Chapter 3

Methodology

3.1 Specimen used in this study

Gallus gallus gallus

Samples G1-10, representing the typical subspecies, were collected from wild caught and from F1 birds born from wild parents at the Kao Soi Dao Wildlife Breeding Station, Chantaburi province.

Gallus gallus spadiceus

Samples S83-92 were collected from 10 wild caught and F1 red earlobe Junglefowls (*G. g. spadiceus*) kept at the Phu Khieo Wildlife Breeding Station in Chaiyabhum province, north-eastern Thailand.

3.2 Materials

3.2.1 Instruments

Omni Gene Thermal Cycler PCR	HYBAID, UK
Sequi-Gen^{R} II Nucleic acid Sequencing Cell	BIORAD Laboratory, USA
Automated DNA sequencer ABI^{R} 310	Perkim-Elmer Cetus, USA
Microcentrifuge 12000rpm	Eppendorf

3.2.2 Chemicals

Distilled water	Gibco^{R} BRL
Agarose ultra pure	Gibco^{R} BRL
Polyacrylamide	Promega, USA

Gelmix^{R} polyacrylamide	Gibco^{R} BRL
TRIS	AMRESCO, USA
EDTA	BIORAD Laboratory, USA
Urea	Promega, USA
Chelex^{R} rasin bead	BIORAD Laboratory, USA
Phenol	Sigma
Chloroform	Sigma
Bovine Serum Albumin (BSA)	Sigma
Mineral oil	Sigma
NaOH	Sigma
Boric acid	Sigma
^{32}P radioisotope	
4 dNTPs 40 micromole each	Promega, USA
Taq DNA polymerase (storage buffer A)	Promega, USA
∅X174 Hinf I DNA marker	Promega, USA
Sequenase PCR product sequencing kit	Amersham Life Science, USA

3.3 Methods

3.3.1 Morphometric data collection

All specimens were measured for 5 parameters; the wing length, tarsometatarsus length, head, third digit length and the beak length (Figure 3.1 and 3.2) and weight as well as were identified for their age level and sex.

