III. MATERIALS AND METHODS

POPULATIONS

Populations studied came from four parts of Thailand: the north, central, south, and northeast comprising 60 to 62 individuals for each locus in CTT triplex and 79 to 109 for each one in FFv triplex.

Multiplex	Locus	Total number
CTT	CSF1PO	62
	TPOX	62
	THO1	60
FFv	F13AO1	102
	FESFPS	79
	vWA	109

SAMPLE PREPARATION

Buccal cells or blood were collected from Thai populations according to suitable conditions.

- BUCCAL CELLS

For the collection of buccal cells, applicators (Life Technologies™) were used and then followed up by the following protocol:

1. Suspend collected cells in 1 ml. normal saline in 1.5 ml. microvials.
2. Centrifuge at 5,000 rpm for 5 minutes.
3. Discard supernate, and collect buccal cells.

- EDTA BLOOD

20 µl of 1% EDTA was added to 200 µl blood, mixed well, then followed up with this protocol:

1. Pellet the cells and wash them with 0.9% NaCl.

2. Pellet the cells again and resuspend them in one volume of cold (4 °C) hypotonic solution (20 mM Tris HCL, pH 8.0, 10 mM EDTA).

3. Pellet the cells at 4,000 rpm for 10 minutes (4 °C).

4. Discard the supernatant and collect leucocytes.

DNA EXTRACTION

0.8-1.0 ml. DNAzol™ reagent (Life Technologies™) was added to the buccal cells or Leucocytes then followed up with this protocol:

1. Mix gently upside-down 3-4 times.

2. Precipitate DNA with 0.5 ml. absoluted ethyl alcohol (mixed gently upside-down).

3. Let it stand for 1-3 minutes at room temperature.

4. Centrifuge at 4 °C, 1,000 x g for 1-2 minutes.

5. Discard supernatant.

6. Wash 3 times with 0.8-1.0 ml. 95% ethyl alcohol. At each wash, suspend the DNA in ethanol by inverting the tubes 3-6 times. Store the tubes vertically for 0.5-1.0 minute to allow the DNA to settle and remove ethanol by pipetting.

7. Dry the DNA by open microvial for 5-15 minutes at room temperature.

8. Solubilize the DNA with 50-100 µl sterile distilled water.

DETERMINATION OF DNA

The extracted DNA was determined by using the following protocols:

1. Apply 2 µl of DNA extracted to 0.8% agarose gel.

2. Run electrophoresis at 50 volts for 1 hour and 30 minutes.

3. Stain with 0.5 µg/ml ethidium bromide solution for 20 minutes and destain with distilled water for 5 minutes.

4. Visualize DNA band by the UV transilluminator.

Sample investigations were processed as :

1. Determination of PCR products from DNA samples by commercial multiplex PCR.

2. Detection of amplification products.

3. Preparation of a denaturing polyacrylamide gel.

4. Determination of the DNA Fingerprinting.

5. Collection and analysis of data

MULTIPLEX PCR

Two commercial multiplex sets, CTT and FFv Triplex (GenePrint™ of Promega) were introduced according to the following provided protocols:

1. 1-3 µl of extracted DNA (volume used was determined by observation of band intensities).

2. 2.5 µl of 10x STR buffer (Product reagent).

3. 2.5 μl of 10x Primers (Product reagent).

4. 0.125 μl of Taq DNA polymerase.

5. Distilled water corrected to 25 μl of total volume.

6. Overlayer all components with 1 drop of mineral oil.

7. Briefly spin down.

8. Perform an amplification reaction in a Thermocycler (COY TEMP- CYCLER II, Model 110SM) to acquire these parameters:

96 °C for 2 minutes, then;

94 °C for 1 minute.

60 °C for 1 minute.

70 °C for 1.5 minutes.

for 10 cycles, then;

90 °C for 1 minute.

60 °C for 1 minute.

70 °C for 1.5 minutes.

for 20 cycles, then stabilize strands at 72 °C for 7 minutes.

