ความแปรผันทางพันธุกรรมของไก่พื้นเมือง *Gallus gallus domesticus* ของไทย โดยไมโครแซทเทลไลท์ดีเอ็นเอ

นางสาวปิยมาศ การสมดี

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GENETIC VARIATIONS OF THAI NATIVE FOWLS *Gallus gallus domesticus* BASED ON MICROSATELLITE DNA

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ปิยมาศ การสมดี : ความแปรผันทางพันธุกรรมของไก่พื้นเมือง *Gallus gallus domesticus* ของไทย โดยไมโครแซลเทลไลท์ดีเอ็นเอ (GENETIC VARIATIONS OF THAI NATIVE FOWLS *Gallus gallus domesticus* BASED ON MICROSATELLITE DNA) อ.ที่ปรึกษา : รศ. วีณา เมฆวิชัย, อ.ที่ปรึกษาร่วม : รศ.ดร.วรวิทย์ สิริพลวัฒน์, 94 หน้า. ISBN 974-333-965-5

จากการวิเคราะห์ลักษณะทางสันฐานวิทยาระหว่างไก่พื้นเมืองของไทย *Gallus gallus domesticus* จำนวน 4 สายพันธุ์ๆละ 15 ตัว และไก่ป่าตุ้มหูขาว *Gallus gallus gallus* จำนวน 10 ตัว พบว่ามีความแตกต่างทางสันฐานวิทยา คือ ความยาวของแข้ง ความยาวของจงอยปาก ความยาวของหัว และความยาวของปีก อย่างมีนัยสำคัญทางสถิติ ($P<0.05$)

การวิเคราะห์ความแปรผันทางพันธุกรรมระหว่าง subspecies ของไก่พื้นเมือง *G. g. domesticus* ของไทย 4 สายพันธุ์ๆละ 25 ตัว และระหว่างไก่พื้นเมืองของไทย กับ ไก่ป่าตุ้มหูขาว *G. g. gallus* 11 ตัว โดยการใช้ไมโครแซทเทลไลท์ดีเอ็นเอ ทำการเพิ่มปริมาณดีเอ็นเอในยีโนมของไก่ในแต่ละสายพันธุ์ด้วยปฏิกิริยาลูกโซ่โพลีเมอเรส (PCR) เมื่อใช้ไพรเมอร์ของไมโครแซทเทลไลท์ดีเอ็นเอ 4 บริเวณ คือ MCW240, ADL23, LEI73 และMCW87 ได้จำนวนอัลลีลในแต่ละบริเวณเป็น 13, 12, 11 และ 6 ตามลำดับ จากการวิเคราะห์ค่าเฉลี่ย observed heterozygosity (H_o) ของสายพันธุ์ ไก่แจ้ ไก่ป่าตุ้มหูขาว ไก่ชนสีประดู่หางดำ ไก่เบตง และไก่ชนสีเหลืองหางขาว พบว่ามีค่าเท่ากับ 0.76, 0.73, 0.72, 0.72 และ 0.66 ตามลำดับ และจากการทดสอบความแตกต่างของค่า expected heterozygosity (H_E) ระหว่างสายพันธุ์ไก่พื้นเมืองของไทย พบว่าไม่มีความแตกต่างกันทางสถิติ

การทดสอบกฎของ Hardy-Weinberg ในไก่พื้นเมืองของไทยทุกสายพันธุ์ และ ไก่ป่าตุ้มหูขาว พบว่าเฉพาะไมโครแซทเทลไลท์ดีเอ็นเอบริเวณ MCW240 ของสายพันธุ์ไก่ชนสีเหลืองหางขาวมีการเบี่ยงเบนไปจากกฎดังกล่าว และจากการทดลองพบว่าสายพันธุ์ไก่ชนสีเหลืองหางขาวพบอัลลีลที่มีแนวโน้มที่สามารถจะนำมาใช้เป็นตัวบ่งชี้ในการจำแนกสายพันธุ์ไก่ชนิดนี้ได้ อย่างไรก็ตามเมื่อทำการศึกษาความแตกต่างทางพันธุกรรมโดยไมโครแซทเทลไลท์ดีเอ็นเอ แสดงให้เห็นว่าไก่พื้นเมืองของไทยมีความแตกต่างทางพันธุกรรมภายใน subspecies มาก แต่มีความแตกต่างระหว่าง subspecies น้อย และเมื่อคำนวณค่าระยะห่างทางพันธุกรรมตามวิธี Cavalli-Sforza พบว่าระยะห่างทางพันธุกรรมระหว่างไก่แจ้และไก่ป่าตุ้มหูขาวมีค่ามากที่สุด คือ 0.1340 ในขณะที่ระยะห่างทางพันธุกรรมระหว่างไก่ชนสีประดู่หางดำและไก่เบตงมีค่าน้อยที่สุด คือ 0.0618

ภาควิชา.....-.....ลายมือชื่อนิสิต.....ปิยมาศ การสมดี.....

สาขาวิชา.....เทคโนโลยีทางชีวภาพ.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2542..... ลายมืออาจารย์ที่ปรึกษาร่วม.....

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PIYAMAS KARNSOMDEE : GENETICVARIATIONS OF THIA NATIVE FOWL *Gallus gallus domesticus* BASED ON MICROSATELLITE DNA. THESIS ADVISOR: ASSOC. PROF. WINA MECKVICHAI. THESIS CO-ASVISOR: ASSOC. PROF. VORAVIT SIRIPHOLVAT, Ph.D., 94 pp., ISBN 974-333-965-5

Morphometric analysis of tarso-metatarsus length, beak length, head length and wing length of Thai Native Fowls *Gallus gallus domesticus* 15 individuals per variety and 10 individuals of Red Jungle Fowl *Gallus gallus gallus* are significant differences ($P<0.05$).

Genetic variations between subspecies of 4 varieties of Thai Native Fowls *G. g. domesticus*, and Red Jungle Fowl *G. g. gallus* were analyzed by using 4 chicken microsatellite loci are 25 individual per varieties. Genomic DNA was extracted from bloodstain and amplified by polymerase chain reaction (PCR). Amplification products of 4 microsatellite genotyping at MCW240, ADL23, LEI73 and MCW87 the original from loci have total alleles number at each locus were 13, 12, 11 and 6, respectively. Mean of observed heterozygosity (H_O) of Bantam, Red Jungle Fowl, Praduhangdam Fighting Cock, Betong Chicken and Luenghangkhoa Fighting Cock were 0.76, 0.73, 0.72, 0.72 and 0.66, respectively. The analysis of mean of expected heterozygosity (H_E) was not different between varieties.

All populations of Thai Native Fowls and Red Jungle Fowl were test for Hardy-Weinberg assumption. It is found that only Luenghangkhoa Fighting Cock at MCW240 locus deviated from this assumption. Luenghangkhoa Fighting Cock found an unique allele that has trend to use for allele specific of this population. Genetic differentiation among four varieties of Thai Native Fowl was higher than genetic differentiation between subspecies. Genetic distance was analyzed by Cavalli-Sforza method. It found that Bantam - Red Jungle Fowl genetic distance was lowest, and Praduhangdam Fighting Cock - Betong Chicken genetic distance was highest.

ภาควิชา.....-.....ลายมือชื่อนิสิต.....Piyamas Karnsomdee.....

สาขาวิชา.....เทคโนโลยีทางชีวภาพ.....ลายมือชื่ออาจารย์ที่ปรึกษา.....Wina Meckvichai.....

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List of Abbreviations

$AgNO_3$	=	Silver nitrate
A T C G	=	nucleotide containing the base adenine, thymine, cytosine and guanine, respectively
bp	=	base pair
°C	=	degree cencleus
dNTP	=	deoxyribinucleotide containing the base adenine, thymine, cytosine and guqnine, respectively
DNA	=	deoxyribonucleic acid
EDTA	=	ethylene diamine tetraacetic acid
Kb	=	kilobase
mg	=	milligram
mL	=	millilitre
mM	=	millimolar
$MgCl_2$	=	magnesium chloride
ng	=	nanogram
Na_2CO_3	=	sodium carbonate
NH_4OH	=	ammoniumhydroxide
rpm	=	round per minute
SDS	=	sodium dodecyl solphate
S.E.	=	standard error
TE	=	tris-ethylene diamine tetraacetic acid
TBE	=	tris-boric- ethylene diamine tetraacetic acid
TEMED	=	N, N, N', N'-tetramethylethylenediamine
µL	=	microlitre
µM	=	micromolar

Chapter 1

Introduction

Thailand is an agricultural country. Most of the incomes of the country come from agriculture and livestock production. In the poultry industry, chicken products come from both commercial and hybrid strains. These strains often mate between native fowl breed (domestic chicken) and exotic fowl breed (imported chicken) has been carried out by breeders. The chicken products, both eggs and meats, which are important protein sources for people make a high benefit for Thai farmers. Some other varieties of domestic chickens are bred for pet and game birds. Domestic chicken has high utility for breeding program, because it has many good characteristics. For example, the males domestic chickens have beautiful appearance, and females are good at parental care their the chicks. In addition, domestic chickens have advantages over exotic chicken that they tolerate extremely high temperature. They also resist to several diseases and violence microorganism. Therefore, domestic chickens are suitable to use for parent stock and important native resources. In Thailand, there are three common varieties of Thai Native Fowl, which are very well known among Thai farmers, first variety is Fighting Cock. It is the most common Thai Native Fowl, because of the male of this variety has an attractive appearances, courage, strong beak, strong wings and strong legs. Therefore, it is suitable for cock fighting game as well as for breeding to hybrid strains. Second variety is Bantam. This variety is bred for pet and fancy chicken, because they are miniatures in size, friendly and colorful plumage patterns. Some Bantams have unique color and characteristic that are not found in other varieties. The last variety is Betong Chicken. This variety is commonly found in Southern Thailand, because Betong Chickens prefer and grow well at a high humidity climate. In addition, it is often bred with other local varieties for a good hybrid strain. Therefore, pure variety of Betong Chicken is difficult to find. As a result, population of this variety is normally smaller than other varieties. However, Betong Chicken is famous for white meat

source, because it is meaty and delicious. Therefore, Betong Chicken's meat is more expensive than other meats.

Previously, genetic diversity of Thai Native Fowls has not been studied and without any published report. Thus, the present status of genetic diversity of Thai Native Fowls is still unknown. Genetic diversity is the basic requirement for animal breeding and development of the breed and variety for a long-term sustainable of livestocks. It is important to study genetic variability among individuals and population of Thai Native Fowls.

In general, genetic variability can be studied by two methods. First is by an indirect method, morphological characteristics (e.g.: body dimension, color and pattern of plumages). This method is relatively inexpensive, simple and less time consuming. However, morphological characteristic is phenotype, which is a product of the gene expression and gene-environmental interaction. So, environment may affect the expression of the gene, and effect to morphological traits. Furthermore, the result of morphological characteristic may be different from molecular genetic. As a result, the studies of genetic variation by this method may not be sufficient. Second is by molecular genetic technique that is a direct method. Although It is complex, expensive, demanding expertise and time-consuming, this technique has availability for the studies of genetic variations because it can detect variation at DNA level and explain the genetic variation better than morphological characteristics. Therefore, this method is now appropriate for solving many problems in many fields such as evolution, taxonomy, phylogeny, speciation, genetic diversity and animal breeding.

Recently, a new genetic marker has been discovered as microsatellite DNA. It is very useful for studying the genetic variation, because it is a hypervariable genetic marker, high mutation rate and it can be detected genetic variation among species, populations and individuals. When polymerase chain reaction (PCR) has been

developed, some problems such as very small amount or even degraded sample of DNA have been eliminated. The small amount of DNA from the field can be amplified at a specific DNA region by thermal cycling machine. In addition, PCR is very easy to handle sample for fresh and preserved tissue, oral-swabs, bloodstain and even museum specimens. Moreover, we can chose non-invasive method by collecting hair follicles or feces as DNA source for PCR amplification.

Genetic variations among three common varieties of Thai Native Fowl *Gallus gallus domesticus*, Fighting Cock, Bantam and Betong Chicken, were studied by using four microsatellite DNA markers. The research gives some basic information about the genetic variation. This result shows the present status of genetic diversity of Thai Native Fowls, and can be planed to conserve these genetic resources in order to maintain genetic diversity for sustainable livestock and be used for genetic information for future research in breed development.

Objective

1. To study genetic variations among and within four varieties of Thai Native Fowl are that Praduhangdam and Luenghangkhoa Fighting Cock, Bantam and Betong Chicken, using four microsatellite DNA markers.
2. To study the relationship between morphometric and molecular genetic analysis of four varieties of Thai Native Fowl.

Anticipated benefit

1. Knowledge of genetic variation within and among four varieties of Thai Native Fowl.
2. Knowledge of morphological characteristic among four varieties of Thai Native Fowls.
3. Knowledge of the present status of genetic diversity of Thai Native Fowls.
4. The result of this study is a basic information for the research of breed development.

Chapter 2

Literature Review

The native chickens *Gallus gallus domesticus* are believed to be selected and domesticated by human from its ancestor, Red Jungle Fowl *Gallus gallus gallus*, in the Southeast Asia since 2500 BC (Crawford, 1990; Siegel, 1993; Rose, 1997). They have been adapted and modified morphology, physiology and behavior via genetic and non-genetic procedures (Siegel, 1993; Moreng and Avens, 1996). Today, domestic chicken is commonly used for raising in animal farms. In addition, Red Jungle Fowl is still found in bamboo forest in Southeast Asia and can be seen roaming freely through the small villages as well as large cities in Phillipines, Indonesia, Malaysia, Vietnam and Thailand (Crawford, 1990; Moreng and Avens, 1996).

At present, domestic chickens have been selected and developed to serve human demand and have varieties of strains (Dettlaff and Vassetzky, 1991). Crawford cited in Sossinka (1982), They describe adaptation and predisposition of domestic chicken. He listed four traits that are common to most domestic chicken species. First, in early stage of domestication, chickens would have to forage on their own, favoring species that was seed or grass eaters. Second, it had to be able to reproduce in captivity, giving an advantage to species that was not highly dependent on specific climatic and environmental factors for breeding. Third, the ability to imprint was important in initial taming. Finally, it had to have a social order that permitted the keeping for large number of individuals. Previously, domestic chickens were unconsciously selected long before intentional selection for the specific traits. Later, developmental process of domestication of chicken has accelerated. Because of intensive selection for production traits and changes in the environment in which chicken are maintain (Siegel, 1993). Thus, the development of domesticated chickens was viewed not only as successful or unsuccessful but with awareness that there was genetic variability among

those individual considered to be successful in adapting (Siegel, 1993). Domestic chicken has good properties that this can be maintained and hereditary. It is genetic property, which index genetic diversity.

In Thailand, native fowls have been adapted and modified in characteristic and behavior for the development and maintenance of the standard breeds, varieties and strains which through both natural and artificial selection (Sawat Thammabud, 1996; Charan Chantalukkana, 1996).

2.1 Classification of domesticated chickens

A recent taxonomic status for domestic chicken *Gallus gallus domesticus* belongs to:

Kingdom Animalia
Subkingdom Metazoa
Phylum Chordata
Subphylum Vertebrata
Class Aves
Order Galliformes
Family Phasianidae
Genus Gallus
Species *Gallus gallus domesticus* (Fumihito *et al.*,1995)

Common name: chicken, poultry, fowl

2.2 Characteristic of Thai Native Fowl

Purebred chicken may be identified according to their placement into a specific class, breed, variety and strain (Moreng and Avens,1992). Many breeds are subdivided into varieties, distinguished by the difference in comb type, plumage colors and pattern (Crawford, 1990).

Three common varieties of native fowls are raised in Thailand. There are Fighting Cocks, Bantam and Betong Chickens. The morphology of three common varieties of Thai Native Fowls are shown in figure 2.1, 2.2 and 2.3 and described as follows:

1. Fighting Cock, they have several plumage colors for identify the varieties. For example, Praduhangdam, Praduhangkhoa, Luenghangdam, Luenghangkhoa. The most common varieties are raised among Thai farmers are that Praduhangdam and Luenghangkhoa. The name of this variety is based on plumage color of the male, but the female is entirely in black color (Wu, Gusman and Peterson, 1991).

Praduhangdam, in adult males have dark-brown fringed feathers on the saddle, neck hackles, beak and wings bow. Rectric, primary and secondary wing feathers are black. The ventral part of the breast down on to the abdomen and belly are also black. Its comb is pea type.

Luenghangkhoa, in adult males have mainly black color on ventral part, while dorsal plumage including the neck, hackle, saddle, beak and wing bow region are yellow. Some feather at the middle of rectric and primary wing feathers are whitish color on the web. Its comb is pea type.

AP

AL

Figure 2.1 Fighting Cock (Praduhangdam, AP and Luenghangkhoa, AL)

Figure 2.2 Bantam (B)

Figure 2.3 Betong Chicken (C)

2. Bantam is miniature chicken. They are approximately one-fourth to one-fifth the weight of larger chickens (Moreng and Avens, 1996). They are originated in the Orient and are also many different types in color and plumage patterns. Plumage colors of males and female are alike. This variety is a single comb type.

3. Betong Chickens are named by the town, which believed to have originated. Plumage colors of adult males are all reddish-yellow, while females are all whitish-yellow. There is some white on the web of the primaries and the rectrics of females. Betong Chickens show no development of the tail feathers and only 4-8 secondary wing feathers have developed (Wu, Guzman and Peterson, 1991).

2.3 The chicken genome

Chickens have 39 pairs of chromosomes. The genome comprises of all the genetic materials present in the haploid cell and include all the nuclear and mitochondrial genes (Cooke and Buckley, 1989).

