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MICROSATELLITE DNA VARIATION OF RED JUNGLEFOWLS *Gallus gallus spadiceus* IN NORTHERN AND SOUTHERN THAILAND

Mr.Pramong Begthaisong

A Thesis Submitted in Partial Fulfillment of the Requirements

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By	Mr. Pramong Begthaisong
Program	Biotechnology
Thesis Advisor	Associate Professor Wina Meckvichai

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ประมง เบกไธสง : ความแปรผันของไมโครแซทเทลไลท์ดีเอ็นเอของไก่ป่าตุ้มหูแดง *Gallus gallus spadiceus* ในตอนเหนือและตอนใต้ของประเทศไทย (MICROSATELLITE DNA VARIATION OF RED JUNGLEFOWL *Gallus gallus spadiceus* IN NORTHERN AND SOUTHERN THAILAND) อ.ที่ปรึกษา : รศ. วีณา เมฆวิชัย; 47 หน้า. ISBN 974-332-396-1.

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาความเป็นไปได้ในการใช้ไพรเมอร์ (primer) ที่ใช้เพิ่มปริมาณไมโครแซทเทลไลท์ดีเอ็นเอ (microsatellite DNA) ของไก่เลี้ยงมาใช้เพิ่มปริมาณไมโครแซทเทลไลท์ดีเอ็นเอไก่ป่าตุ้มหูแดง *Gallus gallus spadiceus* และใช้ตรวจสอบความหลากหลายทางพันธุกรรมในประชากรทางตอนเหนือ (จังหวัดแพร่ และพะเยา) และทางตอนใต้ (จังหวัดชุมพร) จากการตรวจสอบไมโครแซทเทลไลท์ดีเอ็นเอทั้งหมด 6 ตำแหน่งคือ HUJ1 HUJ2 HUJ7 ADL37 LEI73 และ LEI92 โดยอาศัยกระบวนการลูกโซ่โพลีเมอร์เรส (polymerase chain reaction) พบว่าไพรเมอร์จากไก่เลี้ยงทั้งหมดที่เลือกใช้สามารถเพิ่มปริมาณไมโครแซทเทลไลท์ดีเอ็นเอจากดีเอ็นเอต้นแบบ (DNA template) ของไก่ป่าตุ้มหูแดงที่สกัดด้วย Chelex®100 ได้

จากไมโครแซทเทลไลท์ดีเอ็นเอที่เลือกใช้ทั้งหมดพบว่า 5 ตำแหน่ง คือ HUJ1, HUJ2, HUJ7, LEI73 และ LEI92 มีความหลากหลายสูง และพบว่ามีจำนวนอัลลีล (allele) แต่ละตำแหน่งเป็น 9, 13, 8, 10 และ 8 ตามลำดับ และพบว่าทุกตำแหน่งมีอัลลีลร่วม (shared allele) ระหว่าง 2 แหล่ง ความถี่อัลลีลที่ตำแหน่ง HUJ1, HUJ2, HUJ7 และ LEI92 ในประชากรทางตอนเหนือเป็นไปตามกฎของ Hardy-Weinberg แต่มีเพียง 2 ตำแหน่งคือ HUJ2 และ LEI92 ในประชากรจากจังหวัดชุมพรที่เป็นไปตามกฎนี้ จากการวิเคราะห์ geographic heterogeneity แสดงให้เห็นว่าไม่มีความแตกต่างกันอย่างมีนัยสำคัญระหว่างประชากรทางตอนเหนือและจังหวัดชุมพรที่ตำแหน่ง HUJ1 ($P = 0.109$) HUJ2 ($P = 0.313$) HUJ7 ($P = 0.065$) และ LEI92 ($P = 0.465$) แต่พบว่ามีความแตกต่างกันอย่างมีนัยสำคัญที่ตำแหน่ง LEI73 ($P = 0.013$) ระหว่างสองประชากร

ภาควิชา – ลายมือชื่อนิสิต .....

สาขาวิชา เทคโนโลยีทางชีวภาพ ลายมือชื่ออาจารย์ที่ปรึกษา .....

ปีการศึกษา 2541 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

C827161 : MAJOR BIOTECHNOLOGY
KEY WORD: *Gallus gallus spadiceus* / RED JUNGLEFOWL / MICROSATELLITE DNA / GENETIC VARIATION

PRAMONG BEGTHAISONG : MICROSATELLITE DNA VARIATION OF RED JUNGLEFOWLS *Gallus gullus spadiceus* IN NORTHERN AND SOUTHERN THAILAND. THESIS ADVISOR : ASSOC. PROF. WINA MECKVICHAI. 47 pp. ISBN 974-332-396-1.

The purposes of this study were to validate possibility to using chicken microsatellite marker for investigation of microsatellite variation in Red Junglefowl genome and used these marker to determine genetic diversity of this chicken ancestor in northern and southern Thailand. Six available microsatellite-flanking PCR primers which developed from genomic libraries of domestic chicken were used to amplify microsatellite DNA from Chelex®100 extracts.

Five loci, HUJ1, HUJ2, HUJ7, LEI73 and LEI92 shown polymorphic amplified product (with number of allele at each locus of 9, 12, 8, 10 and 8 respectively) whereas ADL37 shown only two observed allele. Shared allele between northern (Phrae Prayao and Chaiyabhum province) and southern (Chumphon province) local were found at all loci. Conformity with Hardy-Weinberg expectation was found at almost loci (HUJ1, HUJ2, HUJ7 and LEI92) in northern local. Unlike, only HUJ2 and LEI92 loci in southern local conformed this expectation. Geographic heterogeneity analysis shown non significant difference between two populations at HUJ1 (P = 0.109), HUJ2 (P = 0.313), HUJ7 (P = 0.065) and LEI92 (P = 0.465) but significant difference at LEI73 (P = 0.013).

ภาควิชา..... ลายมือชื่อนิสิต.....

สาขาวิชา เทคโนโลยีทางชีวภาพ ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2541 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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Chapter 1

Introduction

Like the other tropical countries, forest degradation and fragmentation have been progressive in Thailand. Consequently, most wildlife habitats were degraded or eliminated. The surviving population which is been small and fragmented potentially losses the future ability to survive and evolve. Their conservation will require far more management than was necessary in the past. To develop an effective conservation management, various biological disciplines is necessary. Unfortunately, the Thai biota is still rather poorly known, making it difficult to set conservation priorities (Woodruff, 1991).

The Red Junglefowl, *Gallus gallus*, an important bird in evolution of human civilization and the main genetic stock for the world's lineage of domestic chicken (Fumihito et al., 1996), has stood on this situation. The Red junglefowl is one of four species of the world's Junglefowl naturally distributed in only the warmer part of Asia. Two out of five subspecies of this species which are distinguishable by their earlobe color and their distribution, partially inhabit in Thailand. *G. gallus gallus* with whitish ear spot has been found in the eastern part of the country whereas other red earlobe, *G. gallus spadiceus* inhabits in the northern forest from Lao border down to southwest along the Myanmar border through the Malay peninsula. Habitat degradation and fragmentation, hunting as well as geographical difference in habitat between Indo-China continental and Malay peninsula may be the causes of random genetic drift and genetic differentiation between local populations of the red ear lobe subspecies in northern and southern habitat..

The understanding of population genetic is necessary for evolution biology, breeding management and conservation of interested species. When combined with demographic, ecological, behavioral, and physiological characteristics of interested species, genetic data has emerged as a unifying component for interpreting past history, present status, and future prospects of threatened population.

To study the population genetics, molecular method has replaced the conventional method which concentrate on easily detected variations including morphometric characters, blood groups or blood proteins and chromosomal inversion. Although these variations are important variation but they do not allow the estimation of total genetic variation of natural population. On the contrary, molecular techniques involve the examination of the direct translated products or the genes themselves so that the total genetic variation is clarified. In addition,interpretion of molecular data is relatively simple and well understood.

Unfortunately, previuos studies of the Red Junglefowl had been concentrated on taxonomy, morphology, ecology, behavior and its relationship among domestic chicken. Although, molecular techniques are now available to address the genetic questions in various species but molecular studies of this representative species of order Galliformes are relatively scarce. There are few researches using molecular techniques to examine the relationship between domestic fowl and the Red Junglefowl (Fumihito et al, 1994; 1996; Okada, 1994). Recently, Boripat Siriaroonrat (1997) used the nucleotide sequence of the mitochondrial control region to investigate genetic variation of these two subspecies of Thailand. Phylogenetic analysis shown a different branches between two subspecies whereas sequence divergence within each subspecies seem to be low. On the other hand, molecular markers and genetic data of chicken, a domestic descendant of this species, are well studied.

Hence, in order to investigate genetic variation among individuals within and between local populations of the red ear lobe subspecies exist in northern and southern habitat, chicken microsatellite markers have been used for two reasons. First, there are a number of available microsatellite markers of commercial domestic fowl, a closely related descendant of the Red Junglefowl. Using these markers to investigate genetic diversity of natural populations of the Red Junglefowl is highly possible. Thus the expensive and laborious laboratories for the development of microsatellite-flanking PCR primer from genomic libraries of Red Junglefowl are disregarded. Second, there are several technical and analytical advantages of microsatellite marker over other markers for instance; microsatellite alleles are inherited in co-dominant fashion so all alleles can be decriminated.

