Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Equipment

- Disposable syringe 1.0 ml
- Whatman® filter paper
- Autoclave (19 & 29 series, Combraco Industrice Inc., Mass. U.K.)
- Water bath (Uni-Bath model RU-2,Sakura Finetecnical co.Ltd., Tokyo Japan)
- Microcentrifuge tube 0.5, 1.5 ml
- Surgical knife, scissors and forceps
- Centrifuge 5410 (eppendof)
- MS1 minishaker (IKA-works Inc, USA)
- Automatic Micropipette P10, P20, P200, and P1000 (Gilsen Medical Electronic S.A, France)
- Pipette tip P10,P200, P1000
- Spectrophotometer DU 650 (Beckman, U.S.A)
- Laminar flow Hood with UV light
- PCR Thermal cycler : Omnigene (Hybriad Limited, U.K)
- Vertical sequencing gel electrophoresis apparatus (Bio-RAD Laboratories, U.S.A.)
- Power supply (Power pac 3000 - Bio-RAD Laboratories, U.S.A)
- Gel dryer (Model 583 Bio-Rad Laboratories, U.S.A)
- X-ray film (AGFA CURIX, AGFA -GEVERT S.A.)
- Autoradiography cassette (Kodak X-Omatic cassette, Eastman Kodak company, U.S.A.)
- A-20^{0}C Freezer
- pH meter
- Bunsen burner

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3.1.2 Chemicals

- Chelex ® 100 (Bio-RAD Laboratories, U.S.A)
- Sequenase ™ PCR Product sequencing kit (Amercham Life Science USB, U.S.A)
- 100 mM of dATP, dCTP, dGTP, dTTP (Promega corporation,U.S.A.)
- Tris-(hydroxy methyl) aminomethane (Phamacia Biotech, Sweden)
- Boric acid (Merck, Germany)
- Ethylene diamine tetraacetic acid, disodium salt (Bio-RAD Laboratories, U.S.A.)
- Urea (Promega corporation, U.S.A)
- N, N-methylene-bis-acrylamide (Promega corporation, U.S.A)
- Amnonim persulfate (Promega corporation, U.S.A)
- N, N, N', N' -tetramethylethylene diamine (Promega corporation, U.S.A)
- Developer G150 (AGFA -GEVERT S.A)
- Fixer G350 (AGFA -GEVERT S.A)

3.1.3 Radioactive

- [γ- ^{32}P] dATP specific activity 3000 Ci /mmol (Amersham Intemation, U.S.A)

3.1.4 Enzymes

- Proteinase K
- T_4 polynucleotide kinase (Promega corporation, U.S.A)
- Taq DNA polymerase (Promega corporation, U.S.A)

3.2 Methods

3.2.1 Tissue samples collection

The Red junglefowls were wild caught from Chumporn provinces (n = 11) for sourthern wild population. Wild caught fowl from Phayuo (n = 3) and Prae (n = 1) provinces as well as captive fowl at the Phu Khieo Wlidlife breeding station in Chiayaphum province

(n = 5) were used as representation from the northern population. Blood was collected by radial venipunction from branchial vien, using Tuberculin syringe with needle (no.25). An amount 0.1-0.2 ml of blood was dropped on Whatman® filter paper. Air dried blood stained paper was place in a labelled plastic bag. Feathers of each blood sampled fowl was plucked and placed in the same labelled plastic bag. Both blood samples and feathers were kept in dessicator.

3.2.2 DNA preparation

(a) Chelex® 100 DNA Extraction

New method which is much simpler, cheapter and involves far fewer opportunities for contaminations than conventional alcohol/chloroform extraction was used to extract DNA from a small number of cell. DNA was either extracted from blood stain or from feather. Protocol for Chelex-based extraction from whole blood/blood stains was described previously (Singer-Sam et al., 1989; Walsh et al., 1991) was use as follows:

1. pipet 1 ml of sterile distrilled water into a sterile 1.5 mL microcentrifuge tube. Add a portion of blood stain about 3-mm square and mix gently.
2. Incubate at room temperature for 15-30 minute. Mix occsionally by inversion or gentle vortexing.
3. Spin in microcentrifuge for 2-3 min at 10,000-15,000x *g*
4. Carefully remove supematant(all but 20-30 µl) and discard. Leave the filter paper in the tube with the pellet.
5. Add 5% Chelex® 100 to a final volume of 200 µl
6. Incubate at 56°C for 15- 30 min.
7. Vortex at high speed for 5-10 s.
8. Incubate in boiling water bath for 8 min.
9. Vortex at high speed for 5-10 s.
10. Spin in microcentrifuge for 2-3min at 10,000-15,000x*g*
11. Tranfer supernatant to a steriller 0.5 microcentrifuge tube.The sample is now ready for PCR amplification. Store the sample at 2° -3°C or frozen untill require

