MITOCHONDRIAL DNA SEQUENCE VARIATION WITHIN AND BETWEEN RED JUNGLEFOWL (*GALLUS GALLUS GALLUS* AND *GALLUS GALLUS SPADICEUS*) IN THAILAND

MR. BORIPAT SIRIAROONRAT

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE IN ZOOLOGY
DEPARTMENT OF BIOLOGY
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ความแปรผันของลำดับนิวคลิโอไทด์บนไมโตคอนเดรียลดีเอ็นเอ ภายในและระหว่างไก่ป่า *Gallus gallus gallus* และ *Gallus gallus spadiceus* ในประเทศไทย

นายบริพัตร ศิริอรุณรัตน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาสัตววิทยา ภาควิชาชีววิทยา
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Thesis title	Mitochondrial DNA sequence variation within and between Red Junglefowl *Gallus gallus gallus* and *Gallus gallus spadiceus* in Thailand
By	Mr. Boripat Siriaroonrat
Department	Biological Science
Thesis advisor	Associate Professor Wina Meckvichai
Thesis co-advisor	Dr. Thaweesak Tirawatnapong

Accepted by the Graduate School, Chulalongkorn University in partial fulfillment of the requirement for the master degree.

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..... Member
(Assistant Professor Dr. Patchara Verakalasa, Ph.D.)

บริพัตร ศิริอรุณรัตน์ : ความแปรผันของลำดับนิวคลิโอไทด์บนไมโตคอนเดรียลดีเอ็นเอภายในและระหว่างไก่ป่า *Gallus gallus gallus* และ *Gallus gallus spadiceus* ในประเทศไทย (MITOCHONDRIAL DNA SEQUENCE VARIATION WITHIN AND BETWEEN RED JUNGLEFOWL *Gallus gallus gallus* AND *Gallus gallus spadiceus* IN THAILAND) อ.ที่ปรึกษา รศ.วีณา เมฆวิชัย , อ.ที่ปรึกษาร่วม อ.ดร.ทวีศักดิ์ ตีระวัฒนพงษ์, 80 หน้า

ไก่ป่า 2 ชนิดย่อยในประเทศไทยได้แก่ ไก่ป่าตุ้มหูขาว (*Gallus gallus gallus) และไก่ป่าตุ้มหูแดง* (*Gallus gallus spadiceus*) ถูกแบ่งตามความแตกต่างของสีที่ปรากฏบนแผ่นหนังด้านข้างแก้ม เก็บตัวอย่างจำนวน 10 ตัวจากแต่ละชนิดย่อย มาเจาะเลือดเพื่อสกัดดีเอ็นเอ ทำการเพิ่มปริมาณยีนในหลอดทดลองด้วยปฏิกิริยาลูกโซ่โพลีเมอเรสแล้วหาลำดับนิวคลิโอไทด์บนส่วน control region ของไมโตคอนเดรียลดีเอ็นเอ และทำการวัดขนาดความยาวแข้ง ความยาวของนิ้วที่สาม ความยาวจงอยปาก ความยาวปีก ความยาวจากโคนปากถึงท้ายทอย ชั่งน้ำหนัก และ ดูเพศ

ผลการวิเคราะห์พบว่ามีเพียงความยาวจงอยปากเท่านั้นที่มีความแตกต่างอย่างมีนัยยะสำคัญทางสถิติ

ผลการวิเคราะห์ความแปรผันของลำดับนิวคลิโอไทด์บนไมโตคอนเดรียลดีเอ็นเอบนส่วนของ control region (D-loop) ความยาว 225 เบส พบว่าสามารถระบุความแตกต่างของตัวอย่างทั้งสองกลุ่มได้เมื่อวิเคราะห์ด้วยค่า genetic distance โดยที่ค่าความแปรผันของนิวคลิโอไทด์ภายในชนิดย่อยมีค่าระหว่าง 0 ถึง 6.54 เปอร์เซนต์ ขณะที่ค่าดังกล่าวมีค่า ระหว่าง 1.34 ถึง 8.0 เปอร์เซนต์ เมื่อเปรียบเทียบระหว่างสองชนิดย่อย

##c725534 : MAJOR ZOOLOGY
KEYWORD : RED JUNGLEFOWL/ *Gallus gallus gallus*/ *Gallus gallus spadiceus*/ MITOCHONDRIAL CONTROL REGION/ DNA SEQUENCE ANALYSIS
BORIPAT SIRIROONRAT:
MITOCHONDRIAL DNA SEQUENCE VARIATION WITHIN AND BETWEEN RED JUNGLEFOWL (*Gallus gallus gallus and G. g. spadiceus)* IN THAILAND
ADVISOR: ASSOC. PROF. WINA MECKVICHAI
THESIS CO-ADVISOR: DR.THAWEESAK TIRAWATNAPONG

Two subspecies of red junglefowl are described using the difference in their earlobe color. *Gallus gallus gallus* carries a white earlobe while that of *G. g. spadiceus* is red.

10 animals from each subspecies were measured for 5 morphomtetric parameters. The analysis found that 4 out of 5 showed nonsignificant differences but one character, the beak length, showed significance difference between two subspecies using the analysis of variance (ANOVA).

DNA sequence analysis of 225 base pairs in the mitochondrial control region (D-loop) showed phylogenetic branching of the two subspecies using genetic distance analysis. Sequence divergence within the subspecies ranged from 0 to 6.54 %, while the divergence between the two subspecies ranged from 1.34 % to 8.0 %.

ภาควิชา...ชีววิทยา.....ลายมือชื่อนิสิต.....
สาขาวิชา....สัตววิทยา.....ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา....2540.....ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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Mom and Dad for four years of patience till I have this day. I love you.

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LIST OF ABBREVIATIONS

A, T, C, G	= nucleotide containing the base adinine, thymine, cytosine and guanine respectively
bp	= base pair
CO I	= cytochrome oxidase subunit I
CO II	= cytochrome oxidase subunit II
CO III	= cytochrome oxidase subunit III
dNTPs	= deoxyribonucleotide triphosphates (dATP, dTTP, dGTP,dCTP)
ddNTPS	= dideoxyribonucleotide triphosphates (ddATP, ddTTP, ddGTP,ddCTP)
DNA	= deoxyribonucleic acid
EDTA	= ethylenediamine tetraacetic acid
hr	= hour
Kb	= kilobase
min	= minute
M	= molar
mM	= millimolar
mg	= milligram
$MgCl_2$	= magnesium chloride
ml	= milliliter
mtDNA	= mitochondrial DNA
PCR	= polymerase chain reaction
rpm	= round per minute

sec	= second
TEMED	= N,N,N',N'-tetramethylethylenediamine
TRIS	= tris (hydroxy methyl) aminomethane
µl	= microliter
µg	= microgram

Chapter 1

Introduction

Red Junglefowl is a common bird, which closely resemble to the domestic chicken we are familiar with. Indeed it is generally considered to be the ancestor of all domestic chicken (*Gallus domesticus*) lineage (Delacour, 1947).

It is defined by terrestrial and arboreal habits; 4 toes; 2 carotids; sternum usually white or monochrome; nidifugous downy young in which remiges grow rapidly and which soon fly (Sibley and Alquist, 1990). In both subspecies which occur in Thailand, the beak is black and the legs are gray and without feather. The male has a dominant seven rayed comb on its head while the female has a very small remnant; some criteria for pure red junglefowl says that the comb must be absent from the female (Brisbin, 1980).

Phylogenetic classification

Red junglefowl:

Kingdom Animalia

Phylum Chordata

Class Aves

Order *Galliformes*

Family *Phasianidae*

Genus *Gallus*

In this genus, 4 species are described (Howard and Moore, 1984);

1) *Gallus lafayettei* (Ceylon junglefowl) found in Srilanka.

2) *Gallus sonnerrati* (Gray junglefowl) found in west and south India.

3) *Gallus varius* (Green junglefowl) found in Java.

4) *Gallus gallus* (Red junglefowlconsists of five subspecies in the different locations.

4.1 *G.g. murghi* , Kashmir to Assam

4.2 *G.g. gallus,* Southern Indochina, Thailand

4.3 *G.g. spadiceus,* Southwest Yunnan, Northern Indochina, Burma and Malaysia.

4.4 *G.g. jabouillei,* Northern Vietnam

4.5 *G.g. bunkiva,* Java (Sumatra)

Red Junglefowl (*Gallus gallus,* Linnaeus) is a common species, which has been observed throughout Thailand. Two subspecies occur in the country, differentiated by their different ear lobe colors. *G.g.gallus* shows the white and *G.g.spadiceus* has the red color. The geographical distribution of these two subspecies is not clear since there is no reliable scientific report or survey have been published. In the Guide to the Birds of Thailand (Lekagul and Round), it is said that the race *gallus* junglefowl with whitish ear spot is found only in the eastern part of the country. It may be distributed in Khao Yai National Park where the subspecies has been reported and photographed (Koonkwamdee, 1995). In the photo, the cock has a yellowish leg which is different from the reported gray slate leg by Lekagul and Round (1991).

The subspecies *Gallus gallus spadiceus* with red earlobe distributed in the northern forest from Laos border down to the southwest along the Myanmar border through the Malay Peninsula. There is an evidence that both subspecies occur in the Khao Yai National Park area though distribution needs confirmation.

There is no study on the taxonomic support for the subspecies' difference so far. And there is only one group from Japan (Fumihito et. al, 1994) reported on their genetic study and concluded from DNA sequence data that it is the oldest genetic stalk for the worlds' lineage of domestic chicken.

In terms of economic and cultural importance to human civilization, the Red Junglefowl is arguably the single most important species of bird. Considering the problems of ornithological conservation in Southeast Asia, molecular genetic techniques may offer the best approach to evaluating both captive and wild populations (Brisbin, 1995).

Figure 1.1 Distribution map of Red Junglefowl, (Beebe, 1921).

