CHAPTER II

Materials and Methods

2.1 Instruments

1. Autoclave HA-30 (Hirayama Manufacturing Co., Japan)
2. Automatic pipette P2, P20, P100 (Gilson Medical Electronics S.A., France)
3. Camera Pentax super A (Asahi Opt. Co., Japan)
4. Electonic balance Alsep EY220A (A&D Co. Ltd., Japan)
5. Electrophoresis apparatus
    - 5.1 Horizontal agarose gel electrophoresis apparatus (9x12 cm. gel)
    - 5.2 Vertical gel electrophoresis apparatus for DNA sequencing (Hoefer, England)
6. -20 °C freezer (Krungthai Ltd., Thailand)
7. -80 °C freezer (Krungthai Ltd., Thailand)
8. Gel dryer 583 (Bio-RAD Laboratories, USA)
9. High speed microcentrifuge MC- 15A (Tomy Seiko, Japan)
10. Heating block BD 17016-26 (Sybron Thermolyne Co., USA)
11. Incubator BM-600 (Memmert Gamblt, Germany)
12. Light box 2859 SHADON (Shandon Scientific Co., Ltd., England)
13. Magnetic stirrer M21/1 (Franz Morat KG GambH, Germany)
14. Microcentrifuge Force 6 (Denver Instrument company, USA)
15. Power supplies (Bio-RAD Laboratories, USA)

15.1 Power PAC 300

15.2 Power PAC 3000

16. Shaking water bath O1PF623 (New Brunswick Scientific Co. Inc., USA)
17. Standard film cassette: Ranex Regular Screen Eastman (Eastman Kodak company, USA)
18. Thermal Cycler:GeneAmp PCR system 2400 (Perkin Elmer Cetus, USA)
19. UV transilluminator 2011 Maccrovue (SanGabriel California, USA)
20. Vortex genic K550-G (Scientific Industries Inc., USA)

2.2 Inventory Supplies

1. Black and white film (TriX-pan 400, Eastman Kodak Company Ltd., USA)
2. Whatman 3 mm. filter paper (Whatman Internation Ltd., England)
3. Hyper-film MP (Amersham International, England)
4. 0.5 ml and 1.5 ml microcentrifuge tubes (Axygen Hayward, USA)
5. 30X40 cm glass plates (Axygen Hayward, USA)
6. Pipette tips (Axygen Hayward USA)
7. 0.2 ml thin-wall microcentrifuge tubes (Axygen Hayward, USA)

2.3 Chemical reagents

Name	Company	Country
Absolute ethanol	Merck	Germany
Acrylamide	Merck	Germany
Agarose gel	FMC Bioproducts	USA
- Metaphor Agarose		
- SeaKem LE Agarose		
Ammonium persulfate	Merck	Germany
Boric acid	Merck	Germany
Bromophenol blue	Sigma	USA
Chelex® 100 resin	Bio-RAD	USA
Developer	Kodak	USA
Ethylenediamine tetraacetic acid disodium salt dihydrate(Na_2EDTA)	Fluka	Switzerland
Ethidium bromide	Sigma	USA
Ficoll 400	Sigma	USA
Fixer	Kodak	USA
GeneAmp PCR core reagents	Perkin Elmer	USA
- Ampli*Taq* DNA polymerase (5U/µl)		
- 10x PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl)		
- 25mM $MgCl_2$ solution		
- 10 mM dNTPs (dATP, dCTP, dGTP and dTTP)		

Name	Company	Country
N,N′-methylene-bis-acrylamide	Sigma	USA
N,N,N′,N′-tetramethylenediamine (TEMED)	Sigma	USA
Geneclean II kit	Bio 101	USA
- Glassmilk		
- 6 M Sodium iodide		
- TBE modifier		
- New wash concentrate		
OmniBase™ DNA cycle sequencing system	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		
(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 mMTris-HCl pH 7.5, 100mM $MgCl_2$,		
50 mM DTT, 10 mM spermidine)		
- 200 ng/µl pGEM3zf(+) control DNA		
- 24 mer pUC/M13 forward primer		
Potassium acetate	Merck	Germany
Potassium chloride	Merck	Germany

Name	Companpy	County
Sodium acetate	Merck	Germany
Sodium dodecyl sulfate (SDS)	Sigma	USA
Tris-(hydroxy methyl)-aminomethane	Fluka	Switzerland
Urea	Fluka	Switzerland
Xylene cyanol FF	Sigma	USA
100 base pair DNA ladder	Promega	USA

2.4 Oligonucleotide primers

Oligonucleotides used for PCR were purchased from Bioservice Unit, National Center for Genetic Engineering and Biotechnology, National Science and Biotechnology Development Agency, Thailand or from Biosynthesis, Inc., USA The primer sequences are shown in Table 2.1 and 2.2 .