For extracting genomic DNA from Feather, protocol modified from Sanger-Sam et al. (1989) and Walsh et al.(1991) with added proteinase K (Ellegren, 1992) was use as follows:

1. Handling feather with clean forceps, wash the calamus of feather to reduce surface dirt and contaminant by sterile distrilled water and 70% ethanol.
2. Use a sterile blade to cut bilaterally and then cut off a 5 mm portion from promial end on a clean piece of white paper.
3. Transfer the proximal portion of feather to 200 µl of 5% Chelex ® 100 in a sterile 1.5 ml microcentrifuge tube.
4. Add 1.5 µl of 10 mg/mL protein K and mix genthy.
5. Incubate at 56°C for at least 4 hrs., and an additional portion of protein K is add one half way through the incubation
6. Vortex at high speed for 5-10 s.
7. Incubate in boiling water bath for 8 min.
8. Vortex at high speed for 5-10 s.
9. Spin in microcentrifuge for 2-3min at 10,000-15,000x*g*
10. Tranfer supernatant to a steriller 0.5 microcentrifuge tube.The sample is now ready for PCR amplification. Store the sample at 2° -3°C or frozen untill require.

(b) Measurement of DNA Concentration

The purify and concentration of extracted DNA was measured by UV absorbance with a spectrophotometer. Double strand DNA sample in concentration of 50 ng/µl have absorbance of 1.0 at 260 nanometer. The purity can be judged by examing the ratio of absorbance at 260 nanometer and 280 nanometer (Aquadra et al., 1992). Pure DNA have ratios of approximately 1.8 whereas RNA or protein contamination have much hiher or lower respectively (Kirby,1992). For use in PCR, the DNA in samples was diluted into of 10 ng/µl.

3.2.3 *In vitro* Amplification of Microsatellite DNA using the Polymerase Chain Reaction (PCR)

(a) Selection of Polymerase Chain Reaction Primers

Since the previous studies using mitochrondrial displacement loop (D-loop) indicated that the Red Junglefowls were closely related to domestic fowl(Fumihito et al, 1994; fumihito et al, 1995 Fumihito et al, 1996). The aviable microsatellite - flanking PCR primers developed for domestic chicken were chosen for studies of polymorphism in the Red Junglefowl.Considerately,ten oligonucleotide primer pairs which amplified a highest allele number and polymorphism in Compton reference family (a backcross base on a cross between outbred (N) and inbred (I_5) White Leghorn line -Bromstead and Palyga, 1992), East Lansing reference family (a cross between a single male of Red Junglefowl origin and four females of a highly inbred White Leghorn line - Crittenden et al. 1993), and Wageningen resuorce population (a cross between two diverse broiler line - Crooijmans et al, 1997) were selected from three main groups (ADL, HUJ LEI) of chicken microosatellite markers. The (TG)n dinucleotide repeat marker, ADL37, developed within the laboratory of Hans Cheng (Cheng and Critteden, 1994; Cheng et al. 1995) and later modified by Crooijmans et al. (1997).The same repeat marker, HUJ1, HUJ2, HUJ7 were designed by Khatib et al (1993) whereas LEI73, LEI92 were designed by Gibbs et al. (1997). The characteristic of all selected primers were listed in table 3-1.

Table 3-1. Characteristic of selected chicken microsatellite-flanking PCR primers.

Locus	Forward primer	Reverse primer	Ta*	L.(bp)#
HUJ1	CCATCCGCTTATACAGAGCACA	CCCTTTGTTAACACCTACTGCA	55	151-180
HUJ2	CATCTCACAGAGCAGCAGTG	GAATCCTGGTGTCAAAGCC	60	124-142
HUJ7	CATAAACTAAAGTCTCAACAC	TTCTTCCACACACATCTTGCTA	55	152-156
ADL37	ATGCCCCAAATCTCAACTCT	TCTCTAAAATCCAGCCCTAA	55	164$^{\pi}$
LEI73	CCATATCATTTGTCAAGCACC	AATTCCTGACCTCCATGATAC	55	163-221
LEI92	GATCTACATTTGTGCAGTGTC	TCCTTGGTCTGACTCTCCATG	55	164-212

* Optimized annealing temperature

\# PCR product size

$^{\pi}$ Mean PCR product size

(b) 5'-End - labelling with T4 Polynucleotide Kinase

The forward primer for each microsatellite loci was end - lbelled by using T4 polynucleotide kinase (PNK) as an enzyme to incorporate [γ- ^{32}P] ATP into the primer .The protocol was to combine:

- primer (10 μM) (approximately 30 ρmol	3 μl
- 10x T4-PNK-buffer(Promega)	2 μl
- T4- PNK (10 U/μl)	2 μl
- [γ- ^{32}P] ATP (3000 Ci /mmol)	4 μl
- sterile deionized distrilled water	9 μl
total volume	20 μl.

