CHAPTER II

Materials and Methods

2.1 Instruments

- Autoclave HA-30 (Hirayama Manufacturing Co., Japan)
- Automatic micropipette P2, P20, P100(Gilson Medical Electronics S.A., France)
- Camera Pentax super A (Asahi Opt. Co., Japan)
- Electronic balance Alsep EY220A (A&D Co. Ltd., Japan)
- Electrophoresis apparatus
  - :Horizontal gel eletrophoresis apparatus (9x12 cm gel)
  - :Vertical gel eletrophoresis apparatus for sequencing (Hoefer, England)
- -20°C Freezer (Krungthai Ltd., Thailand)
- High speed microcentrifuge MC-15A (Tomy-Seiko, Japan)
- Heating block BD 17016-26 (Sybron Thermermolyne Co., U.S.A.)
- Incubator BM-600 (Memmert GambH, Germany)
- Light box 2859 SHANDON (Shandon Scientific Co. Ltd., England)
- Magnetic stirrer M21/1 (Franz Morat KG GambH, Germany)
- Microcentrifuge Force6 (Denver Instrument Company, U.S.A.)
- Microwave oven TRX1500 (Tarbora International Co. Ltd., Korea)
- Power supply (Bio-RAD Laboratories, U.S.A.)
  - :Power PAC 300
  - :Power PAC 3000

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

- Standard film cassette: Ranex Regular Screen Eastman (Eastman Kodak Company, U.S.A.)

- Thermal cycler :GeneAmp PCR system 2400 (Perkin Elmer Cetus, U.S.A.)

- UV transilluminator 2011 Macrovue (San Gabriel California, U.S.A.)

2.2 Inventory Supplies

- Aerosol resistant filter tip 10, 50 µl (Centrified Scienctific Plastics, U.S.A.)

- Black and white print film TriX-pan 400 (Eastman Kodak Company, U.S.A.)

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

- Hyper-film MP (Amersham International., England)

- Microcentrifuge tubes 0.5, 1.5 ml (Axygen Hayward, U.S.A.)

- Mirror for sequencing 30x40 cm (Hoefer Inc., England)

- Pitpette tips 10, 20, 100 µl (Axygen Hayward, U.S.A.)

- Thin-wall microcentrifuge tubes 0.2 ml (Axygen Hayward, U.S.A.)

2.3 Chemicals

- Absolute ethanol (Merck, Germany)

- Acrylamide (Merck, Germany)

- Agarose gel (FMC Bioproducts, U.S.A.)

  : Seakem LE Agarose

  : MetaPhor Agarose

- 100 base pair DNA ladder (Promega Co., U.S.A.)

- Boric acid (Merck, Germany)

- Bromophenol blue (Merck, Germany)

- Chloroform (Merck, Germany)

- Developer (Eastman Kodak Company, U.S.A.)

- Disodium ethylene diamine tetraacetic acid: Na_2EDTA (Fluka, Switzerland)

- Ethidium bromide (Sigma Chemical Company, U.S.A.)

- Ficoll 400 (Sigma Chemical Company, U.S.A.)

- Fixer (Eastman Kodak Company, U.S.A.)

- GeneAmp PCR core reagents (Perkin Elmer Cetus, U.S.A.)

  : 10X PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl)

  : 25 mM $MgCl_2$

  : 10 mM each of dNTPs

- Geneclean kit (Bio101, U.S.A.)

  : glassmilk

  : 6 M sodium iodide

  : TBE modifier

  : new wash concentrate

- Hydrochloric acid (Merck, Germany)

- Isoamyl alcohol (Merck, Germany)

- N,N-methylene-bis-acrylamide (Sigma Chamical Company, U.S.A.)

- N,N,N′, N′-tetramethylethylenediamine (Sigma Chemical Company, U.S.A.)

- OmniBase™ DNA cycle sequencing system (Promega Co., U.S.A.)

