CHAPTER III

MATERIALS AND METHODS

3.1 Instruments

- Autoclave Ken Clave max-26DX (Ikiken Co., Ltd., Japan)
- Automatic micropipette P10, P20, P100, P200 and
  P1000 (Gilson-Medical Electronics S.A., France)
- Balance l80A (Precisa Balances, Switzerland)
- Boekel dry bath incubator (Boekel Scientific, U.S.A.)
- Camera Nikon FM2 (Nikon Co., Japan)
- 20 °C Freezer WCF (Whirlpool Ltd., Thailand)
- Horizontal gel electrophoresis apparatus
  : MGD-402T (C.B.S. Scientific company, Inc., D.S.A.)
  : Taitec Pico-2 (Taitec Co., Japan)
- Incubator BM 400 (Memmert GambH, Germany)
- Magnetic stirrer PC-320 (Corning, Inc., U.S.A.)
- Microcentrifuge Kubota 1120 (Kubota Co., Japan)
- Personal microcentrifuge C9314-3 (National Labnet Co., U.S.A.)
- Polaroid DS-34 camera (Polaroid Co., U.S.A.)
- Shaker VRN-360 (Gemmy Industrial Co., Taiwan)
- Thermal cycler: The MiniCycler Model PTC-150 (M. J. Research, Inc., U.S.A.)
- Ultraviolet Transilluminator model TVC-312R/F (Spectronics Corporation, U.S.A.)

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- Vortex Genie-2 Model G560E (Scientific Industries, Inc., U.S.A.)
- Water bath model SH28L (Polyscience, U.S.A.)

3.2 Inventory supplies

- Black and white paint film TriX-pan400 (Eastman Kodak Company, U.S.A.)
- Filter paper whatman 3 MM (Whatman Internation Ltd., England)
- Microcentrifuge tubes 0.5, 1.5 ml (Treff Lab, Switzerland)
- Pipette tips (Treff Lab, Switzerland)
- Whatman laboratory sealing film (Whatmat Internation Ltd., England)

3.3 Chemicals

- Absolute ethanol (Merck, Germany)
- Agarose, Seakem LE (FMC Bio products, U.S.A.)
- Boric acid (Merck, Germany)
- Bromophenol blue (Merck, Germany)
- Chloroform (Merck, Germany)
- Ethylene diaminetetraacetic acid disodium salt dihydrate (Fluka, Switzerland)
- Ethidium bromide (Fluka, Switzerland)
- GeneAmp PCR core reagent (Sigma, U.S.A.)
  : RED *Taq* DNA polymerase
  : 10X PCR buffer

- 100 mM dNTPs : dATP, dCTP, dGTP and dTTP (Boehringer Mannheim, Germany)
- Isoamyl alcohol (Merck, Germany)
- Phenol saturated solution (Amresco, U.S.A.)
- Sodium acetate (Merck, Germany)
- Sodium chloride (Merck, Germany)
- Sodium dodecyl sulfate (SDS) (Sigma, U.S.A.)
- Tris-(hydroxy methyl)-aminomethane (Fluka, Switzerland)
- Xylene cyanol (Sigma, U.S.A.)

3.4 Oligonucleotide primers

- Bio Service Unit, Thailand
- Biosynthesis Inc., U.S.A.

3.5 Enzymes

- Proteinase K (GibcoBRL Life Technologies Inc., U.S.A.)
- Restriction enzymes (New England Biolabs Inc., U.S.A.)
- RED *Taq* DNA polymerase (Sigma, U.S.A.)

3.6 Sample collections

Adult workers of stingless bees were collected about 10-20 bees per colony from natural colonies in different locations. Total of 407 colonies of five species were from 41 provinces in various parts of Thailand. Species identification was based on morphology using keys of Sakagami (1985) and

Schwarz (1939). Details of the samples collections of *T. collina* (224 colonies), *T. fuscobalteata* (18 colonies), *T. laeviceps* (131 colonies), *T. terminata* (28 colonies), and *T. thoracica* (6 colonies) are shown in Figure 3.1. Samples were preserved in 95 % ethanol and stored at 4 °C until DNA extraction.