Microsatellite sequences are ubiquity through the genome and selective neutrality. Microsatellite DNA can be amplified by PCR that make it possible to analyze the small amount of DNA. May some microsatellite loci exhibit high polymorphism. Thus this marker are suitable for the determination of various fine parameters in population genetic such as genetic diversity in species that show low level of variation, genetic variations in the intraspecific level, population substructure in recently seperated population.

In this study, six highly polymorphic microsatellite marker that developed within the laboratory of Hans Cheng (Cheng and Critteden, 1994; Cheng et al., 1995), Crooijmans et al. (1997) and Gibbs et al. (1997) were screened. The microsatellite - flanking PCR primers which shown polymorphic amplified products were used to examine microsatellite vriation in genomic DNA extracted from feather or blood stain by using chelex ®100.

Objectives

1. To evaluate the possibility of using some chicken microsatellite-flanking PCR primer to investigate the genetic variablility of Red Junglefowl.

2. To use the available domestic chicken microsatellite markers to examine genetic variation within and between the red ear lobe Red Junglefowl inhabits in the northern and southern Thailand.

Anticipated benefit

To obtain basic knowledge about genetic diversity among the red earlobe Red Junglefowl inhabit in fragmented, geographical different of forests in the northern and southern Thailand. This could be used for interpreting the natural history of *Gallus gallus spediceus* population for providing useful clues about population genetic structure that are valuable in developing an effective management strategies. The wild populations of this species are the important genetic resources in the future for the improvement of native chicken.

Chapter 2

Literature Reviews.

2.1 The Red Junglefowl

Red Juglefowl was first described by Linnaes as *Phasianus gallus* in 1758. Its scientific names has been changed several time before the latest revised name, *Gullus gallus* O.Grant (nec.Linn) was accepted (Rothschild, Hartert , and Jordan, 1926). The present classification of Red Junglefowl is:

Kingdom Animalia
Phylum Chordata
Subphylum Vertebrata
Class Aves
Order Galliformes
Family Phasianidae
Genus *Gallus*
Species *Gallus gallus*

The genus *Gallus* (Junglefowl) consists of four distinct species (Wayer, 1969); *Gallus gallus* (Red Junglefowl), *Gallus lafayettei* (Ceylon Jungle), *Gallus sonnerati* (Gray Junglefowl) and *Gallus varius* (Green Junglefowl). Red Junglefowls are also subdivided into five subspecies comprising *G. g. gallus*, *G. g, spadiceus*, *G. g. jabouillei*, *G. g. murghi*, and *G. g. bankiva*.

In general appearance, these junglefowls are similar to each other. The cock has comb on the top of the head and two lappets below the bill. Its tail is much compressed and composed of fouteen or sixteen rectrics. The central pair is elongated and curved downward. The legs are long and armed with a large curve spur. However, It is distinguishable by their plumages colour pattern (Figure2-1). Wayre (1962) detailed the characters of these birds and described the Red Junglefowl as:

The male has a long, pointed crown and neck feathers of golden-brown to fiery red in colour; the mantle and wing coverts are of metallic green and the scapulars, back and median wing coverts dark reddish-brown to orange-red on the rump, where the feathers are elongated and pointed. The primaries are blackish-brown and the secondaries rufous; the

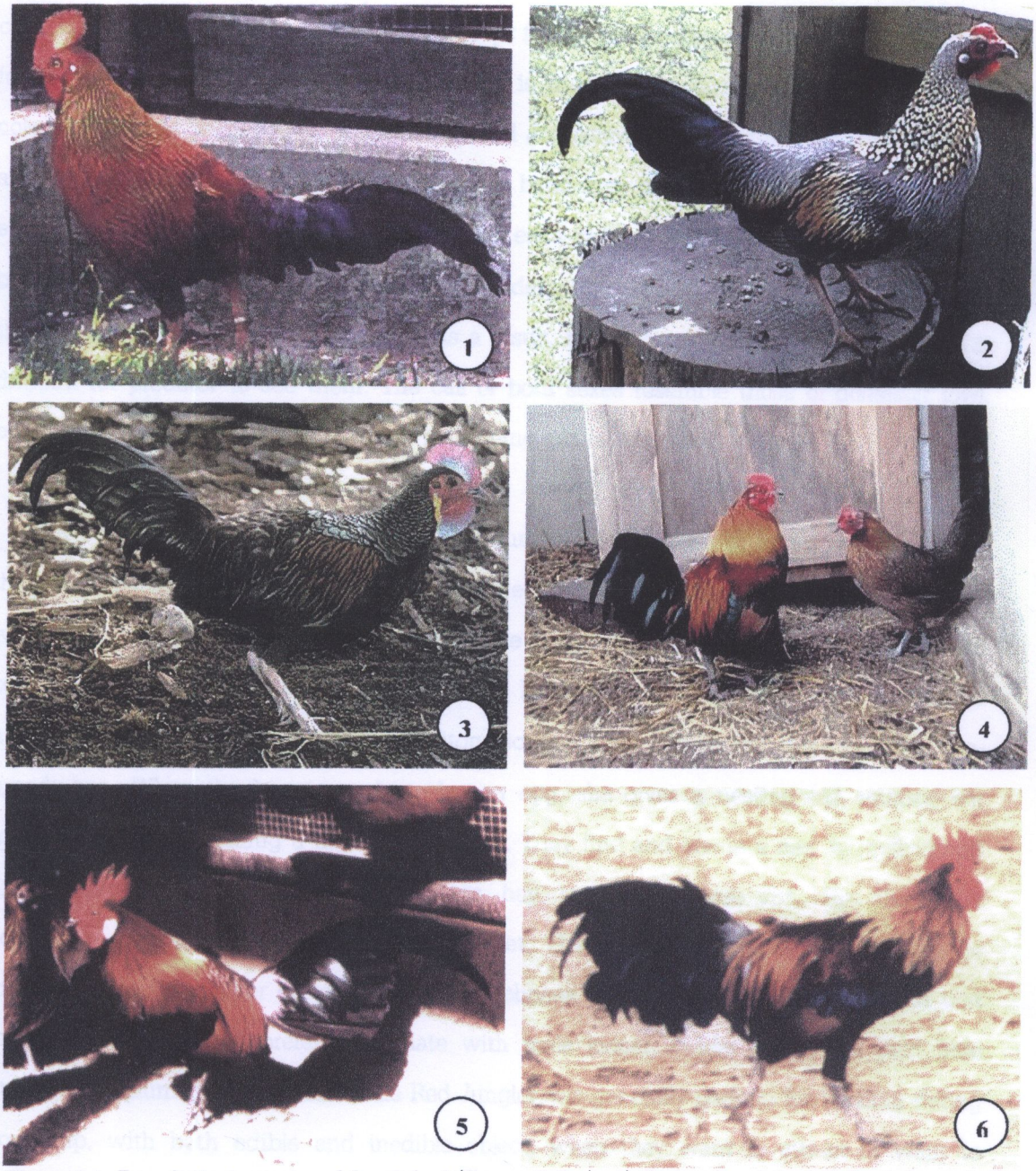

Figure 2-1 Four distinct species of Junglefowl (Genus *Gallus*). 1) Ceylon Junglefowl *Gallus lafayettei* 2) Grey Junglefowl *G. sonnerati* 3) Green Junglefowl *G. varius* and 4) Red Junglefowl *G. gallus* which two subspecies 5) *G. g. gallus* and 6) *G. g. spadiceus* are found in Thailand.

tail and its coverts are dark metallic green. The under parts are dull black. The comb is largish and serrated; there are two wattles which include the naked throat and face are scarlet. He undergoes a moult into eclipse plumage in the summer (breeding season). Since junglefowl's wings are rounded, curved and fit close to body. They are able to fly swiftly, but cannot sustain flight for very long. When flying away the most noticeable feature of this cock is the white patch on a blue black except for the red wattle on the head. Nevertheless, female of this genus is closely resemble and is much duller, brown in basic colour. The colour pattern of precocial downy chick of Red Junglefowl was described by Lord Medway and Well (1976) as: buff, with a broad median band from crown to rump, a band from the eye to the side of the beck and a band on each side of the back, dark reddish brown bordered with black; throat, ear coverts and underparts pale buff, with a darker band across the upper breast; wings rufous brown. As a result, the colour of her and her chick make it difficult for predator to see them. The call of both sexes resemble those of domestic fowl, but the cock's crow is shorter, the last note being cut off abruptly.

In comparison with other species, Red Junglefowl are likely to be polygamous rather than monogamous. While the other species are usually found singly or in pair (sometime with their young), Red Junglefowl generally live in small groups that comprise a dominant cock, one or more hens, and immature birds. During the mating and rearing phase, the hens separate off within the group territory so that the cock is left alone or with non-broody-hen. When the hen is ready to lay an egg, she gives a nesting call, inviting her mate to join her in finding a nest site together. They find and create a nest by pulling and flinging around themselves twings feathers, hay, leaves and loose dirt, after they have scraped a depression with their beaks and feet. The hen usually makes the nest on the ground under the shelter of vegetation. Zuk, Johnsen and Maclarty (1995) pointed out that female Red Junglefowl prefered to mate with male longer, redder comb, but paid little attention to plumage characters. Male Red Junglefowls generally perform food calling during courtship, with both edible and inedible object, when they want to attract or want to contract with a female. The male's courtship behavior is similar to that of domestic fowl, his oblique attitude resulting in a conspicuos display of the bright neck and saddle feathers to

the hen. His sudden circling, accompanied by the rasping sound produced by the movement of the lowered primaries, stimulates the hen to crouch, after which copulation may follow.