Genome size

All cells from a typical eukaryotic have the same amount of DNA per haploid set of chromosome. Chicken genome has 1.2×10^9 basepairs of haploid genome size (Smith and Wood, 1991), and it has 1.41 picograms of average haploid DNA content (Stevens, 1996). The amount of DNA in all the diploid cells of an individual organism including chickens is content and there is only small variation in the amount of DNA per cell within species. DNA in eukaryotic cell is usually divided into three types (Stevens, 1996) as follow: (i) unique sequences, of which there are only single copy, (ii) moderately repetitive sequences (up to about 1000 copies), and (iii) highly repetitive sequences ($10^3$to 10^5copies). Chickens have a smaller proportion of repeat sequence compare with other vertebrates (Nicholas, 1996). The chicken genome is unusual in that it is dispersed over a large number of chromosomes that can be subdivided into 14-16

macrochromosomes and 60-64 microchromosomes. In the domestic chicken, approximately 70% of DNA is distributed amongs the macrochromosomes (pairs 1 to 10) and 30% amongst the microchromosomes (pairs 11 to 39) (Stevens, 1996).

2.4 Genetic variation of Thai Native Fowls

The major requirement of any progress in animal breeding is the genetic variation within and between varieties of the farm and domestic animals (Ciampolini *et al.*,1995). The genetic variation should be maintained for developing sustainable animal production systems (Moran, 1993). Also the genetic variation of Thai Native Fowls should be highly maintained. The status of genetic diversity of Thai Native Fowl is no obvious, because the study about genetic diversity in Thailand recently has been occurred by encouragement of FAO (FAO, 1992; Stene, 1996).

2.5 Molecular genetic method of chicken genome

Since the molecular genetic method and polymerase chain reaction (PCR) technique have been developed. There have been increasingly studied in vertebrate genome, especially in the chicken genome. This method has highly a resolution power. It also permits exceedingly detailed description of genetic variation in DNA and molecular component (Crawford, 1990). Among the ornithologists, molecular genetic method and PCR technique in explosively increase study in systematic and population genetic are mainly at the level of DNA (Mindell, 1995). Furthermore, this method and technique is used to apply for many fields in the chicken genome such as breeding system (Crooijmans et al, 1996), genome mapping (Cheng et al, 1995), marker-QTL (Kaam et al, 1999), and genetic diversity (Zhou and Lamont, 1999).

2.6 Method of determining genetic variation

The most common method exists for determining genetic variation in natural population can be subdivided into two methods.

Phenotypic method

Several animal species are grouped on the basis of their phenotypic attributes. The common parameter taken from different characters is morphological characteristic. It is subdivided into two parameter as morphology and morphometric traits that are compared between different species, populations and individuals (Stevens, 1996). Morphology is a comparative based on plumage color and pattern polymorphism, where as morphometric trait is comparative based on body dimension polymorphism (e.g.: tarso-metatarsus length, beak length, wing length, nape length) (Lovette, 1998). In addition, the phenetic analyses of morphological characteristic might track phenotypic evolution. The evolution process and rate of evolution may not be related with, and differed from molecular variation (Zink and Blackwell, 1996). For example, the study of molecular phylogeny and evolution of morphology of the Piranga genus found that morphology and morphometric trait are not related with genetic variation (Burns, 1998). In addition, the study of allozyme, mtDNA and morphometric variation of sparrow genera found that molecular variation is not related with morphometric variation (Zink and Blackwell, 1996). Thus, the genetic variation by only morphological characteristic may be resulted differently from molecular genetic method. As a result, the study of variation should be used morphological characteristic together with molecular genetic method.

Genotypic method

Molecular genetic is now appropriate for solving important question. It also has been developed for a new perspective in biology research. Many molecular

genetic methods were studied. For example, several ornithological fields provide good illustrations that allozymes have been used intensively in studies of genetic relatedness. Nevertheless, low genetic variability and a paucity of loci make this technique unsuitable for parentage assignment or within group relatedness determination (Ferraris and Palumbi, 1996). DNA-DNA hybridization has clearly contributed to avian molecular and systematic (Sibley and Anlquist, 1990). Also polymorphism pattern of mitochondrial DNA (mtDNA) has aided in differentiation studies of species and populations (Mindell, 1995), it is maternal inheritance. However, it is not suitable for discrimination of closely related population, which seems to be this case study. Polymorphism of random genetic DNA fragment has been used to resolve questions about polymorphism (Ferraris and Palumbi, 1996). In addition, DNA fingerprinting using minisatellite or synthetic simple repeats probes to prove the most sensitive method for determining the genetic relationship of individuals in population (Sililuck Ponsuksiri, 1995). In addition, it is a reliable technique for parentage exclusion within family groups, but it is not suitable for examining larger or across groups, because of the high variability of this technique and difficulties associated with marking comparisons between gel (Ferraris and Palumbi, 1996). Microsatellite DNA marker has received considerable attention in genome analysis (Ellegren, 1992). Furthermore, it is used for parentage assignment and relatedness estimation, ideal marker would be Mendelian fashion, selectively neutral, and high polymorphic. Scores would be unambiguous enough to be evaluated against a standard across gels. Microsatellite meets these criteria (Ferraris and Palumbi, 1996).

2.7 Microsatellite DNA marker

Recently, microsatellite DNA marker is a new genetic tools that applies to study in the population genetics, and it has become the focus of the search for hypervariable single locus marker, being abundant and wildly dispersed in the eukaryotic genomes (Tautz, 1989)

Microsatellite DNA belongs to the family of repetitive non-coding DNA sequence (Koreth, O'Leary and McGee, 1996). It is short tandemly repeating oligonucleotide sequences found to be highly polymorphism in the genome (Mindell, 1995). Microsatellite DNA demonstrates to be inherited in a Mendelian fashion (Sukamol Srikwan, Field and Woodruff, 1996). It is usually 2-6 nucleotide of repeating unit (e.g.: di-, tri-, or tetranucleotide repeats), with variation in numbers of repeating unit that is thought to be due to slippage strand missing, unequal crossing over, slippage during DNA replication and replication error (Wright, 1994). Therefore, it is highly polymorphism genetic markers in the animal taxa (Crooijman, 1993). Microsatellite DNA marker is commonly having more than 12 alleles at a single locus and heterozygosity level up to 90% (Taylor, Sherwin and Wayne, 1994). In addition, the DNA sequences flanking the repetitive arrays can be used to design primers for PCR amplification, because it has small of the total size of the arrays, generally less than 300 basepairs (Taylor, Sherwin and Wayne, 1994). The mutation rate of microsatellite DNA is estimated to range between 10^{-3} to10^{-5} per gamate per generation (Mindell, 1996). The products of PCR amplification are visualized by polyacrylamide gel electrophoresis. The development of microsatellite marker has proceeded for a wide variety of animal, including shrimp (Wolfus, Garcia and Warren, 1991), pig (Moran, 1993), cattle (Machugh *et al.*, 1994) and chicken (Khatib, 1993; Cheng and Crittenden, 1994; Crooijmans, 1997). There are having a range of 1-15 alleles and 40-90% heterozygosity for the avian microsatellite DNA, but it seems to have fewer microsatellites DNA in their genome than other vertebrates (Ferraris and Palumbi, 1996)

Microsatellite markers are used in several scales. For example, among gene, they are used for gender determination in bird by using microsatellite loci on W and Z chromosome (Mindell, 1995). Among individuals, they are good choice for assessing parentage and relatedness among individuals because of the contribution of relative to inclusive fitness (Blouin *et al.*,1996). Among population, microsatellite is the major element analyses in avian, because screening is usually confined to perfect

repeats. In a population level study, validity of a number of repeats concerning the mutation process and the balance between mutation and drift (Allen *et al.*, 1995). The sampling necessary to be examined variation among population. Population genetic of microsatellite DNA marker was used to study about genetic variation of Red Jungle Fowl between northern and southern Thailand (Pramong Begthaisong, 1998). Among species, they are used to study of comparing level of genetic variation in domestic sheep (*Ovis aries*) with those of bighorn sheep (*Ovis canadensis*) (Forbes *et al*,1995). Among Genera and Higher Taxa, they are examined genetic distance that will probably be less studied at lower taxonomic level. Because it has problem about homoplasy that constrains on repeating number, non linear divergence and high mutation rate are likely to make such analysis unrewarding in comparison to those using other molecular more suited to these coarse grained analysis (Mindell, 1995). In addition, microsatellite can be used to investigate for animal breeding and preservation of natural resource, it can be subdivided a breed into subpopulation that are genomic homogeneous, and these can be tested for morphological or functional differences (Ciampolini *et al.*, 1995)

Furthermore, Microsatellite DNA markers are the most likely to conform with the assumption of neutrality and proved to be powerful in differentiation geographically isolated population, sibling species and subspecies (Rico, Ibrahim, Rico and Hewitt, 1997). It is also shown an appropriate tools to detect linkage between markers and genes controlling productive, disease and morphological traits (Ciampolini, 1995). Not only are these markers useful for genome mapping, but they can also be used in animal breeding.

The advantages of microsatellite DNA markers over traditional methods showed that population surveys of microsatellite variations yield 5 or more time higher heterozygosity level than it can be detected by using allozyme electrophoresis (Sukamol Srikwan, Field and Woodruff, 1996). Also, multilocus minisatellite VNTRs resolved by whole genomic DNA fingerprinting, becomes in contrast to microsatellite DNA marker,

which is a small size of amplified products. Thus, temporal changes in allele frequencies can potentially be studied directly through non-invasive DNA sampling methods based on degraded DNA in museum specimens, plucked feathers and faces (Taylor *et al.*, 1993; Mundy *et al.*,1997).

2.8 Utilization of microsatellite genotyping in the domestic chicken

In the domestic chickens, microsatellite DNA markers are used to detect and determine genetic variation in many cases. For example, 29 microsatellite tracts had polymorphism, when it was examined in East Lancing and Compton (reference families of chicken) (Khatib *et al.*, 1993). 19 of 33 microsatellites primer set were examined polymorphism in at least one of three sets of chicken families (Cheng and Crittenden, 1994). Microsatellite 275 markers were polymorphism in Wageningen resource population 93%, in East Lancing 57% and Compton 44% that has the average number of alleles are 4 alleles (Crooijmans et al., 1993). Microsatellite 34 markers of poly (TG) type have polymorphism in genomic library of DNA of white leghorn chicken (Crooijmans *et al.*, 1994). The 151 of microsatellites were developed and used to detect polymorphism for two reference populations and their resource population (Cheng *et al.*, 1995). Microsatellite 42 loci were analyzed in 23 highly inbred chicken lines derived from Leghorn, Red Jungle Fowl, Fayoumi and Spanish breeds that were calculated ban-sharing and proportion of shared alleles distances (Zhou and Lamont, 1999). The 17 microsatellite markers were determined allele frequencies in commercial and layer broilers that average number of allele was 5.8 and 3.0 and heterozygosity was 53% and 27%, respectively (Crooijmans *et al.*, 1996). Nine microsatellite markers were determined genetic variability in eight chicken lines that polymorphism in all markers and number of allele varying from 4 to 13 per locus and 1 to 10 per lines (Vanhala *et al.*, 1998).

Chapter 3

Methodology

3.1 Specimens used in this study

Three common varieties of Thai Native Fowl *Gallus gallus domesticus* are collected from different sources, as follow:

1. Sample number 1A - 50A are Fighting Cock that they are divided into two varieties following plumage color :

Sample number 1A - 20A and 41A - 45A are the Fighting Cock, Praduhangdam, were collected from Thailand Native Fowl Conservative Association, Nongchaok, Bangkok and Thai farmer in Pranakornsriayutthaya province.

Sample number 21A - 40A and 46A - 50A are the Fighting Cock, Luenghangkhoa, were collected from Kabinburi Nationality Poultry Research and Development Center, Praginburi province and Thailand Native Fowl Conservative Association, Bangkok.

2. Sample number 1B - 25B are Bantam, which is in three different feathers color: cocoa, black and white, are collected from Thailand Bantam Conservative Association, Sathupradit, Bangkok.

3. Sample number 1C - 25C are Betong Chicken, were collected from Betong Chicken farm in Yala province.

Red Jungle Fowl, *Gallus gallus gallus*, number 1D - 10D and 11D were collected from Kao Soi Dao Wildlife Breeding Station, Chantaburi province and Kabinburi National Poultry Research and Development Center, respectively.

3.2 Material

Equipments

- Disposable syringe Tuberculin® 1.0 ml. with needle gauge number 25
- Whatman® filter paper
- Dessicator
- Autoclave
- Water bath (Uni-Bath model RU-2, Sakura Finetecnical co. Ltd., Tokyo 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)
- Spectrophotometer DU 650 (Beckman, USA.)
- Laminar flow hood UV light (model DFL 120, Thai Interfil co. Ltd., TH.)
- PCR Thermal cycler: Omnigene (Hybriad Limited, UK.)
- Vertical sequencing gel electrophoresis apparatus (Bio-RAD Laboratory, USA.)
- Power supply (power pac 3000 - Bio-RAD Laboratory, USA.)
- Gel dryer (Model 583 Bio-RAD Laboratory, USA.)
- - 20^{0}C Freezer (Sony co. Ltd., Japan)
- pH meter SP-7 (Sun Tex Digital pH meter)

- Pyro magnèstir (Lab-Line Instrument, Inc.)
- Bunsen Funnel with plate
- Watman® Laboratory sealing film
- Ice box (USA/Scientific plastic co. Ltd.)
- Electronic clock timer Model CT-30 (Canon co. Ltd., Japan)

Chemicals

- Chelex® 100 (Bio-RAD Laboratory, USA.)
- Phenol (Carlo Erba)
- Chloroform (Merck, Germany)
- Absolute Ethanol (Merck, Germany)
- Sodium acetate (Merck, Germany)
- Sodium dodecyl sulfate, SDS (Merck, Germany)
- Sequenase PCR Product sequencing kit (Amercham Life Science USB, USA.)
- 100 mM. dATP, dGTP, dCTP, dTTP (Promega corporation, USA.)
- Tris-(hydroxy methyl) aminomethane (Phamasia Biotech, USA)
- Boric acid (Bio-RAD Laboratory, USA.)
- EATA (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.)
- Fomamide (Merck, Germany)
- Methanol (Merck, Germany)
- Nitric acid (J.T. Baker, USA.)

- Silver nitrate (Nacalai Tesque, Japan)
- Sodiumcarbonate (Merck, Germany)
- Formaldehyde (Merck, Germany)
- Glacial acetic acid (J.T. Baker, USA.)

Enzymes

- Taq DNA polymerase (Promega corporation, USA.)
- Proteinase K (Promega corporation, USA.)

3.3 Method

3.3.1 Morphometric data collection

Morphometric triats of four varieties *Gallus gallus domesticus* (*G. g. domesticus*) and variety of *Gallus gallus gallus* (*G. g. gallus*), is measured by five parameters as followed: wing length (WL), the beak-basement to nape length (HD), beak length (BL), tarso-metatarsus length (TL) and third digit length (TD). Sexes, juvenile or adult are identified and bodyweight is recorded (in Kilograms). Data of five parameters of all specimens is compared among varieties by one-way ANOVA, and each character among varieties is analyzed by cluster program of the canonical discriminant function. The result of measurement shows in Appendix V.

3.3.2 Sample collection and preservation

Blood of all specimens of Thai Native Fowls are collected by radial venipuncture (Khatib and Gruenbaum, 1996), using the tuberculin® syringe with needle gauge number 25. An amount of blood 0.1 - 0.2 ml. is dropped on Watman® filter paper, air-dry and placed into a labeled plastic bag for each sample, then transport in laboratory and keep in a dessicator.

3.3.3 Extraction of nuclear DNA from each blood stains.

The most common method of DNA extraction can be subdivided into two methods. It is used to extract genomic DNA from bloodstain for DNA template in PCR amplification.

5% Chelex® Extraction method

Chelex is a polyvalent chelating agent in resin form, it is used routinely to assay a small number of cells and amount of DNA. Heating over boiling point condition may help to disrupt cell membranes, which it may also help to assure completed denaturation of the DNA template and separate DNA from the cell (Singer-Sam, Tanguay and Riggs, 1989). DNA extraction from bloodstains seems less prone to contain PCR inhibitors when it is prepared by this method (Walsh, Metzger and Higuchi, 1990). Furthermore, this method is easy, inexpensive, less time-consuming and reduce contamination chance. Protocol of this method can be seen in Appendix I.

Proteinase K/phenol-chloroform extraction method

Proteinase K/Phenol-chloroform extraction method is a common used for DNA extraction. This method removes protein and contaminants from the sample of DNA prior to use by Proteinase K. Phenol-chloroform extraction of protein is followed by an ethanol precipitation step, and that DNA is retrieved. This method permits to gain more purity than 5% chelex method. In addition, high molecular weight DNA (>10 Kb) can be seen for this method (Brown, 1991) and it can be preserved for stock DNA solution in suitable buffer at –20°C for long-term storage (Seutin, White and Boag, 1991). In this study, DNA extraction was applied as following Devis, Kuehl and Battery (1994) and protocol can be seen in Appendix I.

3.3.4 Determination quality and concentration of the isolated DNA samples

Determination quality of Genomic DNA

1% Agarose gel is medium for the electrophoresis separation of DNA. It carries out for visualization of the quality of isolated DNA samples. Approximate nucleic acid size for 1% agarose is 0.3 – 10 kb. Genomic DNA is extracted and loaded into the gel. Especially DNA is stained and detected by ethidium bromide dye, and it can be visualized under ultraviolet absorption (Brown, 1991). In addition, PhiX 174 Hinf I digested is loaded into the gel and served as a DNA standard marker for size estimation. Protocol of this method is shown in Appendix II.