3.7 DNA extraction

Total DNA was extracted from individual thorax of adult worker, generally following the procedure of Hall and Smith (1991). Sample as homogenized and placed in 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 pH 8.0). The cells were lysed by adding 20 % SDS, after which 10 mg/ml proteinase K solution was added to a final concentration of 450 μg/ml and the homogenate was incubated at 60 °C for 2 hours. The suspension was then centrifuged at 8,000 rpm for 10 minutes. After centrifugation, the supernatant was removed to new tube and extracted twice with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), mixed gently for 15 minutes and spun at 8,000 rpm for 10 minutes at room temperature. The aqueous phase (upper layer) was transfered to a new tube and extracted once with an equal volume of chloroform: isoamyl alcohol (24:1). Subsequently, 3 M sodium acetate was added to a final concentration of 0.3 M. DNA was precipitated with double volume of cold absolute ethanol, and overnight incubation at -20 °C. DNA was pelleted by centrifugation at 12,000 rpm for 10 minutes. The pellet was briefly washed with 70 % ethanol, air-dried and resuspended in TE buffer (10 Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0) and kept at 4 °C for the next step.

3.8 PCR amplification

PCR amplifications of 16S rRNA gene of mtDNA of five species were carried out by using primers which has been designed from the *A. mellifera* sequence (Costa *et al*., 2003). Amplification was done in a 25 μl volume and 0.04 U/μl of RED *Taq* DNA polymerase (Sigma) were used. Primer sequences, PCR conditions, and appropriate concentrations are shown in Table 2.

Table 2 PCR condition for amplification of 16S rRNA gene of five *Trigona* species.

Primers (Costa *et al*., 2003)	Primer (μM)	$MgCl_2$ (mM)	Running program	No. of cycle
5-CACCTGTTTATCAAAAACAT-3 5-CGTCGATTTGAACTCAAATC-3	0.2	1.1	94°C 1 min 42°C 1.30 min 64°C 1.30 min extension 72°C 5 min	40

3.9 Agarose gel electrophoresis

Agarose gel electrophoresis was performed in order to separate DNA fragments on the basis of their molecular sizes. Agarose gel at 0.7 % and 1.5 % concentration were used to check the result of DNA extraction and the result of PCR amplification, respectively. The appropriate amount of agarose (Seakem LE or MetaPhor) was weighed out and mixed with TBE buffer (89 mM Tris-HCI, 8.9 mM boric acid and 2.5 mM EDTA pH 8.0) and heated until complete solubilisation in a microwave oven, then poured into an electrophoretic mould.

After the gel was completely set, 3 μl of the loading dye (0.25 % bromphenol blue, 40 % ficoll 400 and 0.25 % xylene cyanol) were mixed with 3 μl of each DNA sample before loaded into each well. DNA fragments were electrophoresed at 110 V for 1.5 hours or 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 in distilled water for 30 minutes. DNA was visualized under UV light and photographed. The λ DNA-*Hin*d III digested marker, 1 kb DNA ladder, 100 bp DNA ladder, and 25 bp DNA ladder were used as the standard molecular size.

3.10 Restriction enzyme digestion

PCR products of 16S rRNA gene were singly digested with 12 restriction enzymes (*Afl* II, *Ase* I, *Bam*H I, *Dra* I, *Eco*R V, *Hin*f I, *Hpy*188 III, *Mse* I, *Pac* I, *Rsa* I, *Sau*3A I, and *Ssp* I) according to the manufacturer's instructions.

Digestion was carried out in a 10 μl volume containing approximately 500 ng of DNA, appropriate concentration of restriction enzymes, 1 μl of 10X reaction enzyme buffer and appropriate amount of sterile deionised water. The reaction mixture was incubated at 37 °C for 5-6 hours or overnight.

The restriction fragments were separated on 3.0 % agarose gel (Metaphor) at 110 V for about 2 hours, stained with ethidium bromide and detected on UV light.

Table 3 List of restriction enzymes used to analyse mtDNA variation of five species of stingless bees.

Restriction enzymes	Recognition site	Restriction enzymes	Recognition site
Afl II	C / TTAAG GAATT / C	*Hpy*188 III	TC / NNGA AGNN / CT
Ase I	AT / TAAT TAAT / TA	*Mse* I	T / TAA AAT / T
*Bam*H I	G / GATCC CCTAG / G	*Pac* I	TTAAT / TAA AAT / TAATT
Dra I	TTT / AAA AAA / TTT	*Rsa* I	GT / AC CA / TG
*Eco*R V	GAT / ATC CTA / TAG	*Sau*3A I	/ GATC CTAG /
Hinf I	G / ANTC CTNA / G	*Ssp* I	AAT / AAT TTA / TTA

3.11 Data analysis

The data of restriction fragments were organized into 1-0 matrix (Appendix III); 1 indicates the presence of fragment, and 0 indicate the absence. The statistical analysis was carried out in Phylip version 3.63 (Felsenstein, 1993) and the phylogenetic reconstruction using UPGMA (unweighted pair group method with arithmetic mean).