The hen lay 6-12 eggs, resemble small domestic hen's eggs. Most eggs are probably laid from March to May, old nest are found up to October, so that the breeding season may be said to extend through out the year. While hen is incubating her egg, She will occasionally leave the nest very briefly to feed, drink, preen or defecate. After 21 days, the eggs hatch, hen and her chick form a unit independent from her original flock and for the large past away from any males although males may occasionally consort with these hens. Chick mortality is extremely high since there are many predators including lizards, birds of prey, and carnivores such as wild cats and civets (Beebe, 1921) Red Junglefowl are almost exculsively ground living, flying only to safety, to roost, or when chasing or being chased by another Junglefowl (Sullivan,1991).

Junglefowls are natural foragers. They have long strong legs with four-clawed feet. The leg and bill are adapted for scratching in the ground for food. They spend long hours in early morning and late afternoon for scratching away at the covering of leaves that hides their favorite food. All junglefowl feed chiefly upon seeds, grain, shoots and bud as well as insects especiallly on the eggs and larvae of termites.

Red Junglefowl has been found from sea level to approximately 2000 metres. Their prefered habitat usually is open forest. Forest edge, lightly logged and particularly bamboo forest are all typical habitats in which they are found (Johnsgard, 1986).

All Junglefowl are found in only warmer parts of oriental region as shown in figure 2-1. Ceylon Junglefowl are endemic to several different habitats on the island of Sri Lanka (formerly Ceylon). Green Junglefowl inhabit in Java and neighboring islands. Gray-Junglefowl are found in western and southern India up to in the north-west and east to the River Godavari, central India and Rajputana (Wayre,1988). The most widespread species, Red Junglefowl, is range from northern India in the Southern edge of Himalayas westward

to southern Sikim, Napal and Kashmir. Eastward it is commonly found in the hilly portion of Bengal and, throughout Burma including Pegu and Tennessarim, Yunnan, Siam (now Thailand), Cochin-China and southward to the Malay Peninsula and Indonesia including Bali, but absent from Island of Singapore and Borneou (Beebe, 1921). Five subspecies of this species are differently found in this area as Wayre (1969) describe as:

1. *Gallus gallus gallus* (L), Cochin-Chinese Red Junglefowl are found in Cochin-China, Cambodia and nearby island, Vietnam (except extreme north) central and lower Laos, eastern Thailand with in northern Vietnam.
2. *Gallus gallus spadiceus* (Bonnaterre), Bermese Red Junglefowl inhabit south-western Yunnan, Burma, Thailand (except extreme east), northern Laos, Malaya and northern Sumatra. Integrates with, *G. g. murghi* and *G. g. bankiva* near their respective boundaries.
3. *Gallus gallus jabouillei* Delacour and Kinnear, Tokinese Red Junglefowl live in Tokin, north of Veitnam, extreme southeast of Yunnan, Kwangsi and. Integrade with *G. g. spadiceus* in north-western Tokin.
4. *Gallus gallus murghi* Robinson and Kloss, Indian Red Junglefowl dwell in northern and north-eastern India, the lower ranges of the Himalayas from southern Kashmir to Assum, northern and eastern central India. Meets *Gallus sonnerati* in the Central Province and hybrid have been found
5. *Gallus gallus bankiva* Temminck, Javan Red Junglefowl exist on southern half of Sumatra, Java and Bali.

In Thailand, subspecies with whitish ear spot, *Gallus gallus gallus* exist in north-eastern and eastern parts of country. Burmese Red Junglefowl that have clear red earlobe distributed form Laos border down to southwest.

2.2 Habitats of the Red Junglefowl are on Progressively Declined

The biodiversity crisis in Thailand is similar to those prevailing in many other tropical countries. Deforestation is the one of the most important problem, causing loss of biodiversity. Thailand's forest cover been reduced from 53% in 1960's to about 22.8% or

111,010 km^2 at present (Bhumibamon, 1986; FAO, 1997; cited in Forest Restoration Research Unit,1998), proceeding the rate of more than 2% per year. A ban on commercial logging since 1989, has help to slow down the destruction, but the deforestation rate then increase and still exceeds 1,000 km^2 per year or about 0.8% annually (RFD statistic), due to illegal logging, agricultural expansion and development projects. Consequently, progressive loss of wildlife habitats and food source which are results of deforestation and forest degradation have strong negative effects on wildlife's population viability. Much of remains of Thailand's remarkable flora and fauna are being threaten with extinction in the next decade (Woodruff, 1990). Populations surviving on small and fragmented habitats potentially loss of genetic variation, future adaptation and evolutionary ability. The non-migratory species such as Red Junglefowl are subject to this alteration more severely. Thus conservation activities of these speciesis of immediately concern.

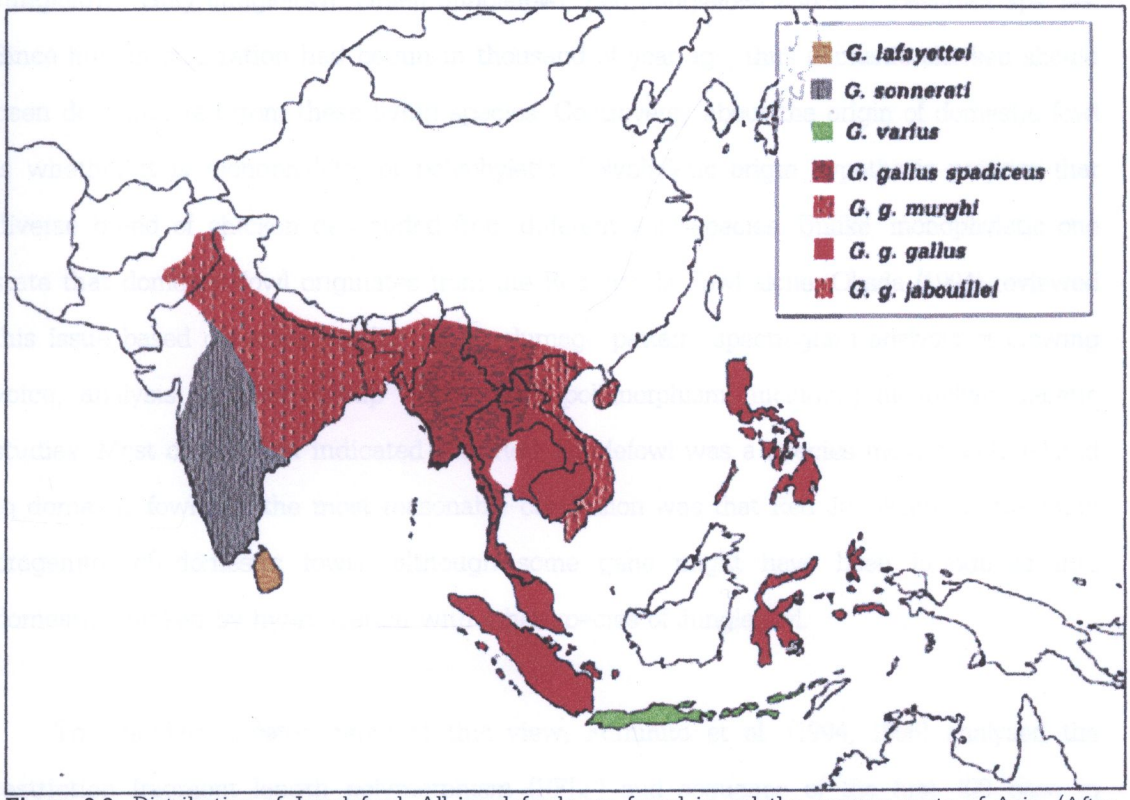

Figure 2-2 Distribution of Junglefowl. All junglefowls are found in onlythe warmer parts of Asia. (After Beebe, 1921)

2.3 All Varieties of Domesticated Chicken are Descendant of the Red Junglefowls.

In terms of economic and cultural importance to mankind, chicken, the single mat obvious species of bird, have played an important role in the evolution of human civilization since prehistoric time. Chicken have been domesticated for their meat, egg as well as for recreational purpose such as cock fighting and ornamentation. Now chicken is the most important economic animal of the world as the main source of food. There are many kinds of domestic chickens exist in all over the world. The progenitor of domestic fowls is seemingly more clear, but there is still no clear answer to question of when and where chicken were first domesticated.

As one would expect, domestic fowls closely resemble Junglefowls in general appearance. Accordingly, a belief that domestic fowls originated from wild resemblance is broadly except. The Junglefowls consist at least of four living species such as Ceylon Junglefowl, Gray Junglefowl, Green Junglefowl, Red Junglefowl and two fossilized species. Since human civilization had begun in thousand of year ago, thus domestic chicken should been domesticated from these living species. Controversy about the origin of domestic fowl is whether it is monophyletic or polyphyletic. Polyphyletic origin hypothesis propose that diverse breed of chicken descended from different wild species. Unlike, monophyletic one state that domestic fowl originates from the Red Jungle Fowl alone. Okada (1994) reviewed this issue based on crossbreeding data, plumage pattern, spectrogram analysis of crowing voice, analysis of blood group and protein polymorphism, including mendelian genetic studies. Most of the data indicated that Red Junglefowl was a species most closely related to domestic fowl and the most reasonable conclusion was that Red Junglefowl is the main progenitor of domestic fowls, although some gene might have been introduced into domestic chicken by hybridization with other species of Junglefowl.

