CHAPTER II

MATERIALS AND METHODS

2.1 Instruments

- Autoclave HA-30 (Hirayama Manufacturing Co., Japan)
- Automatic micropipette P2, P20, P100, P200, P1000 (Gilson Medical Electrical S.A., France)
- Autoradiography cassette (Research Products International Corp., USA)
- Camera (Pentax Super A)
- Electrophoresis apparatus

  : Power supply (Bio-RAD Laboratories, USA)

  : Horizontal agarose gel electrophoresis apparatus (9x12 cm. Gel)

  : Vertical gel electrophoresis apparatus for DNA sequencing (Hoefer, England)
- -20 ^{O}C freezer
- -80 ^{O}C freezer
- Gel dryer Model 583 (Bio-RAD Laboratories, USA)
- High speed microcentrifuge MC-15A (Tomy Seiko, Japan)
- Heating block BD 1761G-26 (Sybron Thermermolyne Co., USA)
- Incubator BM-600 (Memmert Gamblt, Germany)
- Light box 2859 SHADON (Shandon Scientific Co., Ltd. England)
- Microcentrifuge Force 6 (Denver Instrument Company, USA)
- PCR, Gene Amp System 2400 (Perkin Elmer Cetus, USA)
- PCR workstation Model#P-036 (Scientific Co., USA)
- UV transilluminator 2011 Maccrovue (San Gabriel California, USA)

2.2 Inventory supplies

- Black and white paint film TriX – pan400 (Eastman Kodak Company, USA)
- Filter paper whatman 3 MM (Whatman Internation Ltd., England)
- Microcentrifuge tubes 0.5, 1.5 ml (Axygen Harward, USA)
- Mirror for sequencing sox40 cm (Hoefer Inc., England)
- Pipette lips (Axygen Hayward, USA)
- Thin-wall microcentrifuge tubes 0.2 ml (Axygen Hayward, USA)
- X-ray film, X-Omat XK-1 (Eastman Kodak company Rochester, USA)

2.3 Chemicals

- Absolute ethanol (Merck, Germany)
- Acrylamide (Merck, Germany)
- Agarose, Seakem LE (FMC Bio products, USA)
- Ammonium persulfate (Promega, USA)
- Boric acid (Merck, Germany)
- Bromophenol blue (Merck, Germany)
- Chloroform (Merck, Germany)
- Developer (Eastman Kodak Company, USA)
- Ethylene diamine tetraacetic acid, disodium salt dihydrate (Fluka, Switzerland)
- Ethidium bromide (Sigma, USA)
- Fixer (Eastman Kodak Co., USA)
- GeneAmp PCR core reagents (Perkin Elmer Cetus, USA)
  : 10X PCR buffer (100 mM Tris-HCl pH8.3, 500 mM KCl)
  : 25mM $MgCl_2$ solution
  : 10mM dNTPs (dATP, dCTP, dGTP and dTTP)
- Hydrochloric acid (Merck, Germany)
- Isoamyl alcohol (Merck, Germany)
- *N,N* – methylene – bis - acrylamide (Amersham, England)

- *N,N,N′,N′* – tetramethylenediamine (Sigma, USA)
- OmniBase™ DNA cycle sequencing system kit (Promega, USA)
  : 5X DNA sequencing buffer (250 mM Tris-HCl pH 9.0, 10 mM $MgCl_2$)
  : d/ddNTP nucleotide mixes
  : DNA sequencing stop solution (95% NaOH, 10 mM 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
- Phenol crystal, analytical grade (Fluka, Germany)
- Sodium acetate (Merck, Germany)
- Sodium chloride (Merck, Germany)
- Sodium dodecyl sulfate (SDS) (Sigma, USA)
- Tris- (hydroxy methyl) - amminomethane (Fluka, Switzerland)
- Urea (Fluka, Switzerland)
- Xylene cyanol (Sigma, USA)