2.5 Radioisotope

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

2.6 Sample collections

Two parasitic mite species, *T. clareae* and *T. koenigerum* were collected from the geographic ranges of these taxa. The former can be found in two hosts including *A. dorsata* and *A. mellifera* and widely distributed whereas the latter is host-specific to *A. dorsata*. Therefore, *T. clareae* from *A. dorsata* was sampled from Lumpang, Nakhonratchasima, Chanthaburi, Trad, Samutsakhorn, Samutsongkhram, Prachuab Khiri Khan and Chumporn while *T. clareae* from *A. mellifera* was collected from Chiang Mai, Uttaradit, Pisanuloks, Khon Kaen,

Udon Thani, Chanthaburi, Bangkok and Chumporn. Individuals of *T. koenigerum* was collected from Samut Songkhram and Chanthaburi.

An individual of the parasites was separately placed in a 0.5 ml microcentrifuge tube and immediately transferred to a tank containing liquid nitrogen until further required. A number of specimens was approximately 15-50 individuals per location.

Table 2.1 Primer sequences used in PCR and sequencing of an amplified ITS region of ribosomal DNA.

Name	Sequence (5′ to 3′)	Tm (°C)	
ITS5[1]	GGAAGTAAAAGTCGTACAAGG	63	PCR/Sequencing
ITS4[1]	TCCTCCGCTTATTGATATGC	58	PCR/Sequencing
inITS5[2]	TCGTATGTATTCCATTCGTA	54	Internal primer used for sequencing
inITS4[2]	CATAGACACAAGGCATCCAT	58	Internal primer used for sequencing

[1]After White et al., 1990.

[2]Primers designed in this study.

Table 2.2 The sequences of all random primers primirily used for screening of informative primers for *T. clareae* and *T. koenigerum*.

Primer	Sequences (5′ to 3′)
OPA-01	CAGGCCCTTC
OPA-02	TGCCGAGCTG
OPA-03	AGTCAGCCAC
OPA-04	AATCGGGCTG
OPA-05	AGGGGTCTTG
OPA-06	GGTCCCTGAC
OPA-07	GAAACGGGTG
OPA-08	GTGACGTAGG
OPA-09	GGGTAACGCC
OPA-10	GTGATCGCAG
OPA-11	CAATCGCCGT
OPA-12	TCGGCGATAG
OPA-13	CAGCACCCAC
OPA-14	TCTGTGCTGG
OPA-15	TTCCGAACCC
OPA-16	AGCCAGCGAA
OPA-17	GACCGCTTGT
OPA-18	AGGTGACCGT
OPA-19	CAAACGTCGG
OPA-20	GTTGCGATCC

Table 2.3 The number of specimen being sequenced and the geographic origin of specimens used in this study.

Abbreviation	Sampling Area	Host	Number of specimens being sequenced
N2M	Bupphachat Beefarm, Muang, Uttaradit	*A. mellifera*	2
N1M	Supha Beefarm, Maerim, Chiang Mai	*A. mellifera*	1
N1D	Thungkweng Market, Hang chat, Lumpang	*A. dorsata*	2
NE1M	Preservation and Bee Culture Center 3, Khon Kaen	*A. mellifera*	1
NE2M	Phupan Beefarm, Muang, Udon Thani	*A. mellifera*	1
NE1D	Nongnunnak, Nakhon Ratchasima	*A. dorsata*	1
C2M	Naraesaun University, Phisanulok	*A. mellifera*	1
C1M	Chulalongkorn University, Bangkok	*A. mellifera*	1
C1D	Samroy Yod, Prachuap Khiri Khan	*A. dorsata*	1
C2D	Krathumban, Samutt Sakorn	*A. dorsata*	1
E1M	Preservation and Bee Culture Center 4 Chanthaburi	*A. mellifera*	2
E3D	Muang, Trat	*A. dorsata*	2
S1M	Preservation and Bee Culture Center 5 Chumphon	*A. mellifera*	1
S1D	Sawi, Chumphon	*A. dorsata*	3
TKC	Krathumban, Samutt Sakorn	*A. dorsata*	3
TKE	Soy Down, Chanthaburi	*A. dorsata*	2

