Molecular studies on genetic diversity of genus *Galllus* with indels markers

インデルマーカーを用いた *Gallus* 属の多様性に関する分子遺伝学的研究

Doctoral Thesis

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2013

SUMMARY

[Introduction] Indels, as defined as an insertion and/or deletion of one or more nucleotide base pairs into a DNA sequence. The chicken genome was announced for the first time in 2004, December by International Chicken Genome Sequencing Consortium (ICGSC). The draft sequence of chicken genome composed of approximately 1.05 Gb and it was reported that chicken's genome includes 447,388 indels polymorphisms in 2012, October (NCBI). The present study focused on the application of indels polymorphisms as genetic marker. 102 indels markers were developed to analyze the genetic diversity and phylogenetic relation of various chicken populations.

[Materials and methods] The blood samples of various chicken populations including native chicken populations from Myanmar (n = 3), Thai (n = 6), Laos (n = 3), Indonesia (n = 8), Satsuma's chickens from Japan (n = 2), improved chicken breeds (n = 3), commercial chickens (n = 3), subspecies of Red Jungle fowl (n = 4) and Green Jungle fowl (n = 1) were used. Genomic DNA was extracted from blood samples using standard phenol-chloroform extraction protocols. DNA samples were genotyped by using 102 indels markers, which developed at an average separation of 10 Mb of chromosome length. Genotyping was conducted by PCR amplification and agarose gel electrophoresis. Genotype data were used to calculate allele frequency, proportion of polymorphic loci (*Ppoly*), observed and expected heterozygosity ($\overline{H}_O$ and $\overline{H}_E$), test for Hardy-Weinberg Equilibrium (HWE), coefficient of genetic differentiation (G_{ST}) and genetic distance (*Ds*). To construct phylogenetic tree, MEGA software version 4.1 was

used. To analyze population structure, STRUCTURE 2.3.3 and DISTRUCT 1.1 applications were used.

[Results] In this study, all of 102 indels markers were in HWE in respective populations. The *Ppoly* values were ranged from 0.713- 0.989 in 20 native chicken populations, 0.500-0.608 in Satsuma's chickens, 0.549-0.618 in improved chickens, 0.716-0.814 in commercial chickens, 0.218-0.946 in four subspecies of Red Jungle fowl and 0.039 in Green Jungle fowl. The $\overline{H}_O$ and $\overline{H}_E$ values in native chickens, Satsuma's chickens, improved chicken, commercial chickens and Red Jungle fowl were ranged from 0.197-0.268 and 0.229-0.392, 0.147- 0.196 and 0.175- 0.212, 0.153-0.203 and 0.176-0.192, 0.237-0.250 and 0.225-0.247, 0.101-0.231 and 0.078-0.366, respectively. The $\overline{H}_O$ and $\overline{H}_E$ in Green Jungle fowl was 0.003 and 0.012. The G_{ST} among native chicken populations was 0.045. That indicated that they are genetically similar populations. In phylogenetic analysis, they were the same clade to Satsuma's chickens, improved chickens, commercial chickens and three subspecies of Red Jungle fowl (*G. g. spadiceus*, *G. g. jabouilei* and *G. g. gallus*). The Green Jungle fowl and *G. g. bankiva* were remote from all other populations and they are genetically different from domestic chickens and other Red Jungle fowl's subspecies. The present of common origin and genetic admixture was observed among Thai's native chicken, Thai's cross chicken (native chicken X Jungle fowl), *G. g. spadiceus* and *G. g. jabouilei* in STRUCTURE analysis.

ACKNOWLEDGEMENT

First and fore most the author value and honor all that her father, the late U Ohn Mg and her mother Daw San San because they granted me the gift of life. The author would like to express hearty thanks and deepest gratitude to her honorable supervisor, Professor Shin Okamoto, Laboratory of Animal Breeding and Genetics, Faculty of Agriculture, Kagoshima University, for his continuous advice, constant supervision and valuable suggestion throughout the period of the research work.

The author says thanks to former Dean of the Faculty of Agriculture Professor Maeda Yoshizane for his kindly request to Professor Shin Okamoto to accept me as his candidate. The author wishes to convey her warmest thanks to Assistant Professor Shimogiri Takeshi and Researcher Kawabe Kotaro (FSRC) for their positive advice, opinions and constructive comments during the period of the study. The author acknowledges the company of all graduate and undergraduate students from the Laboratory of Animal Breeding and Genetics 2007-2012.