Measurement and calculation of DNA concentration

Concentration of extracted DNA is measured and estimated by ultraviolet (UV) absorption of spectrophotometer model DU 650. Extracted DNA solution can be absorbed UV at wave length 260 nm (OD_{260}), whereas protein can be absorbed UV at wave length 280 nm (OD_{280}) (Brown, 1991). Purity of extracted DNA is assessed by determining ratio of OD at 260 am to OD 280 nm. If this ratio is greater than 1.5, the absorption is probably due to nucleic acid. But, if this ratio is below 1.5, there may be protein or other contaminants in sample (Devis *et al.*, 1994). An OD_{280} of 1.0 corresponds to a concentration of 50 µg/mL double strand DNA.

Therefore, DNA concentration of each sample is calculated by the assumption:

DNA concentration (in mg / mL) = OD_{260} x dilution factor x 50

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

Selection of Polymerase Chain Reaction Primer

Suitable primers should be similar length and melting temperatures, but should not be complementary. In addition, they should be produced product of 100-300 bp in length, but they should not be annealed to one another or form hairpin loops (Ferraris and Palumbi,1966). In addition, products of primer should be easier to score, which they are tested for the presence of correctly sized 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 oliginucleotide primers are chosen from several publications. All primers are selected from chicken genomic libraries that can amplify and show the highest allelic number, high polymorphism and unambiguous amplification pattern in the East Lancing reference family (mating between the Red Jungle Fowl and White Leghorn) (Khatib *et al.*,1993). In addition, they are exhibited in highly polymorphism in the Compton reference family (mating between Inbred White Leghorn X outbred White Leghorn) (Cheng and Crittenden, 1994), and Wageningen resource population (Crooijmans *et al.*, 1997). These microsatellite primers are screened by end-labelled method (by γ- P^{32} dATP), which it modify as follow by Khatib *et al.* (1993). Non-labelled method is silver staining method, which followed by Perkin Elmer Protocol. The protocols of both methods are shown in Appendix IV. This method is used for detecting polymorphism in Thai Native Fowls. Four of eight microsatellite primers were selected to amplify for Thai Native Fowls. Microsatellite loci are that ADL23, LEI73, MCW87 and MCW240, which are purchased from Bioservice Unit, National Center for Genetic Engineering and Biotechnology, Bangkok. The characteristic of all selected primers are listed in table below:

Table 3.1 The characteristics of selected chicken microsatellite-flanking PCR primers

Locus name	Forward sequence / Reword sequence	Motif	Length (bp.)	Tm (°C)	Product size (bp)
ADL23	5' CTTCTATCCTGGGCTTCTGA 3' 5' CCTGGCTGTGTATGTGTTGC 3'	TG	20 20	61	164
LEI73	5' CCATATCATTTGTCAAGCACC 3' 5' AATTCCTGACCTCCATGATAC 3'	AC	20 21	60	163-221
MCW87	5' ATTTCTGCAGCCAACTTGGAC 3' 5' CTCAGGCAGTTCTCAAGAACA 3'	CA	21 21	62	272-287
MCW240	5' CAAAACCGGTGTCACCTACTG 3' 5' GGTTATTTCTTCAGTGACTTCC 3'	AC	21 22	63	172-197

Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a technique for *in vitro* amplification of specific DNA sequence by primer extension of complementary strand of DNA. Two methods, end-labeled primer and non-labeled primer method, are used to screen and examine of microsatellite markers. Generally, the requirements of the reaction are composed: DNA template, buffer containing magnesium chloride, deoxynucleotides, microsatellite primers and DNA polymerase (Mcpherson, Quirke and Taylor, 1991). PCR has great potential for DNA-level studies of conservation and population genetic. PCR amplification of DNA from many individuals is obtained alleles frequency data that variation in allele frequency among different population is occurred (Arnheim, white and Rainey, 1990).

All the selected microsatellite loci are amplified the genomic DNA of each sample that it is extracted by phenol /chloroform method. Approximately 30-50 ng of

genomic DNA from an individual of *G. g. domesticus* is used for DNA template in PCR and a total volume of each reaction is 25 μL.

The PCR reaction mixture is slightly modified from Khatib *et al.*(1993). The mixtures are composed of each at 0.1-0.2 μM, dNTP each at 0.2 mM, 2.0-3.0 mM $MgCl_2$, 1.0 unit of Taq DNA polymerase (Promega) per 25 μL, 30-50 ng template of genomic DNA (prepared from red blood cell). The composition of enzyme storage buffer is 20 mM Tris-HCl (pH 8.0 at 25°C), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50 % glycerol, 0.5 % Tween®20 and 0.5 % Nonnidet®- P40. The PCR mixture is overlaid with sterile mineral oil about one drop and is spin briefly at 10,000 rpm before subjected to the amplification process in Hybraid Omnigene DNA thermal cyclear.

For amplification of all loci, more complicated PCR amplification program are required. The melting temperature (Tm) for each primer is examined by the Wallace's rules. As a result,

$$Tm\ (^{\circ}C) = [4\ (\text{total number of G and C}) + 2\ (\text{total number of A and T})]$$

The temperature cycling is as following. Denaturing is 94°C for 3 minutes. Annealing is for 60 seconds at 53°C - 62°C (depending on primer composition at Table 3.1). The extension is at 72°C for 90 seconds. The reaction is carried out for 35 cycles. The final extension is at 72°C for 10 minutes. When PCR finish the samples in PCR reaction mixture are called PCR products.

Standard size DNA marker

Microsatellite DNA markers are detected and detected by running the PCR products out on the gel (e.g.: denaturing acrylamide gel) through electrophoresis technique, and then stain them with silver staining method and microsatellite allele can be scored. It can be recorded either, homozygote and heterozygote for each locus (Ferraris and Palumbi, 1996). Size standard marker is run on the gel for estimation of alleles of microsatellite loci. Sample may be exhibited a complex banding pattern in which an allele may appear as several bands, particularly if they are dinucleotide repeat motifs.

3.3.6 Eight percents Denaturing Polyacrylamide gel electrophoresis

PCR products are detected with 8 % denaturing polyacrylamide gel that based on electrophoresis technique. The sequencing plate (Bio-rad®) is used for running electrophoresis, the cleaning steps is washed with water, cleaned with 70% ethanol, rinsed with distilled water and wiped with kimwiped® until dry. Let the plate to siliconised with sigmacote® and air-dried.

Standard sequencing gel (8% acrylamide monomer) is prepared for separating PCR products of microsatellite DNA of *G. g. domesticus*, and detected with standard size DNA marker, pBR322 MspI digest

PCR products are mixed with 10X loading dye (0.05% bromophenol blue, 0.05% xylene cyanol, 95% formamide, 10 mM NaOH and 10X TE buffer). They are heated at 95^{0}C for 3 minutes and immediately snap-cool on the ice before loading, PCR products are loaded 3 microlitres. Before loading the PCR product, insert the comb into the upper open end with the teeth pointing into the top of the gel and flush out the well

with running buffer. Standard size marker is loaded 2 microlitres. Electrophoresis is carried out with:

Pre run	400 W constant, 2500 V, 350 mA for 1 hours
Run	400 W constant, 2000 V, 350 mA for 3 - 3.3 hours (depended on sizes of PCR products), temperature at 50^0C

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

3.3.7 Developing PCR products on 8 % polyacrylamide gel by Silver staining

When electrophoresis is finished, the power supply is terminated and the gel is prepared for visualization of PCR products by silver staining. Reagents and preparation protocol is shown in Appendix III and IV.

Bands (alleles) are scored by eye. It is suitable for denaturing gel. Advantages of using silver staining over radioactive labeling is that the concentration of dNTP's does not have to be lowered in order to facilitate incorporation of 32^P-labeled dNTP. In addition, the stutter band is observed, but it is not also much shaper, allowing size discrimination within a much shorter distance on the gel (Luqmani *et al.*, 1997).

Pairwise comparison between two varieties is used for band scoring. pBR 322 MspI digest marker is used for a standard when comparing bands between varieties. The gel aligns well with the fragments of the adjacent molecular weight. Alleles are designed according to PCR product sizes. Numerical (1 to n) is used designate the alleles, where 1 and n represent to the largest and the smallest alleles observed in the gel, respectively. This method is applied from Ciampolini *et al.* (1995).

3.3.8` Data analysis

Assumption

Genotype of microsatellite DNA is scored by eyes for all parameter. Heterozygotes are clearly two bands, whereas homozygote shows one major band (Sukamol Srikwan *et al.*, 1996). Observed of PCR products banding pattern is typically presented the additional or stutter bands beside the microsatellite band differing by 1 or 2 bp. (Mable, Morize and Hillis, 1996). It is resulted form 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 *et al.*, 1996). Therefore, the most intense and clear band of each allele are detected and compared within and between varieties.

3.3.8.1 Allele Frequency

Differences in microsatellite allele frequencies among four varieties of *G. g. domesticus* and variety of *G. g. gallus* are assessed using Fisher's exact test in GENEPOP program that on common versus pooled rare alleles frequencies (Taylor *et al.*, 1994).

Allele frequency In a population for diploid organism can be estimated as followed (Hedrick, 1985):

$$\text{Allele frequency} = \frac{2N_{AA} + N_{Aa}}{2N}$$

Where, N_{AA}, N_{aa} are number of homozygote at allele A and a, and N_{aa} is number of heterozygote, for such an allele, N is number of investigated individuals

In addition, allele frequency can be calculated by software GENEPOP, version 2.0 (Raymond and Rousset, 1995). The mean number of alleles per locus (A) is calculated to compare genetic variation among varieties by a one-way ANOVA (Taylor *et al.*, 1994). The effective number of alleles per locus (n_e) is calculated. It is 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, 1988). For each locus the number of effective allele as followed:

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

Where P*i* is the *i*th allele frequency

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

3.3.8.2 Heterozygosity

Heterozygosity is the statistic parameter used to evaluate the informative of a genetic marker for genetic variation. When the variety is in Hardy-Wienberg equilibrium, heterozygosity can be calculated from heterozygous alleles frequencies at a given locus (Hoelzel, 1992), which is called observed heterozygosity (h_{obs}), by

$$h = 1 - \sum_{i=1}^{k} X_i^2$$

Where X_i is the frequency of the *i*th alleles, H is then given as the mean of h overall loci

Expected from Hardy-Wienberg assumption (h_{exp}) can be calculated and examined using the package GENEPOP, version 2.0 (Raymond and Rousset, 1995).

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

3.3.8.3 Hardy-Wienberg equilibrium

Hardy-Weinberg law is a remarkable theory that is extremely useful in enabling us to understand what happens to gene frequencies and allele frequencies in varieties (Nicolas, 1996). This law assumes that the genes involve are found in an infinite populations of sexually reproducing and random mating diploid organisms 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 *et al.*, 1994) as followed:

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

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

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

This test is carried out using The GENEPOP computer program (Raymond and Rousset, 1995). A sequential Bonferroni correction method is used to adjust significance levels for multiple test (Lessios, 1992).

3.3.8.4 Genotypic linkage disequilibrium test

The genotypic linkage disequilibrium is defined in terms of two-locus genotypic counts. This parameter is a study of association between alleles at different loci when two or more loci are considered together. The null hypothesis is that genotypes at one locus are independent from genotype at the other locus (Viard *et al.*, 1996). This parameter is tested for each locus and each variety by using program GENEPOP, version 2.0. It uses a Markov chain method following the algorithm of Guo and Thomson (1992). Significant level is adjusted by a Bonfferoni method (Lessios, 1992).

3.3.8.5 Differentiation between varieties test

The statistically significant differences in genotypic frequencies between *G. g. domesticus* from a pair of varieties are tested using the exact test of Genic Differentiation of GENEPOP version 2.0. Heterogeniety of allele frequency within varieties of *G. g. domesticus* and *G. g. gallus* are determined by the Fisher test for RxC contingency tables. The probability of type I error (P-value) for rejecting null hypothesis (H_o : no differentiation among varieties) is estimated using a Markov chain method (Raymond and Rousset, 1995). Results are expressed as the probability of homogeneity between compared varieties. To diminish type I error, level of significant is further adjusted using the Bonferroni method.

3.3.8.6 Genetic distance analysis and phylogenetic reconstruction

Genetic distance based on Cavalli-Sforza and Edwards chord distance is calculated (Cavalli-Sforza and Edwards, 1967). This is the gene diversity among varieties expressed as a function of genotype frequency. The genetic distance estimated from this method are appropriated for microsatellite data obtained from various taxa whether they have undergo bottleneck events or not (Takezaki *et al.*, 1996). Practically, Cavalli-Sforza and Edwards chord distance is computational estimate using GENDIST (Felsenstein, 1993). The resulting genetic distance is subject to the phylogenetic reconstruction based on Neighbor-joining (NJ) approach (Saitou and Nei, 1987) using NEIGHBOR. The NJ tree is plotted by DRAWTREE. All computational programs mentioned above are routinely implement in PHYLIP 3.572c (Felsenstein, 1993).

Chapter 4

Results

4.1 Results

4.1.1 Morphometric analysis

All morphometric traits were analyzed for comparing among 15 individual per variety of four varieties of Thai Native Fowl *Gallus gallus domesticus* (*G. g. domesticus*) as follow: Fighting Cock (Praduhangdam, AP and Luenghangkhoa, AL), Bantam (B) and Betong Chicken (C). In addition, morphometric trait was analyzed between 10 individual of varieties of *Gallus gallus gallus* (*G. g. gallus*) Red Jungle Fowl (D). The measured data of the wing length (chord of unflattened wing, WL), beak length (distal tip of the maxilla of the proximal edge of the exposed clumen, BL), tarso-metatarsus length (TL) and head length (the beak-basement to the nape length, HD) were analyzed. But, the third digit length (TD) was accepted because breeder and farmer often to cut this parameter. Therefore, it was permitted the bias to analyse the data. In addition, sexes were identified and weighted the body. Four parameters (WL, BL, TL and HD) of five varieties (Praduhangdam Fighting Cock, Luenghangkhoa Fighting Cock, Bantam, Betong Chicken and Red Jungle Fowl) were analyzed by using one-way ANOVA of SPSS program that was following Lovette, 1998. The result is shown in Table 4.1. There is significant difference among five varieties ($P<0.05$). Five morphometric traits of multiple comparison by Tukey in order to assess the mean difference of each morphometric traits and group the data.

Table 4.1 Four morphometric traits of four varieties of *G. g. domesticus* and variety of *G. g. gallus* were analyzed by using one-way ANOVA.

Morphometric traits	d.f.	Sum of square	Mean of square	F	P-value*
TL Between varieties	4	834.37	208.59	143.95	<0.001
Within varieties	65	94.19	1.44		
Total	69	928.56			
BL Between varieties	4	7.40	1.85	38.12	<0.001
Within varieties	65	3.15	0.05		
Total	69	10.55			
HD Between varieties	4	84.93	21.23	37.53	<0.001
Within varieties	65	36.77	0.57		
Total	69	121.70			
WL Between varieties	4	3447.12	861.78	132.15	<0.001
Within varieties	65	423.89	6.52		
Total	69	3871.01			

* Significant level at 0.05

TL = Tarso-metatarsus length, BL = Beak length, HD = Head length, WL = Wing length

The mean difference of tarso-metatarsus length (TL) between Betong Chicken and Red Jungle Fowl were similar with Praduhangdam Fighting Cock and Luenghangkhoa Fighting Cock, and showed no significant difference. In addition, only Bantam showed significant difference from other varieties ($P < 0.05$).

The mean difference was grouping for the homogeneous subset of TL trait into three groups, (1) Betong Chicken was grouping with Red Jungle Fowl, (2) Bantam, and (3) Praduhangdam Fighting Cock was grouping with Luenghangkhoa Fighting Cock. The result is shown in Table 4.2.

Table 4.2 The homogeneous subset of the mean difference of tarso-metatarsus length (TL), beak length (BL), head length (HD) and wing length (WL) among four varieties of *G. g. domesticus* and variety of *G. g. gallus* by Tukey.

Morphometric traits	Mean difference of each varieties				
	AP	AL	B	C	D
TL	13.31[c]	12.67[c]	4.04[a]	9.19[b]	8.12[b]
BL	2.19[c]	2.33[c]	1.83[b]	1.95[b]	1.30[a]
HD	7.46[c,d]	7.99[d]	4.98[a]	7.02[c]	5.97[b]
WL	43.52[c]	43.56[c]	28.29[a]	29.00[a]	32.46[b]

[a,b,c,d] = group in homogeneous subset of mean difference

AP = Praduhangdam (Fighting Cock, *G. g. domesticus*),

AL = Luenghangkhoa (Fighting Cock, *G. g. domesticus*), B = Bantam (*G. g. domesticus*)

C = Betong Chicken (*G. g. domesticus*), D = Red Jungle Fowl (*G. g. gallus*)

The mean difference of beak length (BL) of Bantam showed no significant difference from Betong Chicken. In addition, Praduhangdam Fighting Cock showed no significant difference from Luenghangkhoa Fighting Cock. In addition, only Red Jungle Fowl showed significant mean difference from other varieties ($P<0.05$).

The mean difference was grouping for the homogeneous subset of BL trait into three groups, (1) Red Jungle Fowl was separated from other varieties, (2) Bantam was grouping with Betong Chicken, and (3) Praduhangdam Fighting Cock was grouping with Luenghangkhoa Fighting Cock. The result is shown in Table 4.2.

The mean difference of head length (HD) of Praduhangdam Fighting Cock and Luenghangkhoa Fighting Cock were similar with Praduhangdam Fighting Cock and Betong Chicken that showed no significant difference. In addition, Bantam and Red Jungle Fowl showed significant difference from other varieties ($P<0.05$).