2.4 Oligonucleotide primers

Oligonucleotide used for PCR were purchased from Bio service Unit, Thailand or from Biosynthesis, Inc., USA. The primer sequences are shown in Table2.1 and 2.2

2.5 Enzymes

- Ampli *Taq* DNA polymerase (perkin-Elmer Cetus, USA)
- Proteinase K (GibcoBRL Life Technologies, Inc., USA)

2.6 Radioactive

- [γ-^{32}P]dATP specific activity 3,000 Ci/mmol (Amersham, England)
- [α-^{35}S]dATP specific activity 1,000 Ci/mmol (Amersham, England)

2.7 Samples

Adult workers honeybee *A. cerana* included 265 colonies were collected from natural colonies and beekeeping's cases from five different areas in Thailand (appendix 1 and 3). The sampling areas were as follows : the North, the North-East, the Central, the South and the Samui Island. Samples were immediately preserved in 99 % ethanol and then stored at 4 ^{O}C for later use.

2.8 DNA extraction

Genomic DNA was extracted from a thorax of each *A. cerana* individual using the modified method of Hall and Smith (1991). A thorax was transferred into a 1.5 ml microcentrifuge tube containing 500 μl of STE buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 mM EDTA) and briefly homogenized with a plastic pestle, then 20 % SDS solution was added to a final concentration of 1.0 %. A proteinase K solution (10mg/ml) was added to a final concentration of 500 μg/ml and incubated at 55 ^{O}C for 2 hours. The supernatant was extracted once with an equal volume of phenol/chloroform/ isoamyl alcohol(25:24:1v/v). And then twice with chloroform/isoamyl alcohol(24:1v/v). After each extraction, the mixture was then centrifuged at 8,000 rpm (High speed microcentrifuge, MC-15A) for 10 minutes at room temperature. The upper aqueous phase was carefully transferred to a new microcentrifuge tube. Subsequently, further purification was performed by one-tenth volume of 3 M sodium acetate pH 7.0 was added following by two volumes of cold absolute ethanol and kept at -20 ^{O}C overnight to ensure complete precipitation. The DNA pellet was recovered by centrifugation at 12,000 rpm (high speed microcentrifuge, MC-15A) for 10 minutes and wash twice with 70 % ethanol(v/v). The pellet was air-dried and resuspended in 40 μl of sterile deionized

water. The DNA solution was incubated at 37 ^{O}C for 1-2 hours for complete redissolved and kept at 4 ^{O}C.

2.9 Measurement of DNA concentration / Agarose gel electrophoresis

The agarose gel electrophoresis is 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 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 molecular or standard size markers. Nevertheless, the concentration of DNA sample can be determined by comparison with the intensity ethidium bromide fluorescent DNA standards (e.g. λ/*Hind* III standard DNA). After staining with ethidium bromide, the intensity of orange-red fluorescence of the DNA bands was observed under ultra-violet light.

Agarose gel was prepared by weighed out an appropriate amount of agarose and mixed with 1XTBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2.5 mM Na_2EDTA pH8.3). The agarose was heated in a microwave oven until complete solubilization and cooled at room temperature before pouring gel into a gel mould that in which the combs was already inserted and sealed with agarose gel. The agarose gel was completely set at room temperature about 1-2 hours before being used. Before sample was loaded into the well of agarose gel, sample was mixed with one-fifth volume of the loading dye buffer (15% ficoll 400, 0.25% bromophenol blue and 0.25% xylene cyanol FF).

The extracted total DNA was electrophoresed on 0.7% agarose gel in 1XTBE buffer and electrophoresis was operated at 100 volts whereas λ/*Hind* III standard DNA was used to compared for the size and concentration of extracted total DNA. For the PCR product of ITS, it was electrophoresed on 1.5% agarose gel at 120 volts using a 100 bp DNA ladder as a DNA standard. When electrophoresis is complete, the gel was stained with ethidium bromide solution and then destained in distilled water until

unbound ethidium bromide was removed from gel. Then, DNA bands were visualized under a UV transilluminator and photographed through a red filter using Kodak Tri-X-pan 400.