DETECTION OF AMPLIFICATION PRODUCTS

Five microlitres of amplification products were detected by 1% agarose gel electrophoresis at 100 volts for 60 minutes. It was then stained in 0.5 μg/ml ethidium bromide solution for 20 minutes and destained with distilled water for 5 minutes. The band of amplification products was visualized by UV transilluminator.

PREPARATION OF DENATURING POLYACRYLAMIDE GEL

The following protocol was for the preparation of a denaturing polyacrylamide gel with the dimensions of 31 cm x 38 cm x 0.4 mm (w x h x thickness).

1. Etch each glass plate on one side in one corner with a diamond pencil to distinguish the treated sides of the glass plates (Note: the gel side is the etched side of the glass plate). Thoroughly clean the shorter and longer glass plates twice with 95 % ethanol and tissues.

2. Using gloves, apply 600 µl of silicone (sigmacote™) onto the etched side of the longer glass plate. With a dry tissue, wipe quickly in a one-way motion over the entire surface.

3. Let it dry for 30 minutes. Remove the excess silicone with soft tissue saturated with 70 % ethanol. Finally, dry the glass plate with a dry soft tissue.

4. Prepare a fresh binding solution by adding 3 µl of bind silane (Methacryloxypropyltrimethoxysilane) to 1 ml of 0.5 % acetic acid in 95% ethanol in a 1.5 ml microcentifuge tube. Wipe the etched side of the shorter glass plate with soft tissues saturated with freshly prepared binding solution. Be certain to wipe the entire surface of the plate with the saturated tissue.

Note: preparation of fresh binding solution should be carried out in a chemical fume hood because the bind silane (Methacryloxypropyltrimethoxy- silane) is toxic.

5. Let it dry for 30 minutes. Wipe the shorter glass plate 3-4 times with a soft tissue saturated with 95% ethanol to remove excess binding solution.

Note: failure to wipe excess binding solution from the shorter glass plate will cause the gel to stick to both plates, and the gel will be destroyed upon separation of the glass plates after electrophoresis.

6. Take special care not to allow the treated surfaces to touch each other. Assemble the glass plates by placing 0.4 mm side spacers. Seal both sides and the bottom of the glass plates with sticky tape and use clamps to hold them in place (4-5 clamps on each side and 3-4 clamps on the bottom of the glass plates). Lean the assembled plates against a supporting box (such as a test tube rack).

7. Prepare a 4 % acrylamide solution (total volume 75 ml.).

8. Pour the filtered acrylamide solution into a squeeze bottle.

9. Add 50 µl of TEMED and 500 µl of 10% ammonium persulfate to the acrylamide soluion and mix gently.

10. Pour the acrylamide solution carefully between the glass plates. To prevent bubble formation, start pouring at one side of the assembled plates and maintain a constant flow of solution.

11. Position the gel horizontally, resting it on two supporting boxes.

12. Insert 14 cm fine 25 point sharkstooth combs , straight side into the gel, between the glass plates.

13. Allow the polymerization to proceed for at least 1 hour.

Note: the gel may be stored overnight by placing soft tissues saturated with deionized H_2O around the well end of the gel and wrap in plastic to prevent it from drying out.

DNA FINGERPRINTING DETERMINATION

Amplification products from multiplex PCR were processed to create the DNA fingerprinting according to these steps:

- Gel Pre-run
- Sample preparation
- Sample loading
- Gel electrophoresis
- Silverstaining of gel
- Gel drying
- Exposure of film.

Details of the above steps are as follows:

Gel Pre-Run

1. Remove the clamps from the polymerized acrylamide gel and clean the glass plates with soft tissues saturated with deionized H_2O.

2. Remove the comb and shave any excess polyacrylamide away from the comb.

3. Add 0.5x TBE to the bottom chamber of the electrophoresis apparatus.

4. Gently lower the gel (glass plates) into the buffer with the longer plate facing out with the well-side on top.

5. Secure the glass plates to the sequencing gel apparatus.

6. Add 0.5x TBE to the top buffer chamber of the electrophoresis apparatus.

7. Using a 50 ml. syringe filled with buffer, remove the air bubbles on the top of the gel. Using a syringe with a bent 19-gauge needle, remove the air bubbles from between the glass plates on the bottom of the gel.