  : 5X sequencing buffer (250 mM Tris-HCl ph 9.0, 10 mM $MgCl_2$)

: d/ddNTP nucleotide mixes

: DNA sequencing stop solution (10 mM NaOH, 95 % formamide, 0.05 % bromophenol blue, 0.05 % xylene cyanol)

: 10 U/μl T4 polynucleotide kinase

: 10X T4 polynucleotide kinase buffer (500 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 50 mM DTT, 1.0 mM spermidine)

: 200 ng/μl pGEM3zf (+) control DNA

: 24 mer pUC/M13 forward primer

- pBR322-Msp I digest (Biolabs, U.S.A.)
- Phenol crystal (Fluka, Switzerland)
- Sodium acetate (Merck, Germany)
- Sodium dodecyl sulfate: SDS (Sigma Chamical Company, U.S.A.)
- Tris-(hydroxy methyl)-aminomethane (Fluka, Switzerland)

-Urea (Fluka, Switzerland)

- Xylene cyanol (Sigma, U.S.A.)

2.4 Enzymes

- *AmpliTaq* DNA Polymerase (Perkin Elmer, U.S..)
- Proteinase K (GibcoBRL Life Technologies, Inc., U.S.A.)
- Restriction endonucleases : *Eco*R I, *Hin*d III, *Hin*f I and *Dra* I (Boehringer Mannhemn, Germany)

2.5 Primers

- Oligonucleotides (Bioservice Unit, Thailand or Biosynthesis, Inc., U.S.A. or Vetrogen, Inc., Canada)

2.6 Radioactive

- [γ-^{32}P] ATP specific activity 3,000 Ci/mmol (Amersham International, England)

2.7 Samples collections

Adult honey bee workers *A. cerana* from natural colonies or beekeeping's cases in Thailand were collected. The sampling areas of *A. cerana* were composed of five geographically different locations indicating 1) North 2) North-East 3) Central 4) South and 5) Samui Island (Figure 2.1). The number of colonies per population was between 20 - 50. *A. cerana* workers were individually collected and immediately put in 95 % ethanol and then stored at 4 °C until further required.

2.8 DNA extraction

Total DNA was prepared individually using a modification of the method of Hall and Smith (1991). Thorax was dissected out from individual specimen, placed in a pre-chilled microcentrifuge tube containing 500 µl of STE buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 mM EDTA) and briefly homogenised using a micropestle. The cells were lysed by adding 20 % SDS to 1% SDS final concentration. A proteinase K solution (10 mg/ml) was added to a final concentration of 500 µg/ml and incubated at 37 °C for 3 hours. The DNA was then extracted using a standard phenol-chlorofrom method. The extraction was carried out twice with an addition of an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), mixed gently for 15

