CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

- Autoclave MLS-3020 (Sanyo, Japan)
- Automatic micropipette P2, P20, P100, P200, P1000 (Gilson Medical Electrical S.A., France)
- Centrifuge, Microfuge® 22R (Beckmen Coulter, USA)
- Dry bath incubator MD-01N (Major Science, Taiwan)
- -20 °C freezer
- Horizontal agarose gel electrophoresis apparatus, GelMate 2000 (Toyobo, Japan)
- Microcentrifuge tubes 0.6, 1.5 ml (Axygen Harward, USA)
- Micro Pulser (Bio-RAD Laboratories, USA)
- Pipette tips (Axygen Harward, USA)
- Power supply (Bio-RAD Laboratories, USA)
- Thermal cycler, Mastercycler gradient (Eppendorf, Germany)
- Thin-wall microcentrifuge tubes 0.2 ml (Axygen Harward, USA)
- UV transilluminator model M-20 (UVP, UK)
- Vertical gel electrophoresis apparatus for AFLP and TE-AFLP analysis, Sequencing system Model SA or Model S2 (GibcoBRL Life Technologies, Inc., USA)
- Vertical gel electrophoresis apparatus for SSCP analysis, Protean II xi Cell (Bio-RAD Laboratories, USA)
- X-ray film, X-O1000 mat film (Eastman Kodak Company Rochester, USA)

2.2 Chemicals

- Absolute ethanol (Merck, Germany)
- Acetic acid, glacial (Merck, Germany)
- Acrylamide (Merck, Germany)
- Agarose, GenePure LE (ISC BioExpress, USA)
- Ammonium persulfate (Promega, USA)
- Bind silane, PlusOne (Amersham Biosciences, Sweden)
- Boric acid (BDH, England)
- Bromophenol blue (Sigma, USA)
- Chloroform (Merck, Germany)
- Ethylene diamine tetraacetic acid, disodium salt dihydrate (Fluka, Switzerland)
- Ethidium bromide (Sigma, USA)
- Formaldehyde (Carlo Erba Reagent, Italy)
- Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan)
- High-Speed Plasmid Mini Kit (Geneaid, Taiwan)
- *N, N*-methylene-bis-acrylamide (Promega, USA)
- *N, N, N', N'*- tetramethylenediamine, TEMED (USB Corporation, USA)
- pGem®-T Easy Vector (50 ng/µl; Promega, USA)
- Phenol, Equilibrated (USB Corporation, USA)
- Repel silane, PlusOne (Amersham Biosciences, Sweden)
- Silver nitrate (Merck, Germany)
- Sodium carbonate (Merck, Germany)
- Sodium chloride (Merck, Germany)
- Sodium dodecyl sulfate (SDS) (Sigma, USA)
- Sodium thiosulfate (Merck, Germany)

- Tris-(hydroxyl methyl)-amminomethane (USB Corporation, USA)
- Urea (Fluka, Switzerland)
- Xylene cyanol (Sigma, USA)

2.3 Oligonucleotide primers

Oligonucleotides used for PCR were purchased from Bio Basic Inc., Canada or from 1st BASE Holdings, Singapore.

2.4 Enzymes and Restriction enzymes

- *Taq* DNA polymerase, DyNazyme™ II DNA polymerase (Finnzymes, Finland) and GoTaq® Flexi DNA polymerase (Promega, USA)
- Proteinase K (Sigma, USA)
- 2X Rapid Ligation Buffer, T4 DNA Ligase (Promega, USA)
- Restriction endonucleases; *Bam*HI, *Eco*RI, *Pst*I, *Rsa*I, *Tru*9I, *Xba*I (Promega, USA)
- RNaseA (Sigma, USA)
- T4 DNA ligase (Promega, USA)
- T4 Polynucleotide Kinase (Promega, USA)

2.5 Radioactive

- [γ-^{32}P] dATP specific activity 100 µCi/mmol (Perkin Elmer, USA)

