CHAPTER III

MATERIALS AND METHODS

3.1 Equipments

- Autoclave, model: Conbraco, Conbraco Ind. Inc., USA
- Automatic micropipette P10, P20, P200, and P1000, Gilson Medical Electronic, France
- Centrifuge, model: Centrifuge 5410, Eppendorf, Germany
- Cuvette, model: 700 µl Semi-micro cuvette, Brand, Germany
- Dessicator for storage specimen
- Disposable gloves, Meditrate, USA
- Disposable syringe Tuberculin® 1.0 ml. with needle gauge number 25
- Electronic clock timer, model: CT-30, Canon Co. Ltd., Japan
- Electronic U.V. transilluminator, Ultra Lum Inc., USA
- Electrophoresis, model: Mupid, Advance Co. Ltd., Japan
- Gel dryer, model: 583, Bio-RAD Laboratory, USA
- Ice box, Scientific Plastic Co. Ltd., USA
- Incubator waterbath, model: 3575-1, Lab-line Instruments Inc., USA
- MetaPhor agarose, FMC Bio Product, Denmark
- Microcentrifuge tube 0.2, 0.5 and 1.0 ml., Treff®, Switzerland
- Microincubator, model: M-36, Taitec, Japan
- Micropipette tip P10, P20, P200 and P1000, Treff®, Switzerland
- Microwave, model: Sharp carousel R7456, Sharp, Thailand

- PCR, model: Perkin-Elmer 2400, PE Applied Biosystem, Singapore
- Fuji film, model: Fp-3000B, Fuji Photo Film Co. Ltd., Japan
- Polaroid camera, model: Direct screen instant camera DS 34 H-34, Peca Products, UK
- Power supply, EC 570-90 LVD CE, E-C Apparatus Corporation, USA
- Pyro magnestir, Lab-line Instrument, Inc., USA
- pH meter, Cyberscan 500, Eutech Cybernetics, Singapore
- Waterbath, model: 20 H, Gallenkamp, Germany
- Whatman® filter paper: number 1, England
- Whatman® Laboratory sealing film, England
- Electrophotometry, mini VE, Hoefer, Pharmacia Biotech, USA
- Spectrophotometer, model: UltraspecII, LKB Biochrom, England
- Surgical knife, a pair of scissors and forceps
- Vertical sequencing gel electrophoresis apparatus, Bio-RAD Laboratory, USA
- -20 ^{0}C Freezer, Sanyo Co. Ltd., Japan

3.2 Chemicals

- 100 % Acetic acid, CH_3COOH, M.W. = 60.05, Merck, Germany
- Absolute ethanol, Merck, Germany
- Acrylamide, M.W. = 71.08, Promega, USA
- Ammonium peroxydisulphate (APS), $(NH_4)_2S_2O_8$, M.W. = 249, BDH Laboratory Supplies, England
- Boric acid, Bio-RAD Laboratory, USA

- 100 mM dATP, dGTP, dCTP, dTTP, Promega Corporation, USA
- Chelex® 100 Resin: 100-200 mesh sodium form, Bio-RAD Laboratory, USA
- Chloroform, Merck, Germany
- Ethylene diamine tetra-acetic acid (EDTA), $C_{10}H_{16}N_2O_8$, M.W. = 292.2, Bio-RAD Laboratory, USA
- 95% (v/v) Ethanol, CH_3CH_2OH, M.W. = 46, Thailand
- Formamide, Merck, Germany
- 37% (v/v) Formaldehyde, CH_2O, M.W. = 30, Merck, Germany
- 99.5% (v/v) Glycerol, $C_3H_8O_3$, M.W. = 92.10, BDH Laboratory Supplies, England
- Glycine, NH_2CH_2COOH, M. W. = 75.07, BDH Laboratory Supplies, England
- Hydrochloric acid fuming 37% (v/v), HCl, Merck, Germany
- Loading dye, Promega Corporation, USA
- Methanol, CH_3OH, M.W. = 32.04, Merck, Germany
- N,N'-methylene-bis-Acrylamide(Acrylamide/Bis), Promega Corporation, USA
- Nitric acid, J.T.Baker, USA
- PCR purification kit, Qiagen, Germany
- Phenol, Carlo Erba, Italy
- QIAamp DNA extraction mini kit (catalog # 51304), Qiagen, Germany
- Silver nitrate, Nacalai Tesque, Japan
- Sodium acetate, $CH_3COONa.3H_2O$, M.W. = 136.09, M&B Ltd., England

- Sodium carbonate, Merck, Germany
- Sodium chloride, NaCl, M.W. = 58.4, Merck, Germany
- Sodium dodecyl sulfate, SDS, Merck, USA
- Sodium hydroxide, NaOH, M.W. = 40, Merck, Germany
- Sodium thiosulfate, $Na_2S_2O_3.5H_2O$, M.W. = 248.2, Sigma, Germany
- TEMED, Promega Corporation, USA
- Tris-(hydroxymetyl)-aminomethane, $NH_2C(CH_2OH)_3$, M.W. = 121.4, Pharmacia Biotech, USA

3.3 Enzymes

- T*aq* DNA polymerase, Promega corporation, USA
- DyNAzyme™ II DNA Polymerase, Finnzymes, Finland
- Proteinase K, Promega Corporation, USA

3.4 Sample collection and preservation

Green peafowl specimens were collected from various regions in the north, south, east and west of Thailand (Figure 3.1). Blood and feather specimens were taken from Huay Hong Krai royal project (HH) in Chiang Mai province, Sri Nan national park (SN) in Nan province, Wieng Lor wildlife sanctuary (WL) in Phrayao province, Doi Phu Nang national park (PN) in Phrayao province, Phatthalung wildlife research and breeding station (PL) in Phattalung province, Khao Soi Dao wildlife research and breeding station (SD) in Chantaburi province and Huay Kha Kaeng wildlife sanctuary (HK) in Uthai Thanee province. All details of specimens are shown in Appendix I.