The mean difference was grouping for the homogeneous subset of HD trait into four groups, (1) Bantam, (2) Red Jungle Fowl, (3) Praduhangdam Fighting Cock was grouping with Betong Chicken, and (4) Praduhangdam Fighting Cock was grouping with Luenghangkhoa Fighting Cock. The result is shown in Table 4.2.

The mean difference of wing length (WL) of Red Jungle Fowl showed significant difference from other varieties ($P<0.05$), whereas Betong Chicken showed no significant mean difference from Bantam. In addition, Praduhangdam Fighting Cock exhibited no significant difference from Luenghangkhoa Fighting Cock.

The mean difference was grouping for the homogeneous subset of WL trait into three groups, (1) Betong Chicken was grouping with Bantam, (2) Red Jungle Fowl was separated from other varieties, and (3) Praduhangdam Fighting Cock was grouping with Luenghangkhoa Fighting Cock. The result is shown in Table 4.2.

Overall morphometric traits of four varieties of *G. g. domesticus* and variety of *G. g. gallus* were combined analysis by using Canonical Discrimination method, and the result is shown in Figure 4.1. This result showed that Praduhangdam Fighting Cock overlapped with Luenghangkhoa Fighting Cock, whereas Bantam and Betong Chicken were separated from Red Jungle Fowl and not overlapped between other varieties.

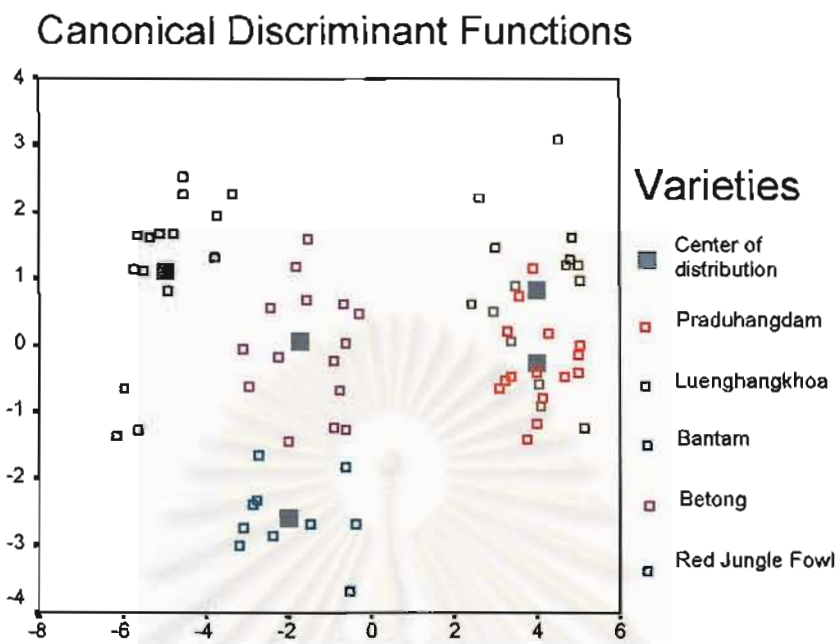

Figure 4.1 Discrimination of four morphometric traits of four varieties of *G. g. domesticus* (Praduhangdam Fighting Cock, Luenghangkhoa Fighting Cock, Bantam and Betong Chicken and variety of *G. g. gallus* (Red Jungle Fowl).

4.1.2 Genomic DNA extraction, visualization quality and determined concentration of extracted DNA

Genomic DNA of all specimens of *G. g. domesticus* and *G. g. gallus* was extracted form bloodstain by 5% chelex and Proteinase K/Phenol-chloroform extraction method. The quality of extracted DNA was visualized via 1% agarose gel. This result showed that the extracted DNA from Proteinase K/Phenol-chloroform method has yielded better quality than 5% chelex method, and several extracted DNA fragments were typically found in 5% chelex method and were called smear band, whereas high molecular weight of genomic DNA was found in Proteinase K/Phenol-chloroform method. The result is shown in Figure 4.2. Extracted DNA of 5% chelex method was not used in this experiment. It was not suitable for microsatellite amplification, because it can not be amplified for specific PCR products and caused the nonspecific products occurred.

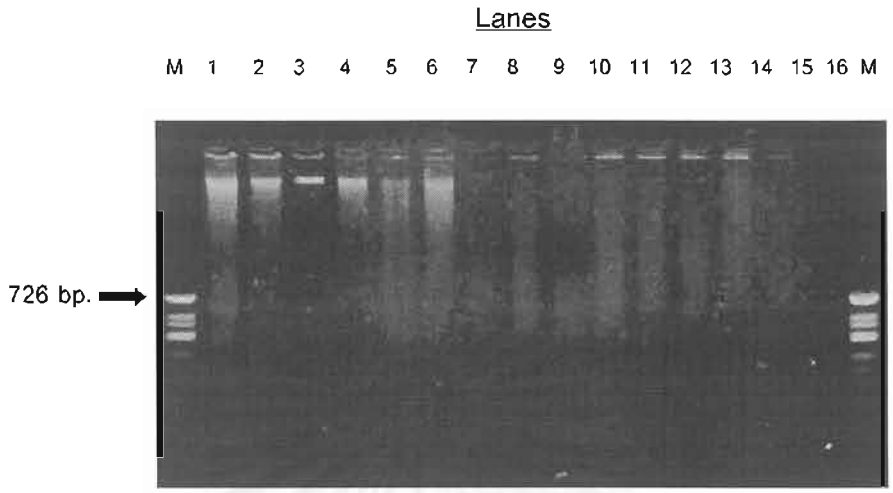

Figure 4.2 Ethidium bromide staining of 1% agarose gel showing extracted DNA from bloodstain of *G. g. domesticus*.

Lane M : Phi X 174 / Hinf I standard marker

Lane 1-6 : genomic DNA of *G. g. domesticus* extract by Proteinase K/Phenol-Chloroform method

Lane 8-13 : genomic DNA of *G. g. domesticus* extract by 5%Chelex method

The genomic DNA of all specimens was measured the DNA absorbent (OD) by spectrophotometer at OD 260 nm, and then the concentration of genomic DNA was calculated. The concentration of extracted DNA from Proteinase K/Phenol-chloroform method has ranged from 20.0 to 116.3 ng/ µL, whereas 5% chelex method has ranged from 6.19 to 87.39 ng/ µL. In addition, ratio of OD260 per OD280 has ranged from 1.5 to 2.3 in Proteinase K/Phenol-chloroform methods, but it has ranged from 0.9186 to 1.4566 in 5% chelex method. The result can be seen in Appendix V.

4.1.3 Optimization of PCR conditions for amplification by microsatellite DNA

Eight microsatellite loci (HUJ2, HUJ7, ADL23, ADL37, LEI73, LEI92, MCW87 and MCW240) were selected from published primer of *G. g. domesticus* and *G. g. gallus*. The microsatellite DNA at loci HUJ2 and HUJ7 were selected from Khatib *et al.* (1993). In addition, microsatellite loci ADL23 and ADL37 were selected from Cheng and Crittenden (1994), whereas microsatellite loci LEI73, LEI92, MCW87 and MCW240 were selected from Crooijmans *et al.* (1997). All selected microsatellite loci were reported, which have high number of alleles per locus (approximate 7 to 9 alleles) from the examination in domestic chicken. These microsatellite loci also showed the polymorphism in the chicken referent family and the chicken resource population. Eight microsatellite loci were screen, it was found that only four microsatellite loci (ADL23, LEI73, MCW87 and MCW240) showed highly polymorphism in Thai Native Fowl. Therefore, these loci were used to examine genetic variation of four varieties of *G. g. domesticus* (Praduhangdam Fighting Cock (AP), Luenghangkhoa Fighting Cock (AL), Bantam (B) and Betong Chicken (C) and a variety of *G. g. gallus* Red Jungle Fowl (D).

The optimized PCR conditions of four microsatellite loci were investigated in oder to determine the genetic variation in *G. g. domesticus* and *G. g. gallus*. The PCR amplification condition, which was reported by Khatib *et al.* (1993) did not work well with Thai Native Fowl. Therefore, this experiment tried to find out the optimal PCR condition by including first, varying annealing temperature in a series of 50, 53, 55, 57, 60, 62°C. When the optimized annealing temperature was successful, selected microsatellite primer was not consisted the non-specific product. Second, level of Mg^{2+} concentration for each locus was examined by varying its concentration in a series of 1, 2, 4, 6, 8 mM in 25 µL of reaction volume. The optimal concentration of Mg^{2+} for ADL23, LEI73, MCW87 and MCW240 was 2.0, 2.5, 3.0 and 3.0 mM, respectively. Finally, the primer concentration was adjusted to use 0.1 and 0.2 mM. The optimal concentration of forward

and reverse primer of ADL23, LEI73, MCW87 and MCW240 was 0.2,0.2, 0.1 and 0.2 μM, respectively. The successful of optimal annealing temperature and reaction mixture are shown in Table 4.3

Table 4.3 The optimal annealing temperature and reaction mixture for PCR amplification of four varieties of *G. g. domesticus* and variety of *G. g. gallus* by four microsatellite loci.

Reaction mixture	Locus			
	ADL23	MCW87	MCW240	LEI73
Buffer (X)	1	1	1	1
$MgCl_2$ (mM)	2	2.5	3	3
dNTP (mM)	0.2	0.2	0.2	0.2
Forward primer (μM)	0.2	0.2	0.1	0.2
Reward primer (μM)	0.2	0.2	0.1	0.2
Taq DNA Polymerase (U.)	1	1	1	1
Reaction volume (μL)	25	25	25	25
Annealing (°C)	53	55	62	60

4.1.4 Genetic variation of *G. g. domesticus* and *G. g. gallus*

The genetic variations within *G. g. domesticus* and *G. g. gallus* were studied by four microsatellite loci. Sample sizes of four varieties of *G. g. domesticus* (Praduhangdam Fighting Cock (AP), Luenghangkhoa Fighting Cock (AL), Bantam (B) and Betong Chicken (C) are 25 individual per variety and *G. g. gallus* (Red Jungle Fowl (D) is 11 individual per variety. All primers generated group of stutter bands, but the

most intense band within group of stutter bands was scored and calculated allele frequencies. The number of alleles and allele frequency of five investigated varieties (Praduhangdam Fighting Cock (AP), Luenghangkhoa Fighting Cock (AL), Bantam (B), Betong Chicken (C) and Red Jungle Fowl (D)) of each microsatellite loci are shown in Table 4.4.

The polymorphic alleles were observed for microsatellite ADL23 locus. Luenghangkhoa Fighting Cock carried 11 of 12 alleles. The Red Jungle Fowl, Praduhangdam Fighting Cock, Betong Chicken and Bantam were found 8, 7, 7 and 6 alleles, respectively.

The microsatellite LEI73 locus showed the total number of alleles per locus 11 alleles. Praduhangdam Fighting Cock was found 11 alleles. In addition, Luenghangkhoa Fighting Cock, Bantam, Betong Chicken and Red Jungle Fowl were found 8, 7, 6 and 4 alleles, respectively.

Only six alleles were observed at microsatellite MCW87 locus from all investigated varieties. Praduhangdam Fighting Cock has 6 alleles. In addition, Luenghangkhoa Fighting Cock, Bantam, Betong Chicken and Red Jungle Fowl carried 5, 5, 5 and 5 alleles, respectively.

The microsatellite MCW240 locus showed the total number of alleles per locus was 13 alleles. Praduhangdam Fighting Cock showed highest number of alleles 11 alleles. In addition, Luenghangkhoa Fighting Cock, Red Jungle Fowl, Bantam and Betong Chicken showed 10, 8, 7 and 6 alleles, respectively.

Table 4.4 The number of alleles and allele frequency of four varieties of *G. g. domesticus* and variety of *G. g. gallus* were analyzed by four microsatellite loci.

Allele Number	Locus ADL23				
	Variety AP	Variety AL	Variety B	Variety C	Variety D
1	0.020	0.020	-	0.020	0.222
2	0.180	0.160	0.270	0.083	0.055
3	0.100	0.220	0.083	0.187	0.055
4	0.040	0.300	0.125	0.104	0.111
5	0600	0.120	0.250	0.333	0.222
6	0.040	0.020	0.166	0.208	0.055
7	0.020	0.040	-	-	0.055
8	-	0.020	-	-	0.222
9	-	0.020	-	-	-
10	-	0.040	-	-	-
11	-	0.040	-	-	-
12	-	-	0.104	0.062	-
Allele Number	Locus LEI73				
	AP	AL	B	C	D
1	0.020	0.120	0.100	0.062	-
2	0.240	0.040	0.160	0.125	0.428
3	0.040	0.060	0.106	0.083	-
4	0.020	0.140	-	-	-
5	0.100	0.060	0.020	0.299	0.071
6	0.080	0.020	-	0.145	0.071
7	0.200	0.120	0.280	-	-
8	0.200	0.440	0.180	0.354	0.428
9	0.060	-	0.160	-	-
10	0.020	-	-	-	-
11	0.020	-	-	-	-

Table 4.4 (continued)

Allele Number	Locus MCW87				
	AP	AL	B	C	D
1	0.300	0.420	0.060	0.280	0.200
2	0.160	0.020	0.200	0.340	0.600
3	0.300	0.300	0.320	0.260	0.050
4	0.080	-	0.040	0.020	0.100
5	0.120	0.120	0.38	0.100	0.050
6	0.040	0.14	-	-	-
Allele Number	Locus MCW240				
	AP	AL	B	C	D
1	-	0.043	-	-	0.062
2	0.022	0.021	-	-	0.125
3	0.090	-	0.020	0.195	0.062
4	0.022	0.217	0.320	-	0.187
5	0.318	0.065	0.040	0.065	0.187
6	0.181	0.108	0.180	0.173	0.087
7	-	0.152	-	-	-
8	0.022	0.021	0.100	0.195	-
9	0.181	0.152	0.240	0.086	0.125
10	0.045	-	0.100	-	-
11	0.068	0.108	-	0.282	0.062
12	0.022	0.108	-	-	-
13	0.022	-	-	-	-

AP = Praduhangdam (Fighting Cock, *G. g. domesticus*)

AL = Luenghangkhoa (Fighting Cock, *G. g. domesticus*), B = Bantam (*G. g. domesticus*)

C = Betong Chicken (*G. g. domesticus*), D = Red Jungle Fowl (*G. g. gallus*)

Four varieties of *G. g. domesticus* and variety of *G. g. gallus* were measured the mean number of alleles per locus (A). The highest of mean number of alleles per locus is 8.75 that was found in Praduhangdam Fighting Cock. In addition, mean number of alleles per locus of Luenghangkhoa Fighting Cock was 8.50, while both Bantam and Red Jungle Fowl showed mean number of alleles per locus as 6.25. The lowest mean number of alleles per locus is 6.00 that found in Betong Chicken. The result is shown in Table 4.6.

Furthermore, the mean number of allele (A) of all varieties was compared by Wilcoxon sign rank test. The result showed significant mean difference among varieties ($P<0.05$). Data can be seen in Appendix VI.

The allele distribution of four investigated loci (ADL23, LEI73, MCW87 and MCW240) of four varieties of *G. g. domesticus* and variety of *G. g. gallus* is shown in Figure 4.3, 4.4, 4.5, 4.6 and 4.7, respectively. In addition, histogram of allele frequencies is shown in Figure 4.8 and 4.9.

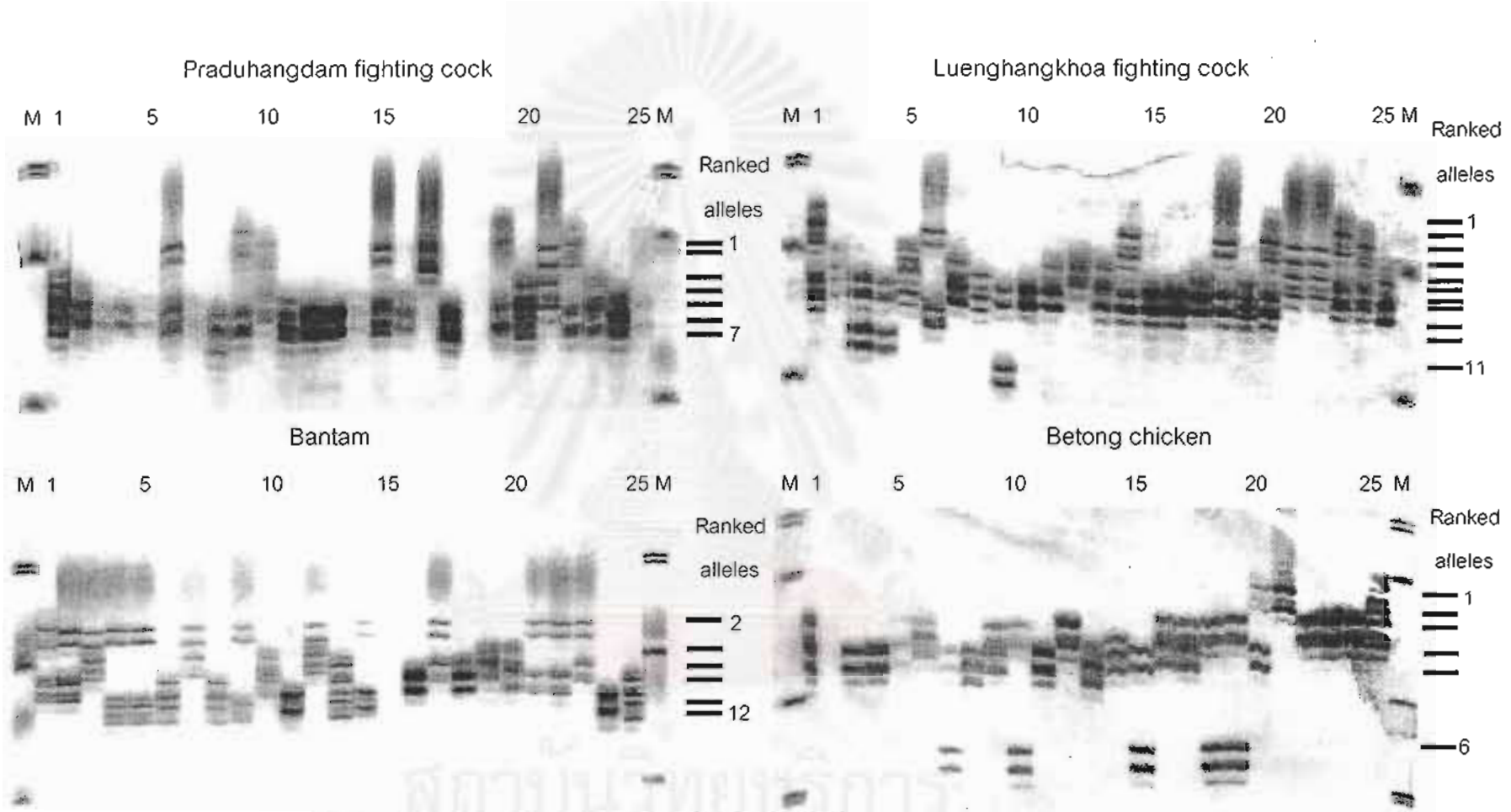

Figure 4.3 Silver staining of 8% polyacrylamide gel showing alleles distribution of the ADL23 locus from 25 individual per variety of *G. g. domesticus* (lanes1-25) under the optimal PCR condition with annealing temperature at 53°C. The size standard marker (M) is pBR 322 MspI digest.