To elucidate greater detail of this view, Fumihito et al. (1994, 1996) analysed the restriction fragment length polymorphism (RFLP) and sequence of the first 400 base in noncoding control region or displacement loop (D-loop) of mitochrondrial DNA extracted from various domestic breeds and all of four species of Junglefowl. They found that both sequences and RFLP haplotype of Red Junglefowl were closer to all breeds of domestic

chicken than another Junglefowl. In conclusion, they suggested that Red Junglefowl alone had been sufficient to yield all the diverse breeds of domesticated chicken.

2.4 Red Junglefowl as Genetic Resources for Future Use in Chicken Breeding.

Genetic diversity is the major requirement of progress in animal breeding. Genetic diversity in commercial line is limited by the selection for production trait whereas those of local animal and wild animal are relatively high.. Under evolutionary consideration, all wild gene pools may hold potential benefits. Wild ancestor of domesticated animal may be an important sources of genetic diversity for future breeding program (Gaston, 1992).

According to chicken lineage describe above, as well as high fertility of artificial hybrids between domestic fowl and Red Junglefowl (Crittenden et al. 1993; Okada, 1994) and the occurrence of hybrid between local native chicken and Red Junglefowl in forest villages indicate that introduction of the Red Junglefowl's genes to domesticated chicken's gene pool is possible. For effective breeding programme and sustainable use of genetic diversity in these birds, evalution of genetic variability and genetic status of both ancestor and domesticated descendant are considerately necessary. Genetic variation within and among different commercial chicken lines including the native breeds were examined (Okada, 1994; Dunnington et al., 1994; Crooijmans et al., 1996; Wei, Dontine and Bitgoud, 1997; Wimmers and Horst, 1998; Takahashi et al., 1998, Vahala et al., 1998; Delany and Krupkin, 1999). For comparative study, their accepted ancestor, Red Junglefowl is usually added to these investigation such as the works of Crittendon et al. (1993) and Dunnington et al., (1994). However, genetic variation within wild ancestor's natural population has scarcely examined. Thus, fulfillment this requirement must be carried out.

2.5 Measurement Genetic Variation in Natural Population

Early studies of general variation in natural population concentrated on easily detected and/or quantifiable variations. Although these were important-variants, they did not allow an estimate of the total amount of genetic variation in the genome of the studied population (Hendrick, 1983). Molecular revolution in population genetics and evolutionary biology, which gained great momentum with the introduction of allozyme (isozyme) method

in the mid-1960s (Avise, 1996), when Lewohtin and Huby estimated the average genomic heterozygosity in population of *Dorsophila pseudoobscur* (O'Brien, 1994). In the late 1970s, attention shifted to method of DNA analysis, primarily through restriction enzymes, and in 1980s, mitichondrial DNA analysis and DNA fingerprint approaches gained immense popularity (Avise, 1994). PCR-mediated DNA sequencing had been widely employed in early of this decade. Recently, analyses of simple tandem repeat loci, microsatellite was just applied into this field.

Barrowclough (1989) pointed out advantages of molecular techniques over more traditional types of characters. First, molecular methods allow us to examine the direct translated products of a gene or the gene themselves. Problem of pleiotropy or complex genetic-environmental interaction during ontogeny are greatly reduced. Second, molecular methods yield information concerning the state of single gene which can be analyzed in term of a well developed population genetic theory. Unlike, thoery of polygenic and quantitative traits for traditional types of characters are more complicated and not yet very well developed.

2.6 Molecular Genetic Markers

Molecular genetic markers, the direct translated products of gene or intact genetic materials, particularly Mendelian inherited, are important to address population genetic questions. The common technique which concerning the direct translated products of genes is protein electrophoresis such as allozyme analysis. To obtain information of more exractly genetic backup of population, nuclear DNA and organelle DNA (mitochrondrial DNA and chloroplast DNA) are analysed by various method such as restriction fragment length polymorphism (RFLP), randomly amplified pholymorphic DNA (RAPD), DNA fingerprinting, DNA sequencing and microsatellite analysis.

2.6.1 Allozyme

This method takes the advantage of the fact that nondenatured proteins with different net charge (rather than size and shape) migrate at different rate through starch or arcrylamide (or other supporting media) to which an electric current is applied (Arise, 1994).

Because of the low cost and safety this method are employed widely. For example, Darrells, Roseberry and Klimstra (1989) used 19 allozymic loci to examine genetic structure and gene flow in the northen Bobwhite (*Colinus virginianus*), Petersen (1992) analyse phylogeny and splitting date of the Aphelocoma Jays (Corvidae): *Aphelocoma coerulescens, A. unicolour* and *A. ultramarina* ,by using 28 loci data; Browne et al. (1993) estimate heterozygosity and genetic distance among population of the Hawaiian Duck, Lysan Duck and Mallard. Genetic variation and distance of various breed of chicken were studied by many researchers. Fortunately, relationship among Junglefowl species was documented by Hashiguchi et al. (1981) (cited in Ponsuksili, 1995). However, only histochemical stains available for investigated enzyme can be carried out. Since requirement of large amount of fresh tissue specimen for protein extraction, investigated animals frequently invasive.Thus,this method is not sutible for studies of endanger species.

2.6.2 Mitochondrial DNA

Since there are several predominant feature of mitochondrial DNA, an extrachromosomal DNA found in mitochondria that make it attractive for use in studying population genetics. First, mtDNA is haploid and transmitted through maternal lines in most species. Thus independent assortment and recombination are absent. Difference in their nucleotide sequence are caused by mutation alone. As a result, interpretion of mtDNA data are easier than those of nuclear DNA. Second, mtDNA evolves rapidly accumulating mutations 5-10 times faster than does single-copy nuclear DNA reflecting its potential to be used for the determination of intraspecific genetic variation among geographically different populations. Finally most mutational difference between mtDNA clones are selectively neutral (Shields and Helmbychowski, 1988)

To detect variation in mtDNA, RFLP or PCR-mediate RFLP and nucleotide sequencing are usually applied. Comparison of the restriction fragment patterns assumes that the great majority of mutations in mtDNA (that due to base substitution) prevent or allows an investigated enzyme to cleave at such position and thus produce the different number and size of DNA fragments from investigated individuals. Different fragments are separated by agarose gel electrophoresis and then detected either by chemical staining or radioactive

labelling (Tegelstrom, 1992). Based on this technique, various avian mtDNA were analysed, such as Chickadee (Mack et al. 1986), Phalaropes (Dittmann and Zink, 1991), Goose (Quin, Shield, and Wilson, 1991), Snow Goose (Avise et al., 1992), Noeth American Crested Titmice (Gill and Slikas, 1992), Francolins (Crowe et al., 1992), Alaskan Song Sparrow (Hare and Shields, 1992), Streaked Saltator (Seutin et al., 1993), Chhpping Sparrow (Zink and Dittmann, 1993), Oilbird (Gutierrez, 1994), and various sibling species (Avise and Zink, 1998). Ball and Avise (1992) to address question of the validity of subspecies designations of six avian species with continental wide distributions in North America. At present, analysis of the entire mtDNA by restriction endonuclease is replaced by PCR-RFLP.

Alternatively, the specific regions of mtDNA are amplified through the polymerase chain reaction (PCR) and further analysed by direct sequencing (Quinn and White, 1987). The hypervariable region such as cytochrome *b* gene and noncoding control region or displacement loop (D-loop) are frequently sequenced. Today, a number of documentation of avian mtDNA sequencing have been obtained by many authors, for example, Krajewski and Fetzner (1994), Lanyon and Hall (1994), Morin, Messier and Woodruff (1994), Leisler et al. (1997), Quin and Wilson (1993), Meckvichai, Malaivijitnond and Tirawatnapong (1997)

In domestic chicken, Desjardins and Morais (1990) cloned and sequenced the entired 16,775 base pair mitochrondrial genome of white Leghorn chicken. Fumihito et al. (1994), Fumihito et al. (1995) and Fumihito et al. (1996) used the first 400 base pairs and RFLP data to elucidate genetic link between domestic chicken and Junglefowl. They found that Thai Red Junglefowls were very close to all breeds of domestic chicken and sufficed as the monophylatic ancestor of all domestic breeds. Boripat Siriaroonrat (1997) amplified and sequenced mtDNA of Thai red Junglefowl. Significant difference in genetic diversity between two subspecies, *G. g. gallus* and *G. g. spadiceus*, was found. However, genetic divergence within these subspecies was small (0-2.25% in *G. g. gallus* and 0-6.54% in *G. g. spadiceus*). Thus, sensitivity of this marker may not be enough for the discrimination of closely related populations which seems to be the case of present study.

2.6.3 High variable nuclear DNA

2.6.3.1 Single copy nuclear DNA

Single copy nuclear DNA is a sequence which occurs with a frequency of one per haploid genome. Restriction analysis of single-copy nuclear DNA or scnDNA traditionally relied on Southern blotting procedure. The raw data are in many respects analogous to those provided by protein electrophoresis. The major advantage over protein electrophoresis is that, in principle, a nearly unlimited pool of genetic variants may be tapped. Despite, disadventage in comparison to protein electrophoresis is that, this method are expensive, both in effort and meterials. The difficulties of genotype determination, evolutionary relationship among scnRFLP allele of a particular locus are seldom accomplished (Avise, 1994). Thus, this technique may not be suitable for intraspecific relationship analysis.