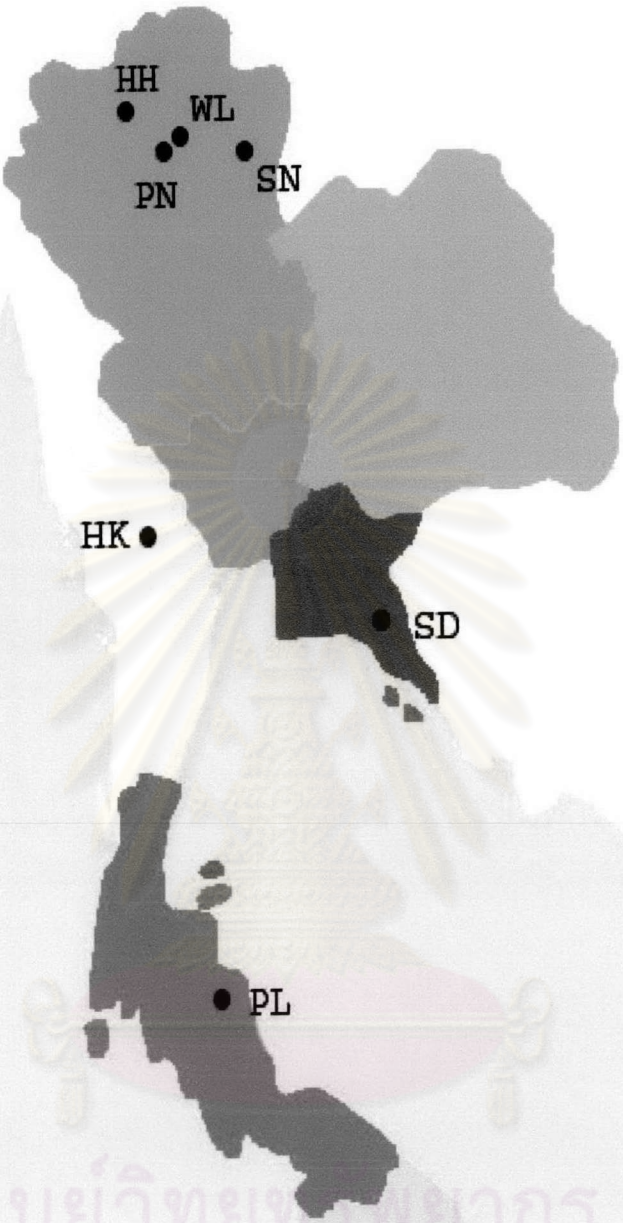

Figure 3.1 Map of Thailand illustrating sample collection sites of *Pavo muticus imperator*; **WL**:Wieng Lor wildlife sanctuary, **PN** :Doi Phu Nang national park **HH**:Huay Hong Krai royal project, **SN** : Sri Nan national park **SD** :Khao Soi Dao wildlife research and breeding station **HK**: Huay Kha Kaeng wildlife sanctuary **PL** : Phatthalung wildlife research and breeding station

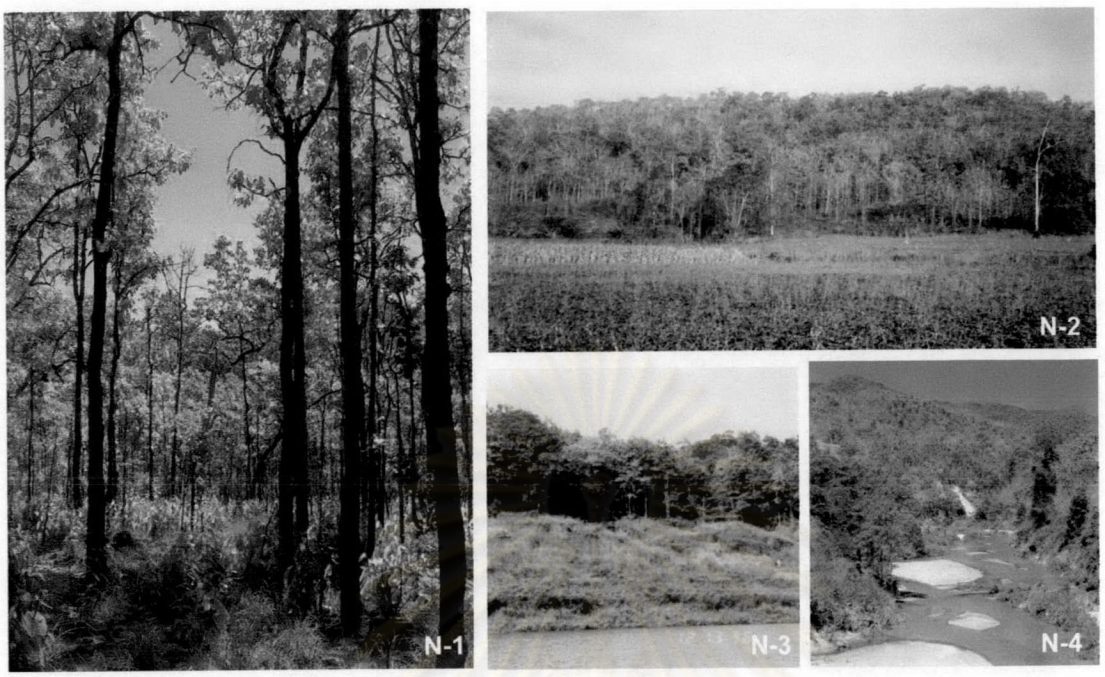

Figure 3.2 Location of *Pavo muticus imperator* in natural resources (N).

(N-1) Wieng Lor wildlife sanctuary.

(N-2) Doi Phu Nang national park.

(N-3) Sri Nan national park.

(N-4) Huay Kha Kaeng wildlife sanctuary

Remark (N-1, N-4) photographed by L.Bruce Kekule, (N-2, N-3) by Wina Meckwichai.