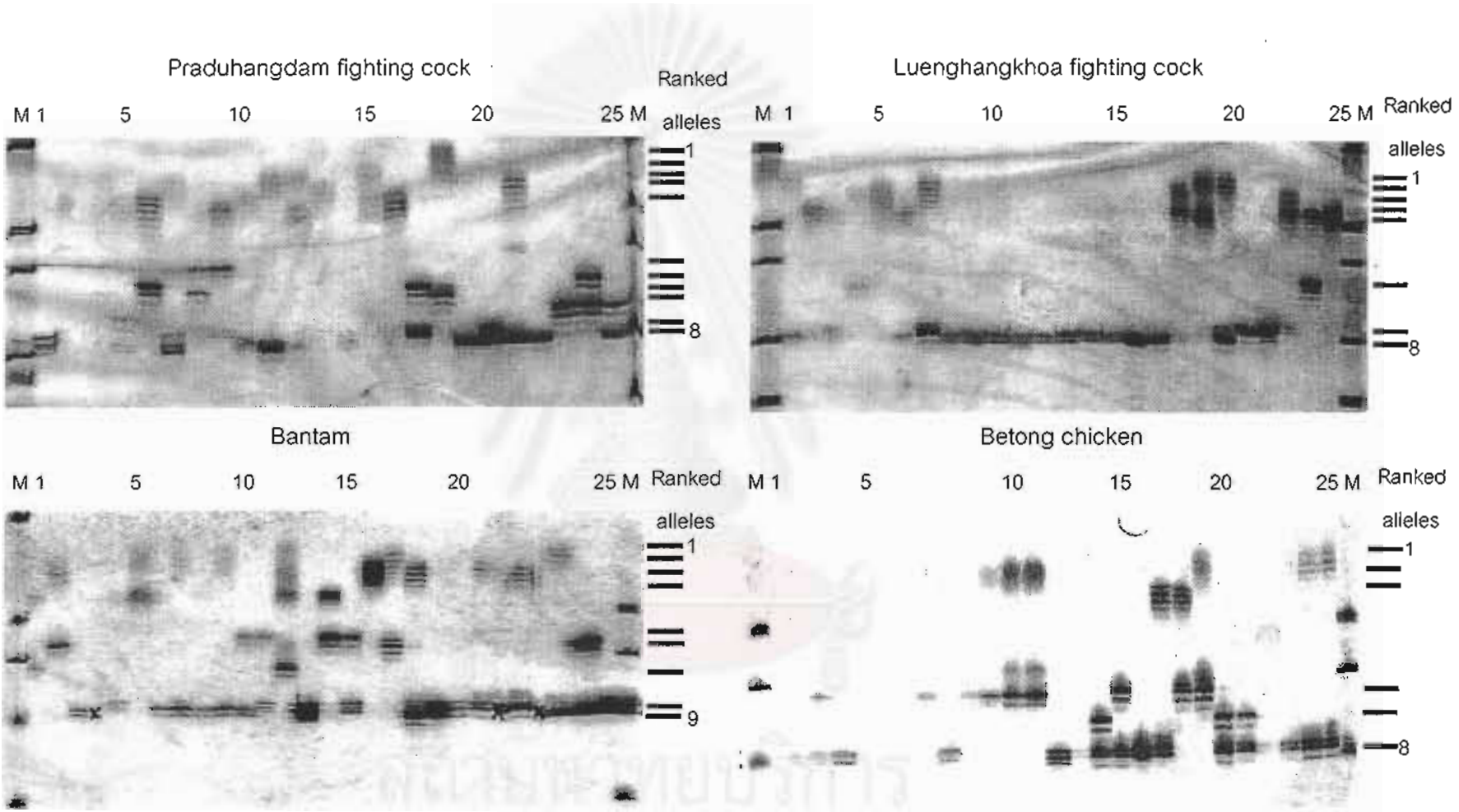

Figure 4.4 Silver staining of 8% polyacrylamide gel showing alleles distribution of the LEI73 locus from 25 individual per variety of *G. g. domesticus* (lanes1-25) under the optimal PCR condition with annealing temperature at 55°C. The size standard marker (M) is pBR 322 MspI digest.

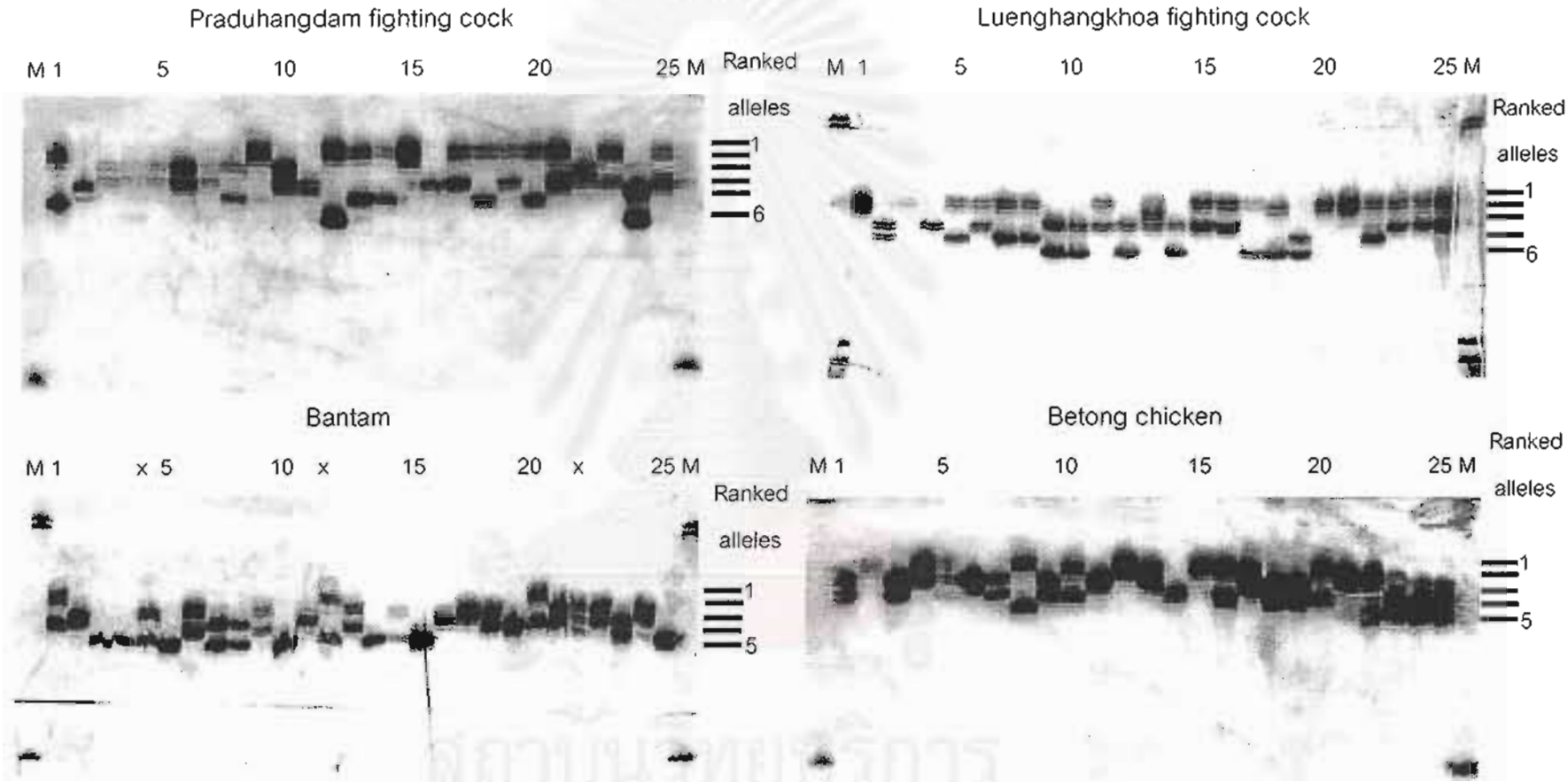

Figure 4.5 Silver staining of 8% polyacrylamide gel showing alleles distribution of the MCW87 locus from 25 individual per variety of *G. g. domesticus* (lanes1-25) under the optimal PCR condition with annealing temperature at 62°C. The size standard marker (M) is pBR 322 MspI digest.

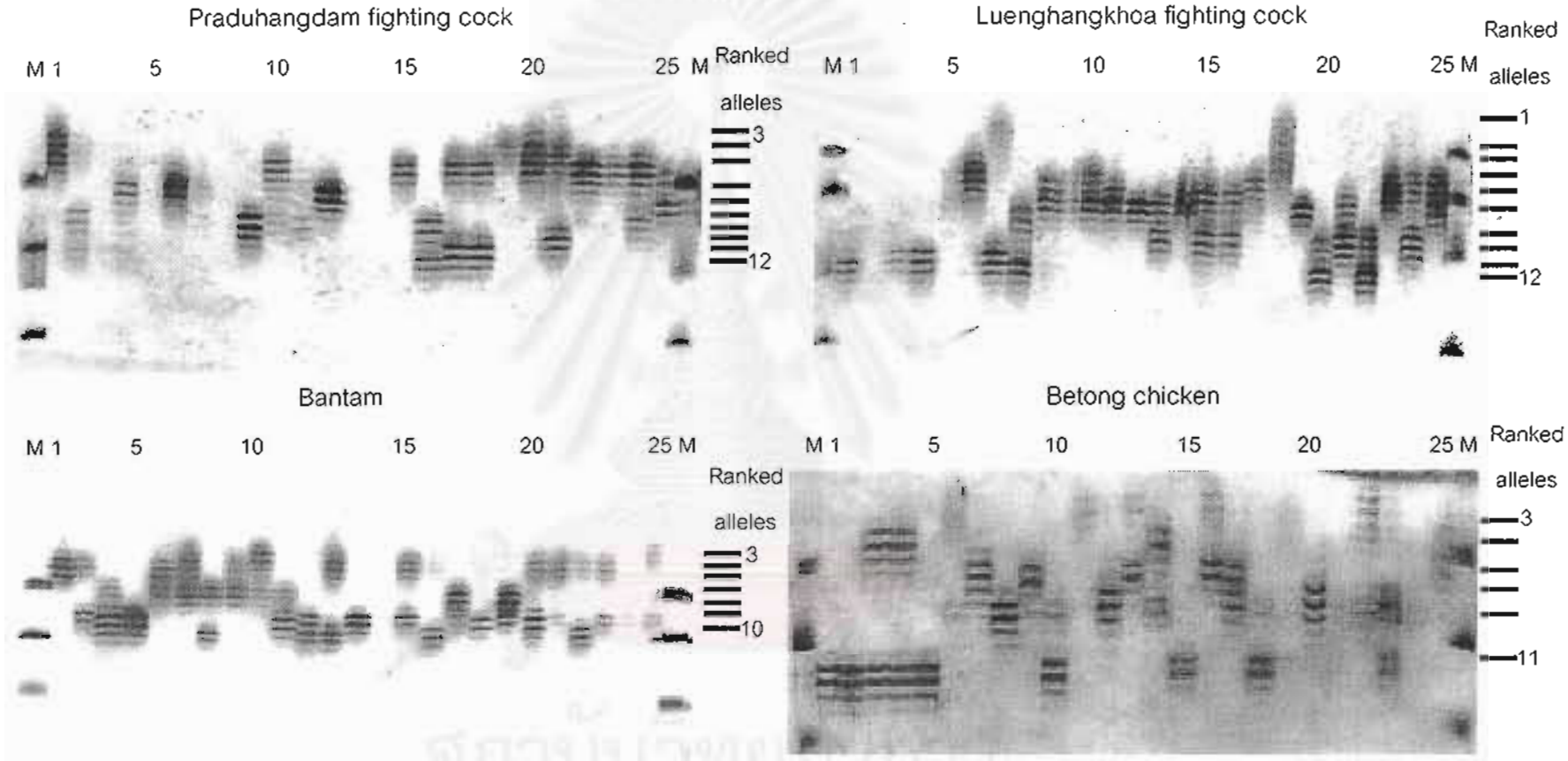

Figure 4.6 Silver staining of 8% polyacrylamide gel showing alleles distribution of the MCW240 locus from 25 individual per variety of *G. g. domesticus* (lanes1-25) under the optimal PCR condition with annealing temperature at 60°C. The size standard marker (M) is pBR 322 MspI digest.

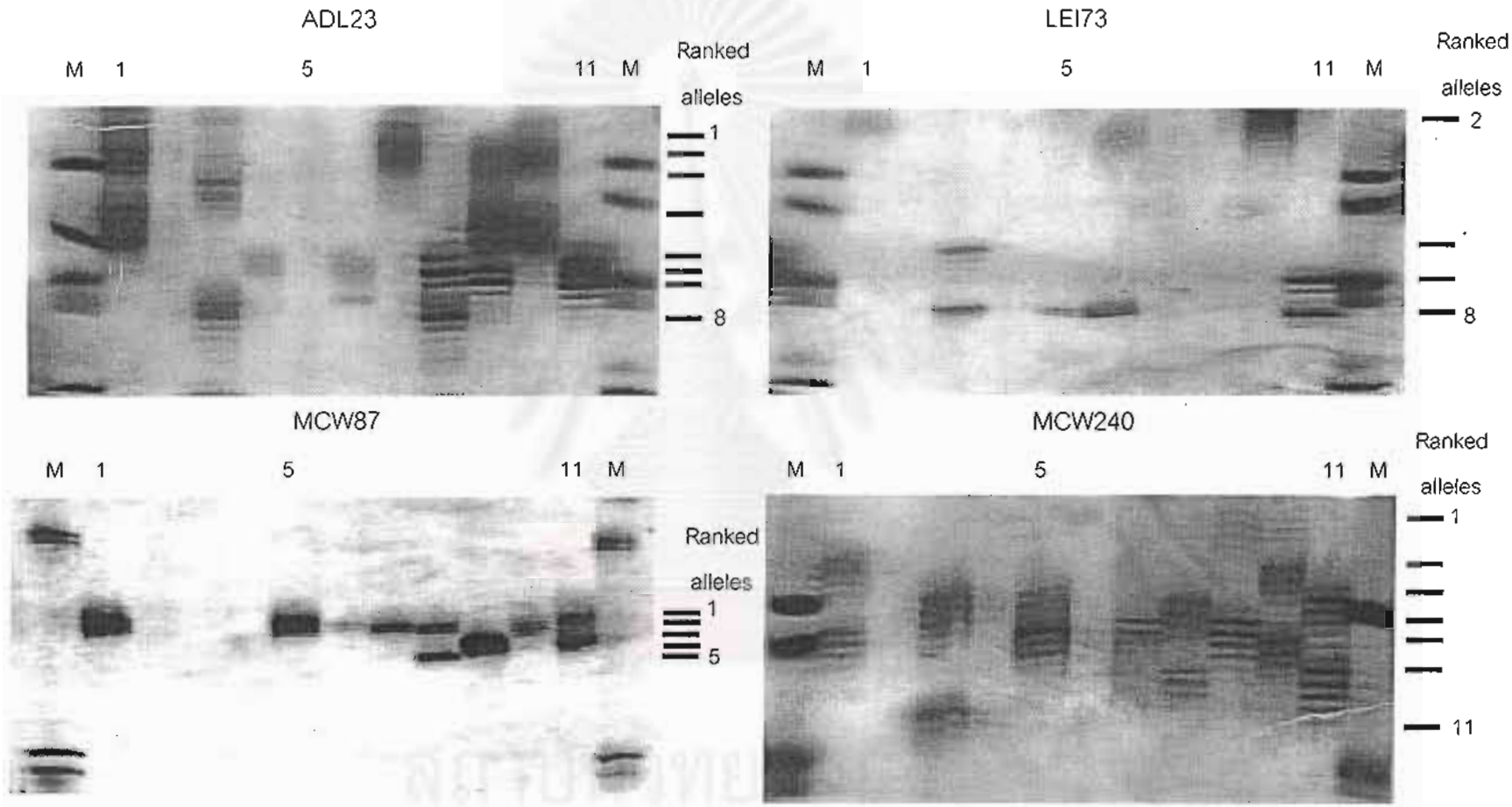

Figure 4.7 Silver staining of 8% polyacrylamide gel showing alleles distribution of ADL23, LEI73, MCW87 and MCW240 locus from 11 individuals of *G. g. gallus* (lanes1-11) under the optimal PCR condition with annealing temperature at 53°C, 55°C, 62°C and 60°C, respectively. The size standard marker (M) is pBR 322 MspI digest.