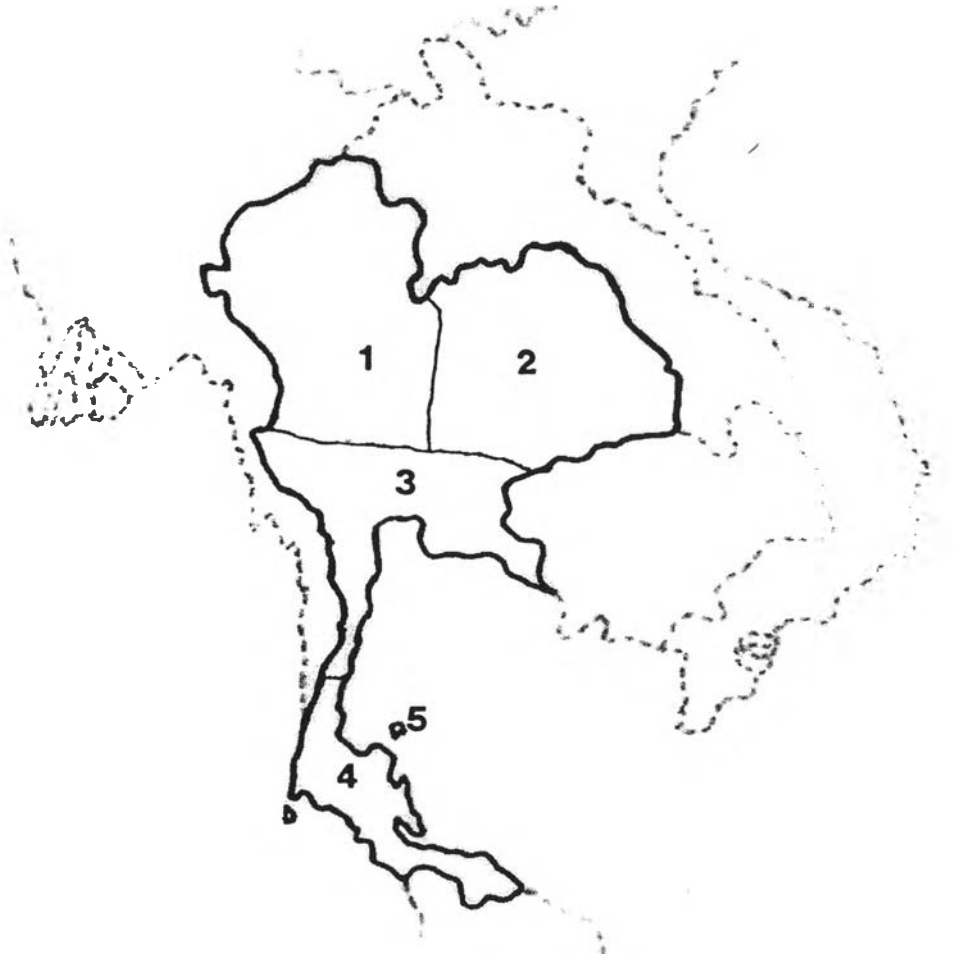

Figure 2.1 Map of Thailand showing five geographic locations of *A. cerana* used in this study. Regions were indicated by number.

(1) North (2) North -East

(3) Central (4) South

(5) The Samui Island

minutes and spun at 4,000xg for 10 minutes at room temperature. The aqueous phase (upper layer) was transfered to a new sterile tube and further extracted once with an equal volume of chloroform-isoamylalcohol (24:1). One-tenth volume of 3M sodium acetate, pH 7.0 was added following by two volumes of cold absolute ethanol. The resulting mixture was kept in a -20 °C freezer overnight. DNA was recovered by centrifugation at 6,000xg for 15 minutes at room temperature and briefly washed with 70 % ethanol, air-dried and resuspended in an appropriate volume of TE buffer (10 mM Tris-HCl pH 7.4 and 1 mM EDTA pH 8.0) or alternatively in sterile deionised water. DNA concentration from each individuals was adjusted to approximately 25 ng/μl by comparing its fluorescence intensity with that of the standard DNA marker(s). This DNA solution was then stored at 4 °C and subsequently utilised as the template for PCR amplification.

2.9 PCR amplification

Three different regions of *A. cerana* mitochondrial genome were *in vitro* amplified by PCR, using conditions modified from Hall and Smith (1991). Amplification reaction was carried out in a 25 μl reaction volume containing 50 ng template DNA, 200 μM each of dNTPs (dATP, dCTP, dGTP, dTTP), 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 2.5-3.0 mM $MgCl_2$, 0.10 μM each of primers and 0.6 unit of *Taq* DNA polymerase (Perkin Elmer). The reaction was then initially denatured at 94 °C for 1 minute following by 35 cycles of a 92 °C denaturation for 1 minute, a 53 °C annealing for 1 minute and a 72°C extension for 2 minutes. The final

extension was carried out at the same temperature for 10 minutes. Appropriate concentrations of each primers and $MgCl_2$ were carfully adjusted as illustrated in Table 2.1. Aliquots of the amplified DNA fragment were eletrophoretically analyzed. If the amplification reaction is sucessful, the remaining solution was then subjected to restriction analysis.

2.10 Agarose gel electrophoresis

Agarose gel electrophoresis was performed in order to separate DNA fragments on the basis of their molecular sizes (Table 2.2). Appropriate amount of agarose (SeaKem LE or Metaphor, FMC) was weighed out and mixed with TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA pH 8.0) and heated until complete solubilisation in a microwave oven. The agarose solution was incubated at 60 °C until all air bubbles was completely eliminated. The solution was then left at room temperature to 50 °C before pouring into an eletrophoretic mould. After the gel was completely set, it was prechilled in a refrigerator for at least 30-60 minutes before being used. The comb and seal of the mould were carefully removed. When ready, one-fifth volume of the loading dye (0.25% bromphenol blue, 0.25% xylene cyanol FF and 15% ficoll 400) was mixed with each DNA sample before loaded into the well. Eletrophoresis was operated at 100 V for 0.7-1.5 % gel or 140 V for 2.5-3.5 % gel until bromphenol blue (lowest dye) migrated to approximately 2 cm. from the bottom of the gel. The gel was stained with a 2.5 μg/ml ethidium bromide solution for 5 minutes and destained to remove unbound ethidium bromide in distilled water for 30 minutes. DNA fragments

Table 2.1 Primer sequences, annealing temperatures, concentrations of primers and $MgCl_2$ used for PCR amplification of *A. cerana* mtDNA genes.