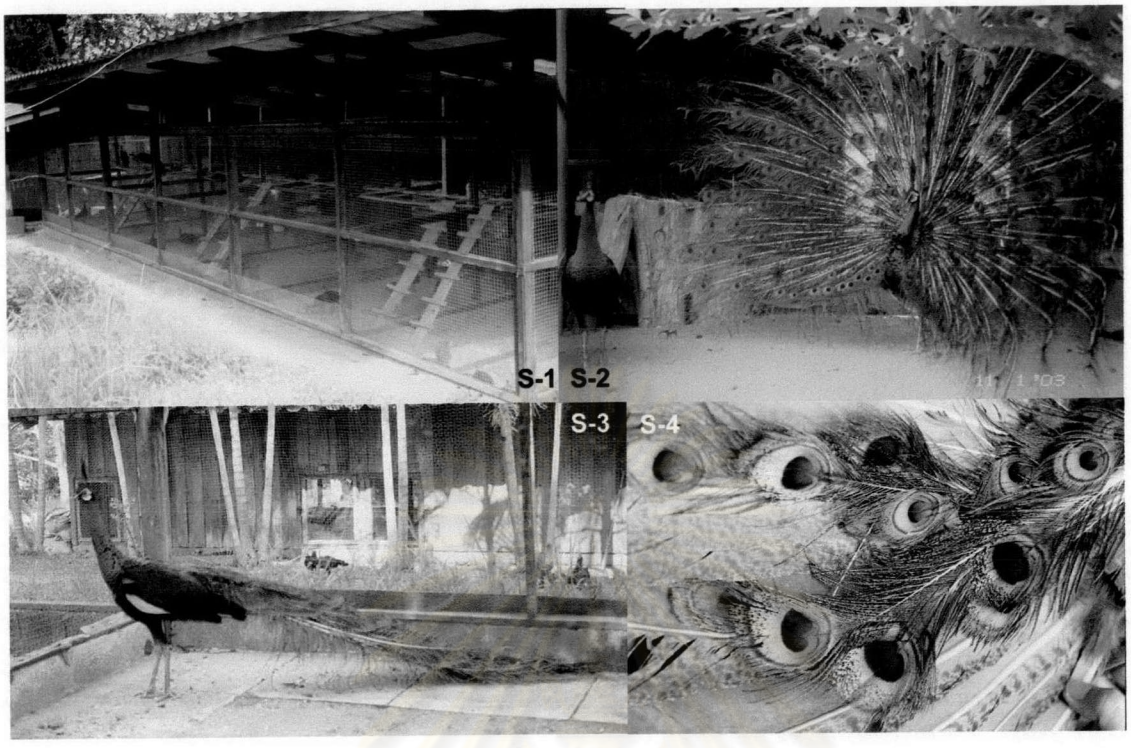

Figure 3.3 Location of *Pavo muticus imperator* in Khao Soi Dao wildlife research and breeding station (S).

(S-1) Cages at the station

(S-2) A couple of green peafowls in the breeding season

(S-3) An adult male green peafowl

(S-4) Feathers

Remark (S-1, S-3, S-4) photographed by Thiti Sukaphan, (S-2) by Waree Wutthivikaikan.

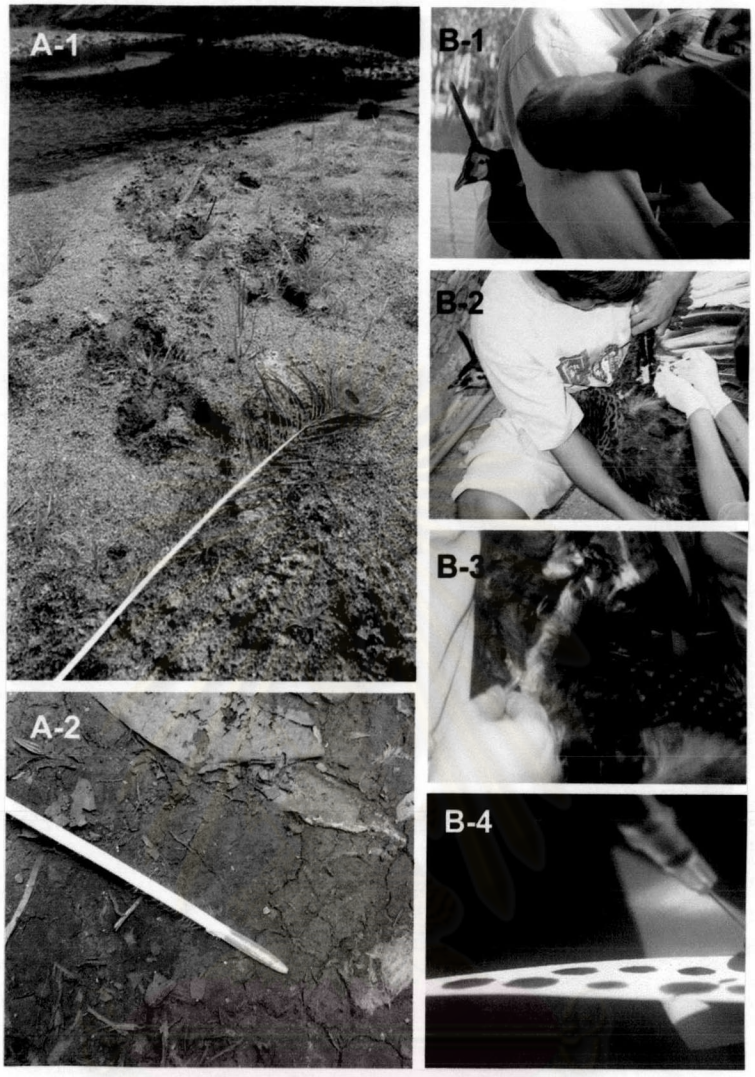

Figure 3.4 Collected samples from *Pavo muticus imperator*. (A-1) feather from a wild source, (A-2) the end of a feather tip for DNA extraction, (B-1) safety method to catch a bird, (B-2, B-3) blood collected from branchial vein from a wing and (B-4) bloodstain.

Remark (A-1) photographed by M.L. Prinyakorn Voravan, (A-2, B-1, B-3, B-4) by Waree Wutthivikaikan and (B-2) by Thiti Sukaphan.

Blood sample was collected by radial venipuncting from a branchial vein with a new Tuburaclin® syringe with needle gauge number 25. About 0.1-0.2 ml of blood was dropped on a piece of autoclaved Whatman® filter paper, air dried and placed into labeled autoclaved paper bag for each sample. Every step must use clean technique and complete air dried to avoid fungal contaminate.

A feather was collected by cutting at the end and placed into a labeled paper bag. Both blood stains and feathers were kept in desiccators.

3.5 Morphological characterization

Morphology of follicle cells from feathers of *Pavo muticus* was examined by Scanning Electron Microscopy (SEM) (JSM-35CF) for comparison with the feathers collected more than 1 year and less than 1 year. The end of the feather tip, about 5 mm, was cut with a pair of sterile scissors. They were stored in 70% alcohol. Then, the samples were washed in a sonicator bath for 5 minutes, 3 times, and treated in 100% alcohol. Later, each feather tip was coated with gold particles (JFC-1100) and photographed under SEM at 15 kilovolt with x35, x50, x75, x2,000 multipliers , respectively.

3.6 Total DNA extraction

Total DNA of *Pavo muticus imperator* was extracted from bloodstain and feathers. In this study, three methods are used. The method giving higher purity and quantity of DNA would be chosen to prepare DNA template for PCR.