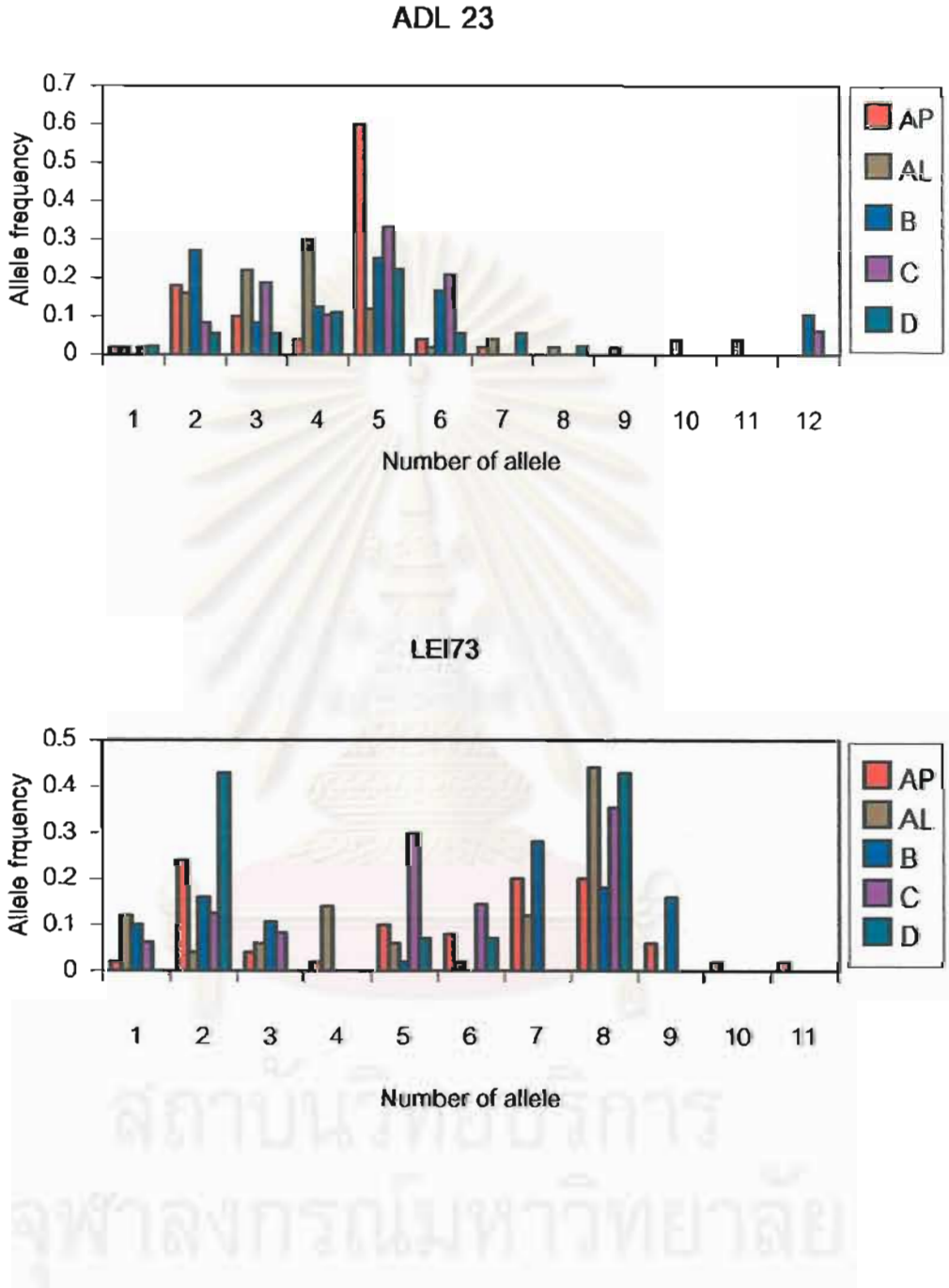

Figure 4.8 Histrogram of allele frequency and number of allele of Praduhangdam Fighting Cock (AP), Luenghangkhoa Fighting Cock (AL), Bantam (B), Betong Chicken (C), and Red Jungle Fowl (D) at ADL23 and LEI73 locus.

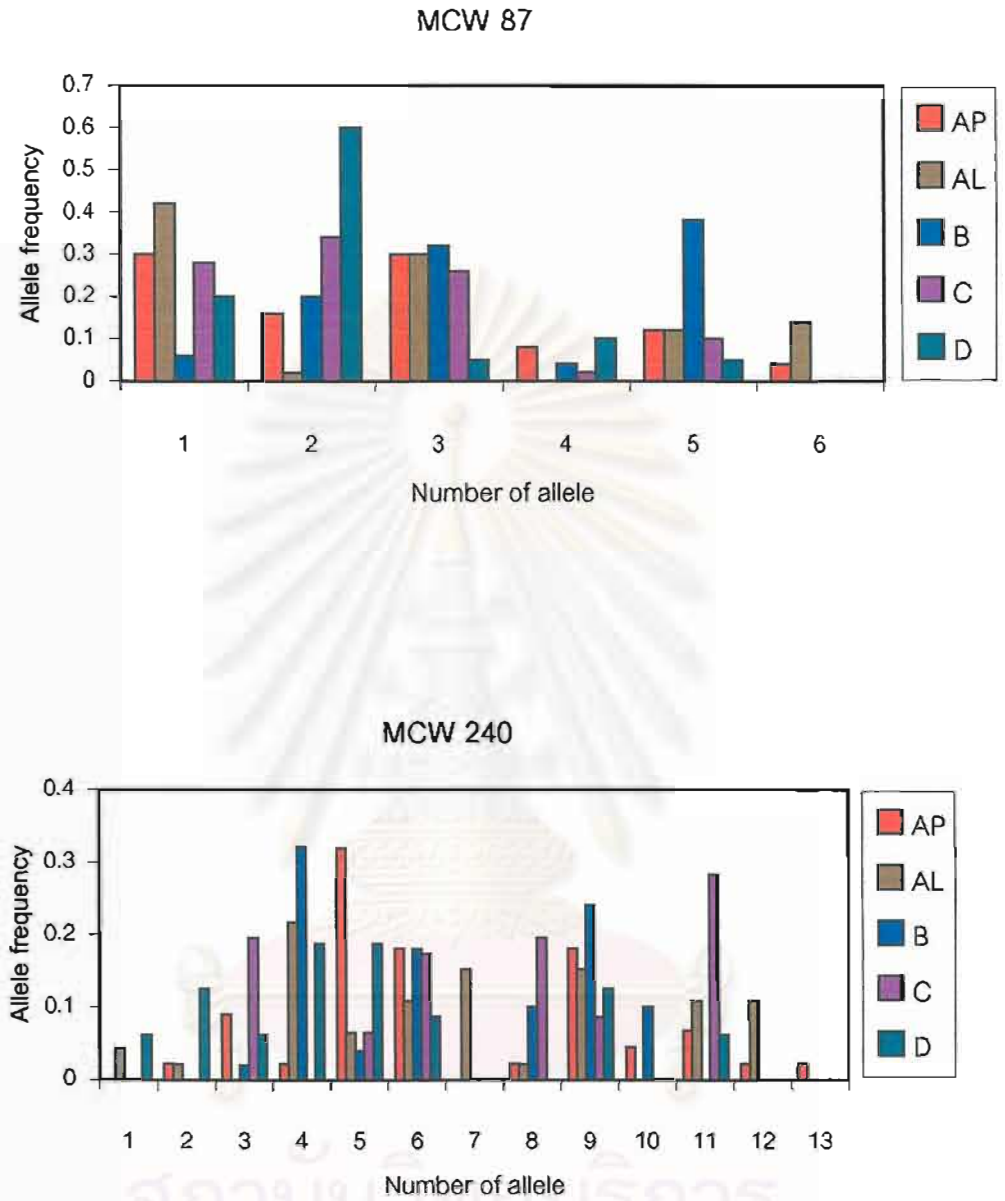

Figure 4.9 Histrogram of allele frequency and number of allele of Praduhangdam Fighting Cock (AP), Luenghangkhoa Fighting Cock (AL), Bantam (B), Betong Chicken (C), and Red Jungle Fowl (D) at MCW87 and MCW240 locus.

The observed heterozygosity ($h_{obs.}$) and that expected from Hardy-Wienberg assumption ($h_{exp.}$) of all microsatellite loci of *G. g. domesticus* (Praduhangdam Fighting Cock (AP), Luenghangkhoa Fighting Cock (AL), Bantam (B) and Betong Chicken (C), and *G. g. gallus*, Red Jungle Fowl (D) were calculated in order to measure the genetic variation. Betong Chicken at ADL23 locus showed the highest of observed heterozygosity. (h_{obs} = 0.89), whereas, Luenghangkhoa Fighting Cock at LEI73 locus showed the lowest of observed heterozygosity. (h_{obs} = 0.52). This result is shown in Table 4.5. Diverse distribution of allele frequency can be compared in term of effective number of alleles (n_e). It was calculated for four varieties of *G. g. domesticus* and *G. g. gallus* was varied from 2.4 in Red Jungle Fowl to 7.4 in Luenghangkhoa Fighting Cock. Data is shown in Table 4.5.

Table 4.5 The sample sizes, number of alleles, effective number of alleles (n_e), observed (h_{obs}) heterozygosity and expected from Hardy-Wienberg assumption (h_{exp}) of five investigated varieties were analyzed using four microsatellite loci

Microsatellite Locus	Varieties	Sample size	Number of alleles/variety	h_{obs}	h_{exp}	n_e
ADL23	AP	25	7	0.64	0.59	2.5
	AL	25	11	0.80	0.81	5.4
	B	25	6	0.79	0.80	5.1
	C	25	7	0.71	0.79	4.7
	D	11	8	0.89	0.83	5.8
LEI73	AP	25	11	0.68	0.69	3.2
	AL	25	8	0.52	0.75	4.0
	B	25	7	0.76	0.82	5.5
	C	25	6	0.87	0.77	4.4
	D	11	4	0.57	0.62	2.6
MCW87	AP	25	6	0.84	0.77	4.4
	AL	25	5	0.80	0.70	3.3
	B	25	5	0.64	0.70	3.4
	C	25	5	0.76	0.73	3.7
	D	11	5	0.60	0.58	2.4
MCW240	AP	25	11	0.72	0.75	3.9
	AL	25	10	0.53	0.86	7.4
	B	25	7	0.88	0.78	4.7
	C	25	6	0.56	0.80	5.0
	D	11	8	0.87	0.85	6.7

AP = Praduhangdam (Fighting Cock, *G. g. domesticus*)

AL = Luenghangkhoa (Fighting Cock, *G. g. domesticus*), B = Bantam (*G. g. domesticus*)

C = Betong Chicken (*G. g. domesticus*), D = Red Jungle Fowl (*G. g. gallus*)

Table 4.6 The mean number of sample size (N), mean number of alleles per locus (A), mean effective number of alleles per locus (a_e), mean observed heterozygosity (H_O) and expected Hardy-Weinberg assumption (H_E) of four varieties of *G. g. domesticus* and variety of *G. g. gallus* were analyzed by four microsatellite loci.

Population	N	A	a_e	H_O	H_E
AP	25	8.75	3.51	0.72	0.70
AL	25	8.50	5.02	0.66	0.77
B	25	6.25	4.66	0.76	0.77
C	25	6.00	4.46	0.72	0.76
D	11	6.25	4.39	0.73	0.72

AP = Praduhangdam (Fighting Cock, *G. g. domesticus*)

AL = Luenghangkhoa (Fighting Cock, *G. g. domesticus*), B = Bantam (*G. g. domesticus*)

C = Betong Chicken (*G. g. domesticus*), D = Red Jungle Fowl (*G. g. gallus*)

The mean of observed heterozygosity (H_O) and expected from Hardy-Wienberg assumption (H_E) of each population for all loci were calculated. Bantam showed the highest mean observed heterozygosity (H_O=0.76), while mean observed heterozygosity of Luenghangkhoa Fighting Cock opposed (H_O=0.66). However, mean expected heterozygosity of Luenghangkhoa Fighting Cock and bantam showed the highest value (H_E=0.77) and followed Betong Chicken (H_E=0.76), Red jungle Fowl (H_E=0.72) and Praduhangdam Fighting Cock (H_E=0.70). This result is shown in Table 4.6. The difference of mean heterozygosity (H_E) was compared among varieties. It was assessed by a Wilcoxon sign rank test. The result showed no significant difference of H_E between varieties (see in Appendix VI).

The mean of effective number of alleles per locus (a_e) was calculated in each variety. The result of the maximum a_e showed in Luenghangkhoa Fighting Cock (5.02). Minimum a_e showed in Praduhangdam Fighting Cock that (3.51). The result is shown in Table 4.5. Furthermore, mean effective number of alleles of all varieties was

compared by Wilcoxon sign rank test. The result showed no significant mean different between varieties (see in Appendix VI).

The Hardy–Wienberg assumption was carried out using an exact test. *G. g. domesticus* (Praduhangdam Fighting Cock, Bantam and Betong Chicken) and *G. g. gallus* (Red Jungle Fowl) conformed to the Hardy-Wienberg assumption all of microsatellite loci (LEI73, MCW87 and MCW240), whereas Luenghangkhoa Fighting Cock conformed to Hardy-Wienberg assumption at LEI 73 and MCW87 locus, but not at MCW240 locus that deviated from this assumption ($P < 0.0025$). The result of Hardy-Wienberg equilibrium test is shown in Table 4.7.

Table 4.7 The estimation under Hardy-Wienberg assumption of four varieties of *G. g. domesticus* (Praduhangdam Fighting Cock, Luenghangkhoa Fighting Cock, Bantam and Betong Chicken) and variety of *G. g. gallus* (Red Jungle Fowl) were analyzed using four microsatellite loci (ADL23, LEI73, MCW87 and MCW240).

Locus	P- value				
	AP	AL	B	C	D
ADL23	0.6439^{ns}	0.3196^{ns}	0.2469^{ns}	0.0809^{ns}	0.7020^{ns}
LEI73	0.0256^{ns}	0.0179^{ns}	0.3198^{ns}	0.9019^{ns}	0.4113^{ns}
MCW87	0.4732^{ns}	0.9168^{ns}	0.2814^{ns}	0.6621^{ns}	0.5885^{ns}
MCW240	0.0851^{ns}	<0.0001*	0.6480^{ns}	0.0153^{ns}	0.5372^{ns}

* Significant level was further adjusted by using a Bonferoni method , ^{ns} no significant

AP = Praduhangdam (Fighting Cock, *G. g. domesticus*)

AL = Luenghangkhoa (Fighting Cock, *G. g. domesticus*), B = Bantam (*G. g. domesticus*)

C = Betong Chicken (*G. g. domesticus*), D = Red Jungle Fowl (*G. g. gallus*)

The exact test for genotypic linkage disequilibrium showed significance between locus pair of ADL23 and LEI73 locus, whereas other locus pairs conformed to the genetic linkage equilibrium. This result is shown in Table 4.8.

Table 4.8 The pairwise comparison of genetic linkage disequilibrium between four microsatellite loci of ADL23, LEI73, MCW87 and MCW240 of five investigated varieties.

Locus pairs	P-value
ADL23&LEI73	0.00044*
ADL23&MCW87	0.33595^{ns}
ADL23&MCW240	0.29180^{ns}
LEI73&MCW87	0.07008^{ns}
LEI73&MCW240	0.46568^{ns}
MCW87&MCW240	0.20613^{ns}

* Significant level was further adjusted by using a Bonferoni method , ^{ns} no significant

AP = Praduhangdam (Fighting Cock, *G. g. domesticus*)

AL = Luenghangkhoa (Fighting Cock, *G. g. domesticus*), B = Bantam (*G. g. domesticus*)

C = Betong Chicken (*G. g. domesticus*), D = Red Jungle Fowl (*G. g. gallus*)

Praduhangdam Fighting Cock showed no significant difference from Luenghangkhoa Fighting Cock at MCW87 locus whereas other loci showed significant difference (Table 4.9). Luenghangkhoa Fighting Cock showed significant difference from Bantam three loci excepted LEI73 locus. Praduhangdam Fighting Cock showed no significant difference from Betong Chicken at ADL23 and MCW87 locus. Comparison between Bantam and Betong Chicken showed significant difference from Luenghangkhoa Fighting Cock at all microsatellite loci. The comparison between subspecies showed Praduhangdam Fighting Cock was different from Red Jungle Fowl only ADL23 locus, whereas Luenghangkhoa Fighting Cock was different from Red Jungle Fowl only MCW87 locus. In addition, Bantam was no different from Red Jungle Fowl only MCW240 locus, but Betong Chicken was no different from Red Jungle Fowl at locus LEI73 and MCW87 locus.

Table 4.9 The pairwise comparison of contingency tests of differentiation among four varieties of *G. g domesticus* and variety of *G .g gallus* by four microsatellite loci.

