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 (57^0, 60^0 and 63^0 C for HUJ 2 ; 53^0 , 55^0 and 57^0C 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.

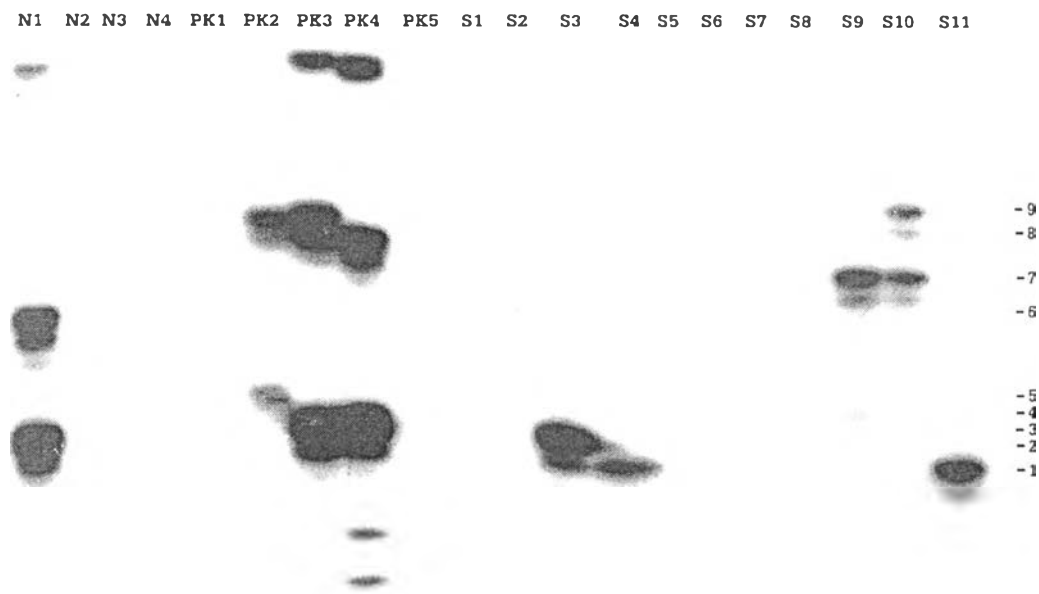

Figure 4-1 Autoradiography of PCR amplified microsatellites of the HUJ1 locus from 20 individual of *G. g. spadiceus* Lane N1, , PK2, PK3, PK4 and PK5 amplified under the condition with anealing temperature at 55 °C, the other lanes were amplified at 53 °C. (N1 and PK1-PK5 extracted ffrom blood stain,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