3.6.1 Chelex® extraction method

Chelex is a polyvalent chelating agent in resin form. It is commonly used to extracted a small amount of DNA. Heating samples over boiling-point condition will help disrupting cell membranes, separating DNA from cells and assuring completed denaturation of the DNA template (Sanger-Sam *et al.*, 1989). DNA extraction from bloodstain seems less prone to contain PCR inhibitors when it is prepared by this method (Walsh *et al.*, 1991). Furthermore, this method is easy, inexpensive, less time-consuming and reduce contamination chance. Protocol of this method is below.

Total DNA was extracted from bloodstain and feathers using Chelex extraction medium (Sanger *et al.,* 1989; Walsh *et al.,* 1991). Bloodstain on filter paper with 2X2 mm^2 size was cut and immersed in a new 1.5 ml microcetrifuge tube containing 1,000 µl of sterile distilled-water, then mixed gently. The tube was incubated at room temperature until blood dissolved, then spined in microcentrifuge at 10,000 rpm for 2 minutes and carefully removed supernatant. The filter paper was taken out off the tube and then 5 % Chelex®100 was added to final volume of 200 µl. The 20 µl of 0.5% (W/V) proteinase K was added and the sample was incubated at 56°C for 40 minutes. After that, it was incubated at 100 °C for 8 minutes to stop reaction. Finally, the mixture was centrifuged to discard cell debris and the Chelex®100 resin from solution. Extracted DNA was kept at -20°C before later used in PCR amplification.

For DNA extraction from feathers, the single feather tip was washed with 70% ethanol and sterile water. Then 5-10 mm of the end of the tips was divided to 2 sides, sliced off with a sterile razor blade, and transferred to a 1.5 microcentrifuge

tube. Then it was chopped into small pieces with a pair of sterile scissors. After feather preparation, 300 µl of 5% Chelex® 100 was added. The proteinase k was added into the sample tube. The sample was vortexed and incubated at 56°C overnight. The sample was vortexed again and then incubated at 100°C for 8 minutes and the mixture was centrifuged to discard cell debris and the Chelex®100 resin from solution. Extracted DNA was kept at -20 ^{0}C.

3.6.2 Proteinase K/ phenol-chloroform extraction method

Proteinase K/phenol-chloroform extraction method is another common technique used for DNA extraction. In this method protein and contaminants from the DNA sample were removed by proteinase K. Then, phenol-chloroform extraction will be performed, followed by an ethanol precipitate step to precipitate DNA.

Two of three mm^2 bloodstain was cut into a small piece and placed in a new sterile 1.5 ml microcentrifuge tube. Then 1.0 ml of TE buffer (10 mM Tris-HCl, pH 7.4, 1mM EDTA) was added to the tube. It was incubated at room temperature for 30-60 minutes. The sample was added with 25 µl of 10% (W/V) SDS and 25 µl of 0.5% (W/V) proteinase K, mixed briefly and incubated at 65 ^{0}C for 1 hour.

To precipitate protein out of the sample, 5M of sodium acetate (pH 8.9) was added to a final concentration of 1M, mixed thoroughly and then hold on ice for 45 minutes. After precipitation, the sample was centrifuged at 12,000 rpm in a refrigerated microcentrifuge for 10 minutes. The supernatant was transferred to a new 1.5 ml microcentrifuge tube. Protein was removed again by extraction with Tris-saturated phenol (pH 8.0) and chloroform as follows. An equal volume of

Tris-saturated phenol was added to the supernatant and mixed gently. The sample was then centrifuged for 5 minutes at 8,000 rpm. The upper aqueous phase was transferred into a new tube, added an equal volume of phenol:chloroform:isoamyl alcohol mixture(25:24:1), mixed gently and centrifuged again. After centrifugation, the upper phase was transferred into another new tube to extract again with chloroform:isoamyl (24:1).

The upper phase was removed and mixed gently with 1/10 volume of 3 M ammonium acetate (pH 5.5). The DNA was precipitated out from the solution by adding 2 volumes of cold absolute ethanol. After the tube was inverted several times, it was placed at -20^{0}C overnight to complete precipitation. The precipitated DNA was removed from the tube, rinsed with 70 % cold ethanol and allowed to air-dry. The DNA was finally dissolved in an appropriate volume of TE buffer or deionized distilled water. The DNA was incubated overnight at 37^{0}C for complete solubilization and kept at -20^{0}C until further use.

3.6.3 QIAamp® Kit extraction method

Genomic DNA was also extracted by QIAamp® DNA Mini Kit. The kit together with its buffer set, are designed to isolate pure genomic DNA direct from various samples including the dried-blood spot samples. Its procedure bases on the a selectivity of QIAGEN anion-exchange resin, which allows isolation of high yields of pure genomic DNA ranges in size from 20-150 kb.

A dried blood spot was cut as 3x3 mm^2 size (1/8 inch) diameter punches with a pair of sterile scissors. The blood punch was transferred into a new 1.5 ml centrifuge tube containing 180 µl of buffer ATL. Then it was incubated at 85^{0}C for

10 minutes and briefly centrifuged to remove solution drops from inside of the lid. Twenty microlitres of proteinase K were added to the sample, mixed thoroughly by vortexing, followed by incubation it at 56°C for 1 hour or until the blood was dissolved out from the paper. After that, 200 µl of buffer AL was added to the sample, mixed well by vortexing, and incubated at 70°C for 10 min. In order to ensure an efficient lysis, it was essential that the sample and buffer AL was mixed immediately and thoroughly. Two hundred microlitres of 96 to 100% ethanol were added, mixed thoroughly by vortexing and briefly centrifuged to remove drops from inside the lid. The mixture was carefully applied to the QIAamp spin column without wetting the rim. The sample was centrifuged at 6,000 x g for 1 minute and the filtrate was discarded. The QIAamp spin column was washed first time with 500 µl buffer AW1, centrifuged at 6,000 x g for 1 minute, and the collection tube containing filtrate was discarded. Then 500 µl of buffer AW2 was added for second washing and centrifuged at full speed or 20,000 x g for 3 minutes. The column was centrifuged again at maximum speed for 1 minute to completely remove any buffer AW2 because residual buffer AW2 in the elute may cause problems in downstream applications. After centrifugation, the bound genomic DNA was eluted with 150 µl of buffer AE or distilled water to a new 1.5 ml centrifuge tube. The QIAamp spin column was incubated at room temperature for 1 minute, and then at 6,000 x g for 1 minute. DNA extracts were kept at -20°C.