Varieties pairs	P-value			
	ADL23	LEI73	MCW87	MCW240
AP&AL	<0.00001*	0.00039*	0.01959ns	0.00003*
AP&B	0.00116*	0.04747ns	0.00166*	0.00001*
AP&C	0.00497ns	0.00107*	0.21730ns	0.00014*
AL&B	0.00033*	0.00005*	<0.00001*	0.00008*
AL&C	0.00027*	0.00017*	0.00003*	<0.00001*
B&C	0.15014ns	<0.00001*	0.00063*	<0.00001*
AP&D	0.00019*	0.54110ns	0.00674ns	0.32285ns
AL&D	0.00569ns	0.00654ns	<0.00001*	0.20491ns
B&D	0.00014*	0.00220*	0.00009*	0.01006ns
C&D	0.00119*	0.16734ns	0.06622ns	0.00034*

* Significant level was further adjusted by using a Bonferoni method, ns no significant

AP = Praduhangdam (Fighting Cock, *G. g. domesticus*)

AL = Luenghangkhoa (Fighting Cock, *G. g. domesticus*), B = Bantam (*G. g. domesticus*)

C = Betong Chicken (*G. g. domesticus*), D = Red Jungle Fowl (*G. g. gallus*)

The allele frequency at four microsatellite loci in each pair of varieties was used to calculate the genetic distance. The genetic distance based on the Cavalli-Sforza and Edwards' chord distance among each pairwise comparison of varieties was observed by using GENDIST (Felsenstein, 1993). The lowest genetic distance was found between Praduhangdam Fighting Cock and Betong Chicken (0.0618), whereas the highest was observed between Bantam and Red Jungle Fowl (0.1340). The result is shown in Table 4.10. The resulting genetic distance was subjected to phylogenetic reconstruction based on the neighbor-joining approach (Saito and Nei, 1987).

Table 4.10 The pairwise comparison of genetic distance between four varieties *of G. g. domesticus* and variety of *G. g. gallus* by the Cavalli-Sforza and Edwards' chord method.

	Praduhangdam Fighting Cock	Luenghangkhoa Fighting Cock	Bantam	Betong Chicken	Red Jungle Fowl
Praduhangdam Fighting Cock	-				
Luenghangkhoa Fighting Cock	0.0843	-			
Bantam	0.0669	0.1082	-		
Betong Chicken	0.0618	0.1108	0.0996	-	
Red Jungle Fowl	0.0829	0.1248	0.1340	0.0911	-

The Neighbor-joining tree allocated all investigated varieties to three different groups consisting of Luenghangkhoa Fighting Cock and Bantam (group1), Praduhangdam Fighting Cock (group2) and Betong Chicken (group3), whereas Red Jungle Fowl was out group (see in Figure 4.10)

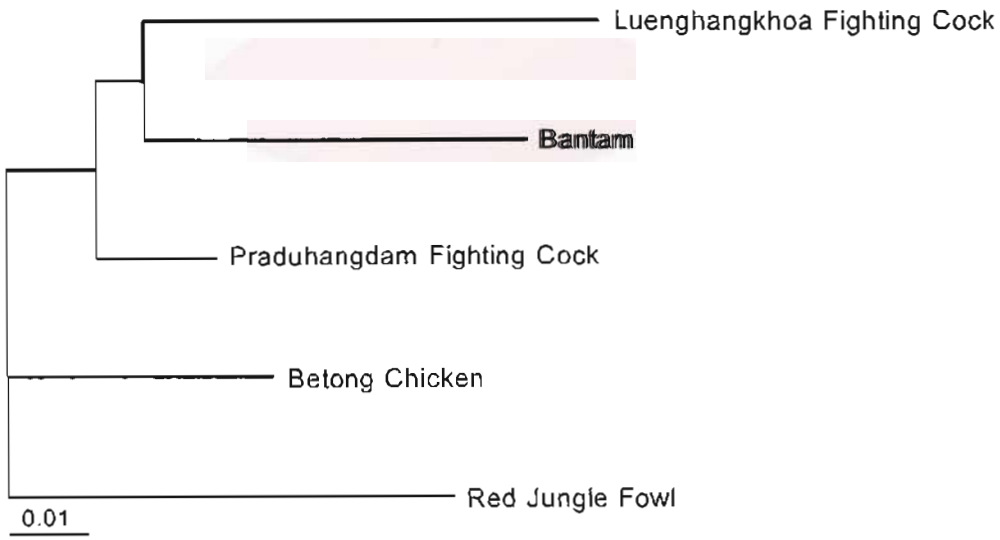

Figure 4.10 The phylogenetic tree of four varieties of *G. g. domesticus* (Praduhangdam Fighting Cock, Luenghangkhoa Fighting Cock, Bantam and Betong Chicken) and out group (*G. g. gallus*, Red Jungle Fowl) by the Neighbor-joining method.

Chapter 5

Discussion

The phenotypic polymorphism of Thai Native Fowls and Red Jungle Fowl were analyzed by morphometric traits. Fighting Cock, Bantam, Betong Chicken and Red Jungle Fowl were morphological selection changes in beak length (BL) associated with pecking in the ground for food (Zink and Blackwell, 1996). Due to Fighting Cock was selected the structures that fit for fighting such as tarsal length (TL), wing length (WL) and beak length (BL). Therefore, the sizes of all morphological characters of this variety were more longer than other varieties. On the other hand, Bantam was developed for pet. It was selected to be a miniature chicken. As a result, Bantam traits are usually small size. The body size of Betong Chicken is nearly the same as Fighting Cock, but the wings of this variety is different from Fighting Cock by having short wings and tail feathers, because it was selected for rearing in a hot and humid weather condition in Southern Thailand. The head length (HD) of each variety was different, while it depend on the body sizes.

The Canonical discriminant function is suitable for heterogeneity data. It also adjusts the heterogeneity data to the homogeneity data for reducing the bias of morphometric data (Zink and Blackwell, 1996). In addition, it combines the data of all morphometric traits, and calculates the center of distribution of each variety, and then morphometric distance is calculated and clustered. This discriminant analyses revealed that each variety was well separated in morphometric space, it explained that the morphometric traits of each variety of Thai Native Fowl were different, and it was also different from Red Jungle Fowl

In this experiment, bloodstains of each sample were collected for DNA extraction, because it is convenient for collecting specimens in a farm the field. It is

appropriated for avian blood, because red blood cells of avian have nucleus that contains genomic DNA. Furthermore, bloodstain can be kept for a long time.

The DNA extraction method in this study was divided into two methods. Although Proteinase K/Phenol-chloroform method need many steps for extraction, but it allow more purify of extracted DNA (OD_{260}:OD_{280} is more than 1.5) than 5% chelex method (OD_{260}:OD_{280} was less than 1.5), because Proteinase K/Phenol-chloroform method removes protein and other substance (e.g. hemoglobin) prior use, which it can be interfered PCR amplification (Davis *et.al.*, 1994). Although 5% chelex method permits easily step and less time consuming, it is no suitable for microsatellite marker. In this study, extracted DNA from bloodstains have high enough of DNA concentration and the extracted DNA by 5% chelex method can be amplified only some of specimen but not all of them. The difficulty of getting good PCR products because its always cause non specific products even it was used the some optimized condition with Proteinase K/Phenol-chloroform method. However, extracted DNA from 5% chelex method can be used to amplify for DNA sequencing (Boripat Siriaroonrat, 1997) and degraded DNA such as museum specimens, because it prevent lost of extracted DNA (Singer-Sam *et al.*, 1989).

The optimal annealing temperature at ADL23, LEI73, MCW87 and MCW240 locus did not get along with annealing temperature that calculated from the melting temperature, because each microsatellite loci was not developed from the same investigated variety (Thai Native Fowl and Red Jungle Fowl in Thailand). As a result, a pair of the primer was not annealing temperature correctly with different DNA template, because there was different sequence in microsatellite-flanking region, which it may be prevented template-primer annealing as a result of these sample can not amplified by calculated annealing temperature (Wolfus *et al.*, 1997). Therefore, if it was not successful to amplify and get a product at this annealing temperature, the PCR should be adjusted at higher or lower temperature in 2-3 degree step until a product of the

correct size was obtained. In addition, Mg^{2+} and primer concentration were adjusted for getting the highest intensity of target band, but the lowest shadow band and non-specific products was not shown on the gel. At MCW87 locus was found intense of the target band, and showed weak shadow of the stutter band less than other loci, which it is possible that this locus has more optimal PCR condition than the others.

The visualization of microsatellite alleles in this experiment was used the silver staining method. It is not handled with radioactive material (Luqmani *et al.*, 1997). Therefore, this method is more safety and less expansive method than radioisotope. The silver staining method offer advantage of sensitivity over ethidium bromide (detecting in picograms of quantities of DNA), the result of this method can also be detected very quickly and can be used for qualitative assessment of microsatellite allelic bands. Nevertheless, this method still cause a problem with variable background (Koreth *et al.*,1996).

Eight percent of polyacrylamide gel electrophoresis was used to separate the sizes of PCR products. It is the effective ranges of separation from 60 to 400 base pairs (Smith and Wood, 1991). PCR Products of two varieties were loaded into the same plate of denaturing sequencing gel and ran electrophoresis together (for pairwise comparison between varieties), according to compare the similar sized of PCR products (similar alleles) of every variety with the same primer. It can be helped error estimation of band scoring, and the pBR 322 MspI digested standard marker was used to reference sizes.

The band scoring of microsatellite was detected by eyes, but the sequencing marker was not used. Therefore, numerical symbol was designated to score the alleles. This method can not be estimated the real sizes of each observed alleles. Recently, sequencing marker for silver staining method has been developed from

Promega Corporation (Zink and Blackwell, 1996), which help to indicate the real sizes of observed alleles.

In this experiment, although number of microsatellite loci was low, the mean number of alleles per locus (A) of all varieties was higher than many other avian. For example, Partridge bird has mean number of alleles per locus between 1.2 and 2.5 (Randi and Laurent, 1999), and Shrike bird has mean number of allele per locus between 2.1 and 5.6 (Mundy *et al.*, 1997). However, this result may be caused by selected the high polymorphism primer. Therefore, each locus of this study showed many different alleles and often shows many rare alleles, which have low allele frequency. The comparison of mean number of alleles between the varieties showed significant mean difference. It can be explained that within subspecies of *G. g. domesticus* and between subspecies of *G. g. gallus* had difference of allele diversity. Moreover, the mean effective number of alleles was compared within and between subspecies showed no significant mean difference. It was explained that genetic diversity level in next generation of *G. g. domesticus* and *G. g. gallus* are not different. However it should be maintained number of alleles as equal as this generation for maintenance genetic diversity of each varieties.

The mean observed heterozygosity (H_O) and that expected from Hardy-Wienberg (H_E) showed no significant difference. Therefore, H_E can be used to compare genetic diversity within and between subspecies, because it reduced estimation error of rare alleles. The result showed no significant difference of H_E both within and between subspecies. It explained that genetic diversity was no different in subspecies level. This result was caused by microsatellite DNA has highly mutation rate, it permitted high polymorphism of alleles and it has different sizes of alleles, which these alleles increased the chance to find heterozygous allele. Moreover, microsatellite DNA is still permitted the band sharing between varieties. Thus, each examined variety can be found the same heterozygous alleles and it was highly heterozygosity (Mindell, 1995; Rico *et al.*, 1997).

Anyone variety, which has high the number of alleles and high heterozygosity, this variety shows highly genetic variation. However, heterozygosity can be calculated only heterozygous allele frequency but not assessed the number of alleles. In addition, number of alleles per locus is not assessed the allele frequency of rare allele, which can permitted the bias. Although the number of alleles per locus and heterozygosity value can be determined the genetic variation, the effective number of alleles is likely to be a good parameter for the study of genetic variation because it can calculated heterozygous allele frequency of all observed alleles (Wolfus *et al.*, 1997).

Luenghangkhoa Fighting Cock, which has shown unique allele (allele number 7^{th}) and has allele frequency higher than other unique alleles at MCW240 locus, is 0.152, whereas Praduhangdam Fighting Cock was found unique allele that has allele frequency 0.022. Unique allele is specific region of each organism (this study is varieties). These results caused from inbreeding, which Luenghangkhoa Fighting Cock at MCW240 locus was low observed heterozygosity and deviated from Hardy-Wienberg assumption (Hartl, 1988). Thus, allele number 7^{th} at this locus may be possible to be selected when inbreeding occurred, which this microsatellite may not be neutral locus. At ADL23 locus, it was found three unique alleles in Luenghangkhoa Fighting Cock that has allele frequency 0.02, 0.04 and 0.04. In addition, LEI73 locus was found two unique alleles that have low allele frequency of 0.02 and 0.02. Generally, if the unique allele frequency was approximate 0.9, this allele is possible to be used for identifying its own out from others (Lovette, 1997). However, most of unique alleles in this study have low allele frequency. Thus, this result should be confirmed by increasing the sample sizes of all varieties in order to increase allele frequency observing.

The Hardy-Wienberg assumption was tested for all loci and all varieties. The result showed only Luenghangkhoa Fighting Cock at MCW240 locus deviated from this assumption. The departure from Hardy-Wienberg of Luenghangkhoa Fighting Cock may be due to several parameters including (1) mutation process at this locus not

conforming to the infinite allele model of mutation (Rico *et al.*, 1997). Given the high mutation rate of microsatellite locus and individual may be homozygous for a pair of alleles of the same size, which may not be the product of a single mutation event. (2) Non-amplification of one of the two alleles presented in heterozygote, because the locus is not suitable for amplification this varieties. Therefore, further study should be developed for species-specific primers (Lessios, 1992; Wolfus *et al.*, 1997). For other varieties, all microsatellite loci conformed to Hardy-Weinberg assumption. It explained that observed and expected heterozygosity was no different, and microsatellite loci were selected to random association.

The microsatellite DNA at ADL23 locus locate on chromosome 5^{th}, LEI 73 locus locate on chromosome 4^{th}, MCW87 locate chromosome 2^{nd} and MCW240 locate on chromosome 4^{th}. The different region of microsatellite loci can effect to genetic linkage on the allelic distribution (Ciampolini *et al.*, 1995). Genetic linkage disequilibrium was used for proving the different region of microsatellite loci. For locus LEI73 and MCW240 locus, which locate on the same chromosome, showed no significance of the linkage disequilibrium. It explained that both loci may locate on far distance region (distance between LEI73 and MCW240 locus approximated 43 cM) (Burt, Bamstead and Critenden, 1993). Therefore, LEI73 locus is independent from MCW 240 locus, and show that the alleles of the locus are random association. Generally, closely region of locus is often found genetic linkage disequilibrium more than far region (Ciampolini *et al.*, 1995).

Nine of sixteen pairs of pairwise comparison between subspecies were that *G. g. domesticus* and *G. g. gallus* showed no significantly genetic differentiation, whereas five of twenty-four pairs within subspecies of *G. g. domesticus* showed no significantly genetic differentiation. It may explained that each variety of *G. g. domesticus* has been evolved from *G. g. gallus* that has period of time not enough for clearly revealed genetic differentiation between subspecies. In addition, rate of evolution of each variety of *G. g. domesticus* may be different. Furthermore, selected

microsatellite loci exhibited many different alleles, so it increased chance to show significant genetic differentiation within and between subspecies. Thus, genetic differentiation within subspecies (*G. g. domesticus*) is more than between subspecies (Mindell, 1995). It supported that Red Jungle Fowl *G. g. gallus* was ancestor of domestic chicken *G. g. domesticus* (Crawford, 1990; Fumihito *et al.* 1996).

According to this study, the results of morphometric analysis and microsatellite analysis were not correlated. The morphological character of Praduhangdam and Luenghangkhoa fighting cock are in the same group, whereas other varieties are separated. On the other hand, molecular approach found that Luenghangkhoa fighting cock and bantam are in the same group (genetic distance between varieties 0.0669), but Praduhangdam fighting cock is group in others. This result of molecular approach should not be occurred, because both Praduhangdam and Luenghangkhoa (genetic distance between varieties 0.0843) are fighting cock, so genetic distance should be less than between Luenghangkhoa fighting cock and bantam. It may be resulted from the selected microsatellite loci have many different sizes of alleles, high mutation rate, and small number of sample sizes. Thus, it is possible to find the difference of genetic distance between the same fighting cock.

In this study, only four microsatellite loci were determined for genetic variation analysis. In addition, each microsatellite loci showed high number of alleles, and that several alleles showed low allele frequency (lower than 0.1). Beside microsatellite DNA markers are neutral marker that not under the selection process and show a high mutation rate, leading to many different alleles, which low allele frequency (Wolfus *et al.*, 1997). Moreover, different mutation process between microsatellite markers may be provided opportunities and probabilities of complicated for analysis of population. The stepwise mutation process whereby repeats was usually added one at a time suggested that similarity of length reflected allelic relatedness and showed band sharing between population (Mindell, 1995). However, microsatellite will exhibit length

homoplasy that derived from variation in flanking region, and many involved point mutation as well as the more frequency slippage event that presumably drive the stepwise process. Because of the high slippage mutation rate of microsatellite, a given time period will involve more divergence of the repeat units than that expected from point mutations in the flanking regions (Ellegren, Primmer and Sheldon, 1995). As a result, the study at subspecies level over which microsatellite provided sufficient resolution may be narrower than that mutate more slowly, such as mitochondrial DNA. It may be showed clearly genetic differentiation between population or subspecies level.

In further study, the number of microsatellite loci at different linkage group or different chromosome should be increased as far as number of sample size, to lift the potential of analysis. For example, allele number 7^{th} was found only in Luenghangkhoa Fighting Cock at a half of maximum allele frequency but only 0.152, which may be effected by number of sample size. Therefore, following the commentary in this thesis should be examined.

Chapter 6

Conclusion

1. Genetic variations within variety of Thai Native Fowls

There are highly observed heterozygosity and highly number of alleles per locus. Therefore, Thai Native Fowls, Praduhangdam Fighting Cock, Luenghangkhoa Fighting Cock, Bantam and Betong Chicken, are high genetic variations.