2.6 Samples

Adult workers of *Tetragonilla collina* from 159 colonies were collected from geographically different locations in Thailand. Other species of stingless bees ;*Tetrigona apicalis* (n = 12), *Lophotrigona caniform* (n = 1), *Lepidatrigona doipaensis* (n = 1), *Homotrigona fimbriata* (n = 3), *Tetragonula fuscobalteata* (n = 6), *Heterotrigona itama* (n = 4), *Tetragonula laeviceps* (n = 6), *Tetrigona melanoleuca* (n = 1), *Tetragonula melina* (n = 1), *Tetragonula minor* (n = 8), *Tetragonula pagdeni* (n = 51), *Lepidatrigona terminata* (n = 7), *Geniotrigona thoracica* (n = 3), *Lisotrigona furva* (n = 2), were included in the experiment (appendix A). Specimens were placed in 95% ethanol and kept at 4 °C until required. Taxonomic identification of collected

stingless bees was examined based on the nest architecture and morphology according to Sakagami (1978) and Sakagami et al. (1983). Species identifications of specimens were kindly confirmed based on external morphology by Dr. Charles D. Michener (University of Kansas). These specimens were used to develop species-specific AFLP marker, to study genetic diversity and population structure of *T. collina* in Thailand using nuclear DNA and mitochondrial DNA polymorphisms.

2.7 DNA preparation

2.7.1 DNA extraction

Genomic DNA was extracted from each stingless bee per nest using a phenol-chloroform-SDS method (Smith and Hagen, 1996). A stingless bee was homogenized in 1.5 ml microcentrifuge tube containing 500 µl of STE extraction buffer (100 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA), then 20 % SDS solution was added to a final concentration of 1.0 %. A proteinase K solution (10 mg/ml) was added to a final concentration of 500 µg/ml and incubated at 65 °C for 3 hours. After that, 25 µl of RNase A (10 mg/ml) was added and incubated at 37 °C for 1 hour. Then, the supernatant was extracted twice with an equal volume of phenol/chloroform (1:1v/v) gently and once with an equal volume of chloroform. After each extraction, the mixture was then centrifuged at 10,000 rpm for 10 minutes at room temperature. The upper aqueous phase was carefully transferred to a new microcentrifuge tube and mixed with double volume of chilled absolute ethanol and kept at -20 °C overnight to precipitate DNA. The DNA pellet was recovered by centrifugation at 12,000 rpm for 20 minutes at 4 °C and washed twice with 70 % ethanol (v/v). The pellet was dried and dissolved with 1X TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA). Genomic DNA was kept at 4 °C until use.

2.7.2 Measurement of DNA concentration / Agarose gel electrophoresis

The concentration of DNA samples was estimated by comparison with the intensity of ethidium bromide fluorescent DNA standards (e.g. λ/*Hin*dIII standard DNA) on agarose gel electrophoresis (Sambrook and Russell, 2001). After staining with ethidium bromide, the intensity of orange-red fluorescence of DNA bands was observed under UV light.

Agarose gel was prepared by weighting out an appropriate amount of agarose and mixing with 1X TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2.5 mM Na_2EDTA, pH 8.3). The agarose was heated in a microwave oven until complete solubilization and cooled at room temperature before pouring gel into a gel tray containing a comb. The agarose gel was completely set at room temperature. Before sample was loaded into the well of agarose gel, sample was mixed with one-fifth volume of the loading dye (0.25 % bromophenol blue and 25 % Ficoll in water). A 100 bp DNA ladder or λ/*Hin*dIII was used as the standard marker.

The extracted total DNA was electrophoresed on 0.8 % agarose gel in 1X TBE buffer at 100 volts whereas λ/*Hin*dIII standard DNA was used to compare for size and concentration of extracted total DNA. When electrophoresis is complete, the gel was stained with ethidium bromide solution and then destained in distilled water to remove unbound ethidium bromide from the gel. DNA bands were visualized under a UV transilluminator and photographed.

2.8 Development of *T. collina*-specific marker using AFLP (Amplified Fragment Length Polymorphism) and SSCP (Single Strand Conformational Polymorphism) analysis

2.8.1 AFLP analysis

2.8.1.1 Digestion and adaptor ligation

The AFLP procedure was carried out as described by Vos et al. (1995) with a few modifications. Each genomic DNA (250 ng) of different *Trigona* species was digested with 5 units of *Pst*I in a 25 µl reaction mixture consist of 1X O-Phor-All buffer (10 mM Tris-acetate, pH 7.5, 10 mM magnesium acetate, and 50 mM potassium acetate) at 37 °C for 3 hours. Then, the digestion was inactivated at 65 °C for 15 minutes. After that, *Tru*9I (3 units) was added in a final volume of 40 µl and incubated at 65 °C for 3 hours. The double stranded adaptors (Table 2.1) were ligated to the restriction fragments in a total volume of 50 µl consist of 5 µM *Pst*I adaptor and 50 µM *Mse*I adaptor, 1X O-Phor-All buffer, 0.4 mM ATP and 1 unit of T4 DNA ligase at 12 °C for 16 hours.