2.10 PCR amplification

The polymerase chain reaction (PCR) is a rapid proceduce for *in vitro* enzymatic amplification of a specific segment of DNA. A typical PCR reaction contains the required components : enzyme *Taq* polymerase, appropriate buffers, ample dNTP, template DNA, primers, and cofactors such as magnesium. The mix is allowed to work repeatedly, copying the DNA strand between two specific primers, with a reaction speed and specificity determined largely by temperature. Successful amplification within this reaction mix depends on efficient interaction of all these components. One cycle of PCR reaction is always contains 3 phases. In denaturation phase, heat is used to stop all enzymatic reaction and denature the DNA from double to single strands. In Annealing phase, the temperature is lowered so that oligonucleotide primers can bind the appropriate sites in the template DNA. Extension phase allows the enzyme to work, synthesizing the target DNA segment. PCR has spawned a multitude of experiments that were previously impossible. The number of application of PCR seems infinite and is still growing.

2.10.1 Amplification of the internal transcribed spacer region (ITS)

The ITS of *A. cerana* was amplified by PCR using primers of fungal ribosomal RNA genes that shown in Table 2.1 (White *et al.*, 1990). Amplification reaction was performed in 50 μl reaction mixture containing 30 ng template DNA, 1XPCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 1.2 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP and dTTP, 0.2 μM of each of primers and 0.5 unit of *Taq* DNA polymerase. Amplification was predenaturated at 94 ^{O}C for 5 minutes by 35 cycles of a denaturating step at 94 ^{O}C for 1 minutes, an annealing step at 52 ^{O}C for 1 minute and

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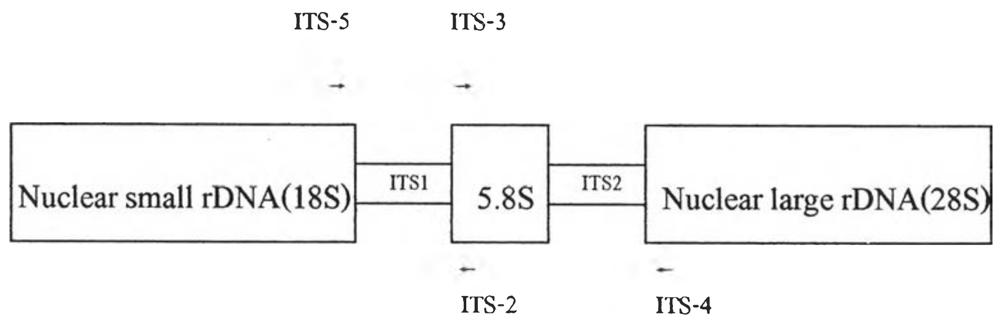
an extension step at 72 ^{O}C for 2 minutes, the last extension at 72 ^{O}C for 10 minutes. The PCR products was analyzed by agarose gel electrophoresis and then purified by recovering from the gel for sequencing.

Table 2.1 Primer sequences used for PCR amplification and sequencing of ITS region in *A. cerana*. Primer s equences were taken from White *et al.*(1990). The relative position of each primer is shown in diagram below.

	Sequence (5′ to 3′)	Tm (^{O}C)	Note
ITS-2	GCTGCGTTCTTCATCGATGC	62	internal primer for DNA sequencing
ITS-3	GCATCGATGAAGAACGCAGC	62	
ITS-4	TCCTCCGCTTATTGATATGC	58	
ITS-5	GGAAGTAAAAGTCGTAACAAGG	63	



2.10.2 Amplification of microsatellite DNA

Various loci of microsatellite DNA of *A. cerana* were radioactively amplified by PCR by direct incorperation with [α-^{35}S] dATP using *A. mellifera* microsatellite primers.