For DNA extraction of feather, single feather tip was washed with 70% ethanol and sterile water. Then 5-10 mm of the end of the tips was divided to 2 sides, sliced off with a sterile razor blade, and transferred to a 1.5 microcentrifuge tube. They were chopped with a pair of sterile scissors. This step was importance for DNA extraction from feathers. One hundred and eighty microlitres of buffer

ATL were added to the microcentrifuge tube. After lysis complete, other buffers were added respectively as suggested. The later extraction steps for feathers are like those for blood spots.

3.7 Concentrated total DNA

Genomic DNA from extracted feathers is normally in lower yield than DNA from blood samples. Low concentration of template DNA may cause a problem in downstream PCR amplification.

Extracted DNA from feathers would be further concentrated by adding 3M sodium acetate (pH 5.2) 1/10 of total volume. Then, 2 folds of total volume of 70% chilled ethanol were added. After the tube was inverted several times, the mixture was frozen at -20^{0}C about 20 minutes or overnight to complete DNA precipitation. After freezing sample, the mixture was centrifuged and discarded supernatant. The pallet was complete dried in air. At the final step, the DNA was finally dissolved in an appropriate volume of sterile deionized distilled water or TE buffer. The DNA was incubated at 37^{0}C for complete solubilization and kept -20^{0}C until further use.

3.8 Determination quality and concentration of genomic DNA

3.8.1 Determination quality of genomic DNA

Electrophoresis is a standard method used for estimation of DNA quality on the basis of its direct relationship between the amount of DNA and the level of fluorescence after ethidium bromide staining. DNA was run in a 1% agarose gel prepared in 1X TBE buffer (89 mM Tris HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) at 100 V. Approximate nucleic acid size suitable for 1% agarose gel electrophoresis is 0.3–10 kb. After electrophoresis, the gel was stained with ethidium bromide. In addition, PhiX 174/*Hinf* I digested DNA was used as a DNA standard molecular size marker. Protocol of this method is shown in Appendix III.

3.8.2 Measurement and calculation of DNA concentration

Concentration of extracted DNA was measured by an ultraviolet (UV) absorption at 260 nm using spectrophotometer model UltraspecII. Extracted DNA solution can absorb UV at wavelength 260 nm (Optical Density: OD_{260}), whereas protein can absorb UV at wavelength 280 nm (OD_{280}) (Brown, 1991). Purity of extracted DNA was assessed by determining ratio of OD at 260 nm to OD at 280 nm. If the ratio is below 1.8, there maybe protein or other contaminants in sample (Devis *et al.,* 1994). An OD_{260} of 1.0 corresponds to a concentration of 50 µg/ml double strand DNA. Therefore, DNA concentration of each sample was calculated by this equation:

$$\text{DNA concentration} = OD_{260} \times \text{dilution factor} \times 50$$

3.9 *In vitro* amplification of microsatellite DNA using the polymerase chain reaction (PCR)

3.9.1 Selection of polymerase chain reaction primers

Selecting the proper primers was one of the most important steps in PCR experiment for this study. The selected primers should give high polymorphism and high number of alleles. They can amplify all in the East Lancing, the Compton and Wageningen resource population of chicken. Primers should be between 18 and 30 basepairs long.(Alkami Quick Guide™, 1999) and have similar lengths and melting temperatures, but not be complementary. In addition, they should produce PCR product of 100-300 bp in length, but not anneal to one another or form secondary structures (Ferraris and Palumbi, 1996). Moreover, try to avoid Gs and Cs at the 3'ends because this may increase the chance of forming primer artifacts. If PCR fails at one temperature, it should be repeated at higher and lower temperature (Arnheim *et al.*, 1990).

Twenty-three pairs of oligonucleotide primers were chosen from several publications and the Poultry Subcommittee of the National Animal Genome Program (NAGRP). In Galliformes, microsatellite primers have been developed in some farm species such as chicken (Khatib *et al.*, 1997; Cheng *et al.*, 1995; Gibbs *et al.*,1997). Although no microsatellite locus has been developed in green peafowls but many studies have demonstrated that sequences flanking the repeats of microsatellite loci are often conserved between closely related taxa, thus allowing cross-species amplifications. All microsatellite primers used in this study were selected from chicken genomic libraries that can amplify the highest allelic

number, high polymorphism and an unambiguous amplification pattern in the East Lancing reference family (mating between the Red Jungle Fowl and White Leghorn) (Khatib *et al.*,1993). They were also exhibited highly polymorphism in the Compton reference family (mating between the inbred White Leghorn and the outbred White Leg horn) (Cheng and Crittenden, 1994), and Wageningen resource population (Crooijmans *et al.*, 1997).

These microsatellite primers were screened by nonlabelled silver staining method, further which it modified by Perkin Elmer Protocol or by Alexander Binder Protocol. Both protocols are shown in Appendix V. The silver staining method was used to detect microsatellite polymorphism in green peafowl. Primer for 10 microsatellite loci (ADL23, ADL37, LEI73, LEI92, LEI126, MCW87, MCW240, HUJ1, HUJ2 and HUJ7 loci) were obtained from Bioservice Unit, National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand. Other primers for another 13 microsatellite loci (ADL102, ADL136, ADL158, ADL171, ADL172, ADL176, ADL181, ADL210, ADL267, MCW305, LEI80, LEI136 and HUJ12 loci) were purchased from PROLIGO Primers & Probes, Singapore.

The WS marker group (Crooijmans *et al.*, 1997) was not chosen because they were monomorphic chicken microsatellite markers. The characteristics of all selected *Gallus gallus* primers are listed in the Table 3.1. Sequence data of all primer is shown in Appendix VI.

Table 3.1 Characteristics of all selected *Gallus gallus* microsatellite-flanking PCR primers

