Chapter III

Methodology

3.1 Material

Equipments

- Dessicator
- Autoclave
- Water bath (Uni-Bath model RU-2, Sakura Finetechnical co. Ltd., Japan)
- Microcentrifuge tube 0.5 and 1.0 ml. (Treff® Switzerland)
- Centrifuge models 5410 (Eppendorf)
- MS1 minishaker (IKA-works Inc., USA.)
- Automatic Micropipette P10, P20, P200 and P1000 (Gilsen Medical Electronic S.A., France)
- Micropipette tip P10, P20, P200 and P1000 (Treff® Switzerland)
- GENESYS 6 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA.)
- Laminar flow hood UV light (model DFL 120, Thai Interfil co. Ltd., TH.)
- 2400 GeneAmp PCR System Thermal Cycler (Perkin-Elmer, USA.)
- Vertical sequencing gel electrophoresis apparatus (Cleaver Scientific Ltd, UK.)
- Power supply (power pac 3000 - Bio-RAD Laboratory, USA.)
- -20°C Freezer (Sony co. Ltd., Japan)
- pH meter SP-7 (Sun Tex Digital pH meter)
- Pyro magnestir (Lab-Line Instrument, Inc.)
- Watman® Laboratory sealing film
- Electronic clock timer Model CT-30 (Canon co. Ltd., Japan)

Chemicals

- Absolute Ethanol (Merck, Germany)
- Sodium acetate (Merck, Germany)

- QIAamp® DNA Mini Kit (QIAGEN, USA.)
- 100 mM. dATP, dGTP, dCTP, dTTP (Promega corporation, USA.)
- Tris-(hydroxyl methyl) aminomethan (Phamasia Biotech,USA.)
- Boric acid (Bio-RAD Laboratory, USA.)
- EDTA (Bio-RAD Laboratory, USA.)
- Urea (Promega corporation, USA.)
- N, N-methylene-bis-acrylamide (Promega corporation, USA.)
- APS (Promega corporation, USA.)
- TEMED (Promega corporation, USA. and Amesco)
- Loading dye (Promega corporation, USA.)
- Methanol (Merck, Germany)
- Nitric acid (J.T. Baker, USA.)
- Silver nitrate (Nacalai Tesque, Japan)
- Sodium carbonate (Merck, Germany)
- Formaldehyde (Merck, Germany)
- Glacial acetic acid (J.T. Baker, USA.)
- Glass Free™ (National Diagnostics, USA.)

Enzymes

- Taq DNA polymerase (Promega corporation, USA.)

3.2 Specimen used in the study

There were two varieties of Green Peafowl, *Pavo muticus*, were collected from different sources, as follow:

1. Sample number 1A – 25A were Green Peafowl, *Pavo muticus* specimens from the natural history museum of Chulalongkorn university that were collected from northern part of Thailand. (Huai Hong Krai Royal Development Study Center, Chiang Mai Province).

2. Sample number 1B - 25B were Green Peafowl, *Pavo muticus* specimens from the natural history museum of Chulalongkorn university that were collected from western part of Thailand. (Huai Kha Khaeng Wildlife Sanctuary, Uthai Thani Province and the northern part of Srinakharindra Dam, Kanchanaburi Province).

3. Sample number 1C – 12C were Red Jungle Fowl, *Gallus gallus spadiceus* specimens from the natural history museum of Chulalongkorn university that were collected from western part of Thailand. (Huai Kha Khaeng Wildlife Sanctuary, Uthai Thani province) for outgroup.

3.3 Method

3.3.1 Sample collection and preservation

Twenty five Green Peafowl feather specimens from the natural history museum of Chulalongkorn University were collected from each area; northern part of Thailand (Huai Hong Krai Royal Development Study Center, Chiang Mai Province) and western part of Thailand (Huai Kha Khaeng Wildlife Sanctuary, Uthai Thani Province and the northern area of Srinakharindra Dam, Kanchanaburi province). The feathers were cleaned by 70% ethanol, dried and cut at its tip about 1-2 centimeters and placed into a plastic bag, labeled the number of specimens to use in laboratory and storage in the dessicator.

3.3.2 Extraction of nuclear DNA from each feather.

Nuclear DNA was extracted from the feather tip using QIAamp® DNA Mini Kit (by dried blood spot protocol). The DNA extraction protocol is in Appendix I.