2.7 DNA extraction

Total DNA was individually extracted from each specimen using the modification of the method of Walsh et al. (1991). When needed, an individual mite was removed from a storage tank, placed in a microcentrifuge tube (1.5 ml) containing 35 μl of 5% chelex and homogenized with a micropestle for a few strokes. The homogenate was gently vortexed at low speed (3-4 degree) for 30 seconds and incubated at 55 °C for at least 3 hours. The mixture was then incubated at 95-100 °C for 7 minutes before centrifugation at 8,000x*g* for 10 minutes at room temperature. The supernatant was carefully transferred to a new sterile microcentrifuge tube. The DNA solution was stored at 4 °C and utilized as the template for PCR amplification.

2.8 PCR amplification

2.8.1 Amplification of the internal transcribed spacer region (ITS)

The ITS of *T. clareae* and *T. koenigerum* was amplified by PCR using a pair of universal primers previously successful used for amplification of the ITS in fungi (White et al., 1990). PCR was carried out in a 25 μl reaction mixture constituting 4 μl of DNA template, 200mM each of dNTPS (dATP, dCTP, dGTP and dTTP), 2.5 mM of $MgCl_2$, 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 0.2 μM each of the forward and reverse primers and 0.6 unit of *Taq* DNA polymerase.

The reaction mixture was firstly predenatured at 94 °C for 1 minute followed by 35 cycles consisting of a denaturation at 92 °C for 1 minute, an

annealing at 52 °C for 1 minute and an extension at 72 °C for 2 minutes. The final extension was performed at 72 °C for 7 minutes. After amplification, the resulting product was examined by agarose gel electrophoresis and further purified for sequencing.

2.8.2 Random amplified polymorphic DNA analysis of *T. clareae* and *T. koenigerum*

Four microliters of total DNA extraction from each individual mite was used as DNA template for PCR in a total volume of 25 μl. An appropriate amount of other constituents composing of 100 mM each of dNTPs (dATP, dCTP, dGTP and dTTP), 2.0 mM of $MgCl_2$, 1x PCR buffer (10mM Tris-HCl pH 8.3, 50 mM KCl), 0.2 μM of a particular primer and 1.0 unit of *Taq* DNA polymerase was added.

The thermo-cycler profile (Perkin Elmer Model 2400) was composed of predenatured at 94 °C for 1 minute followed by 40 cycles of a 92 °C denaturation for 30 second, a 36 °C annealing for 45 second and a 72 °C extension for 2 minutes. The final extension was carried out at 72 °C for 5 minute. The amplification products were electrophoretically analyzed by appropriate concentrations of agarose gels.

2.9 Agarose gel electrophoresis

An appropriate amount of agarose (SaeKem LE or Metaphor) was weighed out and mixed with Tris-Borate-EDTA buffer (TBE; 89 mM Tris-HCl, 89 mM boric acid and 2.5 mM EDTA, pH 8.3) to make the desired gel concentration (1.5% for SeaKem LE and 1.8% Metaphor for detection of ITS

and RAPD-PCR products, respectively). The dissolved agarose was heated until complete solubilization and cooled at room temperature to 50 °C before poured into a gel mould in which a comb was already inserted. When the gel had solidified, the comb was carefully removed. The agarose gel was submerged in a chamber containing an enough amount of 1x TBE buffer that cover the gel for approximately 0.5 cm.

An appropriate amount of ITS or RAPD-PCR amplified DNA was mixed with one-fifth volume of the loading dye buffer (15% ficoll 400, 0.25% bromophenol blue and 0.25% xylene cyanol FF) before carefully loaded into the well. Three microlitters (390 ng) of a 100 bp ladder was used as a DNA standard. Electrophoresis was operated at 100 volts until bromophenol blue moved to approximately 3 cm (for ITS) and 0.5 cm (for RAPD) from the bottom of the gel. The electrophoresed gel was stained by immersed in 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 were visualized under a UV transilluminator and photographed through a red filter using Kodak Tri-X-pan 400.