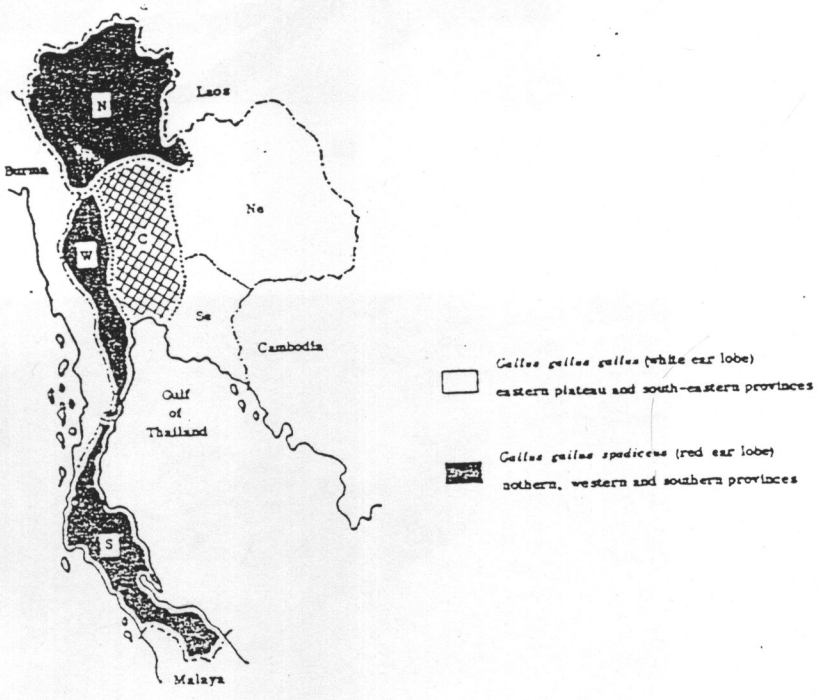


Figure 1.2 Distribution map of *Gallus gallus gallus* and *G. g. spadiceus* in Thailand.

a)

b)

Figure 1.3 The photographs of two subspecies of Red Junglefowl in Thailand.
a) *Gallus gallus gallus* male and female have white earlobe.
b) *gallus gallus spadiceus* male shows red earlobe.

Objectives

This study focuses on the genetic variation of the two subspecies of *G. gallus*, which occur in Thailand. This will serve as a tool to investigate, characterize and use the nucleotide sequence of the mitochondrial control region as a basis for systematic taxonomy and, if possible, get some idea about the species' population biology which could assist the conservation of the species.

Expected results

1. Morphometric analysis data for two subspecies of Red junglefowl in Thailand
2. Knowledge of their partial mitochondrial gene sequence.
3. Molecular systematic differentiation of the two subspecies based on the DNA sequence analysis.

Chapter 2

Literature Review

The species was first described by Linnaeus as *Phasianus gallus* in 1766 and changed to *Gallus gallus* in 1758. At least eight other synonyms have been published since then. The subspecies *spadiceus* was described by Bonaterre, possibly in the 1940's ."Junglefowl from Pacific Islands" by Ball (1933) described chicken specimens collected throughout the small islands of the Pacific Ocean. At that period, Um Pang district, Siam now Thailand was listed as one of the places where specimens had been collected. The specimens were also measured for some morphometric characters. A reference cited in this paper (Beebe, 1921) described the estimated distribution of this species, which was said to range from northern India in the southern edge of the Himalayas westward to southern Sikkim, Nepal and Kashmir. Eastward it is commonly found in the hilly portions of Bengal and Assam, throughout Burma including Pegu and Tennessarim, Yunnan, Siam, Cochin China and southward to the Malay Peninsula and Indonesia including Bali, but absent from the Island of Singapore. .

Delacour (1947) noted that the Red Junglefowl is an ancestor of domestic poultry and Beebe (1921) emphasized the constant interchange of blood between wild and domestic birds resulting in variation of habits and coloration unique among pheasants. Smithies (1986) said that the cock in flight is easily recognized by the white patch over the tail. Medway and Wells (1976) noted that the Red junglefowl has been seen in Phuket and Langkawi Islands in Andaman Sea.

Austin Jr. (1963) said that the junglefowl is different from other members of the family Phasianidae in having a comb and wattles about the head, and in having an arched and curved tail.

Ecology and life history

Red Junglefowl are found from sea level to approximately 2000 meters, in tropical and subtropical habitats. The name Junglefowl is a slight misnomer, as they prefer secondary growth to dense primary forest. Forest edge, lightly logged and particularly bamboo forests are all typical habitats in which they are found. (Johnsgard, 1986) Their preferred habitat usually is open forest. There have been no observations of either the bird or its call in deep forest and/ or at high altitude (Collias and Saichuae, 1967). This sexually dimorphic, polygamous species is almost exclusively ground living, flying only to safety, to roost, or when chasing or being chased by another Junglefowl (Sullivan, 1991).

The species feeds on various seeds, fruits, grass, leaves, and insects. Invertebrates from a small but consistent proportion of the diet, particularly caterpillars, termites and dung associated insects (Collias and Saichuae, 1967). Scratching at the ground to find food occupies a large part of the birds' time in the wild.

Red Junglefowl live in flocks with different numbers of cocks and hens. Collias and Saichuae (1967) observed that the sex ratio of flocks ranged from lone males to the groups of 2 males and 6 females.

Brisbin (1969) suggested that there is a behavioral difference of wildness in Red Junglefowls, including wariness, increased in flight distances and a tendency to avoid the presence of man, all of which are greater than in their domestic counterparts. He also suggested that the male red junglefowl always undergoes

eclipse molting after the breeding season. Sexual maturity is reached in the first year, although the males do not develop fully grown spurs and plumage until their second year. The breeding season is from March to September, depending on locality, with 6-12 eggs being laid in nests which are simple scrapes on the ground, hidden in the undergrowth. While incubating her eggs, the hen will occasionally leave the nest very briefly to feed, drink, preen and defecate. After 21 days the eggs hatch and the hen and chicks form a unit independent from her original flock and for the large part away from any males, although males may occasionally consort with these hens. Chick mortality is extremely high since there are many predators including snakes , lizards, birds of prey, and small and large carnivores such as wild cats and civets (Beebe, 1921).

Genetic factors are one of the priorities to be studied in pheasants (Gaston, 1992) since wild Red Junglefowl may be an important source of genetic diversity for future breeding programs especially of domestic *Gallus gallus.* The entire wild gene pool holds enormous potential benefits to the poultry industry.

Mitochondrial DNA of birds

The avian mitocondrial DNA (mtDNA), like that of most eukaryotes, is an extrachromosomal DNA, which is found in the mitochondria, the powerhouse of every cell. It is a single circular molecule, approximately 16000-20000 base pairs long. Unlike nuclear DNA, mtDNA is maternally inherited and undergoes rapid evolutionary change in its nucletide sequence compared to nuclear DNA. The rate of nucleotide substitution on the mitochondrial genome has been estimated to be about 5-10 times more rapid than that of nuclear DNA (Brown, 1979). Mitochondrial DNA is usually monoclonal and does not seem to undergo recombination in vertebrates. Once a variant is established in a female, all

descendants of that individual carry it and, therefore, the inheritance pattern is cloned through the maternal lineage.

Mitochondrial DNA from animals has been well characterized over the past decades. The complete nucleotide sequence of mtDNA of humans (Anderson *et al.*, 1981), cattle (Anderson *et al.*, 1982), mouse (Bibb *et al.*, 1981) and clawed frog (*Xenopus laevis*, Roe *et al.*, 1985) have been reported. These studies reveal that the gene content and genomic organization has remained stable since the divergence of the mammalian and amphibian lineage, approximately 350 million years ago (Brown, 1983). Data from fish mtDNA suggested that a gene content and genomic organization similar to mammals and amphibians exist in fish mtDNA.

The gene order of chicken (*Gallus domesticus*, Desjardin and Morais 1990) in mitochondrial DNA is ND5, cytochrome b, $tRNA^{Thr}$, $tRNA^{Pro}$, ND6, $tRNA^{Glu}$, the control region (D-loop), $tRNA^{Phe}$ and srRNA. This order is identical to that of Snow Goose (Quinn and Wilson, 1993), Japanese quail (*Coturnix japonica*, Desjardin and Morais 1990), and duck mtDNA but differs from that of mammals and frog (*Xenopus).* Within the control region, several short sequences common to mammals are also conserved in birds.

Desjardin and Morais (1990) reported the sequence and gene organization of chicken mitochondrial DNA by cloning and sequencing the whole mitochondrial genome of a domestic chicken, variety white Leghorn. They found that the 16755 base pairs of avian mitochondrial genome encodes the same set of genes (13 protein genes, 2 rRNA genes and 22 tRNA genes) as do other vertebrate mtDNA (Figure 2.1) and is organized in a very similar economical fashion.

Despite these highly conserved features, the chicken mitochondrial genome displays two distinctive characteristics. First, it exhibits a novel gene order, the contiguous $tRNA^{Glu}$ and ND6 genes are located immediately adjacent to the

displacement loop region (D-loop) of the molecule, just ahead of the contiguous $tRNA^{Pro}$, $tRNA^{Thr}$ and cytochrome b genes, which border to the D-loop region in other vertebrate mitochondrial genomes. This unusual order is conserved among all studied galliform birds. Second, a light strand replication origin, equivalent to the conserved sequence found between the $tRNA^{Cys}$ and $tRNA^{Asn}$ genes in all vertebrate mitochondrial genomes thus far, is absent from the chicken genome. These observations indicate that the galliform mitochondrial genome departed from its mammalian and amphibian counterparts during the course of evolution of vertebrate species.

The chicken displacement loop region (D-loop) is delimited on its 3'(prime) end by the gene for $tRNA^{Phe}$ and on its 5' end by the $tRNA^{Glu}$ gene. In the other vertebrate mtDNA sequenced thus far, the 5' end of the D-loop region is bordered by the gene for $tRNA^{Pro}$. This species difference reflects the transposition of the $tRNA^{Glu}$- ND6 mtDNA fragment that has occurred in chicken. (Fig.2.3). The length of the entire control region is the most variable. The D-loop region in chicken mtDNA is slightly larger (1227 bp) than the corresponding sequence found in human (1122 bp), mouse (879 bp), rat (898 bp) or cow (910 bp) mtDNA but is much shorter that of *X. laevis* (2134 bp).

Sequence analysis of vertebrate mtDNA has revealed that the D-loop region is the most rapidly evolving part of the genome. Together with many reasons above, mtDNA is potentially useful for population genetics and molecular systematic studies of animal taxa due to its high mutation rate. Therefore, this study used the mitochondrial D-loop sequence variation as a marker to look at the intraspecific level between two subspecies of Red Junglefowl in Thailand.

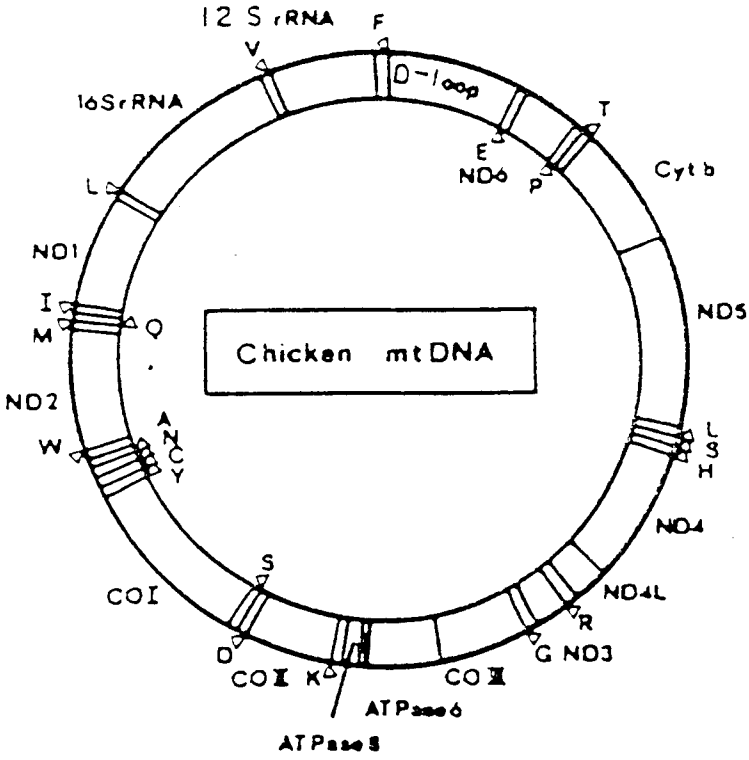


Figure 2.1 Chicken mitochondrial DNA; its genes and gene organization.