2.6.3.2 Ribosomal RNA genes and other Middle-Repetitive Gene families.

The ribosomal DNAs in the nuclear genome of the eukaryotes usully exist as tandem repeated unit. It is classified as one of the moderately repetitive gene families. The coding sequences of these genes are approximately 6 kb in total length and highly conserved, whereas the non-coding sequence are more variable. The most important factor which causes difficulties in the use of rDNA as a genetic marker is the mechanism of concerted evolution. The concerted evolution refer to the tendency of the copies of rDNA sequence to become homogeneous initially among gene copies within a genome and among individuals within population through unequal cross over or gene conversion. Hence, the extent to which different family members can be view as providing independent phylogenetic information (Avise, 1994).

2.6.3.3 Highly repetitive DNA sequence

The highly repetitive DNA sequences are devided into two groups on the basis of their arrangements in the genome. The first group is the dispersed repetitive DNA which contains the repeat unit dispersed throughout the genome. The second class is clustered repetitive DNA made up of repeat sequence arranged into long tendam array. This type of repetitive DNA is generally devided into three categories depending on the length of the

clusters and the short nucleotide sequence motif or repeat unit (Brown, 1992; Koreth, O'Leary and McGee, 1996):

(a) Classical satellite DNA. This is the first type of satellite DNA to be identified. The sequence arrays with repeat size ranging from 5 to 100 bp, characteristically organized in clusters between 100 and 5000 kb in length. They are located in the heterochromatin near chromosomal centromeres and telomere and are not as variable in size within population as the other of this family.

(b) Minisatellite DNA. Minisatellite DNAfrom shorter clusters, between 100bp and 30kb which array with repeat size of 15-70bp.Minisatellites are found in euchromatic region of the genome and are hightly variable in repeat size within population.

(c) Microsatellite DNA. There is overlap between the lengths of minisatellite and microsatellite cluster, but the latter are distinguished by the short length of repeat unit. Microsatellite contains a repeat motif of 1-6 bp which array in tandemly repeated manner for approximately 10-50 copies (Hearne, Ghosh and Todd, 1992), and subsequently, also called as simple sequences length polymorphism (SSLPs), simple sequence repeats (SSRs) or short tandem repeats (STRs), (McDonald and Potts, 1997). These repititive sequences are highly variable in size, ranging around the mean value of 100 bp (Koreth, O'Leary and McGee, 1996) Microsatellite are highly abandant and randomly dispersed in euchromatin and allele size of populations characteristically exhibit multiple size classes distributed about the population mean. It was estimated that one microsatellite locus may be found in every 10 kb in eukaryotic genome (Taulz, 1989; Koreth, O'Leary and McGee, 1996).

Due to variation in the number of tandemly repeated element, hence, a general designation for both micro and minisatellite is the variable number of tandem repeat loci (VNTRs).

The mutational mechanism causing VNTRs loci to be hypervariable is completely understood. Several models have been hypothezised including strand slippage, gene conversion and unequal crossing over between homologous chromosome during miosis (Wolff et al,1989). The predominant means which new length alleles are generated, is

thought to be intra-allelic polymerase slippage during replication (Schlotterer and Taugz,1993).A replicating DNA strand can slip one or more repeat units within a repeat and resume perfect base pairing. The resulting bulge can then be repaired, resulting in the the addition or deletion of the nonpaired based. There is evidence that the addition or deletion usually invloves a single repeat unit, which some evidence for rarer event of larger effect. This type of stepwise mutational process means that allelic variants of similar size are more closely related. VNTR alleles differ in length (number of repeat), are easier to identify than markers differing only by sequense since they can be readily discriminated on the basis of their differential electrophoretic mobility (Mcdonald and Potte,1997). Conventional Southern blotting techniques have been used to reveal minisatellite variation at multiple loci simultaneously to produce the bar code-like multilocus DNA fingerprints or using specific probes to reveal variation at single loci (single locus-DNA fingerprint). An obvious advancement in the efficiency of VNTR analysis utilized the PCR and system were developed for application to specific mimisatelite loci (also called minisatelite variant repeat-PCR, MVR-PCR or digital DNA fingerprinting Jeffreys et al,1991) However, only a limited subset of variation can be analysed by PCR due to the large size of many minisatellite alleles.

A system of highly polymorphic sequences having allele size smaller than 1 kb and varying over narrow size range was desirable because variability in this loci could be assayed by PCR combined with gel electrophoresis, avoiding the need for Southern blotting. Thus, variation could be assessed from minute amounts of material that might contain highly degrade DNA such as forensic or ancient samples. Microsatellite sequence fitted these criteria well, and due to their ubiquity,Mendelian co-dominant inheritance and extreme polymorphism have made them become the most important tool for addressing question at variety of scale, ranging from the extremely fine grained to the fairly coarse grained.(Bruford and Wayne,1993,McDonald and Potts,1997)

Microsatellites can serve as genetic markers at the level of genes in at least two ways: sex determination (Hanotte et al., 1997) and gene mapping (Bailey et al., 1997;Crooijmans, 1995; Zheng et al., 1993; Dietrich et al., 1992 and Wissenbach et al.,1992). At individuals

level, microsatellites have been sucessfully applied to parentage and relateness testing in diverse organism (Ellegren, 1992; Keame, Dittus and Melnick, 1997 and Bowling et al., 1997). Because they are non-functional, they are not subjected to strong selection or selectively neutral. and, accompanying with their characteristic described above, have made microsatellite as an ideal class of genetic markers for population genetic studies. In recent years, population servey of microsatellite variability have been done in many organism (Paetkau and Strobeck, 1994; Lade et al., 1996; Mundy et al., 1997; Valsecchi, 1997 and Estoup et al., 1995). An additional, microsatellite data can provide useful evidence in assessing relationship among species or at above species level (McDonal and Potts, 1997).

Generally, developing microsatellite loci for new species requires that one constructs a genomic library of clones bearing one or more tandem repeats. At present, numeruos microsatellite survey of diverse organisms especially domestic species have been carried out (e.g. Swinburne et al., 1997; Dolf et al., 1997; Goodman, 1997 and Dawson et al.,1997)

2.7 Avialable chicken microsatellite marker

In chicken, the number of microsatellite markers have been characterized and mapped on international reference population. Khatip and Soller (1992a and 1992b) and Cruuigmans et al. (1993) have started to develop microsatellite markers in pooling. The former studies have reported one mono- and dinucleotide repeat polymorphism in functional gene. While Moran (1993) attemping to search for mono-, di-, tri- and tetranucleotide repeat sequence from genebank database, polymorphic (TG/AC)n markers obtained from those database and genomic library screening in the East Lansing and the Compton reference population carried out by Khatib et al. (1993). The East lansing poultion is a cross between a single male of junglefowl origin and four females of highly inbred White Leghorn line (Critteden at al., 1993). The Comton population a cross between an out breded (N) and in breded (I_5) White Leghorn line (Burnstead and Palyga, 1992). Coincidence study of Crooijmans et al. (1993) also screened for polymorphic (TG/AC)n in White Leghorn line. Later, a large number of microsatellite markers of domestic chicken was additionally reported (Cheng and Crittenden, 1994; crooijman et al., 1994, 1995, 1996; Gib et al., 1997, 1997; Ruyter-spira et al., 1996; Dufour et al.,1997). In addition, Crooijmans et al. (1997) optimized a number of

microsatellite markers for automated fluoresent genotyping. Investigation of the use of utility chicken microsatellite markers in other galliformes was carried out by Hanotte et al. (1997)

2.8 Detection of microsatellite variability

Once microsatellite-flanking PCR primers are obtained, PCR can be used to identify genotype of interested individuals. After amplification, the products are fractionated by their different lengths using agarose (usually for tetranucleotide repeat) or polyacrylamide gel (for mono-,di-, and trinucleotides repeats) with radioactive and non-radioactive methods (Kareth,O; Leary and Mcgec, 1996)

2.8.1 Radioactive method

Triditional autoradiography detection (labelling amplification, electrophoresis of products, fixed, exposed gel with x-ray film, and film developing) of PCR-amplified microsatellite, either by the direct incorporation of single labelled deoxynucleoside triphosphate like [α-^{32}P] dCTP during thermal cycle (internal labelling) or a single 5' [γ-^{32}P] ATP end labelled primer in the PCR mix (end labelling) are more sensitive and common. The internal labelling method is easier to perform and more sensitive whereas the end-labelled approach minimize addition (stutter) band and produces cleaner result.