3.3.3 Determination concentration and quality of the isolated DNA samples

Measurement and calculation of DNA concentration

Concentration of extracted DNA was measured and estimated by UV absorption of spectrophotometer. Extracted DNA solution was absorbed by UV at wave length 260 nm (OD_{260}) (Brown, 2002). An OD_{260} of 1.0 corresponded to a concentration of 50 ng/μL double strand DNA.

Therefore, DNA sample concentration was calculated by the assumption:

DNA concentration (μg/mL) = OD_{260} x dilution faction x 50

Determination quality of genomic DNA

1% Agarose gel was medium for the electrophoresis separation of DNA. It carried out for visualization of the quality of isolated DNA samples. Extracted genomic DNA was loaded into the gel. DNA was stained and detected by 0.25 μg/ml Ethidium bromide, and could be visualized under UV absorption (Brown, 2002). In addition, PhiX 174 Hinf I digested was loaded into the gel and served as a DNA standard marker for size estimation. Protocol of this method is shown in Appendix II.

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

Selection of polymerase chain reaction primer

Suitable primers should have similar length and similar melting temperatures, but should not be complementary. In addition, the product should be 100-300 bp in length, and should not be annealed to one another or form hairpin loops (Ferraris and Palumbi, 1966). In addition, products of primer should be easy to score, which they are tested for the presence of correctly size product. If PCR fails to the yield of product at one of temperature, it should be repeated at higher and lower temperature (Arnheim, White and Rainey, 1990).

Eight pairs of oligonucleotide primers were chosen from several publications. The first HUJ002 microsatellite locus was chosen from Wutthivikaikan, 2003 which were used chicken derived microsatellite DNA to study in Green Peafowl population. The other microsatellite loci: LEI166, MCW080, MCW098, MCW034, MCW069, MCW295 and MCW330 which were selected from the studying in screening of Peafowl microsatellite primers and analysis of genetic diversity (Bao *et al.*, 2006). All primers were from Bioservice Unit: BSU, Bangkok. The characteristics of all selected primer are shown in table 3.1

Table 3.1 Characteristics of 8 selected Green Peafowl microsatellite PCR primers.

Locos name	Forward sequence/ Reward sequence	Length (bp)	Tm (°C)	Product size (bp)
HUJ002	5' CATCTCACAGAGCAGCAGTG 3'	20	55	124-142
	5' GAATCCTGGATGTCAAAGCC 3'	20		
LEI166	5' CTCCTGCCCTTAGCTACGCA 3'	20	55	254-267
	5' TATCCCCTGGCTGGGAGTTT 3'	20		
MCW034	5' TGCACGCACTTACATACTTAGAGA 3'	24	50	223-245
	5' TGTCCTTCCAAATTACATTCATGGG 3'	25		
MCW069	5' GCACTCGAGAAAACTTCCTGCG 3'	22	55	159-168
	5' ATTGCTTCAGCAAGCATGGGAGGA 3'	24		
MCW080	5' GAAATGGTACAGTGCAGTTGG 3'	21	55	272-282
	5' CCGTGCATTCTTAATTGACAG 3'	21		
MCW098	5' GGCTGCTTTGTGCTCTTCTCG 3'	21	55	260-262
	5' CGATGGTCGTAATTCTCACGT 3'	21		
MCW295	5' ATCACTACAGAACACCCTCTC 3'	21	55	94-107
	5' TATGTATGCACGCAGATATCC 3'	21		
MCW330	5' TGGACCTCATCAGTCTGACAG 3'	21	55	260-290
	5' AATGTTCTCATAGAGTTCCTGC 3'	22		

Polymerase chain reaction

PCR mixture contained 0.2 μM dNTP, 1.5 mM $MgCl_2$, 1.0 unit of Taq DNA

polymerase (Promega), 30-50 ng template of genomic DNA (prepared from feather). The composition of enzyme storage buffer was 20 mM Tris-HCl (pH 7.4 at 25°C), 0.1 mM EDTA, 1 mM DTT, 100 mM KCl, stabilizers, 200 µg/ml BSA and 50% glycerol and a total volume of each reaction was 25 µL. The reaction mixture are shown in Table 3.2

Table 3.2 The optimal annealing temperature and reaction mixture for PCR amplification of Green Peafowl by eight microsatellite loci.