(Desjardin and Morais, 1990)

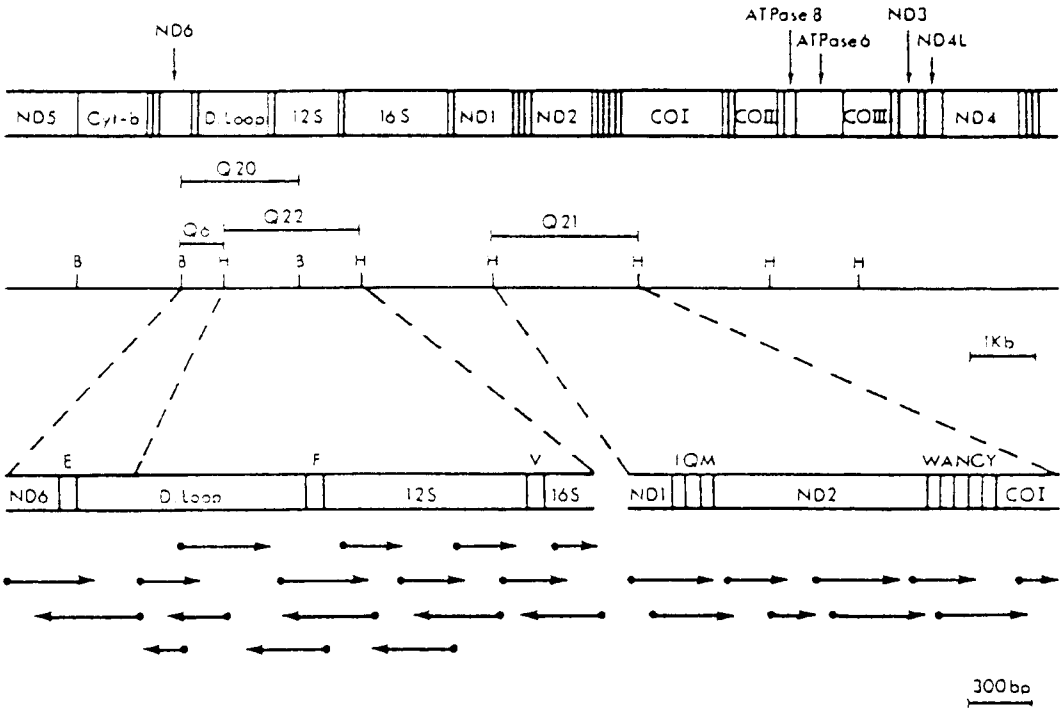


Figure 2.2 A linear presentation of the chicken mitochondrial DNA molecule showing the localization of the genes for cytochrome oxidase subunit I,II and III, (CO I, COII and COIII), ATPase subunit 6 and 8, apocytochrome b (cyt-b), NADH dehydrogenase 1-6 (ND1,2,3,4,4L,5,6), the small (12S) and large (16S) ribosomal DNA subunit, 22 tRNAs (narrow boxes) and the control region (D-loop).

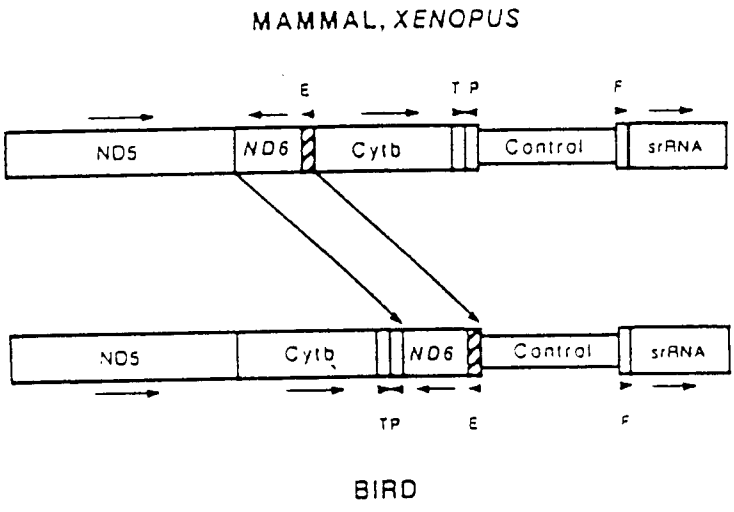


Figure 2.3 Gene order in birds compared to mammals and *Xenopus*. Horizontal arrows indicate the coding strand (to the right, encoded on the heavy strand) Control: control region. (Quinn and Wilson, 1993)

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.

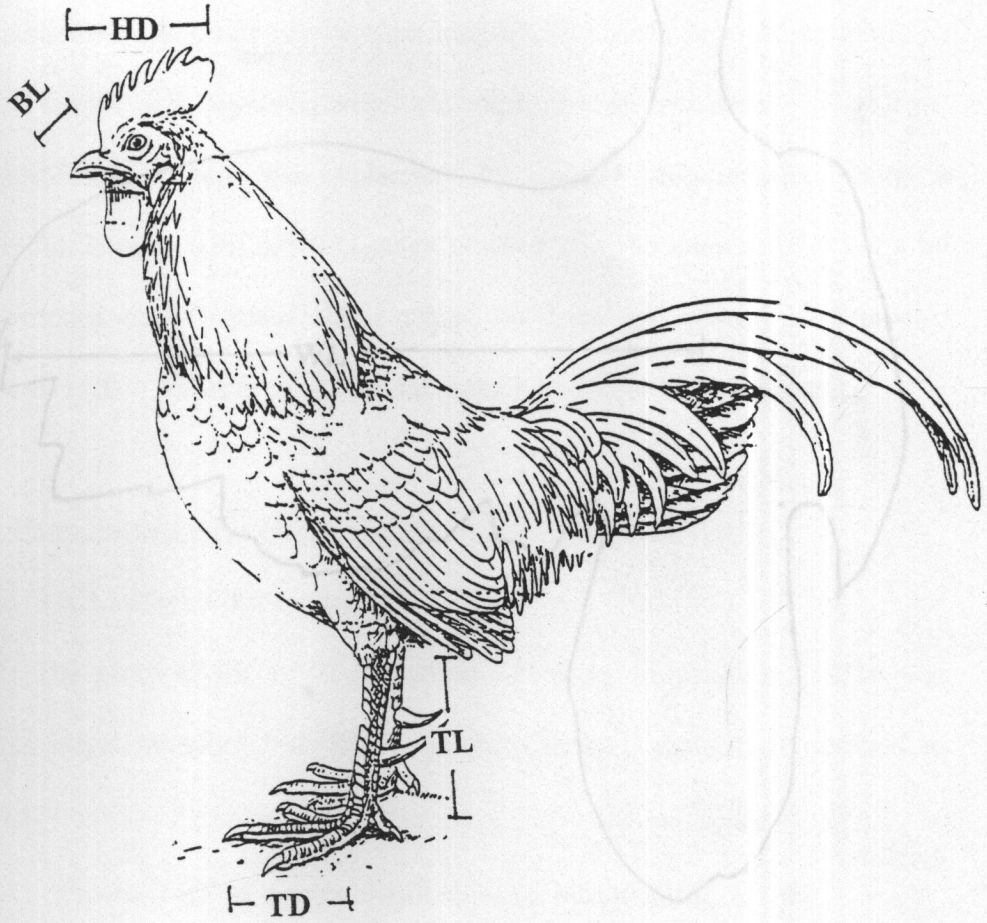


Figure 3.1 Morphometric measurement of basement of upper peak to occipital (HD), beak length (BL), tarsometatarsus length (TL), third digit length (TD)

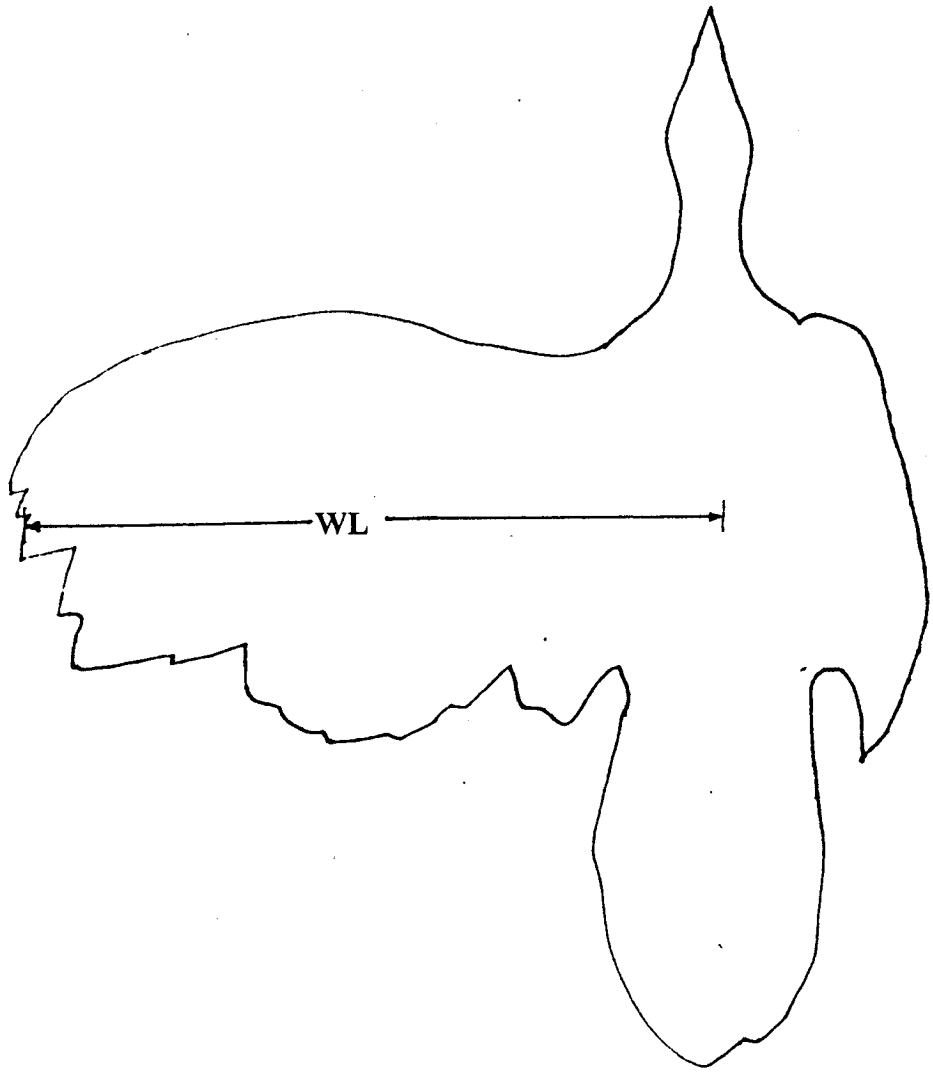


Figure 3.2 Morphometric measurement of wing length (WL)

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 degree 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.