For the optimal PCR condition, the microsatellie DNA of *A. cerana* was amplified with the primer in Table 2.2 under standard condition used in *A. mellifera* (Estoup *et al.*,1995) and the $MgCl_2$ concentration was optimized to used for PCR reaction of each microsatellite locus.

Therefore, radioactive PCR amplifications were carried out in 10 μl of a mixture containing approximately 20 ng of genomic DNA isolated from each individual of *A. cerana*, 400 nM of each primer, 75 μM each dGTP, dCTP and dTTP, 6 μM dATP, 0.7 μCi [α-^{35}S]dATP, 1XPCR buffer (100 mM Tris-HCl pH8.3, 500 mM KCl), 0.4 unit of *Taq* DNA polymerase and an optimal concentration of $MgCl_2$. The PCR was performed by preheated the DNA for 5 minutes at 94 ^{O}C, 30 amplification cycles of a denaturating step for 30 seconds at 94 ^{O}C, an annealing step for 30 seconds at an optimal annealing temperature and an extension step for 30 seconds at 72 ^{O}C. The reaction was terminated by a last extension step at 72 ^{O}C for 10 minutes.

-Size estimation of amplified microsatellic allele using denaturating polyacrylamide gels

After the amplification process was completed, the size of amplified microsatellite DNA (microsatellite alleles) was estimated using denaturating polyacrylamide gels electrophoresis. Whereas, 4 μl of a formamide dye mix solution (10mM NaOH, 99% formamide, 0.1% bromophenol blue and 0.1% xylene cyanol) was added into each amplification reaction. Then, the mixture was heated for 10 minutes at 85 ^{O}C and immediately snap-cooled on ice. For electrophoresis, 4 μl of the denatured mixture was electrophoresed on a 6 % denaturating polyacrylamide gel with M13 standard sequencing marker was run on the same gel as size standards (see 2.13).

-Standard marker of microsatellite allele

The M13 sequencing marker was prepared using OmniBase™ sequencing kit. The reaction mixture contained 100 ng of the single stand M13,100 ng of pUC/M13 forward primer, 1μCi of 1,000 Ci/mmol [α-^{35}S]dATP, 5 μl of 5 X DNA sequencing buffer (250 mM Tris-HCl pH 9.0,10mM $MgCl_2$), 3.5 μl of sterile water and 1.0 μl of OmniBase™ sequencing enzyme mix. Then four microlites of the enzyme/primer/ template mix was added in four 0.5 ml microcentrifuge tubes which each tube contained d/ddNTP mix. The reaction tube were placed in a thermal cycle that has been preheated to 95 ^{O}C for 2 minutes and start the cycling program. The cycling program were performed by 30 amplification cycles of 30 seconds at 95 ^{O}C and 30 seconds at 70 ^{O}C. When the thermal cycling was completed, 3 μl of DNA sequencing stop solution (95% NaOH, 0.05% bromophenol blue,0.05% xylene cyanol) was added into each tube. The M13 sequencing marker was heated and loaded on the same gel of sample as standard size marker.

2.11 Preparation of DNA template for DNA sequencing

The amplified ITS fragments were purified by extracted from agarose gel using QIAEXII gel extraction kit. After electrophoresis was completed, the agarose gel was strained and visualized under UV light. Then the desired DNA band was excised from the 1.2% agarose gel with a clean, sharp scalpel and placed in 1.5 ml microcentrifuge tube. Then 3 volumes of buffer QX1 was added to 1 volume of gel and added 10 μl of QIAEX II. The sample was incubated at 50 ^{O}C for 10 minutes to solubilize the agarose and bind the DNA. During the incubation, the sample was mixed by vortexing every 2 minutes to keep QIAEX II in suspension. The sample was centrifuged at 12,000 rpm(high speed microcentrifuge, MC-15A) for 30 seconds and carefully removed supernatant with a pipette. The pellet was washed once with 500 μl of buffer QX1 and washed twice with 500 μl of buffer PE. Then the pellet was dried at 37 ^{O}C in incubator until the pellet becomes white. Finally 20 μl of water was added to elute DNA through

Table 2.2 Primer sequences and PCR conditions for the 13 *A. mellifera* microsatellite loci used to screen for polymorphic loci in *A. cerana*. Primer sequences were taken from Estoup *et al.*(1994,1995) and Oldroyd *et al.*(1997,1998).