Table 2.1 Adaptor sequences and AFLP primers used for the ligation and PCR amplification

Primer	**Sequences**
Adaptor sequences	
*Pst*I adaptor	5'-CTCGTAGACTGCGTACATGCA-3'
	5'-TGTACACAGTCTAC-3'
*Mse*I adaptor	5'-GACGATGAGTCCTGAG-3'
	5'-TACTCAGGACTCAT-3'
Preamplification primers	
P_{+A}	5'-GACTGCGTACATGCAGA-3'
M_{+C}	5'-GATGAGTCCTGAGTAAC-3'
Selective amplification primers	
P_{+3}-1	P_{+A}-AG
P_{+3}-2	P_{+A}-AC
P_{+3}-3	P_{+A}-GA
P_{+3}-4	P_{+A}-GT
P_{+3}-5	P_{+A}-CG
P_{+3}-6	P_{+A}-CT
P_{+3}-7	P_{+A}-TC
P_{+3}-8	P_{+A}-TT
M_{+3}-1	M_{+C}-AA
M_{+3}-2	M_{+C}-AC
M_{+3}-3	M_{+C}-AG
M_{+3}-4	M_{+C}-AT
M_{+3}-5	M_{+C}-TA
M_{+3}-6	M_{+C}-TC
M_{+3}-7	M_{+C}-TG
M_{+3}-8	M_{+C}-TT

2.8.1.2 Pre-amplification

The ligated DNA was used as a template. Preamplification was carried out utilizing adaptor-specific primers with a single selective base at 3' end on each primer (5'-GACTGCGTACATGCAGA-3' and 5'-GATGAGTCCTGAGTAAC-3'). Each 25 µl of reaction contained 1X PCR buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM each dNTP, 1.5 mM $MgCl_2$, 30 ng of each primer, 1.5 units of DyNazyme™ II DNA polymerase and 1 µl of ligated DNA. PCR was performed consisting 20 cycles of denaturation at 94 °C for 30 seconds, annealing at 56 °C for 1 minute and extension at 72 °C for 1 minute. The final extension was 72 °C for 5 minutes.

2.8.1.3 Selective amplification

The pre-amplification product was diluted 25-fold with sterile deionized water and selectively amplified with primer combinations having three selective bases at the 3′ end of each primer shown in Table 2.1. The selective amplification was performed in a 25 µl reaction volume including 1X PCR buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM each dNTP, 1.5 mM $MgCl_2$, 30 ng of P_{+3} and M_{+3} primers, 1.5 units of DyNazyme™ II DNA polymerase and 5 µl of the diluted preamplification product. PCR was carried out consisting 2 cycles of denaturation at 94 °C for 45 s, annealing at 65 °C for 60 s, and extension at 72 °C for 90 s, followed by 10 cycles of a touchdown phase with lowering of the annealing temperature 0.7 °C in every cycle and additional 25 cycles of 94 °C for 45 s, 56 °C for 60 s, and 72 °C for 90 s. The final extension was carried out at 72 °C for 5 min.

2.8.2 Preparation of polyacrylamide gel and gel electrophoresis

The AFLP fragments were size-fractionated through denaturing polyacrylamide gel. The gel was run at constant power and then the banding pattern was revealed with silver staining.

A pair of glass plates (the long and the short glass plates) was cleaned with deionized water to eliminate impurities, twice washed with 2 ml of 95% ethanol in one plane of glass. Then, the long plate was coated with 1 ml of freshly prepared binding solution consisting 4 µl of bind silane, 995 µl ethanol and 5 µl glacial acetic acid, and left for 10 minutes. The excess binding solution was eliminated by cleaning

the coated long glass with 95% ethanol for 3 times. The short glass plate was also treated as the long one except for coating step. It was coated by the Rapel silane (2% dimethyldichlorosilane in octamethylcyclotetrasitoxone). The coated glass plates were assembled to each other with a pair of spacer in between. The bottom and both sides of assembled glass plates were sealed with tape.