2.8.2 Non-radioactive methods

Non-radioactive methods are composed of ethidium bromide staining, silver staining, and fluorescence detection. Resolution of the products on acrylamide gel either non-denaturing or denaturing sequencing type gels followed by ethidium bromide staining is the simplest means of visualization. The disadvantages include low sensitivity (only > 10 ng of double stand DNA can be detected) and subjective quantitation unless additional image processing is undertaken. The use of silver stains to visualize DNA offers advantages of sensitivity over ethidium bromide (detecting pg quantities of DNA) but there are problem with variable background and non linear deposition of silver. Fluorescent labelling of the PCR products in combination with automated fluorescent DNA fragement analysers yeild significantly more rapid and reliable result and allows data to be recorded automatically for multiple markers (Ziegle et al., 1992)

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Equipment

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

3.1.2 Chemicals

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

3.1.3 Radioactive

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

3.1.4 Enzymes

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

3.2 Methods

3.2.1 Tissue samples collection

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

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

3.2.2 DNA preparation

(a) Chelex® 100 DNA Extraction

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

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

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

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

(b) Measurement of DNA Concentration

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

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

(a) Selection of Polymerase Chain Reaction Primers

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

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

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

* Optimized annealing temperature

\# PCR product size

π Mean PCR product size

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

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

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

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

(c) Polymerase Chain Reaction

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

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

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

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

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

3.9 Data analysis

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

3.9.1 Allelic frequencies

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

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

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

3.9.2 Hardy-Weinberg equilibrium

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

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

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

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

3.9.3 Geographic heterogeneity analysis

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

CHAPTER 4

Results and Discussion

4.1 Tissue sampling and DNA Extraction

As theoritically expected in proposal, blood stains and feathers of the Red Junglefowl were collected from natural population in each local population for at least 10 sample. More sample and more multiple location were disirable, but they were not obtained in this study. As the result of the Red Junglefowl is protected under WARPA (1992), the invasive method such as blood sampling from wild caught birds were avoid. Thus, these difficulties limited a sample size collection and the most samples in this study were feathers.

Genomic DNA was extracted from approximatly 3 mm^2 blood stain by rapid and simple method as describd by Singer-Sam et al. (1989) and Walsh et al. (1991) yeilded 70-130 ng/μl at total volume 200 ul and the ratio of absorbance at 260 nm and 280 nm was 1.2-1.9. However, extraction of chopped proximal end of single feather quill by procedure for extraction mammal hair follow as these author provide a minute amount of DNA. Alternatively, modification by adding proteinase K (Ellegren, 1992) provided more DNA with concentration of 80-200ng/μl which was approximately as obtained from blood stain, but with lower purified (OD_{260}/OD_{280} = 0.7-0.9) as show in Table 4-1).

4.2 Optimization of PCR Conditions for Amplifying Red Junglefowl Microsatellites by Chicken Microsatellite-Flanking PCR Primers.

For each selected chicken' s microsatellite-flanking PCR primer pair, PCR condition described by Crooijmans et al. (1997) were tried on HUJ 1, HUJ2, HUJ7, ADL37, LEI73, and LEI 92. When extracts of blood stain were used for this screening, condition of each primer were fitted to those of previously used. Suitable annealing temperature for almost all of the primer pairs was 55° C, except for HUJ2 was 60° C.Extracts of feather were rather not successful in amplification with these conditions. 0The varying annealing temperature (57°, 60° and 63° C for HUJ 2 ; 53° , 55° and 57°C for HUJ1, HUJ7, ADL37, LEI73 and LEI92) and concentration of $MgCl_2$ (1.0, 1.5 and 2.5 mM) were subsequently used. Unfortunately, only

some of these samples were amplified under these condition (see Figure 4-1-4-5). For a reasonable possibility, non amplifying reaction is througth to be a result of faltering template-primer annealing and/or inhibitory contaminant of unpurifeid DNA extracts. Different sequence in microsatellite-flanking region between rereference population of domesticated chicken which used for primer developmetn and wild Red Jungle Fowl may prevent template-primer anealing. Thus, sample with more different in this region can not amplified whereas less defferent can obtained high concentration of inhibiting protein frequncing cause an extension of DNA polymerase.

Table 4-1 Sample locality, Tissue Source, concentration and absorbance ratios of samples used in this study.

ample	Locality	Tissue source	Concentration (ng/μl)	OD_{260}	OD_{280}	OD_{260}/OD_{280}
N1	Phayoa	Blood stain	129.85	0.52	0.41	1.26
N2	Phayoa	Feather	77.5	0.31	0.44	0.70
N3	Phayoa	Feather	71.38	0.29	0.37	0.79
N4	Prae	Feather	84.16	0.34	0.41	0.83
PK1	Chaiyapum	Blood stain	124.53	0.50	0.38	1.32
PK2	"	"	70.40	0.28	0.15	1.90
PK3	"	"	125.1	0.50	0.36	1.37
PK4	"	"	79.03	0.32	0.20	1.54
PK5	"	"	86.33	0.34	0.22	1.50
S1	Chumphon	Feather	122.58	0.49	0.57	0.86
S2	"	"	261.38	1.04	1.06	0.99
S3	"	"	127.4	0.51	0.60	0.84
S4	"	"	164.43	0.66	0.80	0.84
S5	"	"	176.15	0.70	0.87	0.81
S6	"	"	221.38	0.89	1.02	0.87
S7	"	"	105.13	0.42	0.55	0.76
S8	"	"	99.73	0.40	0.46	0.86
S9	"	"	116.55	0.47	0.59	0.78
S10	"	"	79.75	0.32	0.42	0.75
S11	"	"	1.44.05	0.58	0.72	0.79

4.3 Variability of Selected Microsatellite Loci.

Highly allelic variability were found in most loci, excepting for ADL 37 locus which only 2 distinguishable allele were found. However, individual variation among samples were observed for homozygous or heterozygous genotype. The most polymorphic locus was HUJ2 followed by LEI73, HUJ1, HUJ7 and LEI92 with allele number of 12, 10, 9, 8 and 8 respectively (as show in Table 4-2). Total number of 2, 4, 2, 2, and 2 allele were shared between both locality at HUJ1, HUJ2, HUJ7, LEI73 and LEI92 respectively.

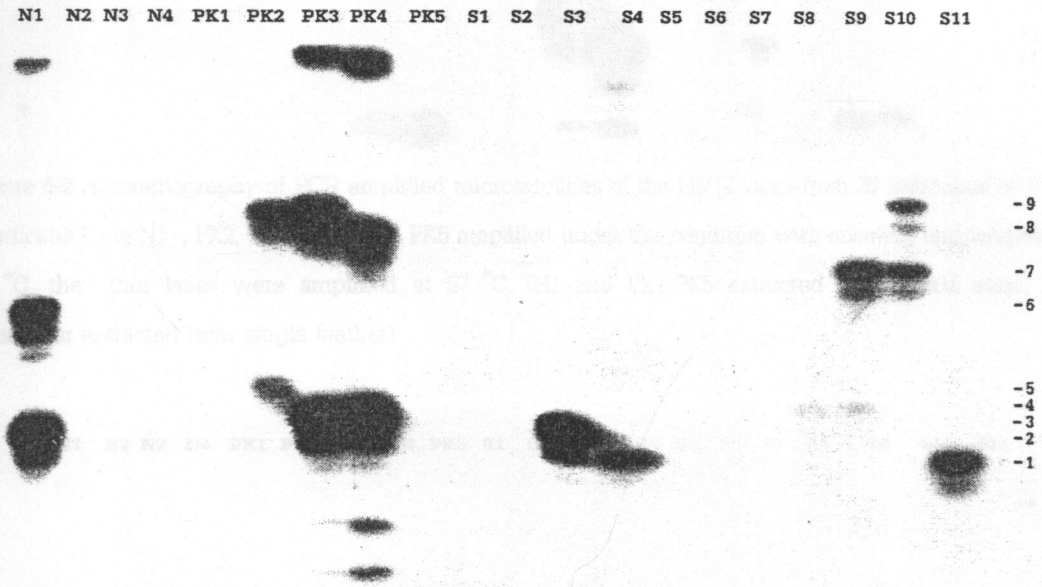

Figure 4-1 Autoradiography of PCR amplified microsatellites of the HUJ1 locus from 20 individual of *G. g. spadiceus* Lane N1, , PK2, PK3, PK4 and PK5 amplified under the condition with anealing temperature at 55 °C, the other lanes were amplified at 53 °C. (N1 and PK1-PK5 extracted ffrom blood stain, the remainder extracted from single feather)

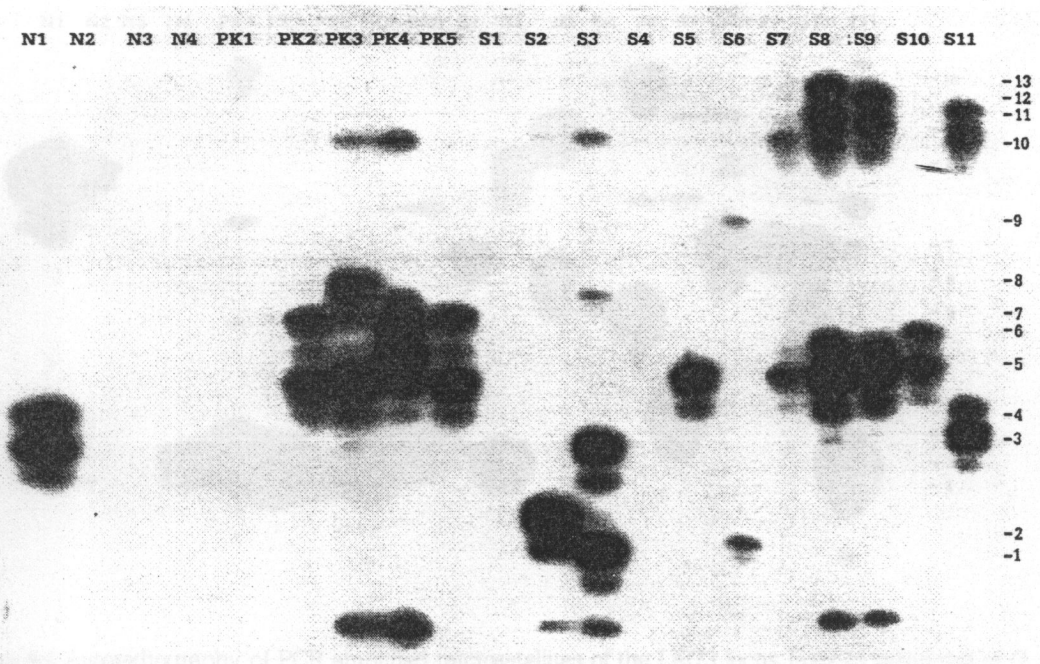

Figure 4-2 Autoradiography of PCR amplified microsatellites of the HUJ2 locus from 20 individual of *G. g. spadiceus* Lane N1, , PK2, PK3, PK4 and PK5 amplified under the condition with anealing temperature at 60 °C, the other lanes were amplified at 57 °C. (N1 and PK1-PK5 extracted ffrom blood stain, the remainder extracted from single feather)