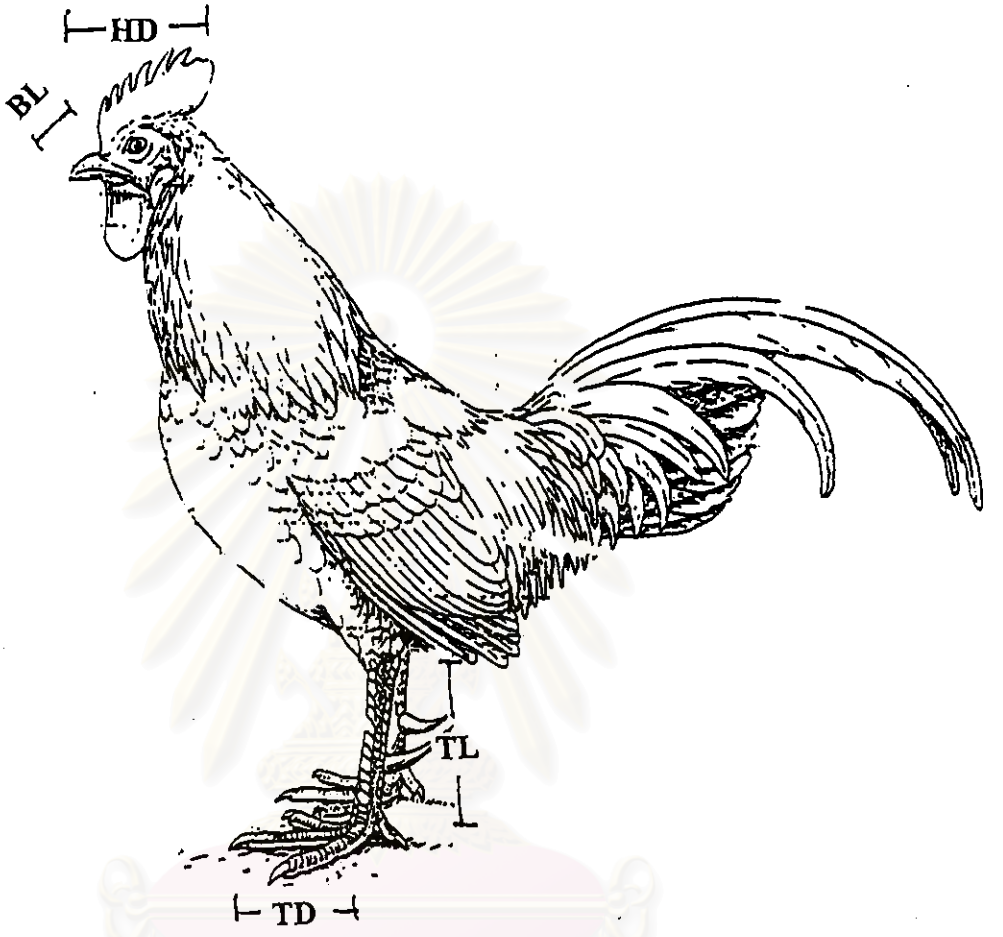

Figure 3.1 Morphometric measurement of basement of upper peak to occipital (HD), beak length (BL), tarsometatarsus length (TL), third digit length (TD)