Reaction mixture	Locus							
	HUJ 002	LEI 166	MCW 034	MCW 069	MCW 080	MCW 098	MCW 295	MCW 330
Buffer (X)	1	1	1	1	1	1	1	1
$MgCl_2$ (mM)	1.5	1.5	3.0	3.0	2.5	1.5	3.0	3.0
dNTP (mM)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Forward primer (µM)	0.3	0.1	0.2	0.2	0.3	0.1	0.2	0.1
Reward primer (µM)	0.3	0.1	0.2	0.2	0.3	0.1	0.2	0.1
Taq DNA Polymerase (U.)	1	1	1	1	1	1	1	1
Reaction volume (µL)	25	25	25	25	25	25	25	25
Annealing (°C)	55	53	53	53	53	53	55	53
Allele size (bp)	118, 151	346	230, 234, 236	144, 160	270, 274, 276, 278, 129	240, 250, 258, 274	86, 93	268, 278
Chromosome number	17	5	2	E46C08 W48	15	4	4	17

PCR reaction contained denaturing temperature 94°C for 3 minutes, annealing temperature at 53°C – 55°C for 60 seconds (depending on primer composition on Table

3.1) and extension temperature 72°C for 90 seconds, 35 cycles of PCR reaction, and final extension at 72°C for 10 minutes.

3.3.5 Eight percents denaturing polyacrylamide gel electrophoresis

PCR products were separated by 8% denaturing polyacrylamide gel electrophoresis. The sequencing plates (Cleaver Scientific Ltd.) which were used for running electrophoresis, were washed with water then cleansed with 70% ethanol, rinsed with distilled water and wiped with Kimwipes® until dry.

Standard sequencing gel (8% acrylamide monomer) was prepared for separating PCR products of microsatellite DNA of Green Peafowl, and detected with standard size DNA marker, 100bp+1.5 kb DNA Ladder with stain for estimation allele size.

PCR products were mixed with 6X Blue/Orange Loading Dye contained 0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400, 10mM Tris-HCl pH 7.5 and 50mM EDTA pH 8.0. They were heated at 95°C for 3 minutes and immediately snap-cool on the ice. Before loading the PCR product, pull the comb from the top of the gel and flushed out the well with running buffer. 5 microlitres PCR products and 1 microlitre standard size marker were loaded in to the gel. Electrophoresis was carried out with:

Pre run 200 V constant, 10 W, 48 mA for 15 minutes

Run 200 V constant, 10 W, 48 mA for 3.15 hours (depended on sizes of PCR products), temperature at 40°C

When electrophoresis was completed, the gel was stained with silver staining method as following describe as in the Perkin Elmer Protocol.

3.3.6 Developing PCR product on 8% polyacrylamide gel by silver staining

When electrophoresis was finished, the gel was visualized for PCR products by silver staining. Reagents and preparation protocol are shown in Appendix III and IV.

Bands (alleles) were scored by naked eye and using program Image - Pro Express to analysis bands. It could be recorded either, homozygous and heterozygous for each locus (Ferraris and Palumbi, 1996). In addition, the stutter band was observed, but it was not also much shaper, allowing size discrimination within a much shorter distance on the gel (Luqmani *et al.*, 1997). Pairwise comparison between two varieties was used for band scoring. The standard marker was used when comparing bands between varieties.

3.3.7 Data analysis

Assumption

Genotype of microsatellite DNA was first scored by naked eyes and follows by Image - Pro Express program for all parameter. Heterozygotes were clearly two bands, whereas homozygote showed one major band (Srikwan *et al.*, 1996). Observed of PCR products banding pattern was typically presented the additional or stutter bands beside the microsatellite band differing by 1 or 2 bp. (Mable, Morize and Hillis, 1996). It was resulted from slippage strand mispairing, the multi-repeats permitting slippage of the copied strand on the template, producing fragment with two-nucleotide spacing and failure of the polymerase to read through the repeat (Koreth, O'leary and McGee 1996). Therefore, the most intense and clear band of each allele were detected and compared within and between varieties.

3.3.7.1 Allele frequency

Difference in microsatellite allele frequency between two varieties of Green

Peafowl were assessed using Fisher's exact test in GENEPOP program that on common versus pooled rare allele frequencies (Taylor, Sherwin and Wayne, 1994).

Allele frequency in a population for diploid organism could be estimated as followed (Hedrick, 2004):

$$\text{Allele frequency} = \frac{2NAA+NAa}{2N}$$

Where, N_{AA}, N_{aa} were number of homozygote at allele A and a, and N_{Aa} was number of heterozygote, for such an allele, N was number of investigated individuals.

