 °C for 5 minutes followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for 60 seconds. After cycle-sequencing was finished, 2 μl of formamide loading dye was added into each reaction. The sample was finally loaded onto a gel based-automated DNA sequencer and operated for 12-16 hours.

For clones from RT-PCR, the universal forward and reverse primers were initially used for sequencing by an automated sequencer at Bioservice unit (BSU), Thailand. Internal sequencing primers were subsequently designed by Oligo program and used for sequencing along the entire length of the insert.

Nucleotide sequences obtained were blasted against those deposited in the GenBank using the BlastN and BlanstX programs (http://www.ncbi.nlm.nih.gov). Significant probabilities were considered when the E value was less than 10^{-4}. For the full length RJ genes, nucleotide sequences were translated to amino acid sequence using ProtParam tool (http://ca.expasy.org/tools/protparam.html). Domain of the protein molecule was then analyzed (http://bmerc-www.bu.edu).

2.20 Phylogeny reconstruction

Nucleotide and deduced amino acid sequences of AcMRJPs and AmMRJPs were aligned using Clustal X. Genetic distance (*d*) between sequences was calculated

based on a Kimura's method using Prodist in PHYLIP. The original data of nucleotide and deduced amino acid sequences was bootstrapped 1000 times and 200 times, respectively using Seqboot. Genetic distances of multiple data sets were calculated using Prodist. Neighbor-joining trees were constructed using Neighbor. The treefile obtained was analyzed by Consense. All phylogenetic program were implemented in PHYLIP 3.56c (Felsenstein, 1993). The bootstrapped treefile was properly viewed using TREEVIEW.

PART IV: QUANTIFICATION OF EXPRESSION LEVELS OF ACMRJPS USING SEMI-QUANTITATIVE PCR

For determination of the expression level of AcMRJP1, AcMRJP2 and AcMRJP3 in the hypopharyngeal glands of *A. cerana* nurse bees, mRNA was extracted as describe in 2.14 and used to prepare the 1st strand template.

In addition, the expression level of the same AcMRJP family in the hypopharyngeal glands of different developmental stages of worker bees (newly emerged, nurse and forager bees) were also examined. In this case, total RNA was extracted and converted to the 1st strand template.

2.21 Total RNA isolation

Total RNA was extracted from hypopharyngeal glands of each stage of *A. cerana* workers using a SV total RNA isolation kit (Promega). Twenty glands from the same stage of worker bees were dissected out and placed into a microcentrifuge tube containing 175 µl of the SV lysis buffer (4 M guanidine isothiocyanate, 0.01 M Tris-HCl pH 7.5 and 0.97 % β-mercaptoethanol) and homogenized for a few times using a micropestle. The homogenate was thoroughly mixed by inversion and diluted by the addition of 350 µl of the SV RNA dilution buffer. The mixture was placed in the heating block at 70 °C for 3 minutes and centrifuged at 12,000xg for 10 minutes. Clear cell lysate was collected and transferred to a new microcentrifuge tube. Two hundred microliters of absolute ethanol was added and mixed by pipetting. This mixture was transferred to a spin column and centrifuged at 12,000 g for 1 minute. The spin column was washed with 600 µl of the SV RNA wash solution (60 mM potassium acetate, 10 mM Tris-HCl pH 7.5 and 60 % ethanol) and centrifuged at 12,000xg for 1 minute. After that, the spin membrane was treated with 50 µl of the DNase I solution and incubated at 20-25 °C for 15 minutes before the addition of 200 µl of the SV DNase stop solution (2 M guanidine isothiocyanate, 4 mM Tris-HCl pH 7.5 and 57 % ethanol). The spin column was centrifuged at 12, 000xg for 1 minute and rewashed once with 600 µl and 250 µl of the SV RNA wash solution, respectively. Finally, total RNA was eluted out by the addition of 100 µl of nuclease

free water and centrifugation at 12,000xg for 1 minute. Total RNA was stored at –70 ° C until used.

When needed, extracted total RNA was reverse transcribed to the 1st template as describe in 2.16.1 and used for optimization of PCR condition.

2.22 Optimization of PCR conditions

Sequences of AcMRJPs derived from 2.1 were aligned using Clustal X and searched for the specific primer sites of each MRJP family. A pair of primers of each AcMRJP family was designed using Oligo 4.0.

For optimization of PCR conditions, appropriate concentrations of each primer and $MgCl_2$ were adjusted and illustrated in Table 2.2. Typically, PCR was carried out in the total volume of 25 µl composing 1 µl of the 1st stranded template, 1X PCR buffer (20mM Tris-HCl pH 8.4, 50 mM KCl), 100 mM of each dNTP, 1.0-1.5 mM $MgCl_2$, 0.15 - 0.20 µM of each primer and 0.6 units of Ampli*Taq* DNA polymerase (Perkin Elmer). The temperature profiles were predenatured at 94 °C for 5 minutes followed by 30 cycles of denaturation at 94 °C for 50 seconds, annealing at 53-54 °C for 50 seconds and extension at 72 °C for 1.50 minutes. The post-extension was performed at 72 °C for 10 minutes. After amplification, an aliquot of 5 ml of PCR product was electrophoretically analyzed on 2.0 % Metaphor agarose gel. Additionally, PCR product was also digested with restriction endonucleases to verify specificity of primers (Table 2.3). Digested DNA fragment was electrophoretically analyzed as above.