Gene	Sequence	Position	Annealing Temperature (°C)	Primer conc. (μM)	$MgCl_2$ Conc. (mM)
16S [1]	5′CTA TAG GGT CTT ATC GTC CC 3′ 5′TTT TGT ACC TTT TGT ATC AGG GTT G3′	13708 14447	53	0.10	3.0
12S [2]	5′AAA AAG CTT CAA ACT GGG ATT AGA TAC CCC 3′ 5′TGA CTG CAG AGG GTG ACG GGC GGT GTG T 3′	14588 14206	53	0.10	3.0
inter CO I-CO II [3]	5′TTG ATT TTT TGG TCA TCC AGA AGT 3′ 5′CCA CAA ATT TCT GAA CAT TGA CC 3′	2492 4213	53	0.10	2.5

[1,3] Primer sequences and their position were inferred from *A. mellifera* (Crozier and Crozier, 1993)

[2] Primer sequences and their position were inferred from *Drosophila yakuba* (Clary and Wolstenholme, 1985)

Table 2.2 Optimal concentration of agarose in 1xTBE buffer used for separation of double stranded DNA in this study.

Gel percentages	Range of fragment sizes to be separated (bp)	
	SeaKem LE agarose	MetaPhor agarose
0.50	1,000-23,000	-
0.70	800-10,000	-
0.85	400-8,000	-
1.00	300-7,000	-
1.25	200-4,000	
1.75	100-3,000	-
2.00	-	100-600
3.00	-	50-250
4.00	-	20-130
5.00	-	< 80

were visualized under a long wavelength UV light and photographed through a red filter using Kodax Tri X pan 400 film.

2.11 Restriction pattern of amplified DNA fragments

An amplified DNA fragment from each individual was singly digested with a set of restriction endonucleases (*Dra* I, *Hin*d III, E*co*R I, *Hin*f I) according to the manufacturer's recommendations. Digestion was carried out in a 20 µl containing 500 ng of DNA, 5 units each of restriction endonucleases, 2 µl of 10x reaction enzyme buffer and appropriate amount of sterile deionised water. The reaction mixture was incubated at 37°C for 2-3 hours. At the end of incubation period, one-fifth volume of a loading dye was added to stop the reaction. The resulting mixture was electrophoretically analysed as soon as possible.

2.12 Purification of amplified DNA fragments for DNA sequencing

An amplified DNA fragment was separated by submerged agarose gel electrophoresis as described in 2.10 along with standard size markers. After eletrophoresis was complete. The marker lane was chopped off and stained with ethidium bromide. The position of desired amplification DNA fragment to be purified could then be able to locate. Interested DNA band was excised from the gel and placed in a pre-weight 1.5 ml microfuge tube. Four and a half gel slice volumes of 6 M NaI solution and 1/2 volume of TBE modifier were added. The mixture was incubated at 55 °C for 5 minutes. Subsequently, 5 µl of glassmilk suspension was added and mixed thoroughly every 1-2 minutes. The tube was placed on ice for 5 minutes to allow

binding of the DNA to glassmilk and centrifuged at 5,000xg for approximately 5 seconds. NaI was decanted and discarded. The white pellet was firm enough to remain intact after pouring out of the liquid content from the tube. The pellet was washed 3 times with a new wash solution. Finally, the pellet was resuspended with TE buffer and incubated at 55 °C for 2-3 minutes to elute DNA from glassmilk. The resulting solution was centrifuged at 5,000xg for 15 seconds. The supernatant containing the eluted DNA was collected for sequencing.