In addition, allele frequency could be calculated by software GENEPOP, version 3.4 (Raymond and Rousset, 2003). The mean number of alleles per locus (A) was calculated to compare genetic variation between varieties by a one-way ANOVA (Taylor *et al.*, 1994). The effective number of alleles per locus (n_e) was calculated. It was the number of equally frequent alleles in an ideal population that would be required to produce the same homozygosity as in an actual population (Hartl, 2000). For each locus the number of effective allele as followed:

$$n_e = \frac{1}{\sum P_i^2}$$

Where P_i was the i^{th} allele frequency

The unique allele was observed in each locus and all varieties. It was used to search for population-specific markers, when the unique allele had high allele frequency, approximate 0.90 (Wolfus, Garcia and Warren, 1997).

3.3.7.2 Heterozygosity

Heterozygosity was the statistic parameter used to evaluate the informative of a genetic marker for genetic variation. When the variety was in Hardy-Wienberg

equilibrium, heterozygosity could be calculated from heterozygous alleles frequencies at a given locus (Hoelzel, 1998), which was called observed heterozygosity (h_{obs}), by

$$h = 1 - \sum X_i^2$$

Where X_i was the frequency of the i^{th} allele, H was then given as the mean of h overall loci.

Expected from Hardy-Wienberg assumption (h_{exp}) could be calculated and examined using the package GENEPOP, version 3.4 (Raymond and Rousset, 2003).

Generally, mean of observed (H_{obs}) heterozygosity and expected from Hardy-Wienberg assumption (H_{exp}) of overall locus was calculated for comparison of genetic variation between varieties. Difference in H_{obs} and H_{exp} were assessed by a Wilcixon signed rank test (Taylor, Sherwin and Wayne, 1994).

3.3.7.3 Hardy-Wienberg equilibrium

Hardy-Wienberg law was a remarkable theory that was extremely useful in enabling us to understand what happen to gene frequencies and allele frequencies in varieties (Nicholas, 2003). This law assumed that the gene involve were found in an infinite populations of sexually reproducing and random mating diploid organism not affected by selection, mutation, migration and random genetic drift (Majerus, Amos and Hurst, 1996).

Basically, deviation from Hardy-Wienberg assumption was tested using the Chi-square test with polling (Taylor, Sherwin and Wayne, 1994) as followed:

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

Where O and E represent observed and expected genotype frequencies, respectively.

Practically, genotype frequencies were tested against Hardy-Wienberg expectation for each locus and all varieties. The probability of type I error for rejecting null hypothesis (H_o : samples were in Hardy-Wienberg assumption) was estimated using a Markov chain method "approximation to exact test" following the algorithm of Gao and Thompson (1992).

This test was carried out using the GENEPOP computer program (Raymond and Rousset, 2003). A sequential Bonferroni correction method was used to adjust significance levels for multiple test (Lessios, 1992).

3.3.7.4 Genotypic linkage disequilibrium test

The genotypic linkage disequilibrium was defined in terms of two-locus genotypic counts. This parameter was a study of association between alleles at different loci when two or more loci were considered together. The null hypothesis was that genotypes at one locus were independent from genotype at the other locus (Viard *et al.*, 1996). This parameter was tested for each locus and each variety by using program GENEPOP version 3.4. Markov chain method following the algorithm of Gao and Thomson (1992) was used. Significant level was adjusted by a Bonferroni method (Lessios, 1992).

3.3.7.5 Differentiation between varieties test

The statistically significant differences in genotypic frequencies between Green Peafowl from a pair of varieties were tested using the exact test of Genetic Differentiation of GENEPOP version 3.4. Heterogeneity of allele frequency within varieties of Green Peafowl and Red Jungle Fowl were determined by the Fisher test for RxC contingency table. The probability of Type I error (P-value) for rejecting null hypothesis (H_o: no differentiation among varieties) was estimated using a Markov chain method (Raymond and Rousset, 2003). Results were expressed as the probability of homogeneity between

compared varieties. To diminish type I error, level of significant was further adjusted using the Bonferroni method.

3.3.7.6 Genetic distance analysis and phylogenetic reconstruction

Genetic distance was calculated and computational estimate by using GENDIST (Felsenstein, 1993). This was the gene diversity between varieties expressed as a function of genotype frequency. The genetic distance estimated from this method was appropriated for microsatellite data obtained from various taxa whether they had undergone bottleneck events or not (Takezaki et al., 1996). The resulting genetic distance was subject to the phylogenetic reconstruction based on Neighbor-joining (NJ) approach (Saitou and Nei, 1987) using NEIGHBOR. The NJ tree was plotted by DRAWTREE. All computational programs mentioned above were routinely implemented in PHYLIP 3.572c (Felsenstein, 1993).