No.	Locus name	GenBank Acc.no.	Length of forward primer/ reverse primer(bp)	Motif	Product size (bp)
1	ADL23	L23905	20 / 20	$(CA)_5(CG)_4(CA)_9$	164
2	ADL37	L23912	20 / 20	$(CAG)_3N_9(CA)_8$	178
3	HUJ1	L05542	22 / 22	(CA)	151-180
4	HUJ2	L10228	20 / 20	(AC)	124-142
5	HUJ7	H93646	21 / 21	(AC)	152-156
6	HUJ12	M13756	20 / 20	(CA)	120-140
7	LEI73	X82871	21 / 21	$(AC)_9CC(AC)_{23}$	163-221
8	LEI80	X82863	22 / 21	$(CA)_{18}$	191-211
9	LEI92	X82810	21 / 21	$(CA)_{15}$	164-212
10	LEI126	X82799	21 / 21	(GA)	189-231
11	LEI136	X82865	21 / 21	$(AC)_{15}(AT)_8$	160-280
12	MCW87	G31949	21 / 21	(CA)	272-287
13	MCW240	G32009	21 / 22	(AC)	172-197
14	MCW305	G32061	21 / 21	(TG)	258-268
15	ADL102	G01547	20 / 20	$(GT)_{18}$	122
16	ADL136	G01561	20 / 20	$(TG)_{10}TC(TG)_{10}$	145
17	ADL158	G01582	20 / 20	$(CA)_{12}$	216
18	ADL171	G01593	20 / 20	$(TG)_{18}$	104
19	ADL172	G01594	20 /20	$(AC)_{18}$	154
20	ADL176	G01598	20 /20	$(GT)_{12}$	192
21	ADL181	G01603	20 /20	$(CA)_{12}$	178
22	ADL210	G01630	20 / 20	$(AC)_{15}$	130
23	ADL267	G01687	20 / 20	$(CA)_{12}$	117

Remark: Primers number 1-14 were selected from publications and number 15-23 were chosen from the population tester kit of NAGRP.

bp: base pairs, **Acc. No.**: access number of GenBank

3.9.2 Polymerase chain reaction variables

The polymerase chain reaction (PCR) is a technique for *in vitro* amplification of specific DNA sequence by primer extension of complementary strand of DNA. PCR has great potential for DNA-level studies of conservation and population genetics. Generally, the required components of the reaction are DNA template, enzyme buffer containing magnesium chloride, deoxynucleotides, microsatellite primers and DNA polymerase (Mcpherson *et al.*, 1991). Usually a combination of variable modification is necessary to produce maximum specificity (selective reactivity), efficiency (yield) and fidelity (accuracy) in PCR reactions. Each variable can be modified to improve results.

In this study, DNA extraction method, DNA template quantity, preincubation time, annealing temperature-time, magnesium concentration, primer concentration and dNTPs concentration were adjusted for the approximate PCR condition of each microsatellite loci.

A negative control is commonly used along with many reactions PCR. It is a PCR reaction tube prepared without adding template DNA. Presence of amplified PCR products in the negative control tube means a DNA contamination in one or (usually) more reagents. In the other hand, positive controls from 1-2 chickens are also important. These are reactions that are guaranteed to work as long as the basic PCR cocktail is functional. Thus, positive control was used for this PCR experiment.

A 25 µl PCR reaction mixtures composed of 0.1-0.3 µM of each different primer, 0.1-0.2 mM dNTPs, 1.5-3.0 mM $MgCl_2$, 1.0 unit of *Taq* DNA polymerase (Promega) and 30-150 ng template of genomic DNA. The composition of enzyme storage buffer (buffer B) was 20 mM Tris-HCl (pH 8.0 at 25°C), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50 % glycerol, 0.5 % Tween®20 and 0.5 % Nonidet®-P40. The PCR mixture was spinned briefly at 10,000 rpm before subjected to the amplification process in Perkin-Elmer 2400.

The temperature cycling was as following.

The predenaturation at 94^0C for 2 or 5 minutes

(The reaction was carried out at 35 cycles)

The denaturation at 94^0C for 30 second - 3 minutes

The annealing at 45^0C – 62^0C for 45 seconds – 1 minute

The extension at 72^0C for 1-1.30 minutes

The final extension at 72^0C for 10 minutes

3.9.2.1 Quantity of template DNA

Generally, if the DNA template concentration is low, the concentration of PCR product should also be low. However, 20 ng of genomic DNA is enough to amplify microsatellite DNA in chicken (Cheng, 1994). In this study, specimens were hardly taken. Therefore, an investigation of appropriate template DNA quantity was important.

The selected microsatellite loci were amplified from the genomic DNA extracted by QIAGEN method. Approximately 20, 50, 100, 150 ng of genomic DNA from an individual of *P. m. imperator* were tested for DNA template in PCR and a total volume of each reaction was 25 µl.

3.9.2.2 Preincubation temperatures and times

In this study, the PCR product yields were compared at 94^0C between 2 and 5 minutes.

3.9.2.3 Annealing temperatures and times

The annealing temperature at T_m-5^0C was used in the first amplification of each primer. The melting temperature (T_m) for each primer is examined by the Wallace's rules: T_m (^{0}C) = [4 (total number of G and C) + 2 (total number of A and T)]. If the PCR failed to yield products at Tm-5^0C temperature, it should be repeated at lower temperature. If faint bands of PCR products are found, the annealing temperature would be decreased by 2^0C. But if the PCR products disappear, the temperature would be decreased by 5^0C. On the contrary, if non-specific products appear, the PCR reaction should be repeated at higher temperature (Table 3.2). To improve PCR specificity, an annealing temperature should be increased since it reduces the possibilities of nonspecific priming and therefore nonspecific product formation. Increasing annealing time should be avoided because very long annealing times normally do not improve yield, but increase spurious priming and thus greater amounts of nonspecific PCR products (Alkami Quick Guide™, 1999).

Table 3.2 Annealing temperatures screened for each microsatellite primer loci

Number	Locus name	Annealing Temp. (^{0}C)	T_m of Fw / Rw primer
1	ADL23	45, **50**, 53, 55, 60, 62	52 / 47
2	MCW87	55, **57**, 60, 62	62 / 62
3	MCW204	55, **60**, 62	64 / 62
4	LEI73	48, 50, **55**, 58, 60	60 / 60
5	ADL37	45, 49, **52**	58 / 56
6	HUJ1	52, 55, **57**	66 / 62
7	HUJ2	50, 52, **55**, 60	62 / 60
8	HUJ7	50, 52, 55, **57**	52 / 60
9	LEI92	53, 55, **57**	60 / 64
10	LEI126	52, **55**, 57	60 / 60
11	MCW305	45, **50**, 55	52 / 49
12	LEI80	**45**, 50, 55, 60	45 / 49
13	LEI136	**45**, 50, 55, 60	49 / 45
14	HUJ12	**55**, 60	60 / 60
15	ADL102	47, 52, **57**	50 / 64
16	ADL136	45, 52, **55**	60 / 62
17	ADL158	47, **52**, 58	58 / 60
18	ADL171	46, **49**	52 / 56
19	ADL172	45, 49, **52**	60 / 50
20	ADL176	52, **55**, 57	60 / 60
21	ADL181	45, 48, **51**	54 / 58
22	ADL210	46, **51**	58 / 54
23	ADL267	48,50, **55**, 60	58 / 60

Remark T_m= melting temperature, F_w = forward primer, R_w = reverse primer

Bold number: first annealing temperature of screening

3.9.2.4 Magnesium concentration

The Mg^{2+} ion is an important cofactor in enzymatic catalysis of the synthesis reaction. Free Mg^{2+} ions should exceed that of total dNTP concentration. The main source of phosphate groups in a reaction is the dNTPs. Any change in their concentration affects the concentration of available Mg^{2+} since Mg^{2+} forms a soluble complex with dNTPs (Hillis, 1996). Varying Mg^{2+} concentration has been a popular method of tinkering with PCR reaction conditions. The 1.5, 2.0, 2.5 and 3.0 mM of $MgCl_2$ were added to the PCR reaction for finding maximum product yield. Generally, 1.5 mM $MgCl_2$ was added to most reactions.