Additionally, different numbers of the amplification cycles (2 cycle increment between 12 – 26 cycles) to ensure the use of the plateau phase of amplification reaction. A PCR reaction was collected according to described cycle numbers and incubated at 72 °C for 10 minutes. PCR products were elcetrophoretically analyzed on a 2.0 % Metaphor agarose gel.

Table 2.2 Primer sequences and PCR conditions for quantification of AcMRJPs expression level in 25 cycles.

	Primer sequence (5' to 3')	$MgCl_2$ concentration (mM)	Primer concentration (μM)	Annealing temperature (°C)
AcMRJP1	For: TCA AGG TAC GAC AAG CAG CAT TC Rev: TTG TCG ATC GCA AGT TTT GTG G	1.5	0.20	53.0
AcMRJP2	For: CTT GGA AAA TTC GTT GAA CGT A Rev: GTA TTT CGA TTT GCT TAA GGT GC	1.5	0.20	53.0
AcMRJP3	For: GCG CAC TGT TGA ACC ATC AAA G Rev: AGA AGA GGT CCA CCT TTG CCT T	1.0	0.15	54.0

Table 2.3 Restriction enzymes specifically digested PCR products ofAcMRJP1, AcMRJP2 and AcMRJP3 amplified with different a pair of primers.

PCR product	Restriction enzymes	Recognition sites
AcMRJP1	*Rsa* I	GT/AC
	Dra I	TTT/AAA
	*Sau*3A I	/GATC
AcMRJP2	*Hinf* I	G/ANTC
	Taq I	T/CGA
AcMRJP3	*Sau*3A I	/GATC
	Rsa I	GT/AC

2.23 Preparation of Target cDNA for competitive PCR

The 2nd stranded cDNA was synthesized using an additional cycle of PCR amplification with family-specific primers. One microliter of the 1st stranded cDNA was used as the template in a 25 µl reaction volume. PCR condition was performed in the optimal condition derived from 2.22. The amplification product (called target cDNA) was further used to simultaneously amplify with the internal standard DNA.

2.24 Preparation of the Internal standard for competitive PCR

An internal standard DNA of AcMRJP1, AcMRJP2 and AcMRJP3 was generated using genomic DNA of *A. cerana*. Size differences between cDNA and genomic DNA of investigated genes were resulted from the existence of intervening sequences of a particular gene. To prepare the internal standard DNA, 25 ng of total DNA in 2.7 was used as the template and amplified using the same primer set for amplification of the cDNA template. The PCR reaction was performed in a 25 µl reaction volume as described in Table 2.2. PCR product was separated on a 1.0 % agarose gels and gel-eluted as described in 2.15.3. One miroclitер of eluted DNA was mixed with 1 µl of 2.5 µg/ml of ethidium bromide and 1 µl of deionized water and dropped on a plastic sheet. The concentration of the PCR product was estimated by comparing its florescence intensity under a long wavelength UV light with that of λ DNA (2 -18 ng).

2.25 Quantitative PCR assay

To perform a PCR reaction, a ten fold dilution between 1 ng/µl, -1 fg/µl of the internal standard DNA of each family was prepared. One microliter of each concentration of the internal standard DNA was added to a microcentrifuge tube and co-amplified with a constant amount of 0.8 - 3.2 µl of the target cDNA. The PCR reaction was performed as described in Table 2.2. The PCR product was electrophoretically analyzed through 2.0 % Metaphor agarose gel and stained with 2.5 µg/ml of ethidium bromide. A range of suitable internal standard DNA amount providing competitive results was chosen and further adjusted in a narrower range to identify a zone showing equivalent band intensity between target cDNA and internal standard DNA.

The PCR reaction was performed in the most appropriate amount of the internal standard DNA. PCR products were electrophoretically analyzed through 2.0 % Metaphor agarose gel. The fluorescence intensity (exhibited as the absorbance unit) was measured from the photographs using a densito-photometer (BioRad). The intensity ratios between the target cDNA and the internal standard DNA amplified product were determined. An amount of initial cDNA was calculated by plotting the log of the ratios of target cDNA to the internal standard DNA amplified products (log [T]/[S]) versus the initial amounts of the standard added to the PCR reaction.

simulation and F_{ST} statistics, Table 3.3). AMOVA indicated significant differences among variance components among groups ($P < 0.0001$) but showed non-significant differences between samples within groups ($P = 0.0893$) and among individuals within geographic samples ($P = 0.0162$) after the significance level was adjusted with a sequential Bonferroni method (Table 3.4).

A neighbor-joining tree based on the percentage of nucleotide divergence between pairs of samples (Figure 3.3) illustrated large genetic differences between *A. cerana* from hierarchical groups A and B. The genetic difference between groups B and C was much lower but still greater than that within each group.

Results from PCR-RFLP analysis revealed that *Dra* I was an informative restriction enzyme for identifying population origins of *A. cerana*. Geographic origins of six *A. cerana* colonies from three populations (north to central Thailand, peninsular Thailand and Samui Island) were verified before further used to produce RJ.