Chapter 4

Results and Discussions

4.1 Morphometric analysis

All specimens were measured for 5 parameters and weight as well as were identified for their age level and sex. The data were shown in Table 1. Results comparing the two subspecies were obtained using analysis of variance

The wing length, tarsometatarsus length, base of beak to nape length, third digit length showed no difference between two groups. But the beak length showed a significant different between two groups (Beak length mean= 1.410, F value=10.06 at $p<0.05$). See Appendix III.

The beak length compared between two subspecies showed the difference, but all specimens are caged birds in captivity, so the result might have some bias due to their changed feeding behavior from their wild habitat, or differences in husbandry between the two sample groups.

Morphometric analysis using more characters should have been done for a larger number of animal both in the wild and captive for differentiation of their morphology, and might have produced more significant taxonomic differences.

Table 1. Morphometric data of two subspecies (G=*Gallus gallus gallus*, S=G. g. *spadiceus*)

No.	Wgt	BL	WL	TM	HD	TD	AGE	SEX
G1	0.85	1.2	29.0	7.7	5.7	5.3	A	F
G2	1.20	1.1	36.8	9.2	6.2	4.1	A	M
G3	1.30	1.3	33.7	8.5	6.8	5.5	A	M
G4	0.67	1.2	29.5	7.4	6.0	4.6	A	F
G5	0.84	1.5	30.2	7.8	5.8	5.0	A	F
G6	0.79	1.2	32.2	7.6	5.8	4.2	A	F
G7	0.66	1.3	30.5	7.5	5.8	5.0	A	F
G8	0.77	1.4	30.5	7.7	5.7	4.7	A	F
G9	1.20	1.5	35.7	8.9	6.4	4.9	A	M
G10	1.30	1.4	36.7	9.3	6.3	5.3	A	M

No.	Wgt	BL	WL	TM	HD	TD	AGE	SEX
S83	0.70	1.4	30.4	7.0	5.5	5.0	A	F
S84	1.20	1.7	38.0	8.9	6.2	5.4	A	M
S85	1.10	1.6	36.3	9.1	6.4	5.6	A	M
S86	0.84	1.4	29.5	7.2	5.2	5.0	A	F
S87	1.10	1.6	35.7	8.7	6.0	5.7	A	M
S88	0.93	1.6	34.5	7.2	5.7	4.9	A	F
S89	0.75	1.2	27.5	7.3	5.8	5.1	J	F
S90	0.73	1.5	28.0	7.0	5.8	5.2	J	F
S91	0.68	1.5	29.0	6.7	5.3	4.6	J	F
S92	1.05	1.6	32.2	8.9	6.4	5.3	J	M

Wgt = Weight (kg)
BL = Beak length (cm)
WL = Wing length (cm)
TM = Tarsometatarsus length (cm)
HD = Head (Basement of upper beak to occipital, cm)
TD = Third digit length (cm)
A = Adult M= Male
J = Juvenile F= Female

4.2 Protocol used for DNA extraction.

2 protocols for mtDNA extraction were used; first the Chelex extraction and second, the classical phenol-chloroform extraction. Ethidium bromide stained agarose gel (Figure 4.1) shows the DNA extraced from Chelex compare to phenol-chloroform extracted product. Chelex ectracted product can not be observed on the gel while concentrated Phenol-Choroform extracted product gave a clear signal of DNA quantity obtained from the extraction.

The comparative result was also observed by the first PCR product on 2% agarose gel electrophoresis. Using the Chelex extract to add PCR mixture as template DNA gave no signal from PCR. Total of 20 samples has been tried and double extraction trials were done.

PCR signals were successfully obtained using phenol-chloroform extracts as an alternative template in PCR mixture. One microliter of Bovine Serum Albumin (BSA) needs to be added as an inhibitors terminator(Cooper, 1994) to all PCR mixtures, otherwise no signals can be obtained.

Cooper (1994) mentioned that the Heme and cytochrome from blood are the major PCR inhibitors. Meckvichai (1997) succeeded in using Chelex extracts for cytochrome b gene amplification but it could not be used for D-loop amplification in this study.

A concentrated purified phenol-chloroform extract is recommended to use as a template DNA for chicken D-loop amplification if sample used are bloodstains.

4.3 DNA amplification from D-loop region

Optimization of PCR condition, using the universal cytochrome b primer for amplification of the some samples of Chelex extracted product and the positive bands were obtained. The PCR mixture and cycles was described in chapter 2.

Using the Chelex extracted solution as template DNA for the PCR reaction gave all negative results when using D-loop primer for amplification.

The alternative phenol-chloroform extracted products were used as an improved substitution. These samples gave positive signals of the D-loop amplified product (Figure 4.2 a and b) but Bovine Serum Albumin (BSA, Sigma) was needed for every PCR reaction tube as inhibitor terminators.

PCR primers was a chicken specifically designed and could amplify the entire 1254 bp fragment, the whole D-loop region.

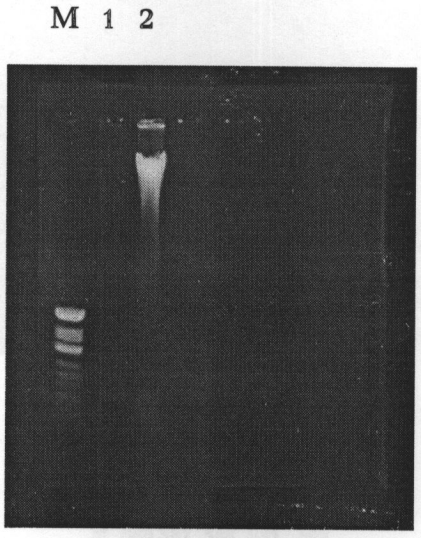
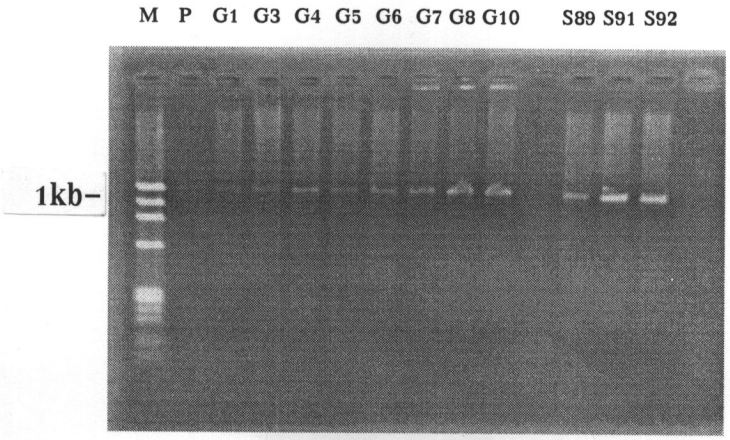
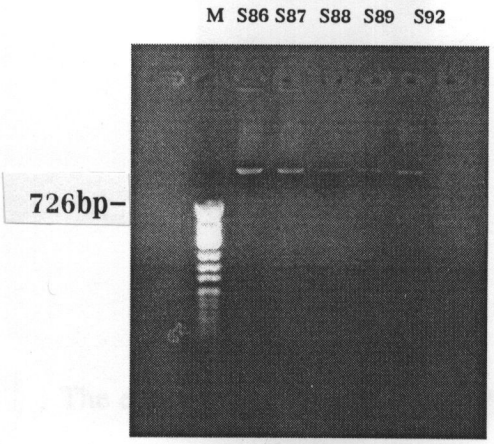


Figure4.1 Ethidium bromide stained agarose gel (1.5%) show the DNA extraced from Chelex compare to phenol-chloroform extracted product. M=ϕX174 DNA marker, Lane 1= Chelex ectracted product can not be observed on the gel, Lane 2= Phenol-Choroform extracted product (concentrated) gave a clear signal of DNA quantity obtained.



a)



b)

Figure 4.2 Ethidium bromide stained gel showing the positive PCR signal of amplified Chicken D-loop gene product.

a) M=∅X174 Hae III DNA marker, G1, 3, 4,5, 6, 7, 8 and 10= *Gallus gallus gallus* samples, S89, 91, 92= *G. g. spadicues* samples, P = positive control.

b) M=∅X174 Hinf I DNA marker, S86, 87, 88, 89 and 92= *G. g. spadiceus* samples.

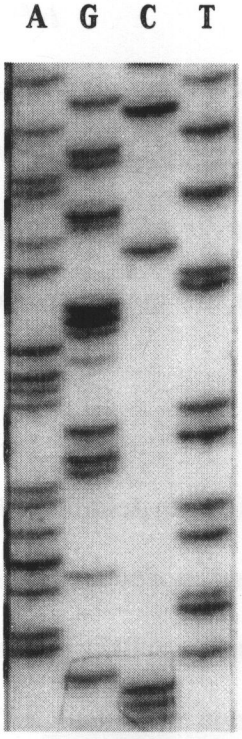


Figure 4.3 The exposed X-ray film from ^{32}P-labelled chicken D-loop DNA sequence.

4.4 Second PCR generating ss-DNA

For manual DNA sequencing using chain-terminating inhibitor (Sanger, 1977), it is better to generate the single-stranded DNA from the first PCR product. Gyllensten (1988) described the protocol for asymmetric PCR that it could generate the single-stranded DNA for the sequencing. The result is not shown here.

4.5 DNA sequence analysis

225 bp sequence from 16 samples, 6 *Gallus gallus spadiceus* and 10 *G. g. gallus*, were analyzed. The obtained sequences were mitochondrial D-loop L-chains (base position L41-L323 compare to *Gallus domesticus* by Desjardins and Morais, 1990 with 3 bases missing). The nucleotide composition of the D-loop sequence were reported by Desjardins and Morais (1990) as; A=26.7, G=13.3, C=26.3 and T=33.7.