The gel was prepared by 40 ml of 6% denaturing polyacrylamide gel including 19% acrylamide, 1% bisacrylamide, 7 M urea and 10X TBE (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3). The solution was degassed for 20 minutes, then added 240 µl of freshly prepared 10% ammonium persulfate and 24 µl TEMED, gently mixed and poured between the glass plated using a 50 ml syringe. The gel comb was inserted and allowed to polymerize at room temperature for 1 hour. The gel was covered by water-soaked tissue paper after it had polymerized. To complete polymerization, the gel was left at room temperature for 4 hours or overnight. The sealing tape and gel comb were removed when gel was required.

The assembled gel was placed in the gel running apparatus. The upper and lower buffer chambers were added with 1X TBE buffer. The comb was reinserted with the teeth on the top of gel. The gel was prerun at 35 W for 15 minutes.

The amplified products (6 µl) was mixed with 3 µl of a loading buffer (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol) and heated at 95 °C for 5 minutes, immediately cooled on ice. For electrophoresis, 6 µl of the denatured mixtures were loaded. The gel was run at 35 W for 2 hours.

2.8.3 Silver staining

After electrophoresis, the short glass plate was removed out of gel. The gel on the long glass plate was soaked and agitated in 2 litres of the fix/stop solution (20% glacial acetic acid) for 30 minutes. The gel was placed in deionized water and agitated 3 times for 2 minutes. The gel was incubated in 0.1% silver nitrate (1.5 liters) with agitation at room temperature for 30 minutes. The gel was immersed in deionized water (1.5 liters) and shaken no longer than 10 seconds and quickly transferred to 1.5 liters of the chilled developing solution (3% sodium carbonate, 0.15% formaldehyde, 0.02% sodium thiosulphate). The gel was well agitated until first band was observed and then placed in another chilled developer and shaken until all bands were

visualized (usually 2-3 minutes). One liter of the fix/stop solution was directly added to the developing solution and continuously shaken for 3 minutes. The stained gel was soaked in deionized water at least 3 minutes. The gel was left at 80 °C for 2-3 hours (for AFLP gels) or at room temperature (for SSCP gels).

2.8.4 Cloning of species-specific AFLP fragment

2.8.4.1 Elution of DNA from polyacrylamide gels

AFLP fragment found in *T. collina* but not in other screened species was excised from the gel using a sterile razor blade. The gel fragment was twice washed with 500 µl of sterile deionized water for 2 hours at room temperature. Twenty microliters of water was added and incubated at 50 °C for 30 minutes and at 37 °C overnight. Reamplification of the target fragment was carried out using the original primer pairs used in selective amplification according to the same PCR recipes with the exception that 100 ng of each primer and 5 µl of the eluted AFLP product were used. The PCR conditions were performed consisting 5 cycles of 94 °C for 30 seconds, 42 °C for 45 seconds and 72 °C for 1 minute followed by additional 35 cycles at a higher stringent annealing temperature at 50 °C. The final extension was performed at 72 °C for 7 minutes. The reamplified product was electrophoresed through 1.5% agarose gel at 100 volt for approximately 40 minutes.

2.8.4.2 Elution of DNA from agarose gels

The required DNA fragment was run through agarose gels in duplication to avoid contamination of ethidium bromide and UV damage. One was run side-by-side with a 100 bp DNA marker and the other was loaded into the distal well of gel. After electrophoresis, lanes of DNA standard and its proximal DNA sample were cut and stained with EtBr for 1 minute. Positions of DNA markers and the EtBr-stained fragment were used to align the position of the non-stained target DNA fragment.

The DNA fragment was excised from gel and eluted out from agarose gel using Gel/PCR DNA Fragments Extraction Kit (Geneaid) according to the protocol recommended by the manufacture. The purified sample was stored at -20 °C until required.

2.8.4.3 Ligation of PCR product to vector

The ligation reaction was set up in the total volume of 10 µl containing 3 µl of the gel eluted PCR product, 25 ng of pGem-T easy vector (Promega), 5 µl of 2X Rapid ligation buffer (60 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10% PEG 8000) and 3 units of T4 DNA ligase. The ligation mixture was incubated at 4 °C overnight.