2.13 DNA sequencing

The OmniBase™ DNA cycle sequencing system (Promega) was used for direct sequencing of purified mtDNA fragment. The reaction was depended on an ability of DNA polymerase to extend ^{32}P end-labelled primer, which was annealled to the template and required the four deoxyribonucleotide triphosphates as building-blocks. Newly synthesised DNA chain was made until the growing oligonucleotides were selectively terminated at respective dideoxynucleotide analog in the reactions. After sequencing reaction, resulting fragments were fractionated by high resolution denaturing polyacrylamide gel electrophoresis. The sequences obtained were directly visualised from an autoradiograph. Initially, the external primer was used for sequencing of about 150-200 bp in both direction. Oligonucleotide sequences overlapping to the previous sequences (the internal primer) were designed by Oligo program (version 5.0) and used for further sequencing.

2.13.1 End-labelling of the primer

The primer was end-labelled with γ ^{32}P ATP (specific activity 3,000 Ci/mmol). The reaction mixture of 10 µl contained 10 pmol primer, 10 pmol γ ^{32}P ATP, 1 µl of 10x T_4 polynucleotide kinase buffer (500 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 50 mM DTT, 1.0 mM spermidine) and 5 units of T_4 polynucleotide kinase. The reaction mixture was incubated at 37 °C for 10 minutes. The T_4 polynucleotide kinase was then inactivated at 90 °C for 2 minutes. The mixture was centrifugued briefly and stored at -20 °C until used.

2.13.2 Primer extension

A set of nucleotide reaction (A, T, G and C) was perpared by adding 2 µl of d/ddNTP mix into four thin - wall microcentrifuge tube. Primer premix was performed in a 16 µl reaction containing 2 ng template DNA, 5 µl of 5x sequencing buffer (250 mM Tris-HCl pH 9.0, 10 mM $MgCl_2$), 1.5 µl of end-labelled primer (2.13.1) and 10 units of OmniBase™ sqeuencing enzyme. The reaction constituents were carefully mixed by pipetting. Afterwards, 4 µl of the primer premix was added into each of nucleotide reactions and thoroughly mixed. Complete reaction mixtures were then subjected to cycle sequencing composing of a pre-denaturation step at 95 °C for 2 minutes followed by 30 cycles of a denaturation step at 95 °C for 30 seconds, an annealing step at 45 °C for 30 seconds and an extension step at 70 °C for 1 minute. Three microlitres of stop solution was added. The reactions was

then ready to be sequenced. When the gel was ready for loading, the reactions were heated to 70 ° C for 2 minutes and snap-chilled on ice.

2.13.3 Polyacrylamide gel electrophoresis

A pair of glass plates were used for preparation of sequencing polyacrylamide gel. They must be cleaned thoroughly with soap and extensively rinse with water, ethanol and air dried. The shorter plate was siliconized with Rain-X (ethyl alcohol, ethyl sulfate, isopropyl alcohol and siloxanes) . The glass plates were assembled with a pair of 0.4 mm plastic spacers and sealed with a tape along the sides and the bottom of the gel glass. A standard 6 % sequencing gel (acrylamide: bisacrylamide = 19:1) was prepared with 1xTBE in the presence of 8M urea in a total volume of 60 ml. Before pouring the gel, 280 µl of 10 % ammonium persulfate and 50 µl of TEMED were added and mixed gently. The glass plates were held at a 45 ° angle. The gel mixture was carefully poured between the glass plates, with a smooth flow rate to prevent any air bubbles. After the gel mould was filled, it was placed at the horizontal position. A shark's comb was slid into the top open end with the teeth pointing up. The gel was left at least 2 hours to allow complete polymerisation. The sealing tape and comb was then carefully removed. The gel was washed to remove any exess gel pieces and placed in an electrophoretic apparatus which 1xTBE buffer was already added to the upper and lower tanks. At this stage, the comb was re-inserted with the teeth on the top of the gel. The gel was pre-electrophoresed at a constant power of 35 W for 30 minutes. Before loading the samples, the wells were flushed with 1xTBE buffer by using a Plastuer pipette to ensure

that urea, if any, was removed. The samples were loaded. The gel was run at constant power of 35 W.