There are 5 sites of variation in *G. g. gallus* and 15 sites in *G. g. spadiceus* which means the genetic variation in the observed area of *G. g. spadiceus* is three times higher than that of *G. g. gallus*.

Within subspecies *G. g. gallus* , 5 transitions (TS) were found. There is no transvertional (TV) nucleotide substitution in this subspecies. The genetic distance within this subspecies= 0.0000-0.0225. Sequence divergence varies from 0 to 2.25%. Fumihito et al (1994) reported the variation of D-loop region of 2 *G. g. gallus* from Thailand that there are 1.25 % sequence divergence.

Within subspecies *G. g. spadiceus*, 10 transitions and 5 transversions were found. The TS: TV ratio is 2:1. The sequence number S84 and S85 showed 100% homology along 225 bp alignment so they were treated into the same taxa when using for the parsimonious analysis. The genetic distance within this subspecies=

0.0000-0.0654. Sequence divergence varies from 0 to 6.54% while Fumihito et al (1994) reported 4.25 % from one sample of *G. g. spadiceus* from Thailand.

There are 9 sites variable between two subspecies. The TS: TV ratio is 8:1. The genetic distances between two subspecies= 0.0134-0.0800. Genetic distances between groups' seem to be larger than that of within each group.

Using the published Japanese quail (*Coturnix coturnix japonica*) as an outgroup reference, 20 variation site were found after aligning the sequences by CLUSTAL V in PHYLIP program version 3.57c (Felsenstein, 1993). The obtained sequences showed the CCC base triplets, which are shown underlined in Figure 4.4, that are similar to the sequences published by Fumihito et al. (1994) but were missed from domestic chicken (*Gallus domesticus*) D-loop that was published by Desjardins and Morais (1990).

All sequences were analyzed for their phylogenetic relationship using the genetic distance data (Kimura's 2 parameters) shown in Table 3.2. The tree was constructed with neighbor-joining method using PHYLIP program version 3.572c and was shown in Figure 4.6. The samples were divided in two groups. One with all the *G. g. spadiceus* (S84, 85, 86, 87, 88 AND 92) and *G. g. gallus* number G9, and another with 9 *G. g. gallus* samples (G 1, 2, 3, 4, 5, 6, 7, 8 and 10)

CLUSTAL V multiple sequence alignment

```
JAPQU           CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTCGATACATTT
1994            CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTCGATACATTT
S84             CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
S85             CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
S86             CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGGGCATACATTT
S87             CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGGGCATACATTT
S88             CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGGGCATACATTT
S92             CCCCTTTCCCCCCCGGGGGGGGTATACTATGCATAATCGGGCATACATTT
G1              CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
G2              CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
G3              CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
G4              CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
G5              CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
G6              CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
G7              CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
G8              CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
G9              CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
G10             CCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTT
                ************** ************************   ********

JAPQU           ATATTCCACATATACTATGGTACCGGTAATATATATTATATACGTACTAA
1994            ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
S84             ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
S85             ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
S86             ATATACCACATATATTATGGCACCGGTAATATATACTATATATGCACTAA
S87             ATATACCACATATATTATGGCACCGGTAATATATACTATATATGCACTAA
S88             ATATACCACATATATTATGGCACCGGTAATATATACTATATATGCACTAA
S92             ATATATCCCATATATTATGGCACCGATAATATATACTATATTGCCACTAA
G1              ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
G2              ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
G3              ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
G4              ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
G5              ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
G6              ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
G7              ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
G8              ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
G9              ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
G10             ATATACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAA
                ****  * ****** ***** **** ********* *****    *****
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JAPQU           ACCCATTATATGTATACGGGCATTA-CATATATTCCCCATTTCTCCCCAT
1994            ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
S84             ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
S85             ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
S86             ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
S87             ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
S88             ACCCATTATATGTATACGGGCATTAAATTATATTCCACATTTCTCCCAAT
S92             ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G1              ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G2              ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G3              ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G4              ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G5              ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G6              ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G7              ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G8              ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G9              ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
G10             ACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAAT
                ************************   ******** ********** **

JAPQU           GTACATTA-GTGCATGCTCCAAGACATA----------------------
1994            GTCCATTCTATGCATGATCCAGGACATACTCATTCACCCTCCCCATAGAC
S84             GTCCATTCTATGCATGATCCAAGACATACTCATTCACCCTCCTCATAGAC
S85             GTCCATTCTATGCATGATCCAAGACATACTCATTCACCCTCCTCATAGAC
S86             GTCCATACTATGCATGATCCAAGACATACTCATTCACCCTTCTCATAGAC
S87             GTCCATTCTATGCATGATCCAAGACATACTCATTCACCCTTCTCATAGAC
S88             GCCCATTCTATGCATGATCCAAGACATACTCATTCACCTCTCTCATAGAC
S92             GTCCATTCTATGCATGATCTAAGACATACTCGTTCACCCTTCTCATAGAC
G1              GTCCATTCTATGCATGATCCAAGACATACTCATTCACCCTCCCCATAGAC
G2              GTCCATTCTATGCATGATCCAAGACATACTCATTCACCCTCCCCATAGAC
G3              GTCCATTCTATGCATGATCCAAGACATACTCATTCACCCTCCCCATAGAC
G4              GTCCATTCCATGCATGATCCAAGACATACCCATTCACCCTCCTCATAGAC
G5              GTCCATTCCATGCATGATCCAAGACATACCCATTCACCCTCCTCATAGAC
G6              GTCCATTCCATGCATGATCCAAGACATACCCATTCACCCTCCTCATAGAC
G7              GTCCATTCCATGCATGATCCAAGACATACCCATTCACCCTCCTCATAGAC
G8              GTCCATTCTATGCATGATCCAAGACATACTCATTCACCCTCCCCATAGAC
G9              GTCCATTCTATGCATGATCCAAGACATACCCATTCACCCTCCTCATGGAC
G10             GTCCATTCCATGCATGATCCAAGACATACTCATTCACCCTCCTCATAGAC
                *  ***    ****** ** * ******
```

```
JAPQU           -------AACCA-TAC--GTTCACC
1994            AGCTCCAAACCACTACCAAGTCACC
S84             AGCTCCAAACCACTACCAAGTCACC
S85             AGCTCCAAACCACTACCAAGTCACC
S86             AGCTCCAAACCACTACCAAGTCACC
S87             AGCTCCAAACCACTACCAAGTCACC
S88             AGCTCCAAACCACTACCAAGTCACC
S92             AGCTCCAAACCACTACCAAGTCACC
G1              AGCTCCAAACCACTACCAAGTCACC
G2              AGCTCCAAACCACTACCAAGCCACC
G3              AGCTCTAAACCACTACCAAGCCACC
G4              AGCTCTAAACCACTACCAAGCCACC
G5              AGCTCTAAACCACTACCAAGCCACC
G6              AGCTCTAAACCACTACCAAGCCACC
G7              AGCTCTAAACCACTACCAAGCCACC
G8              AGCTCCAAACCACTACCAAGTCACC
G9              AGCTCCAAACCACTACCAAGTCACC
G10             AGCTCTAAACCACTACCAAGCCACC
                     ***** ***     ****
```

Figure 4.4 L-chain sequences of 225 bases of the mitochondrial control region from *G. g. gallus* and *G. g. spadiceus* using the published Japanese quail (JAPQU, *Coturnix coturnix japonica*) as an outgroup. 1994 is Thai Red junglefowl published sequence by Fumihito et al., 1994. CCC base triplets underlined in the first section were bases missed in two previous publications by Desjardins and Morais 1990 but similar to the sequence by Fumihito el al, 1994. The stars under the blocks mark the homology of nucleotides.

From the DNA sequence analysis, it is shown that *Gallus gallus spadiceus* samples have higher divergence in their nucleotide sequence than that of *G. g. gallus*. Using the assumption that mutation occur through time of evolution, it is possible that the *G. g. gallus* might have evolved from *G. g. spadiceus*.

The wider range of distribution and more number of population and individuals may cause the higher genetic variation in *G. g. spadiceus* even small number of samples were collected.

From tree drawn by neighbor-joining method, sample number G9 that fall into the same group with other subspecies might have had an evidence of genetic hybridization with *G. g. spadiceus* but need confirmation by nuclear DNA profile.

The most parsimonious tree showed the distinctive two groups with the unclear polytomies, which are S84, S85, G1, G2, G8 and G9. These samples might have common sequence characters in the observed 225 bp even their morphology are different. The sequence number S84 and S85 that showed 100 % homology might have common ancestor.

Regarding the conservation genetics, the animal number G8 and G9 may not be appropriate to use as a parent stock for the *gallus* lineage production since their DNA profile were closed to their *spadiceus* counterparts. All other sample would be a good stock and should have been preserved.

Table 2. Estimated genetic distances among 16 samples, published sequence (1994) and outgroup (Japq, *Coturnix coturnix japonica*) obtained from 225 bp of D-loop sequence.

	Japq	1994	S84	S85	S86	S87	S88	S92	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10
Japq	-																	
1994	0.0844	-																
S84	0.0910	0.0181	-															
S85	0.0910	0.0181	0.0000	-														
S86	0.1160	0.0414	0.0226	0.0226	-													
S87	0.1094	0.0366	0.0180	0.0180	0.0045	-												
S88	0.1160	0.0604	0.0411	0.0411	0.0272	0.0226	-											
S92	0.1556	0.0805	0.0605	0.0605	0.0462	0.0414	0.0654	-										
G1	0.0910	0.0135	0.0045	0.0045	0.0272	0.0226	0.0458	0.0654	-									
G2	0.0970	0.0181	0.0089	0.0089	0.0318	0.0271	0.0505	0.0702	0.0045	-								
G3	0.0970	0.0226	0.0134	0.0134	0.0365	0.0317	0.0552	0.0751	0.0089	0.0045	-							
G4	0.0970	0.0365	0.0180	0.0180	0.0411	0.0364	0.0600	0.0800	0.0225	0.0180	0.0134	-						
G5	0.0970	0.0365	0.0180	0.0180	0.0411	0.0364	0.0600	0.0800	0.0225	0.0180	0.0134	0.0000	-					
G6	0.0970	0.0365	0.0180	0.0180	0.0411	0.0364	0.0600	0.0800	0.0225	0.0180	0.0134	0.0000	0.0000	-				
G7	0.0970	0.0365	0.0180	0.0180	0.0411	0.0364	0.0600	0.0800	0.0225	0.0180	0.0134	0.0000	0.0000	0.0000	-			
G8	0.0910	0.0135	0.0045	0.0045	0.0272	0.0226	0.0458	0.0654	0.0000	0.0045	0.0089	0.0225	0.0225	0.0225	0.0225	-		
G9	0.0910	0.0272	0.0089	0.0089	0.0318	0.0271	0.0505	0.0702	0.0134	0.0180	0.0225	0.0180	0.0180	0.0180	0.0180	0.0134	-	
G10	0.0970	0.0318	0.0134	0.0134	0.0365	0.0317	0.0552	0.0751	0.0180	0.0134	0.0089	0.0045	0.0045	0.0045	0.0045	0.0180	0.0225	-