2.8.4.4 Preparation of competent cells

A single colony of *E. coli* JM109 was inoculated in 10 ml of LB broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl) and incubated at 37 °C with 250 rpm shaking overnight. A half of the starter was inoculated to 250 ml of LB broth and then incubated at 37 °C with 250 rpm shaking until the optical density at 600 nm of cells reached 0.5-0.8. The culture was chilled on ice for 30 minutes and then centrifuged at 8,000X g for 15 minutes at 4 °C. The supernatant was discarded. The pellets were washed twice with 1 volume and 0.5 volume of cold sterile water, respectively and centrifuged as above. The supernatant was removed and the pellet was washed with 10 ml of 10% (v/v) ice cold sterile glycerol and finally resuspend in a final volume of 1-2 ml of 10% ice cold sterile glycerol. The cell suspension was divided into 40 µl aliquots and stored at -80 °C until used.

2.8.4.5 Electroporation

The 0.2 cm cuvettes and sliding cuvette holder were chilled on ice. The competent cells were thawed on ice. One microliter of the ligation mixture was added in 40 µl of the competent cells and placed on ice for 1 minute. This mixture was transferred to a chilled cuvette and one pulse was applied. One milliliter of LB broth was immediately added to the cuvette and quickly resuspended the mixture. The mixture was transferred to new microcentrifuge tube and incubated at 37 °C for 1 hour with 250 rpm shaking. Finally, this suspension was spreaded onto the LB agar plate containing 50 µg/ml ampicillin, 25 µg/ml IPTG and 20 µg/ml X-gal and incubated at 37 °C for 16-18 hours. The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

2.8.4.6 Detection of recombinant clone by colony PCR

The colony PCR was performed in a 25 µl reaction volume consisting of 1X PCR buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM each dNTP, 1.5 mM $MgCl_2$, 0.2 µM of primers PUCI (5'-TCC GGC TCG TAT GTT GTG TGG A-3') and PUCII (5'-GTG GTG CAA GGC GAT TAA GTT GG-3') and 0.5 units of DyNazyme™ II DNA polymerase. A recombinant clone was picked up and mixed well in the amplification reaction. The PCR profile was predenaturation at 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 1 minute, 50 °C for 1 minute and 72 °C for 1 minute. The final extension was carried out 72 °C for 7 minutes. The amplified product was analyzed through agarose gel.

2.8.4.7 Plasmid extraction

The recombinant clone was inoculated into 3 ml of LB broth containing 50 µg/ml ampicillin and incubated at 37 °C with 250 rpm shaking overnight. The culture was transferred into 1.5 ml microcentrifuge tube and centrifuged at 10,000X g for 1 minute. The cell pellet was collected and extracted the recombinant plasmid using High-Speed Plasmid Mini Kit (Geneaid) according to the protocol recommended by the manufacture.

2.8.5 DNA sequencing and primer design

The sequence of recombinant plasmid was analyzed by DNA sequencing service (Macrogen, Inc, Korea) with the M13 forward or reverse primers (universal primer) under BigDye™ terminator cycling conditions on automatic sequencer 3730x1. The sequences were analyzed and compared for the homology search using BlastN (nucleotide similarity) and BlastX (translated protein similarity) available at www.ncbi.nlm.nih.gov. A pair of designed primers is shown in Table 2.2.

2.8.6 PCR amplification of candidate *T. collina*-specific AFLP marker and species-specific test

PCR was carried out using primers designed from the sequences of candidate *T. collina*-specific AFLP marker (Table 2.2). The amplification reaction was performed in a 25 µl reaction volume containing 1X PCR buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM each dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer, 50 ng of genomic DNA and 1 unit of DyNazyme™ II DNA

polymerase. The PCR profile consisted of predenaturation at 94 °C for 3 minutes followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 56 °C for 1 minute and extension at 72 °C for 30 seconds. The final extension was performed at 72 °C for 7 minutes. For species-specific test, the primer pairs were used to test the positive amplified product across the representative individuals of *T. collina* and other *Trigona* species. The amplification product was analyzed by 1.5% agarose gel electrophoresis (Sambrook and Russell, 2001). The expected amplified products were purified and further analyzed by single strand conformation polymorphism (SSCP) to determine polymorphisms of the products.