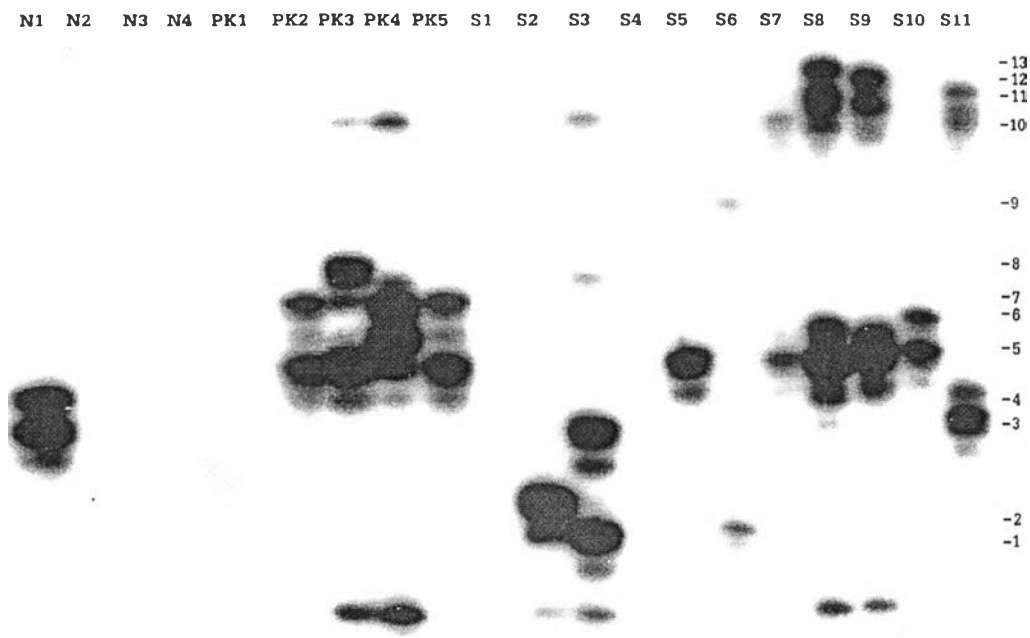

Figure 4-2 Autoradiography of PCR amplified microsatellites of the HUJ2 locus from 20 individual of *G. g. spadiceus* Lane N1, , PK2, PK3, PK4 and PK5 amplified under the condition with anealing temperature at 60 °C, the other lanes were amplified at 57 °C. (N1 and PK1-PK5 extracted ffrom blood stain,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

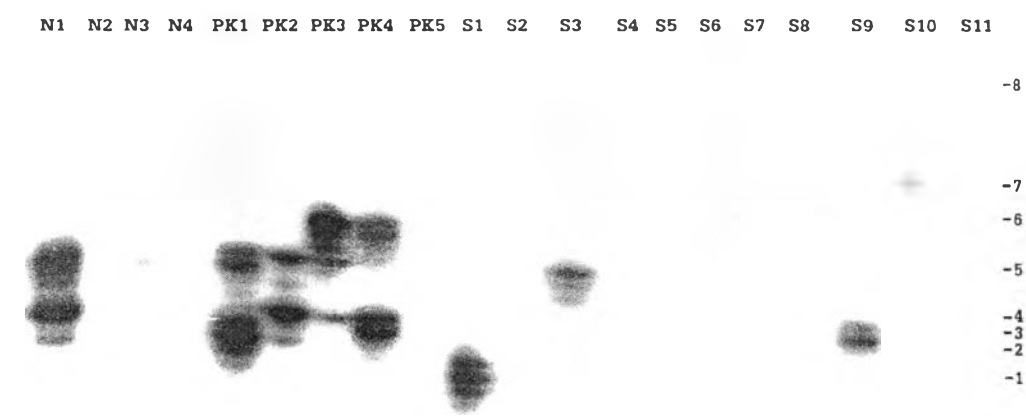

Figure 4-3 Autoradiography of PCR amplified microsatellites of the HUJ7 locus from 20 individual of *G. g. spadiceus* Lane N1, , PK2, PK3, PK4 and PK5 amplified under the condition with anealing temperature at 55 °C, the other lanes were amplified at 53 °C. (N1 and PK1-PK5 extracted ffrom blood stain,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