Locus	Core sequence	Primer sequence (5′ to 3′)
A7	$(CT)_3(T)_7CCTTCG(CT)_{24}$	GTTAGTGCCCTCCTCTTGC
		CCCTTCCTCTTTCATCTTCC
A8	-	CGAAGGTAAGGTAAATGGAAC
		GGCGGTTAAAGTTCTGG
A14	$(CT)_{13}...(GGT)_9$	GTGTCGCAATCGACGTAACC
		GTCGATTACCGATCGTGACG
A24	$(CT)_{11}$	CACAAGTTCCAACAATGC
		CACATTGAGGATGAGCG
A28	$(CCT)_3GCT(CCT)_6(CT)_5TT\ (CT)_4$	GAAGAGCGTTGGTTGCGAGG
		GCCGTTCATGGTTACCACG
A29	$(GT)_{24}$	AAACAGTACATTTGTGACCC
		CAACTTCAACTGAAATCCG
A35	$(GT)_{14}$	GTACACGGTTGCACGGTTG
		CTTCGATGGTCGTTGTACCC
A43	$(CT)_{13}$	CACCGAAACAAGATGCAAG
		CCGCTCATTAAGATATATCCG
A79	$(CT)_{14}$	CGAAGGTTGCGGAGTCCTC
		GTCGTCGGACCGATGCG
A81	-	GCCGAGTTCTTCGACTCCC
		GGACTTTGCCAAATGGGTC
A88	$(CT)_{10}TC(CCTT)_2(CTTT)_3...(GGA)_7$	GCGAATTAACCGATTTGTCG
		GATCGCAATTATTGAAGGAG
A107	$(GCTC)_2(GCT)_2(CT)_{23}$	CCGTGGGAGGTTTATTGTCG
		CCTTCGTAACGGATGACACC
A113	$(TC)_2C(TC)_2TT(TC)_5TT(TC)_8TT$	CTCGAATCGTGGCGTCC
	$(TC)_5$	CCTGTATTTTGCAACCTCGC

the pellet was resuspended by vortexing and incubated at room temperature for 5 minutes. The sample was centrifuged at 12,000 rpm for 30 seconds. The supernatant containing the eluted DNA was carefully pipetted into a clean tube and keep at $-20\ ^{O}C$ until used.

2.12 DNA sequencing

OmniBase™ DNA cycle sequencing system and OmniBase™ sequencing enzyme mix (Promaga) were used for direct - sequencing of the amplified ITS of *A. cerana.*

The sequence of a deoxyribonucleic acid molecule can be elucidated using enzymatic sequencing method is based on the ability of a DNA polymerase to extend a primer, which is hybridized to the template that is to be sequenced, until a chain-terminating nucleotide is incorporated. Sequence determination is carried out as a set of four separate reactions, each of which contains all four deoxyribonucleoide triphosphate (dNTPs) supplemented with a limiting amount of a different dideoxyribonucleotide triphosphate (ddNTPs) per reaction. Because ddNTPs lack the $3'$OH group necessary for chain elongation, depending on the respective dideoxy analog in the reaction. The resulting fragments, each with a common origin but ending in a different nucleotide, are separated according to size by high resolution denaturing polyacrylamide gel electrophoresis.

2.12.1 End labeled primer

The primer was end-labeled with [γ-^{32}P] dATP, specific activity of 3,000 Ci/mmol. For 6 sets of double-stranded sequencing reaction contained 0.01 nM of a particular primer,10 pmol of [γ-^{32}P]dATP, 1 µl of T4 polynucleotide kinase 10X buffer (500 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 50 mM DTT,1.0 mM spermidine), 5 unit of T4 polynucleotide kinase and nuclease-free water was added to final volume of 10 µl. The reaction mixture was incubated at $37\ ^{O}C$ for 10 minutes and then inactivate

the kinase at 90 °C for 2 minutes. The reaction mixture was briefly centrifuged in a microcentrifuge to collect any condensation and stored at -20 °C until used.