Table 2.2 Primers designed from a candidate *T. collina*-specific AFLP marker

Primer	**Sequence**	**Annealing temperature (°C)**	**Expected size (bp)**
CUTc1-F	5′-GGTTCGGATTTGGTTGGCATTG-3′	56	259
CUTc1-R	5′-CGGTGTACGAAGCGCCAG-3′		

2.8.7 SSCP analysis

Non-denaturing polyacrylamide gel was used for fractionation of single stranded DNA secondary structure conformation. A pair of glass plates was prepared as described in 2.8.2. A 40% stock solution (37.5:1 crosslink) was diluted to prepare 12.5% polyacrylamide solution. The acrylamide gel solution (40 ml) was added with 300 µl 10% ammonium persulfate and 30 µl TEMED and poured between the glass plates. The gel was left to polymerize at least for 4 hours.

Six microliters of the purified amplification was mixed with 4 volumes of the loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH). After denaturing at 95 °C for 5 minutes, it was immediately cooled on ice for 2 minutes and electrophoretically analyzed through non-denaturing polyacrylamide gels at 12.5 V/cm for 16 hours at 4 °C. The gels were then visualized by silver staining as decribed in 2.8.3.

2.9 Genetic diversity and population structure analysis of *T. collina* using TE-AFLP (Three Enzymes-Amplified Fragment Length Polymorphism) and TE-AFLP derived markers

2.9.1 TE-AFLP analysis

2.9.1.1 Digestion and adaptor ligation

TE-AFLP procedure was carried out as essentially described in van der Wuff et al. (2000). Genomic DNA (~30 ng) of each stingless bee was simultaneously digested with *Rsa*I, *Xba*I and *Bam*HI and ligated to adaptors having ends complementary to the restricted DNA fragments in a single reaction. Digestion/ligation was carried out in a 20 µl reaction volume containing 1X digestion-ligation buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM ATP), 4 picomol of both *Xba*I and *Bam*HI adaptors (Table 2.3), 0.5 units T4 DNA ligase, 6 units *Xba*I, 1.25 units *Bam*HI and 1 unit *Rsa*I then incubated at 30 °C for 1.5 hours.

2.9.1.2 PCR amplification

The sequence of *Xba*I and *Bam*HI primer for amplification (Table 2.3) are complementary to one strand of each adaptor with arbitrary extensions. The *Bam*HI primer with arbitrary extension-C was end labeled with ^{32}P at the 5' end of primer. The labeling reaction was prepared by 40 µl of 1X T4 kinase buffer (70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT), 10 µM *Bam*HI-C primer, 100 µCi [γ-^{32}P] dATP, and 10 units T4 polynucleotide kinase. The mixture was incubated at 37 °C for 30 minutes and then inactivated at 90 °C for 2minutes (Vos et al., 1995).

Amplification was carried out in a 12.75 µl reaction volume containing 1X PCR buffer (10 mM Tris-HCl, pH 7.6, 50 mM KCl, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 µM each of unlabelled-*Xba*I primer and ^{32}P-labeled *Bam*HI primer and 0.6 units GoTaq® DNA polymerase (Promega). The digestion-ligation mixture of 0.5 µl was used as DNA template. PCR was performed using the following thermal profile: predenaturation at 95 °C for 3 minutes followed by 10 cycles of denaturation at 95 °C for 30 seconds, annealing at 70 °C for 30 seconds and extension at 72 °C for 1 minute, and an additional 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds and extension at 72 °C for 1 minute. The final extension was carried out at 72 °C for 20 minutes.

Each reaction was then electrophoresed through 8% denaturing polyacrylamide gel. The PCR product was mixed to 3 µl loading dye and denatured for 5 minutes at 95 °C. The denatured mixture was loaded on the gel with 0.6X TBE electrophoresis buffer and run for 3 hours at 500 V. At the end of the run, gels were dried on filter paper and exposed X-ray film overnight at room temperature.

2.9.2 Scoring TE-AFLP variation

After autoradiography, the bands were read and recorded manually. AFLP bands were treated as biallelic dominant markers; present (homozygotes or heterozygotes for amplification of the band) or absent (homozygote for lack of amplification) was scored as 1 or 0, respectively, generating a multi-band pattern for each individual. These data were used to calculate genetic diversity and population structure statistics.