This reaction mix was incubated at 37 °C for 10 minutes and then inactivated at 100 °C for approximately 2 minutes. The volume of 3.3 μl was used in PCR mixture.

(c) Polymerase Chain Reaction

Each of the microsatellite loci was singly amplified from genomic DNA in the Chelex ® 100 extract supernatant along with negative control using water instead of supernatant.The PCR mixture for total 25 μl reaction was combined as follow :

- sterile deionized distrilled water	10 μl
- 10x Taq buffer($MgCl_2$ 15 mM)	4 μl
- dNTP mixture(10 mM)	2 μl
- end labelled forward primer	3.3 μl
- reverse primer	0.5 μl
- Taq polymerase (5U/μl)	0.2 μl
- Template DNA (10 ng/μl)	5 μl
total volume	25 μl.

One drop of RNase-and DNase free mineral oil was overlaid on the reaction mixture and then breifly spin in microcentrifuge before incubated in Thermal cycler (Omnigene, Hybriad). The amplification program consists of 94 °C denaturation for 3 minutes followed by thirty-five cycle of denaturation step at 94 °C for 1 minute,primer annealing step at primer-specific annealing tempurature for 1 minute, and an extension step at 72 °C for 1 minute, then complete extension at final cycle of 94 °C, 2 minutes, annealing temperature, 2 minutes, 72 °C for 10 minutes. The primer-specific anealing tempuratue was approximately 55 °- 60 °C depending on each primer sequence and each sample.

3.8 Size Fractionation of Amplfied Microsatellite Alleles using Denaturation Polyacrylamide Gel Electrophoresis

After the primer-radiolabelled amplification was complete, each reaction tube was thoroughly mixed with 5 μl of formamide dye mix solution (95% formamide, 20 mM EDTA, 0.05% Bromphenol Blue, 0.05% Xylene cyanol FF). The mixture were denatured by heating at 95 °C for 3 minutes and immidiately snap cooled on ice. .After loading 5 μl of denatured mixture onto a 6 % polyacrylamide denaturing gel prepared in 10x TBE, electrophoresis was run at 2000 V for approximately three hours. Dried gel were exposed to X- ray film for 2-3 hours. After film developing, electrophoretic allele pattern were scored.

3.9 Data analysis

The genotype of each *G. g. spadiceus* individual at each locus was scored from an electrophoretically observed pattern which could be divided into homozygotic or heterozygotic states. Based on the fact that additional (stutter) bands were commonly observed in dinucleotide microsatellite, scoring of a particular band can be unambiguously carried out by making an assumption that an actual band of given allele was the most intense band compared to the neighbor group of stuttr bands. The smallest size of band was designed as allele no.1 and subsequently bigger band were disigned as allele no. 2, 3, 4, ..., respectively. Allelic states of each single individual was recorded to be either homo and heterozygote for each locus.

3.9.1 Allelic frequencies

The frequency of a given allele in population for diploid organisms can be estimated as :

$$\bar{p} = \frac{2N_{AA} + N_{Aa}}{2N}$$

Where N_{AA} and N_{Aa} are number of homo and heterozygote for such an allele and N is number of investigated individual.

3.9.2 Hardy-Weinberg equilibrium

Once allelic and genotypic froquencies of diploid *G. g. spadiceus* have been estimated, each investigated population were examined against Hardy-Weinberg (H-W) equilibrium for each locus. Theoretically, observed genotypic frequencies are concordant to Hardy-Weinberg rxpertation when there are no significantly disturbing force such as selection, mutation or migration changing allele frequencies over time and mating is actually occurred at random in a large population.

Basically, H-W distribution was test for each locus in each population using the Chi-square for goodness of fit as follow:

$$\chi^2 = \sum \frac{(O-E)^2}{E}$$

Where O and E represent observed and expected genotypic frequencies, Respectively. Practically, Hardy-Weinberg conformation in this study was calculated using a "Markov chain approach for the exact test" implemented in GENEPOP version 1.2 (Raymon and Rousset, 1995).

3.9.3 Geographic heterogeneity analysis

Determination of significant difference in allelic frequencies between *G. g. spadiceus* from northern and southern locations was carried out using the exact test of genic differentiation of GENEPOP version 1.2. Results are expressed as the probability of homogeneity between two population. Probability of smaller than 0.05 are significant difference in confidence interval at 95 percent.