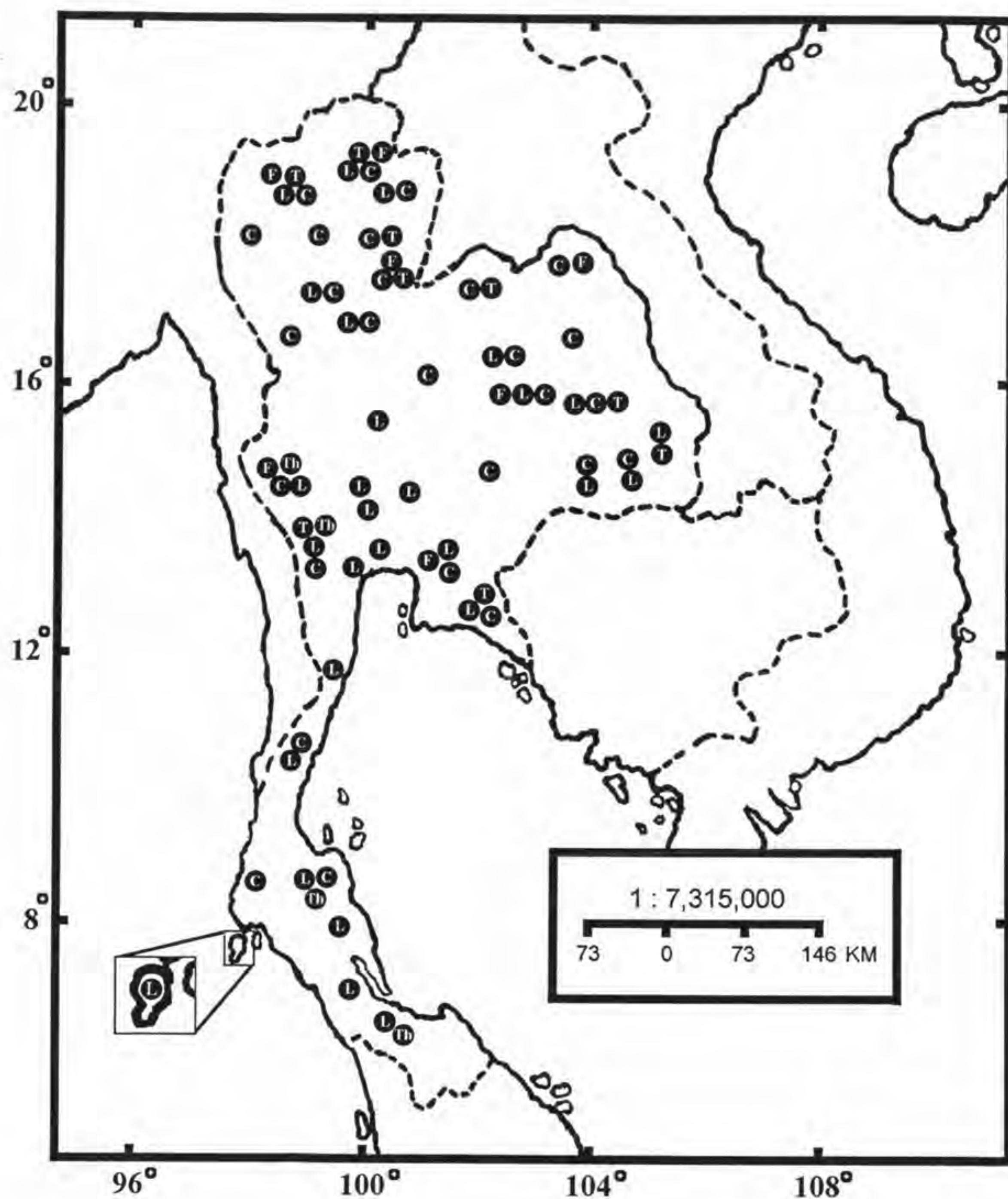

Figure 3.1 Sampling areas of five species of *Trigona* in Thailand: C = *T. collina* (224 colonies), F = *T. fuscobalteata* (18 colonies), L = *T. laeviceps* (131 colonies), T = *T. terminata* (28 colonies), and Th = *T. thoracica* (6 colonies).