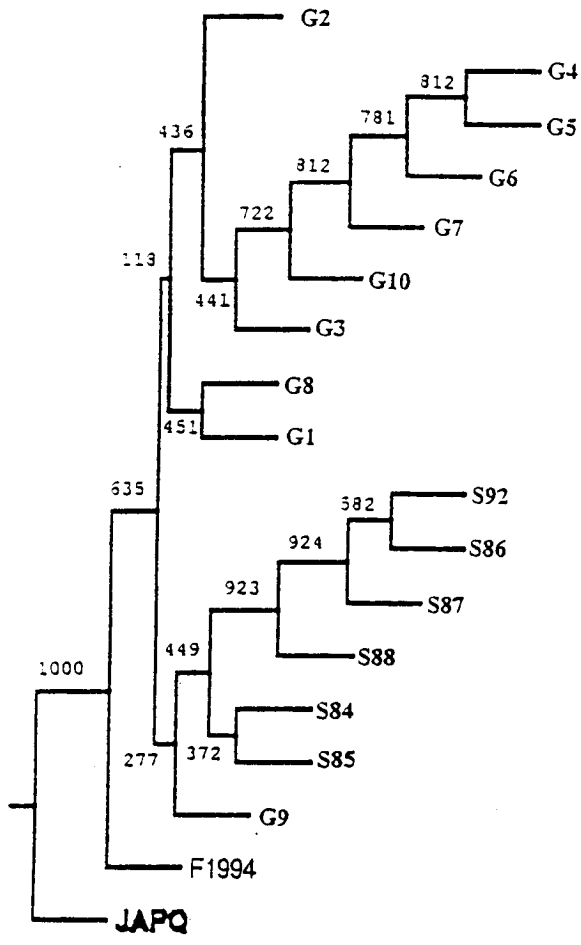

Figure 4.6 The phylogenetic tree inferred from genetic distance data using neighbor-joining method (PHYLIP version 3.572c.) shows the separation of two groups.

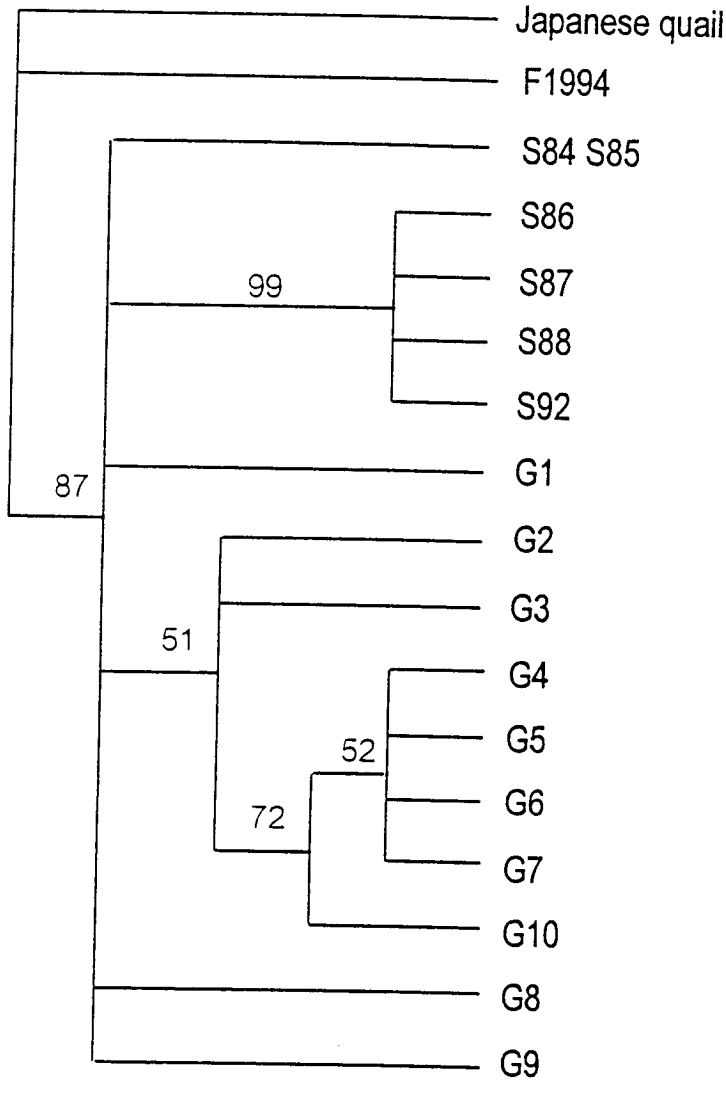

Figure 4.7 The most parsimonious tree drawn from PAUP version 3.0 shows the separation of two group with some polytomies which are not support by 50% bootstrapping criteria.

Chapter 5

Conclusion and Recommendations

Conclusion

This study confirms the anatomical phylogeny which separate one subspecies from another on the basis of earlobe color.

Morphometric analysis of 5 characters gives no significant differences (4 out of 5), but there is one parameter, a beak length, that shows the difference between 2 populations.

DNA sequence analysis from partial D-loop sequencing (225 bp) separated one group from another using statistical analysis. In this principle, it is also found that there is a difference in some DNA sequences between the two groups.

The final conclusion would say that there are two subspecies of Red Junglefowl in Thailand using DNA analysis.

The DNA sequence analysis shows that *Gallus gallus spadiceus* samples have higher divergence in their nucleotide sequence than that of *G. g. gallus*: 15 sites versus 5 sites. Using the assumption that mutations accumulate through time of evolution, it is possible that *G. g. gallus* might have evolved from *G. g. spadiceus*.

In the genetic distance tree, specimen number G9 falls into the same group with another subspecies, which might be evidence of genetic hybridization with *G. g. spadiceus* but this needs confirmation by nuclear DNA profile.

The most parsimonious tree showed two distinctive groups with unclear polytomies, which are S84 and S85, G1, G2, G8 and G9. These samples show common characters in the observed 225 base pairs sequence even though their

morphology is different. The samples S84 and S85 that showed 100% homology might have common ancestor.

From the point of view of conservation genetics, the animal number G8 and G9 may not be appropriate to use as a parent stock for the *G. g. gallus* lineage because their DNA profile was closely related to their *G. g. spadiceus* counterparts. All other specimen would be good future breeding stock. Heterozygosity should be preserved through careful breeding management.

Recommendation

In the further, the study of population genetics will be needed for the evaluation of genetic diversity as well as the status of the natural population in these two subspecies. Further data is still unclear in terms of animal morphology. Pure genetic stocks should be protected for the future as an important sourse of genetic diversity.

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Appendix I
Morphometric analysis data from SAS Program

Analysis of Variance Procedure

Class Level Information

Class	Levels	Values
GENEGR	2	G1 S1

Number of observations in data set = 20

SAS 6:00 Tuesday, September 14, 1993

2

Analysis of Variance Procedure

Dependent Variable: WGT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.01250000	0.01250000	0.24	0.6320
Error	18	0.94812000	0.05267333		
Corrected Total	19	0.96062000			

R-Square	C.V.	Root MSE	WGT Mean
0.013012	24.59879	0.229507	0.93300000

SAS 6:00 Tuesday, September 14, 1993

3

Analysis of Variance Procedure

Dependent Variable: WGT

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GENEGR	1	0.01250000	0.01250000	0.24	0.6320

SAS 6:00 Tuesday, September 14, 1993

4

Analysis of Variance Procedure

Class Level Information

Class	Levels	Values
GENEGR	2	G1 S1

Number of observations in data set = 20

SAS 6:00 Tuesday, September 14, 1993

5

Analysis of Variance Procedure

Dependent Variable: BL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.20000000	0.20000000	10.06	0.0053
Error	18	0.35800000	0.01988889		
Corrected Total	19	0.55800000			

R-Square	C.V.	Root MSE	BL Mean
0.358423	10.00198	0.141028	1.41000000

SAS 6:00 Tuesday, September 14, 1993

6

Analysis of Variance Procedure

Dependent Variable: BL

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GENEGR	1	0.20000000	0.20000000	10.06	0.0053

SAS 6:00 Tuesday, September 14, 1993

7

Analysis of Variance Procedure

Class Level Information

Class	Levels	Values
GENEGR	2	G1 S1

Number of observations in data set = 20

SAS 6:00 Tuesday, September 14, 1993

8

Analysis of Variance Procedure

Dependent Variable: WL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.68450000	0.68450000	0.06	0.8117
Error	18	210.84500000	11.71361111		
Corrected Total	19	211.52950000			

R-Square	C.V.	Root MSE	WL Mean
0.003236	10.59766	3.422515	32.2950000

SAS 6:00 Tuesday, September 14, 1993

9

Analysis of Variance Procedure

Dependent Variable: WL

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GENEGR	1	0.68450000	0.68450000	0.06	0.8117

SAS 6:00 Tuesday, September 14, 1993

10

Analysis of Variance Procedure

Class Level Information

Class	Levels	Values
GENEGR	2	G1 S1

Number of observations in data set = 20

SAS 6:00 Tuesday, September 14, 1993

11

Analysis of Variance Procedure

Dependent Variable: TM

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.64800000	0.64800000	0.88	0.3615
Error	18	13.30400000	0.73911111		
Corrected Total	19	13.95200000			

R-Square	C.V.	Root MSE	TM Mean
0.046445	10.77338	0.859716	7.98000000

SAS 6:00 Tuesday, September 14, 1993

12

Analysis of Variance Procedure

Dependent Variable: TM

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GENEGR	1	0.64800000	0.64800000	0.88	0.3615

SAS 6:00 Tuesday, September 14, 1993

13

Analysis of Variance Procedure

Class Level Information

Class	Levels	Values
GENEGR	2	G1 S1

Number of observations in data set = 20

SAS 6:00 Tuesday, September 14, 1993

14

Analysis of Variance Procedure

Dependent Variable: HD

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.24200000	0.24200000	1.54	0.2303
Error	18	2.82600000	0.15700000		
Corrected Total	19	3.06800000			

R-Square	C.V.	Root MSE	HD Mean
0.078879	6.670577	0.396232	5.94000000

SAS 6:00 Tuesday, September 14, 1993

15

Analysis of Variance Procedure

Dependent Variable: HD

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GENEGR	1	0.24200000	0.24200000	1.54	0.2303

SAS 6:00 Tuesday, September 14, 1993

16

Analysis of Variance Procedure

Class Level Information

Class	Levels	Values
GENEGR	2	G1 S1

Number of observations in data set = 20

SAS 6:00 Tuesday, September 14, 1993

17

Analysis of Variance Procedure

Dependent Variable: TD

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.51200000	0.51200000	3.13	0.0936
Error	18	2.94000000	0.16333333		
Corrected Total	19	3.45200000			