3.9.2.5 Primer concentration

A reduction in primer and dNTP concentration can result in a dramatic improvement of specificity. High concentrations can encourage nonspecific annealing and non specific-product formation as well as formation of primer dimers where primers anneal to themselves and not to the template.

The 1.0, 2.0 and 3.0 μM of forward and reverse primers were tested for PCR amplification of each microsatellite locus and a total volume of each reaction was 25 μl.

3.9.2.6 dNTPs concentration

For a large template sequence, increasing the amount of dNTPs can improve efficiency of PCR amplification. However, low dNTPs concentration can improve PCR fidelity when the Mg^{2+} concentration is high in an equimolar proportion. The high dNTPs concentration can promote misincorporation PCR products by appearing thermodynamic infidelity (Attippahakun, 1996).

The 0.1 and 0.2 mM dNTPs were added in reaction mixtures and yields of PCR products from both dNTPs concentrations were compared.

3.9.3 Touchdown PCR

After the components PCR variation finished, some locus appeared non-specific bands in optimal annealing temperature and could not get rid of them by increasing annealing temperature or decreasing magnesium concentration. Thus, touchdown PCR technique was used for reducing non-specific PCR product in some locus that could not use for normal adjustment.

Standard touchdown PCR: first an annealing process took place at approximately 10^0C above the calculated T_m. During the following cycles, the annealing temperature was gradually reduced by 1-3^0C until reaching a level of approximately 5^0C below T_m (Alkami, 1999).

Moreover, in this study touchdown PCR technique was used to amplify difficult microsatellite loci. Many microsatellite loci could not amplify in normal PCR technique so they were try to amplify in other PCR technique. Because annealing temperature of touchdown PCR in this study were adjusted in wide range between T_m+10 ^{0}C to T_m-12 ^{0}C. Some pale PCR product bands from touchdown PCR may occur in some microsatellite loci. If PCR product disappeared, it is to make sure that particular microsatellite loci could not amplified in *P. m. imperator*.

An example locus of touchdown PCR was LEI 73.

For example, LEI 73 calculated T_m : 60 ^{0}C

The temperature cycling was as following.

The predenaturation at 94 ^{0}C for 3 minutes

Cycle 1-2	The denaturation	at	94^{0}C	for 30	seconds
	The annealing	at	70^{0}C	for 45	seconds
	The extension	at	72^{0}C	for 60	seconds
Cycle 3-4	The denaturation	at	94^{0}C	for 30	seconds
	The annealing	at	67^{0}C	for 45	seconds
	The extension	at	72^{0}C	for 60	seconds

Cycle 5-18 The annealing at 64^{0}C, 61^{0}C, 58^{0}C, 55^{0}C, 52^{0}C, 50^{0}C and 48^{0}C for 45 seconds, respectively. The denaturation and extension were at the same temperature and time.

Cycle 19-35	The denaturation	at	94 ^{0}C	for 30	seconds
	The annealing	at	55 ^{0}C	for 45	seconds
	The extension	at	72 ^{0}C	for 60	seconds

The final extension at 72 ^{0}C for 10 minutes

3.9.4 Eight percents denaturing polyacrylamide gel electrophoresis (8% denat-PAGE)

All PCR products were detected with 8% denaturing polyacrylamide gel electrophoresis technique. Glass plates (Mini VE, Hoefer, Pharmacia biotech) were used to running electrophoresis. Plates cleaning steps before electrophoresis was first washed with water, cleaned with 70% ethanol, rinsed with distilled water and wiped with Kimwiped® until dry. The plates were siliconised with Sigmacote® and air-dried. Four microlitres of samples were mixed with 2 µl of 6x loading dye (0.03% bromophenol blue, 0.03% xylene cyanol FF, 0.4% orange G, 10 mM Tris-HCl(pH 7.5), 50 mM EDTA and 15% ficoll® 400).

A standard marker used in this electrophoresis was PhiX174-λ DNA digested with *Hinf* I. The gel electrophoresis was run at constant 100 volt. Protocol of gel setting and loading was shown in Appendix III.

3.9.5 Developing PCR products by Silver staining method

Traditionally, a microsatellite analysis was performed based on radioactive detection of allelic patterns (PCR products). Despite its high sensitivity, radioactive detection requires special care of hazardous radioisotope. Alternatively, a silver staining detection method offers a non-hazardous and simple method for microsatellite analysis (Schlotterer, 1998) When an electrophoresis was finished, the power supply was turned off and the gel was a silver staining for visualization of PCR products. Reagents and the preparation protocol were shown in Appendix IV and V.

3.9.6 Preparation of PCR products for sequencing

Some microsatellite PCR products from positive loci in *P. m. imperator* were sequenced by Macrogen Inc., Korea and Bioservice unit, Thailand, after they were purified by QIAquick gel extraction kit. Preparation protocol of PCR products for sequencing is in the following paragraph.