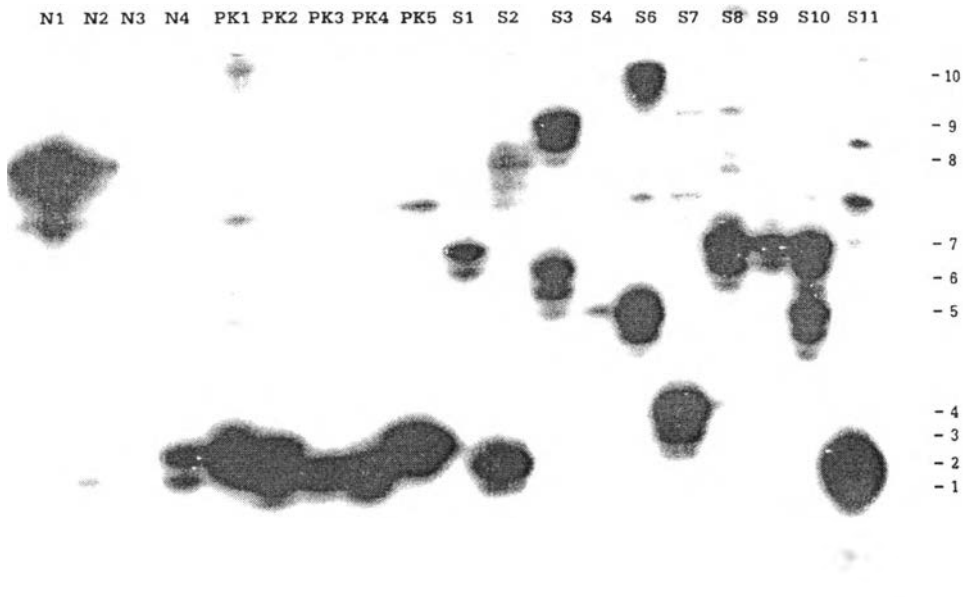

Figure 4-4 Autoradiography of PCR amplified microsatellites of the LEI73 locus from 20 individual of *G. g. spadiceus* Lane N1, , PK2, PK3, PK4 and PK5 amplified under the condition with anealing temperature at 55 °C, the other lanes were amplified at 53 °C. (N1 and PK1-PK5 extracted ffrom blood stain,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

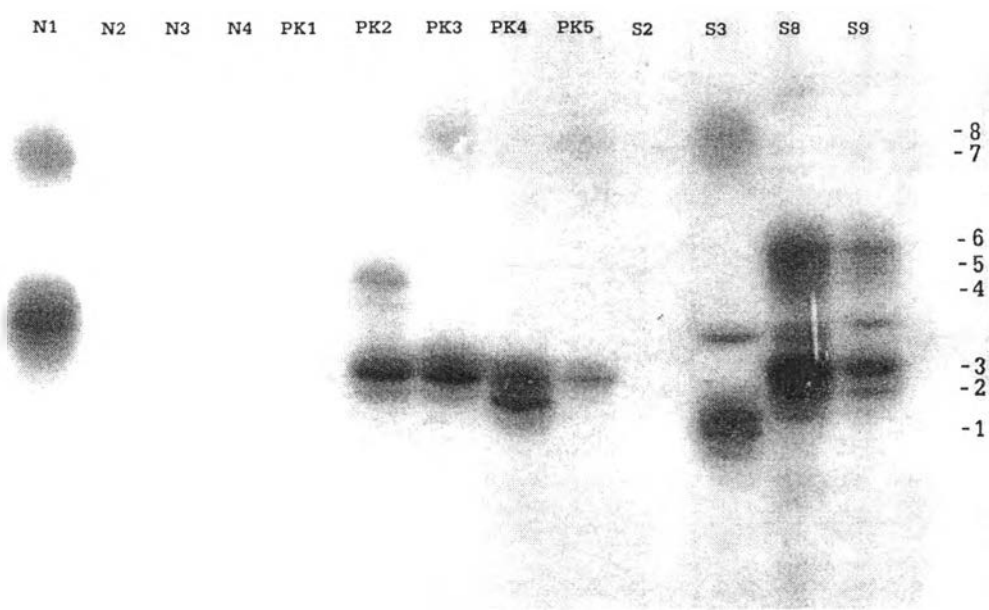

Figure 4-5 Autoradiography of PCR amplified microsatellites of the LEI92 locus from 20 individual of *G. g. spadiceus* Lane N1, , PK2, PK3, PK4 and PK5 amplified under the condition with anealing temperature at 55 °C, the other lanes were amplified at 53 °C. (N1 and PK1-PK5 extracted ffrom blood stain,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