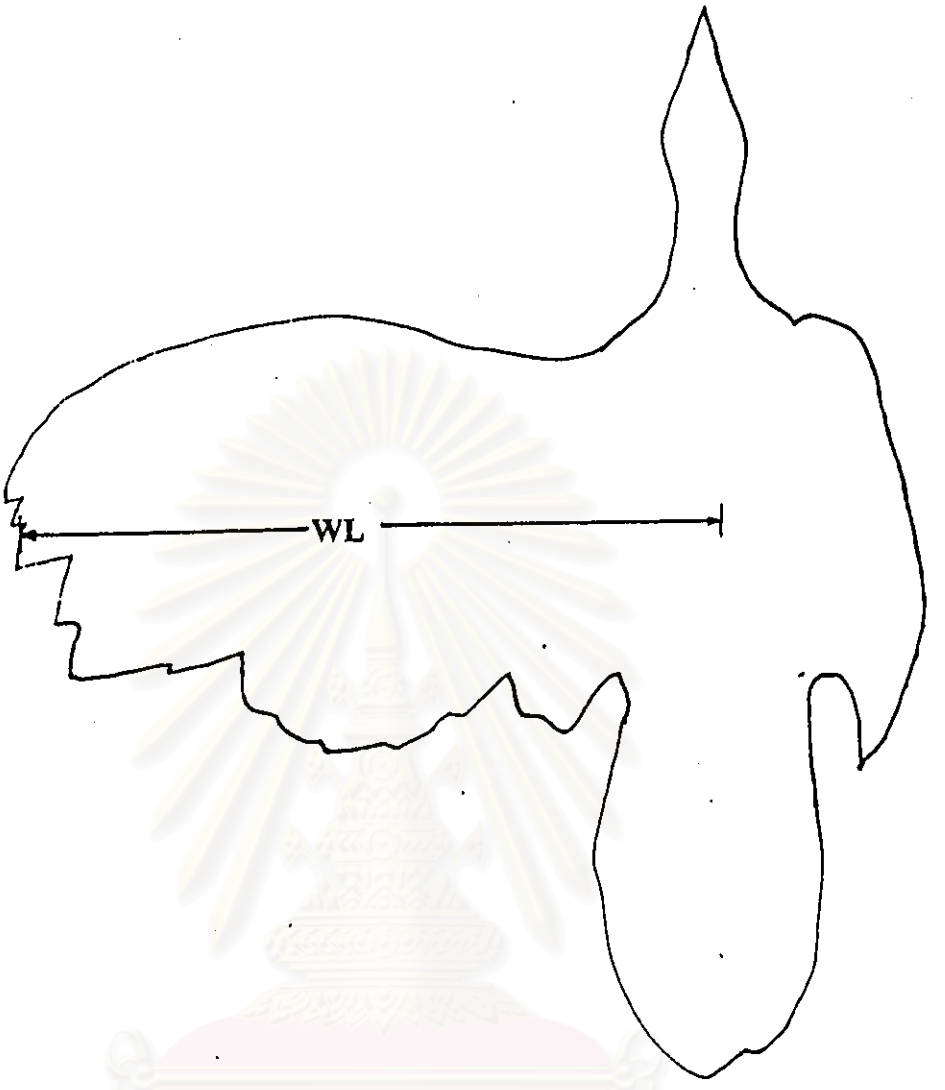

Figure 3.2 Morphometric measurement of wing length (WL) .

3.3.2 Sample collection and preservation

3.3.2.1. Every specimen of caged Red Junglefowl was measured for its wing length (WL), beak length (BL), tarsal length (TL), third digit length (TD) and the beak basement to nape length (HD). Sex, juvenile or adult, as well as weight (in kilograms) were determined for all specimens.

3.3.3.2. Blood was collected by radial venipuncture, using a Tuberculin syringe with needle (guage number 25). An amount of 0.1-0.2 ml was dropped on Whatman^{R} filter paper, air-dried and placed in a labeled plastic bag, then transported to the laboratory and kept in a dessicator.

3.3.3 Extraction of mitochondrial DNA from blood stain

3.3.3.1 Chelex Extraction

The protocol for DNA extraction following Singer-Sam (1989) was used to target mitochondrial DNA via the Chelex^{R} rasin bead method as follows;

- Pipette 1 ml of double distilled water into a sterile 1.5-ml microcentrifuge tubes.
- Cut a small piece of bloodstain (approximately 2mm by 2mm) and drop in the labeled tubes.
- Incubate at room temperature for 20 minutes
- Spin at 15000 rpm for 2 minutes.
- Remove supernatant (leave 20-30 µl) then discard it.
- Add 5% Chelex^{R} to yield a final the volume of 200 µl .
- Incubate at 56 degrees Celsius for 20 minutes.
- Vortex at high speed for 5-10 seconds.

- Incubate in boiling water bath for 8 minutes.
- Vortex at high speed for 5-10 seconds.
- Spin at 15000 rpm for 2 minutes.
- Use 20 µl of the supernatant to add to the PCR mixture.

For the optimization of template DNA quality, an alternative way of DNA extraction was used as follows;

3.3.3.2 Phenol-chloroform extraction.

This protocol start with digesting the blood cell from bloodstain using Proteinase K digestion for mtDNA extraction.

- Pipette 25 µl of 10% SDS to a 1.5-ml microcentrifuge tube.
- Add 500 µl of STE buffer.
- Add 25 µl of Proteinase K (10mg/ml) .
- Add sample (bloodstain on whatman paper).

Then all the samples will be processed in the following way:

- Vortex the incubated sample briefly.
- Add 1:1 Phenol and Chloroform (250 µl each).
- Spin at 12000 rpm for 2 minutes; transport the aqueous phase to a fresh tube.
- Add 500 µl of chloroform
- Concentrate with a microfilter by spinning at 5000 rpm for 3 min.

There is no step for purification of extracted DNA.

3.3.4 Amplification of *gallus* D-loop using Polymerase Chain Reaction (PCR)

After the DNA was extracted from the bloodstain sample, it is necessary to amplify the targeted region so that a visual product can be obtained for the analysis.

3.3.4.1 *Gallus* D-loop primer sequence

PCR primers for D-loop were used following Fumihito et al (1994) ;

Primer1 (L16750: CMD4) 5'AGG ACT ACG GCT TGA AAA GC 3'

Primer2 (H1255: CMD5) 3'CCG TGA CTT CTA CGG TTC TAC 5'

The primer position is referred to that of published chicken mitochondrial sequence (Desjardins and Morais, 1990).