2.9.3 Cloning of TE-AFLP derived marker

The AFLP bands from TE-AFLP analysis (as described in 2.9.1) were investigated for polymorphic AFLP band that was not uniquely or commonly found in investigated *T. collina*. It was excised and eluted out from the polyacrylamide gel. It was then reamplified and the target fragment was eluted from agarose gel (2.8.4.1 and 2.8.4.2). The fragment was ligated to vector and transformed into host cell (2.8.4.3, 2.8.4.5 and 2.8.4.6). The plasmid of recombinant clone was extracted as described earlier (2.8.4.7).

Table 2.3 Adaptor sequences and TE-AFLP primers used for the ligation and PCR amplification

Primer	**Sequences**
Adaptor sequences	
*Bam*HI adaptor	5'-ACGAAGTCCCGCGCCAGCAA-3'
	5'-GATCTTGCTGGCGCGGG-3'
*Xba*I adaptor	5'-ACGTTGTGGCGGCGTCGAGA-3'
	5'-CTAGTCTCGACGCCGCC-3'
Selective amplification primers	
B_{+C}	5'-GTTTCGCGCCAGCAAGATCCC-3'
X_{+CC}	5'-GGCGTCGAGACTAGACC-3'
X_{+CT}	5'-GGCGTCGAGACTAGACT-3'

Table 2.4 Primers of TE-AFLP derived marker

Primer	**Sequence**	**Annealing temperature (^{0}C)**	**Expected size (bp)**
TECU-F	5′-CGTATCAGTGTCGTTCATGGC-3′	56.9	222
TECU-R	5′-CGAGCGCGTGGAATCTC-3′		

2.9.4 DNA sequencing and primer design

The sequence of recombinant plasmid was analyzed by DNA sequencing service (Macrogen, Inc, Korea) with the M13 forward or reverse primers (universal primer) under BigDye™ terminator cycling conditions on automatic sequencer 3730x1. The sequences were analyzed and compared for the homology search using BlastN (nucleotide similarity) and BlastX (translated protein similarity) available at www.ncbi.nlm.nih.gov. A pair of designed primers was shown in Table 2.4.

2.9.5 PCR amplification of TE-AFLP derived marker and SSCP analysis

PCR was carried out using TECU primer (Table 2.4). The amplification reaction was performed in a 25 µl reaction volume containing 1X PCR buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM each dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer, 50 ng of genomic DNA and 1 unit of DyNazyme™ II DNA polymerase. The PCR profile consisted of predenaturation at 94 °C for 3 minutes followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 56.9 °C for 1 minute and extension at 72 °C for 30 seconds. The final extension was performed at 72 °C for 7 minutes. The primer pair was tested against genomic DNA of representative individual *T. collina*. The expected amplified product was purified and further determined on non-denaturing polyacrylamide gel by SSCP analysis as described in 2.8.7.

2.9.6 Scoring SSCP variation

The visible bands in each lane in SSCP gels were read and recorded manually. The bands were treated as present or absent bands and scored as 1 or 0 respectively, generating 0/1 matrix.

2.9.7 Data analysis

The data of scoring TE-AFLP or SSCP variation were used for analysis. Genetic diversity, genetic distance among populations and population structure statistics were calculated using Genetic Analysis in Excel (GenAlEx6; Peakall and Smouse, 2006).

Genetic diversity was estimated as the proportion of polymorphic loci (P) and expected heterozygosity (H_e). A band was considered polymorphic band if samples showed any variation for presence or absence although bands were present or absent in only a single individual. The expected heterozygosity (H_e) was estimated following Lynch and Milligan (1994), which observed each band position as a different locus with two alleles, band amplified (dominant) and band not amplified (recessive); absence of band indicates a recessive homozygote. At each locus, the frequency of the recessive allele (q) is estimated from the frequency of recessive homozygotes (q^2) and the frequency of dominant allele is estimated as $p = 1 - q$. Expected heterozygosity at each locus is $h = 1 - \sum x_i^2$, where x_i is the frequency of the i^{th} allele or $1 - (p^2+q^2)$. Expected heterozygosity averaged over all loci is calculated as:

$$H_e = 1 - 1/m \sum_{y=1}^{m} \sum x_i^2$$

where y represents loci or bands 1 through m

Genetic heterogeneity in allele distribution frequencies between compared geographic samples was examined using the exact test (Guo and Thompson, 1992). F_{ST}-based statistics (Φ_{PT}) between pairs of geographic populations, which generated by Analysis of Molecular Variance (AMOVA; Excoffier et al., 1992 implemented in GenAlEx6.1), were calculated and tested to determine whether Φ_{PT} was statistically different from zero (Weir and Cockerham 1984; Peakall and Smouse, 2006). The significances performing 999 permutations in which individuals are randomly assigned to regions of the same size, was calculated. Unbiased genetic distance between pairs of geographic samples was determined (Nei 1978). The investigated *T. collina* samples from TE-AFLP analysis were divided into hierarchical groups in three ways: (A) geographic region (North, Central, Northeast, and Peninsular Thailand); (B) populations north (North, Central and Northeast) and south (Peninsular Thailand) of the Isthmus of Kra; and (C) Northeast population versus the remaining populations (North, Central, and Peninsular Thailand) while those from TE-AFLP derived marker were grouped into two ways: (A) six populations (North, Central, Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular); and (B) four populations (North+Central+Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular Thailand).

2.10 Mitochondrial DNA diversity of *T. collina* using PCR-SSCP

2.10.1 PCR amplification of the mtDNA gene segments and SSCP analysis

The primers were designed from the sequences of mtDNA genes which were previously deposited in GenBank. The large ribosomal RNA (16S) and cytochrome b (cytb) mitochondrial DNA genes were known from GenBank Accession no. DQ790412 and AY575081, respectively. On the other hand, the primer pair of cytochrome c oxidase I gene (COI) was obtained from sequences of *T. amalthea* (GenBank Accession no. AF214669). These primers (Table 2.5) were tested against genomic DNA of representative individual *T. collina*. The amplification reaction was performed in a 25 µl reaction volume containing 1X PCR buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 µM each dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer, 50 ng of genomic DNA and 1 unit of DyNazyme™ II DNA polymerase. The PCR profiles of each amplification reaction are shown in Table 2.6. All PCR products of each mtDNA region from each *T. collina* were analyzed by SSCP analysis as described in 2.8.7 whereas the amplified products of 16S and COI gene were run on 11% non-denaturing polyacrylamide gel (75:1 crosslink).

Table 2.5 Sequences of primers and size of the expected amplified product mtDNA segment

mtDNA region	Primer	Sequence	Expected size (bp)
16S rRNA	16S-F	5′-ATGGCTGCAGTATAACTGAC-3′	478
	16S-R	5′-ACTTACGTCGATTTGAACTC-3′	
COI	COI-F	5′-CATTCATCTCCTTCTGTTG-3′	497
	COI-R	5′-GCTCGTGTATCAATATCTAATC-3′	
Cyt b	cytb-F	5′-TTGTAGAGTGATTATGAGGAG-3′	316
	cytb-R	5′-GGAGTAACTATAGGATCAGC-3′	

Table 2.6 PCR profile used for amplification of mtDNA segment using primers in Table 2.5

Main genes	PCR profile
16S rRNA	94 °C for 3 minutes 5 cycles of 94 °C for 30 seconds 40 °C for 60 seconds 72 °C for 60 seconds 35 cycles of 94 °C for 30 seconds 56 °C for 60 seconds 72 °C for 60 second 72 °C for 7 minutes
COI	94 °C for 3 minutes 5 cycles of 94 °C for 30 seconds 40 °C for 60 seconds 72 °C for 60 seconds 35 cycles of 94 °C for 30 seconds 50 °C for 60 seconds 72 °C for 60 second 72 °C for 7 minutes
Cyt b	94 °C for 3 minutes 35 cycles of 94 °C for 30 seconds 55 °C for 60 seconds 72 °C for 60 second 72 °C for 7 minutes

2.10.2 Scoring SSCP variation

The bands visible in each lane in SSCP gels were read and recorded manually. The bands for each amplified mtDNA region were treated as present or absent bands and scored as 1 or 0, respectively, generating 0/1 matrix.

2.10.3 Data analysis

Genetic Analysis in Excel (GenAlEx6) was used to calculate genetic diversity, genetic distance among populations and population structure statistics as described in 2.9.7. The investigated *T. collina* samples were divided into 6 hierarchical groups; North, Central, Northeast, Prachuap Khiri Khan, Chumphon, and Peninsular Thailand.