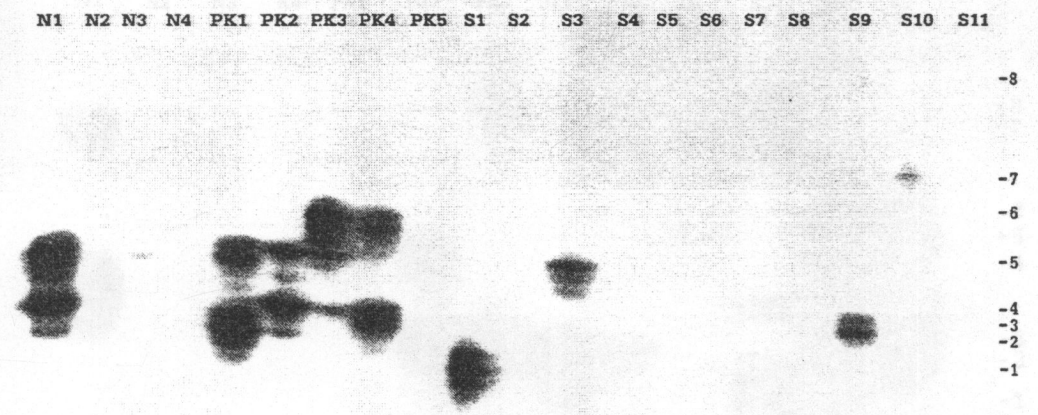

Figure 4-3 Autoradiography of PCR amplified microsatellites of the HUJ7 locus from 20 individual of *G. g. spadiceus* Lane N1, , PK2, PK3, PK4 and PK5 amplified under the condition with anealing temperature at 55 °C, the other lanes were amplified at 53 °C. (N1 and PK1-PK5 extracted ffrom blood stain, the remainder extracted from single feather)

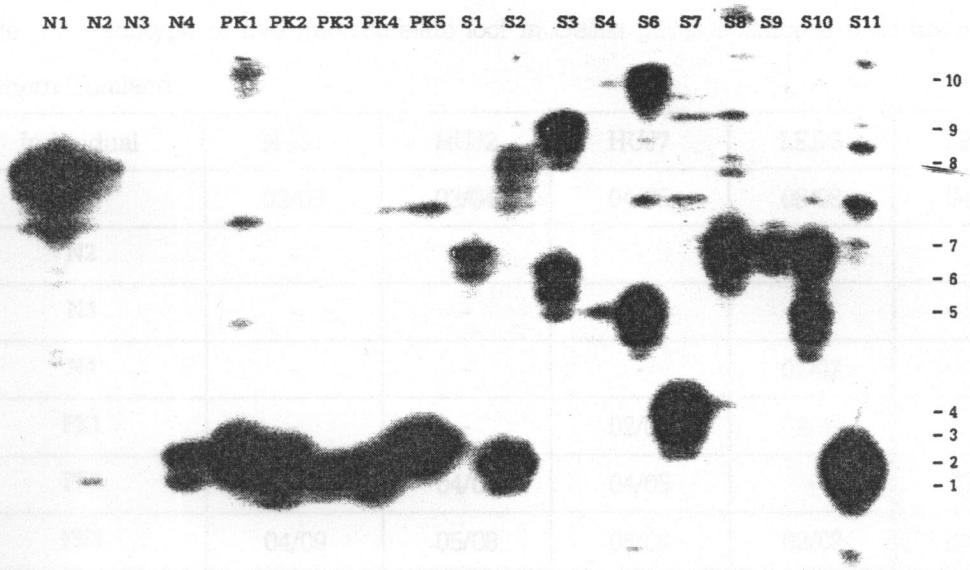

Figure 4-4 Autoradiography of PCR amplified microsatellites of the LEI73 locus from 20 individual of *G. g. spadiceus* Lane N1, , PK2, PK3, PK4 and PK5 amplified under the condition with anealing temperature at 55 °C, the other lanes were amplified at 53 °C. (N1 and PK1-PK5 extracted ffrom blood stain, the remainder extracted from single feather)

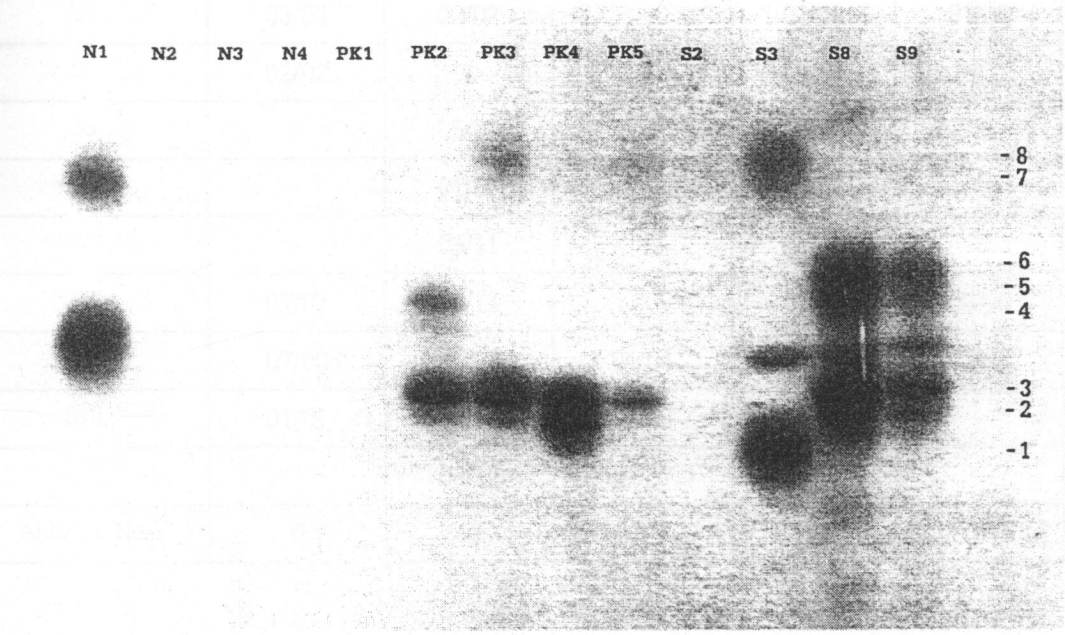

Figure 4-5 Autoradiography of PCR amplified microsatellites of the LEI92 locus from 20 individual of *G. g. spadiceus* Lane N1, , PK2, PK3, PK4 and PK5 amplified under the condition with anealing temperature at 55 °C, the other lanes were amplified at 53 °C. (N1 and PK1-PK5 extracted ffrom blood stain, the remainder extracted from single feather)

Table 4-2 Genotype of five microsatellite loci in *Gallus gallus spadiceus* from northern and southern Thailand.

Individual	HUJ1	HUJ2	HUJ7	LEI73	LEI92
N1	03/03	03/04	04/05	08/08	04/07
N2	-	-	-	-	-
N3	-	-	05/05	-	-
N4	-	-	-	01/02	-
PK1	-	-	02/05	03/03	-
PK2	05/09	04/07	04/05	-	03/05
PK3	04/09	05/08	06/06	02/02	03/07
PK4	04/08	06/07	03/05	01/01	02/03
PK5	-	05/07	-	01/01	03/08
S1	-	-	01/01	03/03	-
S2	-	05/07	05/05	07/07	-
S3	03/03	02/02	-	01/08	01/08
S4	02/02	-	-	06/09	-
S5	-	05/05	-	05/10	-
S6	-	01/09	-	05/05	-
S7	-	05/11	-	07/07	-
S8	07/07	05/14	-	07/07	03/06
S9	07/09	05/13	04/04	05/07	03/06
S10	01/01	05/06	08/08	01/01	-
S11	-	03/12	07/07	-	-
ALLELE No.	9	13	8	10	8

Table 4-3 Summarized allele frequencies of HUJ1, HUJ2, HUJ7, LEI73 and LEI92 locus from northern and southern localities of *Gallus gallus spadiceus*.