2.12.2 Extension – termination reactions

Four 0.2 ml microcentrifuge tubes were labeled with A, C, G, T for each set of sequencing reaction and added 2 µl of the appropriate d/ddNTP nucleotide mix to each tube and stored on ice or 4 °C until used. Mixture of template/labeled primer for each set of four sequencing reactions was prepared in a 0.5 ml microcentrifuge tube. The mixture contained 2 ng of DNA template, 5 µl of DNA sequencing 5X buffer (250 mM Tris-HCl pH 9.0,10 mM $MgCl_2$),1.5 µl of end labeled primer(from 2.12.1) and nuclease-free water to final volume of 16 µl. Finally, 1 µl of OmniBase™ sequencing enzyme mix was added and mixed briefly by pipetting. Four microlitre of the enzyme/ primer/template mix was added into each tube containing d/ddNTP mix. The reaction tubes were placed in a thermal cycle, preheated at 95 °C for 2 minutes followed by 30 cycles of a denaturating 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. After the cycling program has been completed, 3 µl of DNA sequencing stop solution (95% NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol) was added into each tube. Before loading, the reaction were heated at 70 °C for 10 minutes and immediately snap-cooled on ice. Three microliters of each reaction was loaded on a 6 % denaturing polyacylamide sequencing gel.

2.13 Preparation of the polyacylamide gel and gel electrophoresis

The DNA products of sequencing reaction or amplified of microsatellite DNA was separated in denaturing polyacylamide gels. Usually, the distance between small fragments is greater than that of larger fragments. The rate of the migration of DNA fragments in the gel is a function primarity of the voltage gradient (volts/cm of gel length). A good strategy is to run gels at constant power rather than constant voltage or

current. Therefor, the gel was run at constant power and then the banding pattern was revealed with X-ray film.

A pair of glass plates were washed with detergents before rinsed with 95 % ethanol and left to air-dry. The shorter plate was siliconized a glass coating solution (Rain-X). Assemble the gel would with the two spacers, and used gel- sealing tape to seal around the bottom and side of the mould. The gel was prepared by used 60 ml of 6% denaturing acrylamide gel (8 M urea, 0.084 M acrylamide, 2 Mm bis-acrylamide) in 1X TBE (89 mM Tris-HCl, 89 mM boric acid and 2.5 mM Na_2EDTA pH8.3) and then added 300 µl of 10 % (w/v) ammonium persulfate and 42 µl of TEMED (*N,N,N′,N′*-tetramethylene diamine). Mixed and then slowly poured between the glass plates, with a smooth flow rate to prevent any air bubbles. The gel comb was inserted to a depth of about 0.5 cm. The polymerization process were allowed to be completed for at least 1 hours.

Before gel loading and running, the gel-sealing (the close gel) was removed and the gel was cleaned to remove spilled acrylamide solution from plate surfaces with water. The gel mould was placed in the gel running tanks. Added 1X TBE buffer into the upper and lower buffer chambers and then remove the gel comb. The comb was re-inserted with the teeth on the top of the gel. The gel was pre-run at constant power of 45 watts for 30 minutes. Then, the denaturated samples were loaded. The gel was run at constant power of 45 watts for about 2-3 hours dependent on size of DNA fragment.