R-Square	C.V.	Root MSE	TD Mean
0.148320	8.050701	0.404145	5.02000000

SAS 6:00 Tuesday, September 14, 1993

18

Analysis of Variance Procedure

Dependent Variable: TD

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GENEGR	1	0.51200000	0.51200000	3.13	0.0936

□

Appendix II
DNA Sequence data

Data Collection

File:	A9•865
Sample:	865
Inj. Number:	5
Number of Points:	6680
Length:	301
Start Run	9/4/1998, 20:26
Stop Run	9/4/1998, 21:21
Start Collection	9/4/1998, 20:45
Stop Collection	9/4/1998, 21:21
Dyeset/Primer:	DT POP6{BD Set-Any Primer}
Medium Lot #:	
Medium Exp Date:	
Instrument Name:	ABI PRISM™ 310
Collect Vers.:	ABI PRISM 310 Collection 1.0.2

Data Analysis

Base Call Start:	746
Base Call End:	4000
Primer Peak Loc.:	746
Signal:	G (146), A (145), T (148), C (169)
Matrix Name:	BD 22/12/97
Basecaller:	ABI-CE1
Basecaller Version:	Version 3.0
Base Spacing Used:	10.56
Base Spacing Calculated:	10.56
Length to Detector:	47
Tube Position:	A9
Module File Name:	Seq POP6 Rapid (1 mL) E

```
  1  TGNNCCANCT ACGGGNCAAT TTTATTTTTT AACCTAACTC CCCTACTAAG TGTNCCCCNC NTTTCCCCCC CAGGGGGGGN NTACTATGCA TAATCGGGCA  100
101  TACATTTATA TNCCACATAT ATTATGGCNC CGGNAATATA TACTATATAT GGNNCTAANC CCATTATATG TANACGGNCN TTAATNTATA TTCCACATTT  200
201  CTCCCANTGN CCATACTATG CATGANCCAA GACATACTNA TTCACCCTNC TCATANACAG CTCCAAACCA CTACCAAGTC ACCTAACTAT GAATGGGTGC  300
301  A                                                                                                            400
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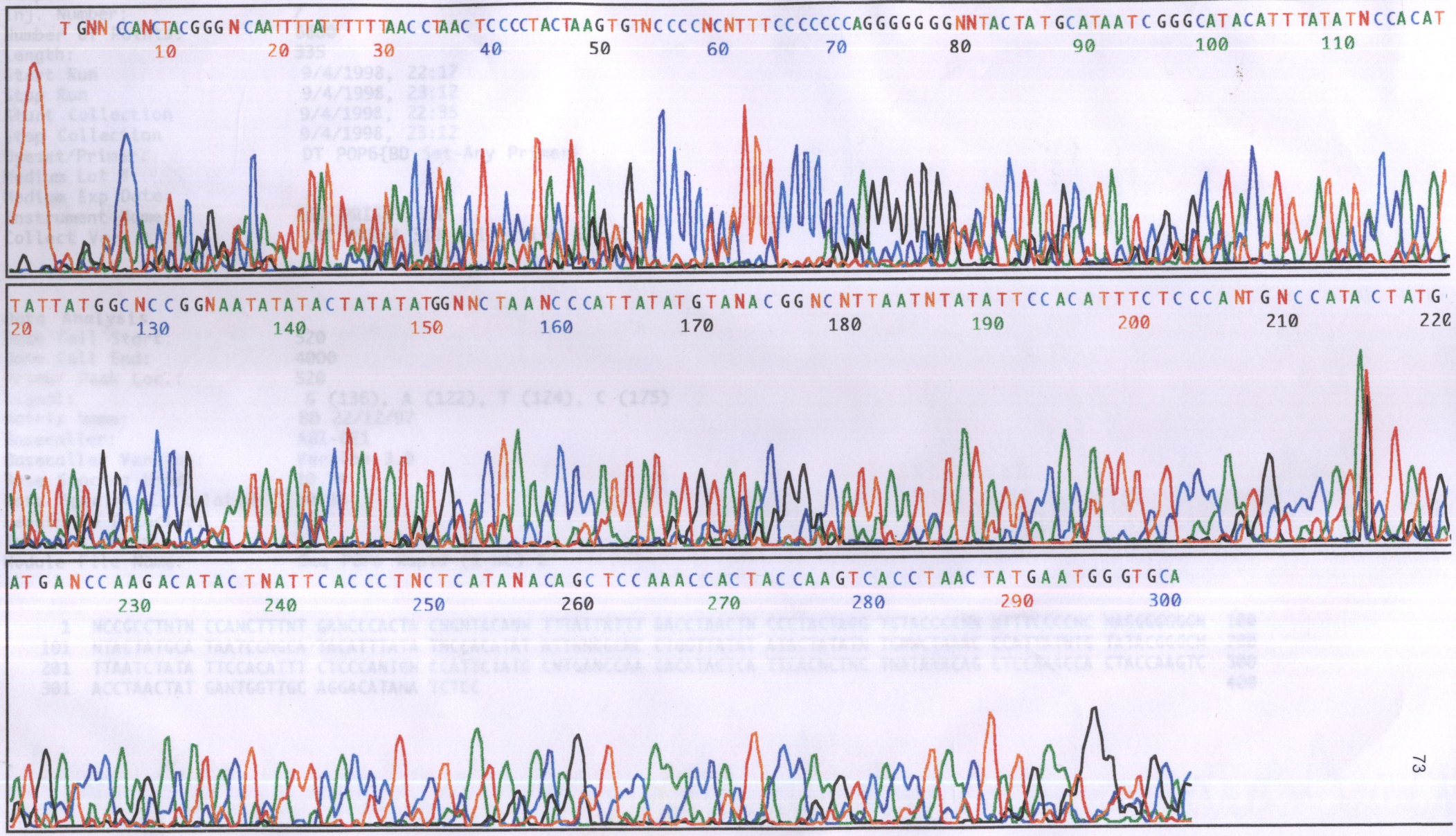

T GNN CCANCTACGGG N CAATTTTATTTTTTAAC CTAAC TCCCCTACTAAGTGTNCCCCNCNTTTCCCCCCCCAGGGGGGGGNNTACTATGCATAATCGGGCATACATTTATATNCCACAT
10 20 30 40 50 60 70 80 90 100 110
TATTATGGCNCCGGNAATATATACTATATATGGNNCTAANCCCATTATATGTANACGGNCNTTAATNTATATTCCACATTTCTCCCANTGNCCATA CTATG
20 130 140 150 160 170 180 190 200 210 220
ATGANCCAAGACATACTNATTCACCCTNCTCATANACAGCTCCAAACCACTACCAAGTCACCTAACTATGAATGGGTGCA
230 240 250 260 270 280 290 300

Data Collection

File:	B2•875
Sample:	875
Inj. Number:	7
Number of Points:	6680
Length:	335
Start Run	9/4/1998, 22:17
Stop Run	9/4/1998, 23:12
Start Collection	9/4/1998, 22:35
Stop Collection	9/4/1998, 23:12
Dyeset/Primer:	DT POP6{BD Set-Any Primer}
Medium Lot #:	
Medium Exp Date:	
Instrument Name:	ABI PRISM™ 310
Collect Vers.:	ABI PRISM 310 Collection 1.0.2

Data Analysis

Base Call Start:	520
Base Call End:	4000
Primer Peak Loc.:	520
Signal:	G (136), A (122), T (124), C (175)
Matrix Name:	BD 22/12/97
Basecaller:	ABI-CE1
Basecaller Version:	Version 3.0
Base Spacing Used:	10.34
Base Spacing Calculated:	10.34
Length to Detector:	47
Tube Position:	B2
Module File Name:	Seq POP6 Rapid (1 mL) E