3.3.4.2 DNA amplification using thermal cycler PCR

20 extracted DNA samples were amplified using a Thermal Cycler PCR. The PCR mixture for the total of 50 µl reaction was prepared as follows;

Distilled water	29.75	µl
10X Taq buffer ($MgCl_2$15 mM)	5.0	µl
dNTP mixture (0.8 mM)	4.0	µl
Primer1 (0.4 mM)	0.5	µl
Primer2 (0.4 mM)	0.5	µl
Taq polymerase enzyme (1 unit)	0.2	µl
Template DNA	10.0	µl

The PCR cycle, for the first PCR, was set for the following temperatures and time. The amplification program was set for the pre-heat of the sampletube at 93 gedree Celsius for 3 min. Then the main PCR cycle was set for denaturation of template DNA at 92 degrce Celsius for 1 min., primer annealing at 55 degree Celsius for 1 min. and primer extension at 72 degree Celsius for 1 min. The mail cycle was repeated for 35 times then all the samples will be extended for complete extension at 72 degree Celsius for 10 min. using the thermal cycler PCR (Biorad).

PCR cycle for the second PCR was used in order to generate a single-stranded DNA for using as a sequencing template. An asymmetric

PCR protocol was used by double the amount of PCR reaction mixture from 50 µl to 100 µl and the PCR cycle was reduced from 35 to 30 cycles.

3.3.5 Agarose gel electrophoresis

After thermal cycling, the PCR products were electrophoretically run in a 2% Agarose gel for the confirmation of positive signals. Those signals are obtained and could be seen by staining the gel in ethidium bromide solution. In practice, the ethidium bromide was mixed with the gel while waiting for the polymerization. Positive bands were observed under ultraviolet light.

3.3.6 Purification of PCR product

Those samples that were used for automated sequencing were purified before being added to the sequencing reaction. The Gene Clean BIO 101^{R} purified PCR products were eliminates the small size DNA such as primers and others. The final samples were measured for DNA quantity using spectrophotometer. The purification protocol was used as follows;

- Put 400 µl of GC spin glass milk into the labeled tube containing GC spin filter.
- Add 15 µl of PCR product , mix well.
- Spin at 14000 rpm for 1 min., discard the solution that was trapped at the bottom of the tube.
- Add 500 µl of GC spin new wash, mix the solution and centrifuge 30 sec then discard the supernate.
- Add 300 µl of GC spin new wash, mix the solution gently then centrifuge 30 sec and discard the supernate.
- The pellet was then dried by centrifugation at 14,000 rpm 1 min .
- Remove spin filter to the elution catch tube.

- To elute the DNA from the Glassmilk, add 20 µl of GC spin elution solution and then centrifuge at 14,000 rpm 2 sec the DNA could be collected from the solution at the bottom of the tube.

3.3.7 Analysis nucleotide sequence by acrylamide gelectrophoresis

- Label ddATP, ddCTP, ddGTP and ddTTP on the lid of each tube.

- Add 2.5 µl of ddNTP (Termination) into each tube, take S35 from the -20 degree Celsius and let stand at room temperature.

- The annealing mixture was prepared by adding 4 µl distilled water with 5 µl purified PCR product and 1 µl of primer CMD5 (1 pmol/ µl). Mix the solution gently and centrifuge at 14,000 rpm 30 sec.

- Denature the solution at 100 degree Celsius for 3 min, then immediately place the tubes on ice and incubate for 5 min.

- Prepare the lable mixture by adding 2 µl of sequenase buffer (kit 70702), 0.1 M of DDT (kit 70706), 2 µl of 1:5 dilution labelmix 4 dNTP (kit 70176-79), 0.5 µl of 35 S-dNTP (1000 Ci/m mole) and 2 µl of sequenase polymerase (kit 70175 blue cap) mix the solution throughly and add 7.5 µl of the label mixture into each tube of annealing mixture. Mix the solution throughly again and leave at room temperature for 5 min.

- Pre-warm 4 termination tubes at 37 degree Celsius for 1 min.

- Alliqout 3.5 µl mixed solution from each tubes into the terminating solution tubes (ddATP, ddCTP, ddGTP and ddTTP), leave at room temperature for 10 min.