2.14 Autoradiography

When electrophoresis was completed, the shorted plate was removed out of the gel. The gel of sequencing reaction was transferred to a piece of Whatman 3 MM filter paper while the gel of microsatellite DNA was fixed with fixing solution (10 % acetic acid, 10 % ethanol) for 30 minutes before transfer the gel to a piece of Whatman 3 MM filter paper. The filter paper containing the gel was covered with cling film and dried the gel under vacuum at $80^{O}C$ for 2 hours. Then the gel was placed in a cassette

next to the X-ray film. Whereas, the gel of sequencing reaction was kept in a -80 ^{O}C freezer for about 36-48 hours and the gel of microsatellite DNA was kept in room temperature for 5 days or until required. Then the X-ray film was developed using the method recommended by the supplier.

2.15 Data analysis

2.15.1 DNA sequence analysis

The nucleotide sequences of the amplified ITS of *Apis cerana* were analyzed by computer programming. To search for similar sequence the BLAST (Altschul *et al.*, 1990) program was used. Searching using the BLAST program was preformed at the National Center of Biotechnology Information (NCBI) in the nonredundant GenBank from the NCBI (updated Feb 24, 1998) using the BLAST network service. Then ClastalX (1.64b) program (Higgins *et al.*, 1988) was used for sequence alignment.

2.15.2 Analysis of microsatellite data

Assumption

An electrophoretic pattern referred to a genotype of each *A. cerana* individuals was inferred from segregation of PCR product size for each microsatellite locus. Therefore, the genotypic state could be divided to homozygotic (single band) and heterozygotic states (double band). Nevertheless, the PCR products, appeared as stutter band, which are common for dinucleotide microsatellits and result from polymerase slippage during PCR amplification. Accordingly, scoring of a particular band can be carried out by making an assumption that an actual band of a given allele was the most intense band among the group of stutter band. The allele sizes were defined in base pair length(bp) by comparing their migration with M13 sequencing marker. For each *A. cerana* individual was recorded to be either homo- and heterozygote. And the allelic stage were recorded from each individual for each locus.

Allele frequency and genetic variation

The frequency of a particular allele in a population for diploid organisms is given by

$$p = \frac{(2N_{AA} + N_{Aa})}{2N}$$

when p is the frequency of the A allele, N_{AA} and N_{Aa} are the number of homo- and heterozygotes for that allele and N is the number of individuals examined.

Genetic variation within populations was measured in terms of heterozygosity (H) which is the proportion of heterozygous individuals in all investigated samples, therefore observed heterozygosity can be estimated as

$$H = \frac{\sum(N_{Aa}/N)}{n}$$

when n is the number of investigated loci. When determined populations conform Hardy-Weinberg expectation, the unbiased estimated of heterozygosity (expected heterozygosity) can be calculated as

$$h = 1 - \sum pi^2$$

when pi is the frequency of ith allele at a given locus, expected heterozygosity across all loci is the mean of h from each locus.

Practically, the number of allele per locus, allelic frequency, the proportion of homo- and heterozygotes individuals were estimated using GENEPOP version 2.0 (Raymond and Rousset, 1995).

Geographic heterogeneity analysis

The statistically significant difference in genotype frequencies between *A. cerana* from a pair of geographic sampling locations were tested using the exact test of differentiation using GENEPOP version 2.0. Results are expressed as the probability of homogeneity between compared populations or regions. To diminish type I error, level of significance was further adjusted using the sequential Bonferroni test (number of population X number of loci).

Estimation of population structure

F-statistics, *Fst* is a standard parameter for measurement of population structure (or interpopulation diversity). It can be calculated using the exact test of genotypic differentiation of GENEPOP version 2.0

Genetic distance and phylogenetic reconstruction

Genetic distance based on Cavalli-Sforza and Edwards chord distance was calculated (Cavalli-Sforza and Edwards, 1967) using GENDIST. The genetic distance model assumes that gene frequencies are changed by genetic drift alone. The population sizes do not assumed to be constant and equal in all population. The resulting genetic distance was subjected to phylogenetic reconstruction based on Neighbor-joining approach (Saitou and Nei, 1987) using NEIGHBOR. The NJ tree was plotted by DRAWTREE. All computational programs mentioned above are routinely implemented in Phylip 3.56c (Felsenstein, 1993).