2. Genetic variations among varieties of Thai Native Fowls

Observed and expected heterozygosity of Praduhangdam Fighting Cock, Luenghangkhoa Fighting Cock, Bantam and Betong Chicken are closely related and not different. On the other hand, number of alleles per locus of Praduhangdam and Luenghangkhoa Fighting Cock is different from Bantam and Betong Chicken. However, Mean effective number of allele per locus is the better parameter for determining genetic variations. It shows Praduhangdam Fighting Cock, Luenghangkhoa Fighting Cock, Bantam and Betong Chicken are not statistic different. Therefore, genetic variations among Thai Native Fowls are not different.

3. Relationship of morphometric and molecular genetic analysis

Morphometric analysis can be divided Thai Native Fowls into three groups (1) Praduhangdam and Luenghangkhoa Fighting Cock (2) Bantam and (3) Betong Chicken, whereas molecular genetic can be divided Thai Native Fowls into three groups (1) Luenghangkhoa Fighting Cock and Bantam (2) Praduhangdam Fighting Cock (3) Betong Chicken. Therefore, morphometric analysis does not get along with molecular genetic analysis.

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APPENDICES

APPENDIX I

DNA Extraction protocol

1. 5% Chelex DNA extraction method

1. Pipette 1 ml of sterile double distilled water into a sterile 1.5 ml micro centrifuge tube
2. Cut a small piecefilter paper containing blood stain (approximately 2-3 mm^2.) and place in a sterile labeled microcentrifuge tube, incubate at room temperature for 15-30 minutes and mixed gently.
3. Spin in a microcentrifuge for 2-3 minutes at 10,000 to 15,000 rpm.
4. Remove supernatant and discard.
5. Add 5 % Chelex to a final volume of 200 µL.
6. Incubate at 56^0C for 15-30 minutes or overnight.
7. Vortex at high speed (DNA to avoid shearing) for 5-10 seconds or mix gently. Then boiled in boiling water bath for 8 minutes.
8. Vortex at high speed (DNA to avoid shearing) for 5-10 seconds or mix gently.
9. Spin in a microcentrifuge for 2-3 minutes at 10,000 to 15,000 rpm.
10. Tranfer supernatant into a new sterile microcentrifuge tube and discard fabric substrate and chelex.
11. All samples were kept at -20^0C. Approximately, 0.5-5 µL of the supernatant was used for the PCR reaction.

2. Phenol/Chloroform DNA extraction

1. Cut a small piece of filter paper containing bloodstain (approximately 2-3 mm^2) and place in a sterile labeled 1.5 ml microcentrifuge tube.
2. Add 1.0 ml of TE buffer and incubate at room temperature for 30-60 minutes.
3. Add 25 µL of 10% SDS and 25 µL of proteinase K and incubate at room temperature for 10 minutes and gentle mix by inversion.
4. Add 1 volume of SS-phenol and 1 volume of chloroform (approximately 250 µL) and mix this sample by repeated inversion (do not vortex)
5. Centrifuge each tube for one minute at 10,000 rpm.
6. Remove upper aqueous layer (do not disturb interphase) and transfer to a new sterile1.5 ml microcentrifuge tube
7. Add 1/10th volume of 3 M sodium acetate pH 7.0
8. Add at 2.5 volumes of 95% ethanol and incubate at -20^{0}C overnight
9. Spin at 10,000 to 15,000 rpm for 10 minutes.
10. Discard supernatant carefully (do not disturb the pellet). To remove residual salt, wash pellet in 300 µL or more of 80% ethanol. Gentle mix by inversion.
11. Spin at 10,000 to 15,000 rpm for 2 minutes and discard the supernatant and air-dried.
12. Add 200 µL of TE buffer and store at -20^{0}C until further needed

APPENDIX II

Quality determination and size estimation can be calculated of DNA

1. Quality determination of DNA

Description previously was used followed :

1. An approximate amount of agarose was weighed out and heated to dissolve in an appropriate volume of 1X TBE buffer.
2. Malted agarose was poured into the gel mould (Mupid set electrophoresis) that the comb was already inserts to gel mould. When the gel completely set (the gel had cooled and solidified), the comb was gently removed.
3. The gel submerged in the gel chamber containing an enough of 1X TBE buffer that covered the gel to a depth about 1-2 mm. Each of extracted DNA was prepared for loading by mixing loading dye buffer and mixed well.
4. The samples were applied into the wells slowly that used Phi X 174 - λ DNA digested with Hinf I was loaded into a well for served as a DNA standard.
5. A gel bath was connected to a power supply and turned on (the gel was run at 100 volt), then DNA migrated into the gel toward the anode. When bromophenol blue had migrated about three-fourths of a gel distance turned off the power supply and stain the gel with 0.25 μg/ml ethidium bromide.
6. The gel was destained in deionise distilled water for 5-10 minutes to leach out unbound ethidium bromide, placed on a long wavelength UV. transluminator and photographed using Polaroid 667 film.

2. Size estimation of DNA

1. The pBR 322 digested with MspI was used as a DNA marker An amount of standard marker is used that concentration was estimated 1 μg / μL
2. The marker is dropped into 0.5 μL microcentrifuge tube.
3. The marker is prepared by proportion of standard marker : 1X of TE buffer : 6X of loading dye was 1 : 2 : 3. It is gently mixed and store at -20^{0}C .

APPENDIX III

Reagent preparation protocol

1. 1% Agarose

An enough amount of ingredients for a 100 ml gel composed of :

- Agarose	1.0	gm
- 1X TBE buffer	100.0	ml

How to applied the description previously is used follow:

1. Agarose powder about 1 gm is mixed into 1XTBE buffer 30 mL.
2. The agarose solution is cooked in microwave for 2 minutes.
3. Prepare gel mould for set the gel. When time is finished, the dissolved gel is transferred about 25-50 mL and is added with 0.2 µL of 1% ethidium bromide into gel. The gel is mixed.
4. The soluble gel is poured into the gel mould, which the comb is already inserted to the gel mould.
5. When the gel has completely cooled and solidified, the comb was removed.
6. The gel is transferred into a gel chamber containing an enough of 1X TBE buffer that covered the gel to about 1-2 mm depth.

2. 8% polyacrylamide gel

An enough amount of ingredients for a 120 ml gel composed of :

- 7M urea	50.4	gm
- 10X TBE buffer	12.0	ml
- 30% stock acrylamide solution (Bio-rad®, acrylamide monomer : bis-acrylamide = 29 : 1)	32.0	ml
- distilled water for added up to	120.0	ml

How to use the description previously is used follow:

1. The dissolved ingredients are added 10% APS 500 µL (fresh prepared) and TEMED 100 µL before used.
2. Then the ingredients are poured into the gel apparatus. the comb is inserted into the upper and pointing out for making a sharp cut edged of the gel.
3. The polymerization process is allowed to complete for 3 hours.
4. The comb is then removed that urea and small pieces of gel are flushed out of the wells prior to loading PCR products.

3. 10X Tris Boric EDTA buffer (10XTBE)

An enough amount of ingredients for a 1000 ml composed of :

- Tris aminomethane	108	gm
- Boric acid	50.4	gm
- EDTA	7.44	gm

The solution is prepared as follow:

1. Tris, Boric and EDTA are mixed into volumetric bottle 1000 mL.
2. Double distilled water is added up 1000 mL.
3. Solutionis stirred until completely dissolve.
4. Store in room temperature and use 10X TBE for acrylamide, while use 1X TBE for running electrophoresis.

4. 40% Methanol

The solution is prepared as follow: Methanol is poured about 400 mL into 1000 mL cylender. Double distilled water was added up to 1000 mL, and mixed gently.

5. 1 M Nitric acid

The solution is prepared as follow:

1. 2 N of Nitric acid is prepared for stock solution and poured into 500 mL cylender about 62.9 mL for 1M working solution (prepared in hood).
2. Deionized water is added up to 500 mL.
3. Mix gently and kept at room temperture.

6. 0.2% Silver nitrate

The solution was prepared as follow:

Silver nitrate 0.2 gm is prepared for 100 mL total volume working solution in 500 mL bottle (freshy prepared and mixed gently).

7. Developer solution (3% Sodium carbonate and 40% formadehyde)

The solution was prepared as follow:

1. Sodium carbonate 3 gm is prepared and added double distilled water about 100 mL.
2. The solution is mixed gently for dissolve.
3. 40% formaldehyde about 50 µL is added into the solution, before use.

8. Stop solution (0.1M Citric acid or 20% acetic acid)

The solution was prepared as follow:

0.1M Citric acid

1M Citric acid is prepared for stock solution and used 0.1 M for working stop reaction.

20% Acetic acid

10 mL. Of Glacial acetic acid is poured into 500 mL cylender and added double distilled water up to 500 mL. Mixed gently.

APPENDIX IV

End labeling and Silver Nitrate staining method

End labeling of primer

Autoradiography detection of PCR product was labelled with $\gamma\ ^{32}P$ dATP at a single 5' end of primer (end labelling) is more sensitive and common use.

Labelling reaction mixture as:

- sterile distilled water	4.5	µL
- labelling buffer	1.0	µL
- 10 µM primer	1.0	µL
- T_4 polynucleotide Kinase (PNK)	0.5	µL
- $\gamma\ ^{32}P$ dATP	3.0	µL

How to use the description previously was used as follow :

1. Labelling reaction was mixed in 0.5 µL microcentrifuge tube.
2. Incubate the mixture at 37 °C for 30 minutes.
3. Heat the mixture for destroy PNK at 65 °C for 15 minutes.

Silver nitrate staining

How to use the description previously was used as follow:

1. Cut the gel as a size of covering PCR products and transfer the gel into the staining chamber.
2. Then the gel was fixed in 40 % methanol for 12 minutes. When finished, discard solution.
3. The gel was rinsed in double distilled water (DDW) and discard.
4. Prepared suitable physical condition of the gel by shoaked in nitric acid for 5 minutes. When finished, discard solution.
5. The gel was shoaked in DDW for 4 minutes and discard.
6. The gel was shoaked in 0.2 % silver nitrate for 13 minutes that this solution was fresh prepared. When finish discard solution.
7. The gel was rinsed in DDW and discard.
8. Then the gel was shoaked in developer solution in developer solution 3 % sodium carbonate, 37 % formaldehyde 50 µL was added before used. When PCR products band were occurred, discard solution.
9. Then stop reaction, the gel was shoaked in 0.1 M. citric acid or 20 % acetic acid for 1 minute, discard solution and shoaked the gel in DDW for 5 minutes, discard solution.
10. Transfer the gel into to fine hard paper, wrapped with by sarun wrap, dried the gel by dryer and the gel was dried at 80^{0}C about 45 minutes, or wrap the gel by cellophane and air-dried for over night. Labelled the gel and take a photography.

APPENDIX V

Morphometric traits data of Thai Native Fowls and Red Jungle Fowl

Table1 Morphometric data of 15 specimens of Fighting cock, Praduhangdam (AP)

Sample number	Sex	Juvenile / Adult	Weight (Kg)	BL (cm)	WL (cm)	TL (cm)	HD (cm)	TD (cm)
1	M	A	3.6	2.05	42.4	13.7	8.2	-
2	M	A	2.9	2.10	41.6	12.8	7.0	-
3	M	A	3.3	2.2	45.5	13.8	8.1	-
4	M	A	3.1	2.15	41.1	13.2	7.2	-
5	M	A	3.2	2.0	43.0	13.6	7.0	-
6	M	A	3.1	2.2	42.7	12.2	7.3	-
7	M	A	2.85	2.1	42.2	12.8	7.4	-
8	M	A	3.4	2.5	42.5	13.2	7.6	-
9	M	A	3.6	2.3	44.9	14.3	8.0	-
10	M	A	3.3	2.4	41.1	13.2	7.7	-
11	M	A	3.35	2.2	45.5	13.8	7.3	-
12	M	A	3.2	2.3	43.8	13.5	7.6	-
13	M	A	3.1	2.0	45.2	13.0	6.8	-
14	M	A	3.5	2.2	46.6	13.8	7.4	-
15	M	A	3.05	2.1	44.8	12.7	7.3	-

Table 2 Morphometric data of 15 specimens of Fighting cock, Luenghangkhoa (AL)

Sample number	Sex	Juvenile / Adult	Weight (Kg)	BL (cm)	WL (cm)	TL (cm)	HD (cm)	TD (cm)
1	M	A	2.4	2.1	44.0	11.7	7.4	9.5
2	M	A	2.8	2.3	43.5	11.9	7.5	9.1
3	M	A	3.25	2.8	43.5	12.8	8.7	9.5
4	M	A	2.4	2.3	43.0	9.6	8.3	9.6
5	M	A	2.73	2.2	41.5	11.1	7.1	9.0
6	M	A	3.2	2.2	43.0	11.4	7.1	9.5
7	M	A	3.4	2.4	41.0	11.8	8.0	9.8
8	M	A	2.9	2.5	44.3	14.2	8.2	10.2
9	M	A	2.9	2.0	43.0	13.4	8.1	10.0
10	M	A	2.9	2.0	44.3	12.7	8.0	9.4
11	M	A	3.0	2.5	44.8	13.8	8.3	10.0
12	M	A	3.2	2.0	44.0	14.7	8.7	9.6
13	M	A	2.73	2.5	44.3	13.6	8.1	9.2
14	M	A	3.4	2.6	44.0	13.8	8.3	10.1
15	M	A	2.75	2.5	45.2	17.4	8.0	10.0

Table 3 Morphometric data of 15 specimens of Bantam, Cocoa (B)

Sample number	Sex	Juvenile / Adult	Weight (Kg)	BL (cm)	WL (cm)	TL (cm)	HD (cm)	TD (cm)
1	M	A	2.4	2.1	44.0	11.7	7.4	9.5
2	F	A	2.8	2.3	43.5	11.9	7.5	9.1
3	M	A	3.25	2.8	43.5	12.8	8.7	9.5
4	F	A	2.4	2.3	43.0	9.6	8.3	9.6
5	M	A	2.73	2.2	41.5	11.1	7.1	9.0
6	F	A	3.2	2.2	43.0	11.4	7.1	9.5
7	F	A	3.4	2.4	41.0	11.8	8.0	9.8
8	F	A	2.9	2.5	44.3	14.2	8.2	10.2
9	M	A	2.9	2.0	43.0	13.4	8.1	10.0
10	F	A	2.9	2.0	44.3	12.7	8.0	9.4
11	M	A	3.0	2.5	44.8	13.8	8.3	10.0
12	M	A	3.2	2.0	44.0	14.7	8.7	9.6
13	F	A	2.73	2.5	44.3	13.6	8.1	9.2
14	F	A	3.4	2.6	44.0	13.8	8.3	10.1
15	F	A	2.75	2.5	45.2	17.4	8.0	10.0

Table 4 Morphometric data of 15 specimens of Betong cock (AL)

Sample number	Sex	Juvenile / Adult	Weight (Kg)	BL (cm)	WL (cm)	TL (cm)	HD (cm)	TD (cm)
1	F	J	0.75	2.08	30.0	4.1	20.6	8.8
2	M	A	0.6	2.1	28.0	4.4	24.0	8.5
3	F	A	0.8	2.06	32.6	4.46	56.0	10.02
4	M	A	0.5	1.28	27.1	3.9	49.0	7.95
5	F	A	0.75	1.9	31.9	4.55	51.0	10.15
6	F	A	0.65	2.2	27.9	4.7	52.5	10.1
7	F	A	0.58	1.95	25.3	4.0	51.0	9.2
8	F	A	0.68	1.95	27.4	4.04	51.0	9.17
9	F	A	0.8	2.02	31.1	4.62	55.0	9.17
10	F	A	1.59	1.18	25.7	3.31	51.9	8.29
11	F	A	0.55	1.3	25.8	3.26	56.0	8.24
12	F	A	0.96	1.97	29.8	3.48	56.2	8.90
13	M	A	0.62	1.89	26.0	3.91	56.0	9.2
14	F	A	0.69	1.7	27.6	4.03	57.5	10.35
15	F	A	0.65	1.86	28.1	3.86	58.7	8.7

Table 5 Morphometric data of 10 specimens of Red Jungle Fowls (D)

Sample number	Sex	Juvenile / Adult	Weight (Kg)	BL (cm)	WL (cm)	TL (cm)	HD (cm)	TD (cm)
1	F	A	0.85	1.2	29.0	7.7	5.7	5.25
2	M	A	1.2	1.1	36.8	9.0	6.2	4.1
3	M	A	1.3	1.3	33.7	8.5	6.1	5.5
4	F	A	0.67	1.2	29.5	7.4	6.0	4.6
5	F	A	0.84	1.5	30.2	7.75	5.8	4.95
6	F	A	0.79	1.2	32.2	7.6	5.8	4.2
7	F	A	0.66	1.3	30.5	7.45	5.8	5.0
8	F	A	0.77	1.35	30.5	7.7	5.65	4.7
9	M	A	1.2	1.5	35.7	8.85	6.35	4.90
10	M	A	1.3	1.35	36.5	9.25	6.25	5.25

APPENDIX VI

Wilcoxon sign rank test

Table Comparison of mean number of allele (A), mean effective number of allele (a_e) and mean expected heterozygosity (H_E) between five investigated varieties by Wilcoxon sign rank test. The significant level at 0.05.

Parameters	P-value
A	0.014
a_e	0.508
H_E	0.593

Biography

Miss Piyamas Karnsomdee was born on the 23th of October 1974 in Pranakornsriayutthaya province. She graduated her bachelor's degree of Science, Kasetsart University. She continued her graduated study for a master's degree of Science in Biotechnology at Chulalongkorn University in 1996.