2.10 Preparation of DNA template for DNA sequencing

The amplified ITS fragment was size-fractionated through a 1.5% agarose gel prepared in 1x TBE buffer and electrophoretically analyzed side by side with a 100 bp ladder. After electrophoresis was completed, the lane corresponding to the DNA marker was excised and stained with ethidium bromide as described above. The ITS fragment could also be excised by comparing its molecular length

corresponding to that of the ladder and placed into a pre-weighed 1.5 ml microcentrifuge tube, one-half volume of TBE modifier and 4.5 volumes of 6M sodium iodide were added. The gel was dissolved by inversion of the tube for several times and further incubated at 55 °C for 10 minutes. Five microliters of glass milk was added to the gel solution. The suspension was incubated on ice for 5 minutes. The glass milk was pelleted by centrifugation at 8000x*g* for 30 seconds. After the supernatant was removed and discarded, 250 µl of a new wash solution was added. The pellet was resuspended and centrifuged at the same speed for 30 seconds. This process was further carried twice. The ITS amplified DNA was recovered from the washed pellet with an addition of the suitable amount of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and incubated at 55 °C for 5 minutes. The solution was centrifuged at 5000x *g* for 30 seconds, the supernatant containing the eluted DNA was collected and used for direct-sequencing.

2.11 DNA Sequencing

OmniBase™ DNA cycle sequencing system and OmniBase™ sequencing enzyme mixes (Promega) were used for direct-sequencing of the amplified ITS of *T. clareae* and *T. koenigerum*. In this sequencing system, a ^{32}P end-labeled primer was annealed to the DNA template and extended by the DNA polymerase activity in the presence of four deoxyribonucleside triphosphates (dNTPs). The reaction also contained one of four dideoxyribonucleoside triphosphates (ddNTPs), which terminated elongation reaction when being incorporated into the DNA synthesizing chain. After sequencing, the products were electrophoretically separated on a high-resolution denaturing polyacrylamide gel. The results were

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visualized by autoradiography. Using the external primers, approximately 200 bp of the sequences could be unambiguously determined, as a result; internal primers were designed to obtained the 3′ downstream sequences of the ITS.

2.11.1 End-labeling of the primer.

The primer was 5′-end-labeled with [γ-^{32}P] ATP (specific activity 3000 Ci/mmol) using the forward activity of T_4 polynucleotide kinase. The labeling reaction mixture in a total volume of 10 μl was set up. This composed of 6 sets of double-stranded sequencing reactions. Each of which contained 10 pmole of a particular primer, 10 pmole of [γ-^{32}P] ATP, 1 μl of 10x buffer (500 mM Tris-HCl pH 7.5, 100 mM DTT, 50 mM spermine) and 5 unit of T_4 polynucleotide kinase. The reaction was gently mixed by pipetting and incubated at 37 °C for 10 minutes. The labeling reaction was terminated by incubated at 90 °C for 2 minutes. The reaction mixture was briefly centrifuged and stored at -20 °C until further needed.

2.11.2 Extension-termination reactions.

The template/primer mix for each of four sequencing reactions was prepared in a 0.5 ml microcentrifuge tube by adding 2 ng of DNA template, 5 μl of 5x sequencing buffer (250 mM Tris-HCl pH 9.0, 10mM $MgCl_2$), 1.5 μl of end-labeled primer (2.11.1) and appropriate amount of nuclease-free water making the final volume up to 10 μl. Finally, 10 units of OmniBase™ Sequencing enzyme was added. The solution was gently mixed by pipetting. Four microliters of each

reaction was added to a 0.5 ml microcentrifuge tube containing 2 µl of appropriate d/ddNTP termination mix and briefly mixed before subjected to cycle sequencing in a thermo-cycler. The cycle sequencing reaction was initially denatured at 94 °C for 1 minute followed by typical PCR steps of a 95 °C denaturation for 30 seconds, a 44 °C annealing for 45 seconds and a 70 °C extension for 60 seconds for 30 cycles. At the end of the cycle-sequencing reaction, 3 µl of a stop solution was added to each sequencing reaction. Prior to loading of these samples on a denaturing sequencing gel, the reactions were heated at 70 °C for 2 minutes and snap-chilled on ice.

2.11.3 Polyacrylamide gel electrophoresis

A pair of glass plates were carefully cleaned with soap and water before thoroughly rinsed with deionized water, 70% ethanol and left to air-dry. The shorter plate was siliconized with a glass coating solution, (Rain-X, Unellco Co., USA). The glass plates were assembled with spacers (0.4 mM thickness). The side and bottom edges of the plates were sealed with plastic tape.