8. Carefully insert the cleaned sharkstooth comb teeth approximately 1-2 mm into the gel.

9. Remove the air bubble from the well area by using the syringe filled with buffer and a 19-gauge needle.

10. Pre-run the gel to achieve a gel surface temperature of approximately 50 °C (about 40 watts).

Sample Preparation

1. Prepare the PCR samples by mixing 2.5 µl of each sample with 2.5 µl of STR 2x Loading solution.

2. Add 2.5 µl of the STR Ladder to 2.5 µl of STR 2x Loading solution for each ladder lane.

3. Briefly spin the samples in a microcentrifuge to bring the contents to the bottom of the tube.

Note: the sample that has the lower band intensities in the PCR product determination step may appear more intense by mixing 3 - 4 µl of each sample with a pre-mix of 2.5 µl STR 2x Loading solution + a corrected volume of STR1x Buffer to a maximum volume of prepared sample in each gel.

Sample Loading

1. Denature the samples by heating at 95 °C for 2 minutes and then chill on ice immediately.

Note: denature the samples just prior to loading the gel. Sample DNA will re-anneal if denatured hours before loading.

2. After the pre-run, use a 50 ml. syringe filled with buffer to flush the urea from the well area.

Gel Electrophoresis

1. At the completion of loading, run the gel using the same condition as the gel pre-run (40 watt) for about 45-60 minutes.

2. Stop electrophoresis any time after the xylene cyanol migrates pass the midpoint of the gel (in a 4% gel, bromphenol blue migrates at approximate- ly 40 bases and xylene cyanol migrates at approximately 170 bases).

Silver Staining

1. After electrophoresis, empty the buffer chambers and carefully loosen the gel clamps. Remove the glass plates from the apparatus.

2. Place the gel (on glass plates) on a flat surface. Remove the comb and the side spacers. Carefully separate the two glass plates. The gel should be strongly affixed to the shorter glass plate.

3. Place the gel (attached to the shorter plate) in a shallow plastic tray.

4. To silver stain, follow these steps:

4.1) Fix/stop solution for 20 minutes

4.2) Deionize H_2O for 2 minutes

4.3) Repeat step 4.2, twice for 2x2 minutes

4.4) Stain solution for 30 minutes

4.5) Deionize H_2O for 10 seconds

4.6) Developer solution (4-10 °C) up to 5 minutes (until alleles and ladders are visible)

4.7) Fix/stop solution (add directly to developer solution to stop causing a reaction.) for 5 minutes

4.8) Deionize H_2O for 2 minutes

4.9) 1% glycerol for 30 seconds

Gel Drying

1. Position the gel (on shorter plate) upright in a hot air oven.

2. Allow the gel to dry at 80 °C for 30-40 minutes.

6. Cover the gel with plastic wrap after it has cooled down.

Exposure of Film

1. Place the dry, stained gel that attached to the shorter plate (gel side up) on a white fluorescent light box.

2. In the darkroom under a safelight, position the APC Film (Automatic Processor Compatible Film, which produces direct mirror images), emulsion side down, over the gel to be copied.

Note: the emulsion side of the film can be identified as the glossy white surface; the non-emulsion side has a gray tint.

3. Turn on the white light box and expose the film for 10 to 30 seconds, depending on the gel background level (if there is very little signal, decrease the exposure time. If the film appears brown or black, increase the exposure time).

4. Develop the film in developing solution until the bands are visible.

5. Fix immediately in fixing solution for 5 minutes.

6. Wash the film in tap water for 5 minutes and let it dry at room temperature.

7. Collect the film as permanent images.

COLLECTION AND ANALYSIS OF DATA

Each genotype was determined case by case in each locus from perma- nent images. The data were collected and analysed in two ways :

- The Chi-square testing of data in each locus for Hardy-Weinberg equilibration
- The Power of Discrimination and Power of Exclusion determinations in each locus and combined locus.