Allele	HUJ1		HUJ2		HUJ7		LEI73		LEI92	
	North	South	North	South	North	South	North	South	North	South
1	-	0.2	-	0.056	-	0.25	0.417	0.050	-	0.167
2	-	0.2	-	0.111	0.083	-	0.25	-	0.1	-
3	0.25	0.2	0.1	0.056	0.083	-	0.167	0.1	0.4	0.333
4	0.25	-	0.2	-	0.167	0.25	-	-	0.1	-
5	0.125	-	0.2	0.389	0.500	0.25	-	0.2	0.1	-
6	-	0.3	0.1	0.056	0.167	-	-	0.05	-	0.333
7	0.125	-	0.3	0.056	-	0.25	-	0.35	0.2	-
8	0.25	0.1	0.1	-	-	-	0.167	0.05	0.1	0.167
9			-	0.056			-	0.05		
10			-	0.056				0.05		
11			-	0.056						
12			-	0.056						
13				0.056						

Table 4-4 Estimation of Hardy-Weinberg expectation in northern and southern local for each microsatellite locus.

Locus	P-value	
	Northern	Southern
HUJ1	1.0000 ^{ns}	0.033
HUJ2	1.0000 ^{ns}	0.0538 ^{ns}
HUJ7	0.1712 ^{ns}	0.0011
LEI73	0.0043	0.0004
LEI92	1.0000 ^{ns}	1.0000 ^{ns}

ns = not significant.

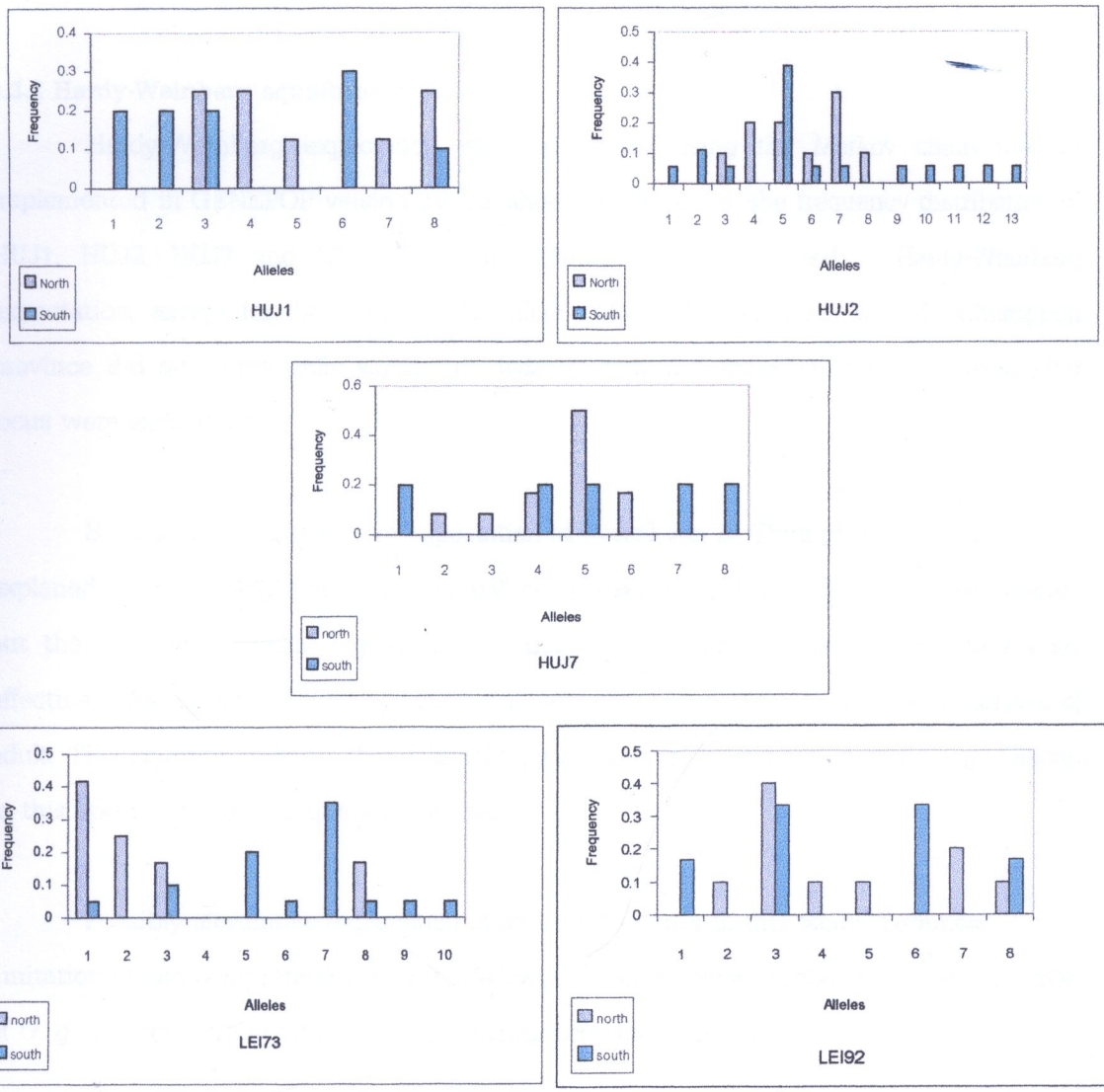

Figure 4- 6 Histogram showing allele frequencies of five microsatellite loci (HUJ1, HUJ2, HUJ7, LEI73 and LEI92) in northern (n=8) and southern (n=11) population of *G. g. spadiceus*.

4.3.1 Allelic frequency

Allelic frequencies of each locus were summarized in table 4-3 and distribution of allelic frequencies were shown in figure 4-7. For HUJ1, frequencies of 9 allele ranged between 0.1-0.3. The lowest frequency were found at 8th allele in Chumphon province. The highest frequency were also found at 6th allele in this location. Twelve allele of the highest polymorphic locus in this study were observe at locus HUJ2. Like locus HUJ1, highest requency was found in southern locally at 5th allele. On the other hand, highest frequency were found in northern locality at locus HUJ7, LEI73 and LEI92. Althrough, these allelic

frequencies did not accurately estimated due to small number of sample size. Trend of allele distribution seemingly depicted (see Figure 4-7) such locus HUJ2 as locus LEI73.

4.3.2 Hardy-Weinberg equilibrium test.

Hardy-Weinberg expectation was carried out using the Markov chain method implemented in GENEPOP version 2.0. As shown in Table 4-4, the frequency distribution of HUJ1, HUJ2, HUJ7 and LEI92 locus in northern locate conformed to Hardy-Weinberg expectation, except for the locus LEI73. Unlike, only HUJ2 and LEI92 locus in Chumphon province did not statistically significant diviated from sich expectation but the remaining locus were exceptional.

Significant deviation from expectation at overall loci in Chumphon province may be explaned as inbreeding effect due to small sample series. Collias and Collias (1996) poined out that related to social organization of population of Red Junglefowl, the genetically effective breeding size of the population was only about 13 percent of the census number of adult. This findings suggest that inbreeding and random differentiation of local population in this species couldbe frequency occurred.

Possibly alternative explanation is an sampling error in this study. Technical limitation of sampling strategies did not allowed to collect large number and diverse locality of *G. g. spadiceus* from wild population during sampling programme

4.3.3 Geographic heterogeneity test

Heterogeneity analysis of allelic frequencies shown non significant different between northern and southern locate at ovrall loci. Althrough, non significant differences in genotype distribution was found in almost loci, including HUJ1 (P = 0.109), HUJ2 (P = 0.313), HUJ7 (P = 0.065) and LEI92 (P = 0.465) locus but significant defferent was found in LEI73 (P = 0.013) locus.

This result indicated that the northern and southern populations were recently separated. So the significant difference of genetic structure between these population was

not detected. However, the small sample size in this study may cause non-significant difference.

To determined more correct significance heterogeneity in distribution of allelic frequencies between these different gergraphically separated populations of *G. g. spadiceus*, larger sample size which as possible as it can obtained and more microsatellite marker are recommend.

Chapter 5

Conclusion and Recommendation

5.1 Conclusion

1. All selected chicken microsatellite-flanking PCR primers can use to amplified microsatellite DNA from the Red Junglefowl's genomic DNA. five of six markers shown polymorphism which allele number of 9, 12, 8, 10 and 8 with number allele for HUJ1, HUJ2, HUJ7, LEI73 and LEI92 in 20 sampling respectively. Otherwise, AD37 locus were not shown a polymorphism, only two observed allele were found. Hence, many chicken microsatellite markers are possible to use for investigation of microsatellite variability of the Red Junglefowl.

2. Analysis of geographic heterogeneity using the marker chain "approximation to exact test" shown nin significant different between northern (n = 8) and southern (Chumphon province, n = 11) at HUJ1 (P = 0.103), HUJ2 (P = 0.313), HUJ7 (P = 0.065) and LEI92 (P = 0.465) locus whereas significant differnet in these locate was found out LEI73 (P = 0.013) locus. Comformity with Hardy-Weinberg expectation was found at almost loci (HUJ1, HUJ2, HUJ7 and LEI92) in northern locate. Unlike, only HUJ2 and LEI92 loci in southern locate (Chumphon province) conformed this expectation.

5.2 Recommendation

In order to determine genetic variation of the Red Junglefowl, chicken microsatellite markers is likely to be possible markers. For more precise-investigation, sample should collected as much as possible. Due to DNA extraction and microsatellite amplification, blood stain collection take advantage over feather samples. Genomic DNA extraction of blood stain appears to be easier and yield better quality of DNA template than that of feather. Optimal PCR condition for amplification of blood stain extracts is uniquely fitted to reference condition. Unlikely amplification from feather extract was difficult to obtain and diverse conritions were required. Consequently, condition optimization for feather extracts may take a lot of labor and cost. Blood stain sampling are recommend.

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Biography

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