- Add 4 µl of stop solution (kit no. 70724) into every tubes above, leave at room temperature, spin briefly then load to the polyacrylamide gel electrophoresis.

The gel has been prepared as follows;

8% polyacrylamide gel electrophoresis protocol

Preparation of 60 ml polyacrylamide gel for 20x60 cm plate.

- Put 25.2 gram of 7M Urea in the beaker
- Add 6 ml of 10X TBE buffer
- Add 20 ml of 24% stock polyacrylamide solution.
- Add distilled water to make the final volume of 60 ml.
- Add 240 µl of 10% APS.
- Add 25 µl of TEMED

Remarks: Preparation should be performed in the cabinet since ingredients are neurotoxic.

The gel and system was set as follows;

- Clean both glasses thoroughly with distilled water before use, then clean again with 70% ethanol, air dried.
- Siliconized one side of the shorter glass plate with 2% dimethyl dichlorosilane in 1,1,1-trichloroethane then assemble the glasses with plastic spacers. Seal both sides and the bottom of the glasses with tape.
- Pour the prepared 8% polyacrylamide gel steadily and carefully between two glass plates to prevent air bubbles.
- Insert the comb into the upper open end with the teeth pointing up for making a sharp cutting edge of the running gel.
- Leave the gel for the complete polymerization for at least 2 hr. Remove the comb and sealing tape.
- The gel was washed the upper end to remove the small pieces of excess

gel and was placed in the electrophoresis system. Add 1X TBE buffer into the upper and lower tank Re insert the comb using the teeth side into the top of the set gel.

- The gel was pre-electrophoresed till the glass temperature went up to 40 degree Celsius.
- Samples were denatured at 95 degree Celsius for 3 min and then loaded (4 lanes for each samples) and run constantly for 3.5 hrs.
- After finishing a run, polyacrylamide gel was removed from the glass onto filter paper
- Dry the gel in the vacuum dryer at 80 degree Celsius for 1 hr.
- The gel was put into the autoradiographic cassette and taken to the dark room. An autoradiographic film was placed over the gel, mark the direction and side on the film. Leave the exposure for 5 days then develop the film.
- The nucleotide sequences were alphabetized by eye and were put in the computer program for genetic analysis.

3.3.8 DNA sequencing by automated capillary electrophoresis

Samples number S86, S87, S88 and S92 were sequenced automatically using ABI 310 capillary electrophoresis machine(Perkin-Elmer Cetus, USA). The cycle sequencing was done in the Thermal cycler PCR model 2400 (Perkin Elmer Cetus, USA) by 3 steps as follows;

- Step 1, for each reaction, add the following reagents to a separate tube:

Reagent	Quantity
Terminator Ready Reaction Mix	8 µl
Template	-
PCR product	30 ng

Deionized water	q.s.
Total volume	20 µl

- Step 2, mix the solution throughly and spin briefely.

- Step 3, place the tubes in a thermal cycler (GeneAmp PCR System 2400) and set the volume to 20 µl.

- Step 4, repeat the following for 25 cycles:

  - 96 degree Celsius for 10 sec

  - 50 degree Celsius for 5 sec

  - 60 degree Celsius for 4 min.

- Setp 5, rapid thermal ramp to 4 degree Celsius and hold until ready to purify

- Step 6, spin down the contents of the tubes in a microcentrifuge.

Analysis of DNA sequence data

The sequences obtained from manual sequencing were read by eye for their nucleotides. Sequences read by the computer attached to the automatic sequencer sometimes were illegible, and then needed to be re- alphabatized by eye from the cumputer generated product.

All the DNA sequences were aligned for comparison by the Clustal V, Phylip program version 3.57c in order to compare bases and identify mutations.

Two phylogenetic trees were constructed. One was obtained from genetic distance data (Kimura's 2 parameters) based on the neighbor-joining method, the other with the PAUP program using the parsimony approach to show the different groupings. The trees produced were run through a bootstrapping analysis for statistical proof.