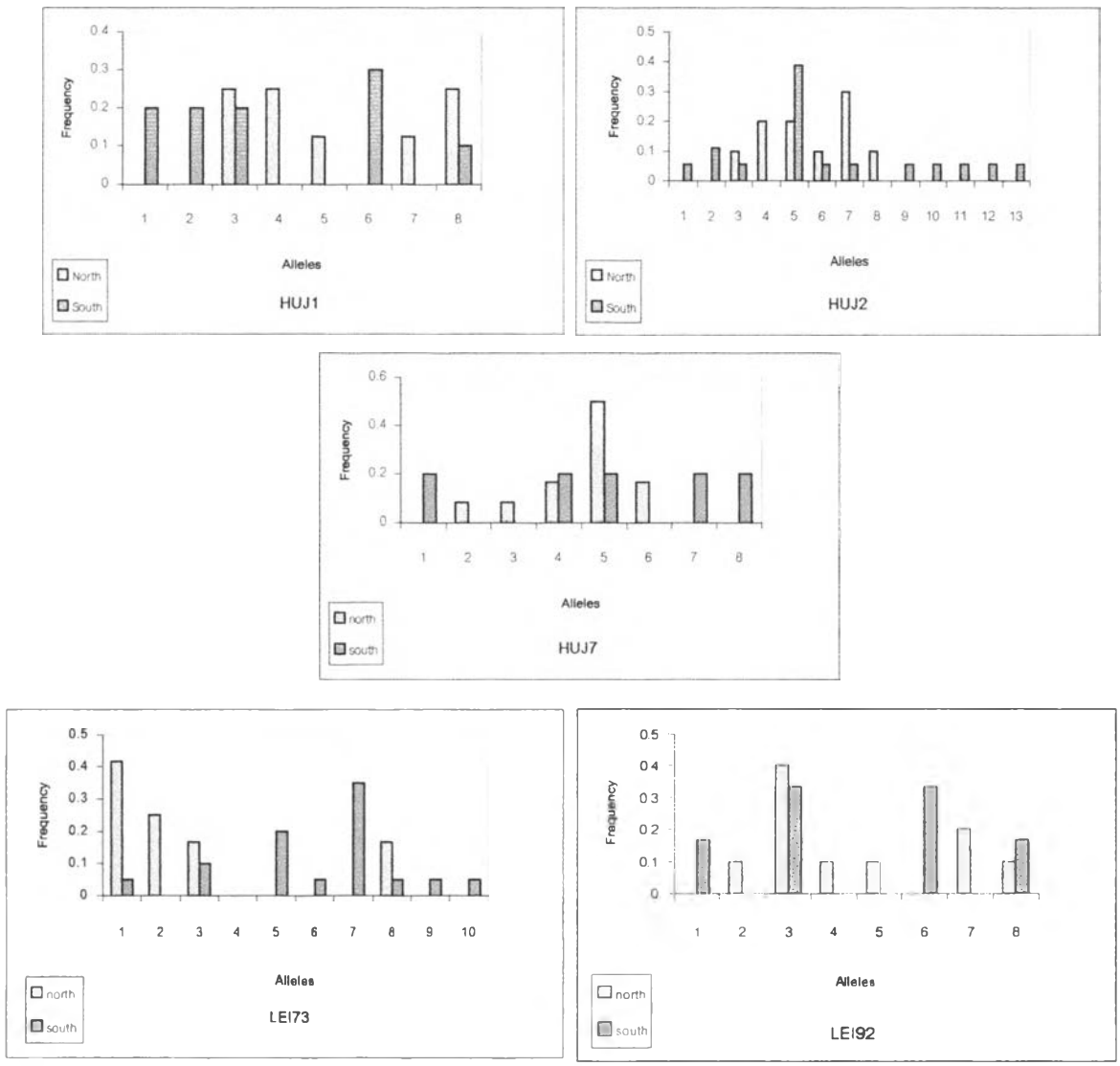

Figure 4-6 Histogram showing allele frequencies of five microsatellite loci (HUJ1, HUJ2, HUJ7, LEI73 and LEI92) in northern (n=8) and southern (n=11) population of *G. g. spadiceus*.

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.