Sixty milliliters of 6% denaturing acrylamide gel (in 1X TBE, 8M urea, 0.084 M acrylamide, 2 mM bis-acrylamide) was prepared in a 100 ml beaker. To initiate gel polymerization, 280 µl of 10% ammonium persulphate and 60 µl of TEMED (N,N,N′,N′-tetramethylethylenediamine) was added to the acrylamide mixture and gently mixed. The resulting solution was immediately poured into the gel apparatus with a short plate face-up. The gel mixture was slowly poured between the glass plates, with a smooth flow rate to prevent any air bubbles. The angle of glass plates was adjusted to allow the gel solution flowed down slowly at

one side. When the gel solution reached the top of the short plate, a 0.4 mm thick shark tooth comb was inserted to approximately 5 mM in depth. The polymerization process were allowed to be completed for 2-3 hours.

When required, the sealing tape was removed. The gel was cleaned to remove spilled acrylamide solution from plate surfaces with water. The gel mould was placed to a sequencing chamber. An enough amount of 1x TBE was added to the upper and lower chamber allowing the gel plates submerged for 2 - 3 cm in the buffer. The comb was removed. The wells were immediately flushed out with a long-stem Pasteur pipette using the buffer in the upper reservoir to remove undesired small pieces of polyacrylamide. A comb was reinserted into the gel. The gel was pre-run at constant power of 35 watts (1.2 kilovolts) for 30 minutes. Prior to loading of the samples, the gel wells were carefully rinsed with the buffer to remove any residual urea. The heat-danatured samples were loaded. The gel was electrophoresed at the same constant power for 2-3 hours.

2.11.4 Autoradiography

After electrophoresis, the glass plates were removed from the electrophoretic apparatus and placed under the running tap water until surfaces of both plates were cooled. The sealing tape and spacers were removed. The shorter glass plate was prized apart using either scissors or a spatula, therefore the gel was remained on the longer glass plate. The gel was transferred to a piece of pre-cut Whatman 3 MM filter paper by laying the paper on top of gel and slowly lower the remaining of the filter paper to cover the entire polyacrylamide gel. The paper was slowly peeled back. At this stage the gel should stick with the filter paper. The 3 MM filter paper containing the sequencing gel was covered with

cling-film and dried at 80 °C for 30-60 minutes in a gel dryer. The dried gel was then placed in an X-ray cassette. In the dark room, an autoradiography film was placed on the dried sequencing gel. The cassette was kept in a -80 °C freezer for 16-24 hours. When required, it was removed from a freezer and left to thaw out to room temperature for approximately 30 minutes. The exposed film was developed and fixed according to the manufacture's instruction. The sequences obtained were visualized manually.

2.12 Statistical analysis of genetic variation

2.12.1 DNA sequence analysis

The sequences obtained from the amplified ITS of *T. clareae* in *A. dorsata* and *A. mellifera* and *T. koenigerum* in *A. dorsata* hosts were aligned using Clustal W. The genetic distance between sequences (*d*) can be calculated by Kimura's two parameter method using the formula;

$$d = (1/2)\ln(a) + (1/4)\ln(b)$$

where a = 1/(1-2P-Q and b = 1/(1-2Q); P and Q represent the proportion of nucleotide differences resulted from transitional and tranvertional mutatations, respectively.

Practically, the genetic distance of investigated sequences was routinely calculated using DNAdist in Phylip 3.56c.

2.12.2 RAPD analysis

The reproducible and well resolvable bands with the molecular length between 265-2040 bp were scored from photographes of the gels. The band intensity differences due to homo- and heterozygotic states were not considered.

Accordingly, the presence (1) and absence (0) of an amplified fragment was treated in a dominant fashion.

The similarity coefficiency between a pair of individuals was calculated using the formula:

$$S_{xy}=2n_{xy}/n_x+n_y,$$

where; n_x and n_y represent the number of scorable fragments from individual x and y, respectively.

n_{xy} is the number of fragments shared by both individuals.

Similarity index within a population (S) is calculated as the average of S_{xy} across all pairwise comparisons between individuals within such a population.

Genetic similarity between populations with a correction for within population similarity is:

$$S_{aij} = 1+S'_{ij}-0.5\ (S_i+S_j),$$

where S_i and S_j represent the S estimates for population i and j, respectively

S'_{ij} is the average similarity between random pairs of individuals across populations i and j (Lynch 1990).

S_{aij} was then converted to the genetic distance (D_{ij}) using the equation:

$$D_{ij} = 1- S_{aij} \quad \text{(Lynch 1991).}$$

2.12.3 Phylogenetic construction

A phenogram based on the distance approach was constructed using the unweight pair-group method with arithmetic average (UPGMA) implemented in Phylip version 3.57c (Felsenstein, 1991).