2.13.4 Gel processing and autoradiography

The gel was removed from the electrophoresis apparatus. The shorter glass plate was gently prized therefore the gel was remained on the longer glass plate. To transfer the gel, A piece of Whatman 3 MM filter paper was placed and pressed against the gel. The paper-gel-glass was then turned over, before the glass plate was lifted off. The gel stuck firmly to the paper support was covered with plastic wrap and placed in an autoradioghaphy cassette and taken to the dark room. An autoradiography film was then placed over the gel. The cassette was left at - 80 °C until required. The exposure period depended on a signal of the radioisotope. When needed, the autoradiography cassette was thawed out to room temperature. The film was developed and fixed in the dark room according to manufacture's instruction. The sequencing data was read manually.

2.14 Data analysis

2.14.1 Restriction patterns analysis

The different restriction patterns of each mtDNA region were assigned and given an alphabets name (A, B, C,...) in the order of their frequencies. The fragment sizes of all restriction profiles were estimated in comparison with those of standard DNA markers. The composite haplotypes were generated from combination of restriction patterns of each region with an alphabetical arranging from the least to the highest polymorphic enzyme.

Binary characters including present (1) or absent (0) of band in each haplotypes were compared for further statistical analysis. All statistical analyses were carried out in Restriction Enzyme Analysis Pakage, REAP (McElroy, 1991).

2.14.1.1 Genetic distance

The relationship of composite haplotype was observed by genetic distance value (d) that could be estimated as the number of nucleotide substitution per site following the equation:

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

where r is the number of recognized sequences at the restriction site and G is $[F\,(3\text{-}2G1)]^{1/4}$ and repeatly calaculated until $G = G1$ then $G1 = F^{1/4}$ is recommended to initial trial value.

F is the similarity index between compared haplotypes estimated by

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

where n_x and n_y are the number of fragments for haplotype X and Y, respectively, and n_{xy} is the number of shared fragment.

2.14.1.2 Haplotype and nucleotide diversity

Genetic diversity within populations was estimated from the haplotype and nucleotide diversity. The former is a function of the frequency of different haplotypes in the same population which can be illustrated as

$$h = n\,(1\text{-}\,\Sigma\, x_i^2)\,/\,(n\text{-}1)$$

where n is the number of individuals investigated and x_i is the frequency of the i^{th} haplotype. Since this value varies enormously with the length of

investigated genome while nucleotide diversity (d) is an average number of nucleotide differences per site between two sequences, which is more appropriate than the haplotype diversity for comparisons of the same DNA markers with difference in length. This estimate can be calculated using

$$d = \frac{n}{n - 1} \Sigma x_i x_j d_{ij}$$

where n is the number of individuals investigated and x_i and x_j are the frequencies of i and j genotype, respectively. d_{ij} is the number of nucleotide differences per site between the i th and j th haplotype.

2.14.1.3 Nucleotide divergence

The nucleotide digervence was used to investigated between population diversity which was calculated from nucleotide diversity between haplotypes in population X, Y (d_x, d_y) following equation in 2.14.1.2. The average nucleotide diversity between haplotypes from population X and Y (d_{xy}).

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

where d_{ij} is nucleotide substitution between the i th and j th haplotype from population X and Y, respectively. The nucleotide divergence between two populations (d_A) is then calculated from

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

2.14.1.4 Geographic heterogeneity analysis

Chi - square (χ^2) test was performed using Monte carlo simulation for analysis of geographic heterogeneity in frequency distribution among

population. This value was indicated the significant difference of the composite haplotype frequencies between population.

2.14.2 Sequence data analysis

DNA sequences of lrRNA gene from investigated individuals were aligned using by Clustal V. The genetic distance (d) between sequence was calculated using Kimura's two parameter method using the formula:

$$d = 3/4 \ ln(1 - 4/3 \ k)$$

where k is the percentage difference in base composition.

2.14.3 Phylogenetic reconstruction

The genetic distance (d) between composite haplotypes or sequences and the nucleotide divergence between populations (d_A) was subjected to phylogenetic reconstruction using UPGMA in Phylip version 3.57 c. The datum was then bootstraped for 2000 times. Genetic distance of multiple data was calculated. A consensus tree based on neighbor-joining method was constructed for statistical support of the tree. Data from sequences was used to construct intraspecific phylogeny both based on genetic distance (Kimura's two parameter method) and maximum parsimony (DNAPARS) approaches. The most parsimonous tree was constructed for 1000 bootstraped data.