Five hundred microlitres of PCR mixtures were amplified with the suitable condition for each locus. PCR products were analyzed by electrophoresed through 4.5% MetaPhor agarose gel at 60 volts about 8 hours, or until Xylene Cyanol FF (in 6x loading dye) moved to approximately 0.5 cm from the bottom of gel. The length of tray box was 15 cm. MetaPhor agarose gels (2% to 4%) can give an approximate the resolution of polyacrylamide gels (4% to 8%). The MetaPhor preparation protocol was shown in Appendix IV. Five hundred microlitres of PCR products were mixed with 100 µl of 6x loading dye in 1.5 microcentrifuge tube. The PhiX 174/*Hinf* I digested DNA merker or 100 bp DNA ladders were used as standard DNA markers. After electrophoresis, the marker lane was excised from the gel and stained with ethidium bromide solution and visualized under UV light. The distances of the DNA fragments size 50 to 500 bp were marked on the gel. After that, marker-lane gel and sample gel were rejoined and then the sample gel with estimated 50 to 500 bp PCR product size was excised. The DNA fragments were eluted from the gel using QIAquick gel extraction kit (QIAGEN) as follows. The gel was chopped into small pieces and transferred into a 1.5 ml microcentrifuge tube (about 0.4 g for each tube). The gel in each tube was dissolved by 400 µl of buffer QG at 50°C. After completely dissolved, 400 µl of isopropanol was added to the tube. The mixture was applied into QIAquick spin column to bind DNA with the column membrane. The columns were centrifuged

for 1 minute at 12,000 rpm. To eliminate all traces of agarose, the columns were added 500 µl of buffer QG and centrifuged again. After that, the columns were washed with 750 µl of buffer PE and centrifuged twice to remove all washed solution. Finally, the columns were added with 7 µl of TE (pH 8.5) and 23 µl of distilled water or 30 µl of distilled water and stood for 1 minute and then centrifuged to collect the eluted DNA.

The extracted DNA yield was estimated by an electrophoresis of the sample with 100 bp ladder markers in 1.5% agarose gel at 100 volts for 1 hour.

3.9.7 Applications of some microsatellite primer loci

Many green peafowls from Khao Soi Dao wildlife research and breeding station at Chantaburi province have been found delibitated, possibly by inclement weather and other conditions in the station. Although cleanness of green peafowl cages and good nutrition for chicks have been carefully maintained by breeders at the station, they usually can not grow up with full speed ahead and some chicks were died by unknown reason.

Blood samples were collected from many birds in the station (Appendix I) and genomic DNA was extracted by QIAamp® kit method (Appendix I). After that, microsatellite primers for the ADL 23 locus and HUJ 2 locus were selected to investigate polymorphism in *P. m. imperator* population.

3.10 RAPD analysis

Under carefully controlled conditions of RAPD, two factors are likely to explain most of the reproducible difficulty, usually found reactions in RAPD experiments. Generation of reproducible and comparable banding patterns is very dependent on template quality and reaction conditions (Hoelzel, 1998).

3.10.1 *Taq* polymerase in RAPD-PCR

Taq polymerase in buffer B from Promega Corporation was compared with DyNAzyme™ II DNA polymerase from Finnzymes. Because both *Taq* polymerase enzymes came from different strains of bacteria and storages in different buffers that could affect the yield of RAPD-PCR.

3.10.2 Source of DNA template in RAPD-PCR

DNA templates from blood and feather sample were amplified by using primer number 6, gaving one pattern of RAPD-PCR products in all templates of blood sample from *P. m. imperator.*

The patterns of RAPD-PCR bands amplified from blood and feather extracted templates were compared.

3.10.3 Template concentrations for all primers

The 10, 50, 100, 150 and 300 ng of DNA templates were added to the PCR reactions to find appropriate template concentrations for generating RAPD-PCR bands.

3.10.4 Amplification conditions

Sixty decanucleotide primers purchased from The Biotechnology Laboratory, University of British Columbia (Canada) were screened. (sequence data of primers were shown in Appendix VI). The amplification reactions were performed in a 15 µl reaction volume containing 10 mM Tri-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% TritonX-100, 200 µM each of dATP, dCTP, dGTP, dTTP, 0.2 µM of the primers, 50 mM KCl, 50 ng of genomic DNA and 0.15 unit of DyNAzyme™ II DNA Polymerase. The reaction mixture was performed in a DNA Thermal Cyclic (Perkin Elmer Model 2400) programmed for 40 cycles consisting of 94^0C predenaturation for 3 minutes, a 94^0C denaturation for 1 minute, a 37^0C annealing for 1 minute and a 72°C extension for 2 minutes. The final extension was carried out at 72 ^{0}C for 5 minutes (Surin, 2002).

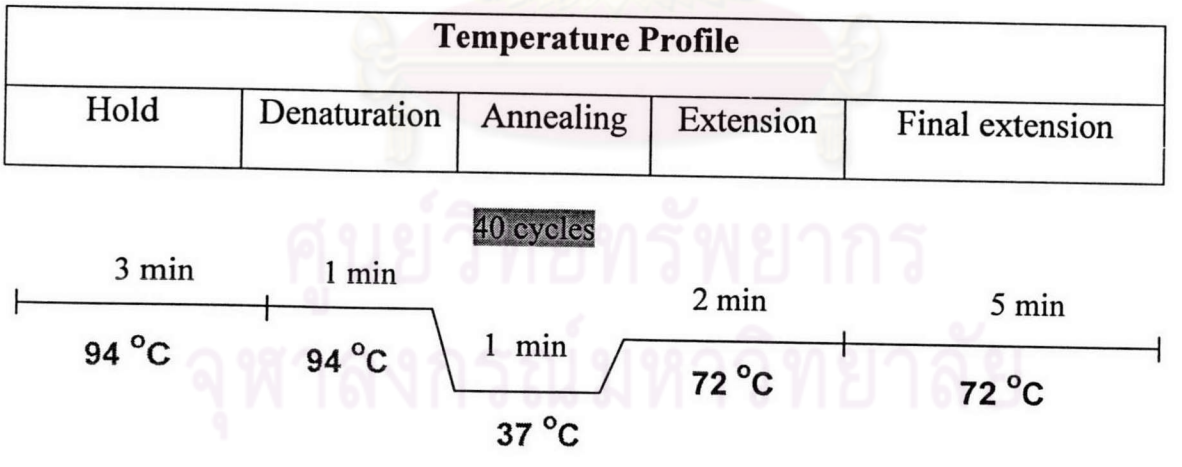

Figure 3.5 Temperature profile for all RAPD primers

3.10.5 RAPD-PCR products

RAPD products were analyzed with an electrophoresis through 1.5% agarose gel. The protocol was shown in Appendix IV. The amount of PCR products were mixed with 6x loading dye by ratio 5:1; that is, 10 microlitres of PCR products were mixed with 2 µl of 6x loading dye (0.03% bromophenol blue, 0.03% xylene cyanol FF, 0.4% orange G, 10 mM Tris-HCl (pH 7.5), 50 mM EDTA and 15% ficoll® 400). The 100 bp DNA ladders were used as standard DNA markers.

3.10.6 Selection RAPD primers

The selected RAPD primers should give 8-10 different-sized fragments of PCR products (Joan and Stephen, 1996). The primers gave high enough polymorphism and a constant pattern was chosen for further study in *P. m. imperator* population.