```
  1  NCCGCCTNTN CCANCTTTNT GANCCCACTA CNGNTACANN TTTATTNTTT AACCTAACTN CCCTACTAGG TGTACCCCNN NTTTCCCCNC NAGGGGGGGN  100
101  NTACTATGCA TAATCGNGCA TACATTTATA TNCCACATAT ATTNNGGCNC CTGGTTATAT ATACTATATN TGNNCTAAAC CCATTATNTG TATACGGGCN  200
201  TTAATCTATA TTCCACATTT CTCCCANTGN CCATTCTATG CNTGANCCAA GACATACTCA TTCACNCTNC TNATANACAG CTCCAAACCA CTACCAAGTC  300
301  ACCTAACTAT GANTGGTTGC AGGACATANA TCTCC                                                                        400
```


ABI PRISM
Model 310
Version 3.0
ABI-CE1
Version 3.0
B2•875
875
Lane 7
Signal G:136 A:122 T:124 C:175
DT POP6{BD Set-Any Primer}
BD 22/12/97
Points 520 to 4000 Base 1: 520
Page 2 o
Fri, Apr 10, 1998 12:04 F
Thu, Apr 9, 1998 10:17 F
Spacing: 10.34{10.3
NC CGCCTNTNC CANC TTTNTG ANCCCA CTAC NG NTACANNTTTATTNTTTAAC CTAACT NCCCT ACTAGGT GTACCCCNNNTTT TCCCCNC NAGGGGGGGNNTACTAT GCATAAT C GN GC ATA
10 20 30 40 50 60 70 80 90 100 110 120
ATTTATATNCCA CATATATTNN G GC N CCTG GTTATATATAC TATAT NT G NNCTAAACCCATTAT NT GTATA C GGGC NTTAATC TATATTCCAC ATTTCTCCC
130 140 150 160 170 180 190 200 210 220
IT G NC CATTC TATGC NT GANCCAAGACATACTCATTCACNCTNCT NATANACA GCTCCAAACCACTACCAAGTCACC TAAC TATGA NT G GTTGCA GGA
230 240 250 260 270 280 290 300 310 320

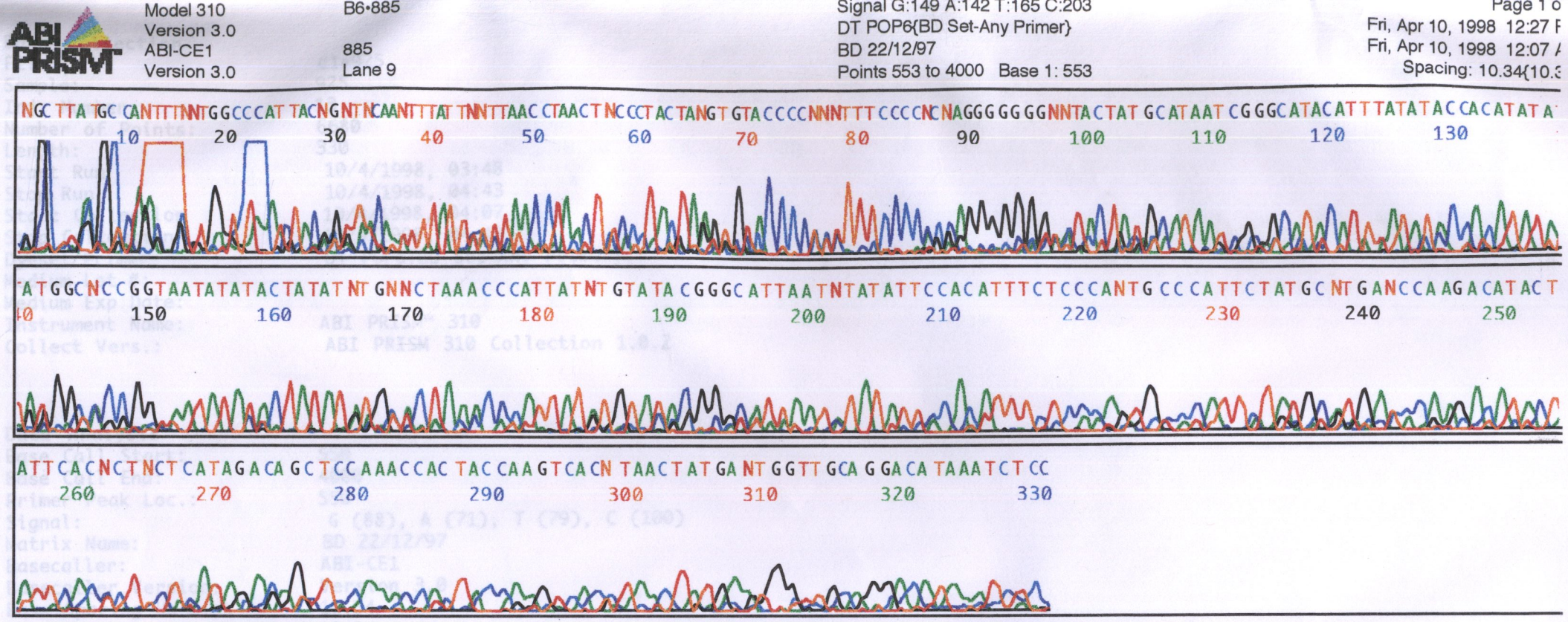
Data Collection

File:	B6•885
Sample:	885
Inj. Number:	9
Number of Points:	6680
Length:	331
Start Run	10/4/1998, 00:07
Stop Run	10/4/1998, 01:02
Start Collection	10/4/1998, 00:26
Stop Collection	10/4/1998, 01:02
Dyeset/Primer:	DT POP6{BD Set-Any Primer}
Medium Lot #:	
Medium Exp Date:	
Instrument Name:	ABI PRISM™ 310
Collect Vers.:	ABI PRISM 310 Collection 1.0.2

Data Analysis

Base Call Start:	553
Base Call End:	4000
Primer Peak Loc.:	553
Signal:	G (149), A (142), T (165), C (203)
Matrix Name:	BD 22/12/97
Basecaller:	ABI-CE1
Basecaller Version:	Version 3.0
Base Spacing Used:	10.34
Base Spacing Calculated:	10.34
Length to Detector:	47
Tube Position:	B6
Module File Name:	Seq POP6 Rapid (1 mL) E

```
  1  NGCTTATGCC ANTTTNNTGG CCCATTACNG NTNCAANTTT ATTNNTTAAC CTAACTNCCC TACTANGTGT ACCCCNNNTT TCCCCNCNAG GGGGGGNNTA  100
101  CTATGCATAA TCGGGCATAC ATTTATATAC CACATATATT ATGGCNCCGG TAATATATAC TATATNTGNN CTAAACCCAT TATNTGTATA CGGGCATTAA  200
201  TNTATATTCC ACATTTCTCC CANTGCCCAT TCTATGCNTG ANCCAAGACA TACTCATTCA CNCTNCTCAT AGACAGCTCC AAACCACTAC CAAGTCACNT  300
301  AACTATGANT GGTTGCAGGA CATAAATCTC C                                                                            400
```


ABI PRISM
Model 310
Version 3.0
ABI-CE1
Version 3.0
B6•885
885
Lane 9
Signal G:149 A:142 T:165 C:203
DT POP6{BD Set-Any Primer}
BD 22/12/97
Points 553 to 4000 Base 1: 553
Page 1 o
Fri, Apr 10, 1998 12:27 F
Fri, Apr 10, 1998 12:07 A
Spacing: 10.34{10.3
NGC TTA TGC CANTTTNNTGGCCCAT TACNGNTNCAANTTTAT TNNTTAAC CTAACT NCCCTAC TANGT GTACCCCNNNTTT CCCCNC NAGGGGGGGNNTACTAT GCATAAT CGGGCATACATTTATATACCACATAT A
10 20 30 40 50 60 70 80 90 100 110 120 130
TATGGCNCCGGTAATATATACTATATNT GNNCTAAACCCATTATNT GTATA CGGGCATTAATNTATATTCCACATTTCTCCCANTG CCCATTCTATGC NT GANCCAAGACATACT
40 150 160 170 180 190 200 210 220 230 240 250
ATTCACNCTNCT CATAGACA GCTCCAAACCAC TAC CAAGTCACN TAACTATGA NT GGTT GCA GGACA TAAATCTCC
260 270 280 290 300 310 320 330

Data Collection	
File:	C1•925
Sample:	925
Inj. Number:	13
Number of Points:	6680
Length:	330
Start Run	10/4/1998, 03:48
Stop Run	10/4/1998, 04:43
Start Collection	10/4/1998, 04:07
Stop Collection	10/4/1998, 04:43
Dyeset/Primer:	DT POP6{BD Set-Any Primer}
Medium Lot #:	
Medium Exp Date:	
Instrument Name:	ABI PRISM™ 310
Collect Vers.:	ABI PRISM 310 Collection 1.0.2

Data Analysis	
Base Call Start:	550
Base Call End:	4000
Primer Peak Loc.:	550
Signal:	G (88), A (71), T (79), C (100)
Matrix Name:	BD 22/12/97
Basecaller:	ABI-CE1
Basecaller Version:	Version 3.0
Base Spacing Used:	10.34
Base Spacing Calculated:	10.34
Length to Detector:	47
Tube Position:	C1
Module File Name:	Seq POP6 Rapid (1 mL) E

```
  1  GNTGCGCCNN TTNCTGGNCC ACTACNGNTA CATTTTATTN TTTAACCTAA CTNCCTACTA NGTGTACCCC NCNTTTCCCC CNNAGGGGGG GNNTACTATA  100
101  GCATAATCGG GCATACATTN ATATNCCNCA TATATTANGG CNCCGAGTAA TATATACTAT ATNTGCNCTA AACCCATTAT ATGTATACGG GCNTTAATCT  200
201  ATATTNCACA TTTCTCCCAN TGNCCATTNT ATGCATGATC TAAGACATAC TCGTTCACNC TNCTCATAGA CAGCTCCAAA CCACTACCAG AGTCACCTAA  300
301  CTATGNAATG NTTGCAGGAC ATAAATCTNC                                                                            400
```

ABI PRISM

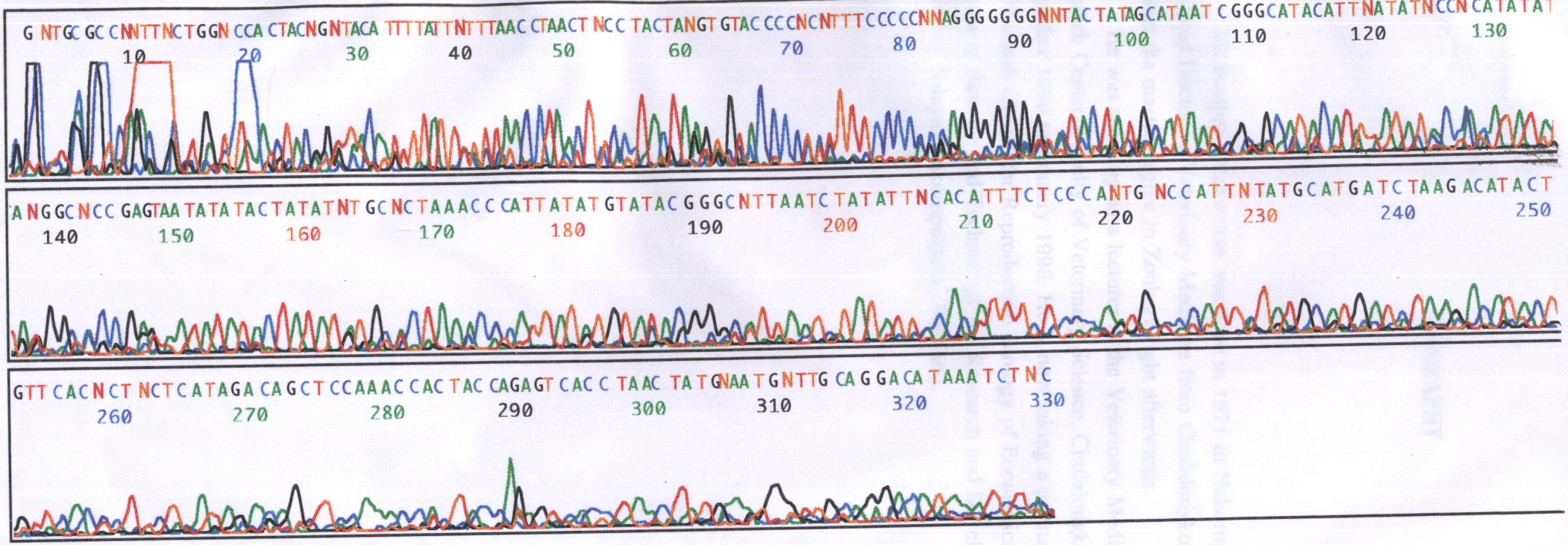
Model 310
Version 3.0
ABI-CE1
Version 3.0

C1•925

925
Lane 13

Signal G:88 A:71 T:79 C:100
DT POP6{BD Set-Any Primer}
BD 22/12/97
Points 550 to 4000 Base 1: 550

Fri, Apr 10, 1998 12:27 F
Fri, Apr 10, 1998 3:48 A
Spacing: 10.34{10.3



BIOGRAPHY

Mr.Boripat Siriaroonrat was born in 1971 in Nakornpathom, Thailand. He graduated Doctor of Veterinary Medicine from Chulalongkorn University in 1994. He start the master degree in Zoology right afterwards.

He was working as a lecturer at the Veterinary Medical Aquatic Animal Research Center, Faculty of Veterinary Science, Chulalongkorn University from November 1996 to January 1998. He is now taking a permanent lecturer position at the Research Center for Reproductive Biology of Economically Valuable Animals, Institute of Science and Technology for Research and Development, Mahidol University Salaya, Nakornpathom, Thailand.