The author is very pleased to recognize her thanks to Professor U Aung Than (former Rector and Head of Department of Animal Science), Professor Dr. Myint Thein;

Director General of Livestock Breeding & Veterinary Department (former Rector), Professor Daw Ni Ni Maw (former Pro-Rector of Academic) from University of Veterinary Science, Myanmar. The author is also expressed her gratitude to Professor Dr. Tin Ngwe (Rector), Professor Dr. New New Htin (former Head of Department of Animal Science and Pro-Rector Academic), Professor Dr. Khin Hnin Swe (Head of Department of Animal Science) and Professor Dr. Tin Tin Myaing (former Pro-Rector Acedemic) of University of Veterinary Science (U.V.S), Myanmar for their enthusiastic permission and supports for further study in abroad.

The author owes a debt of gratitude to Ministry of Livestock and Fisheries of Myanmar and University of Veterinary Science (Yezin), Myanmar for their permission and Ministry of Education Affairs of Japan for their financial support (Monbukagakusho Scholarship) to complete this study.

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LIST OF ABBREVIATIONS

A	Adenine
bp	Base pair
C	Cytosine
CM	Centimorgan
CIA	Chloroform Isoamyl Alcohol
°C	Centigrade
2D	Two dimensional
dNTP	Deoxynucleoside triphosphates
EDTA	Ethylene diamine tetra acetic acid
G	Guanine
Gb	Giga base pairs (10^9)
ID	Indels
IDE	Indels event
kb	Kilo base pair (10^3)
Mbp	Mega base pair (10^6)
min	Minutes
µl	Micro litre
µM	Micro molar

PCI	Phenol Chloroform Isoamyl Alcohol
PK	ProteinaseK
rpm	Rounds per minute
SDS	Sodium dodecyl sulfate
sqKm	Square kilometer
s	Second(s)
T	Thymine
TA	Temperature at annealing
TBE	Tris /Borate/EDTA
TE	Tris/EDTA
UTR	Un translate region

CHAPTER 1

General Introduction

1.1. Genetic diversity

Genetic diversity is a level of biodiversity that refers to the total number of genetic characteristics in the genetic makeup of a species. Genetic diversity at its most elementary level is represented by differences in the sequences of nucleotides (adenine, cytosine, guanine, and thymine) that form the DNA (deoxyribonucleic acid) within the cells of the organism. Genetic diversity protects a species against extinction by providing multiple phenotypes. These phenotypic differences may provide disease resistance, adaptability to a changing climate, or some other trait necessary for the species to survive in the ever-changing world. The study of diversity within species is important to all biologists who use genetic markers. Furthermore the information on genetic diversity is essential in optimizing both conservation and utilization strategies for animal genetic resources.

The simplest parameters for assessing diversity among breeds are the genetic differentiation or fixation indices. Wright (1951, 1965) developed an approach to partition the genetic variation in a subdivided population that is commonly used and provided an obvious description of differentiation. This approach consists of three different "*F*" coefficients of F_{ST}, F_{IT} and F_{IS}, used to allocate the genetic variability to the total population level (T), subpopulations (S), and individuals (I). F_{ST} is a measure of the genetic differentiation over subpopulations and is always positive. F_{IT} and F_{IS} are

the measures of the deviation from Hardy-Weinberg proportions within subpopulations and in the total population, respectively, where positive values indicate a deficiency of heterozygotes and negative values indicate an excess of heterozygotes. Statistical significance can be calculated for the F_{ST} values between pairs of populations (Weir and Cockerham, 1984) to test the null hypothesis of lack of genetic differentiation between populations. Actually, Wright's formulation of fixation indices was developed for the case of two alleles, and for this reason F_{ST} defined for the case of multiple alleles is often denoted by G_{ST}, which was originally called the coefficient of gene differentiation (Nei , 1973). Hierarchial analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992) can be performed to assess the distribution of diversity within and among groups of breeds. The mean number of allele per population (*MNA*), and observed and expected heterozygosity ($\overline{H}_{\mathrm{O}}$ and $\overline{H}_{\mathrm{E}}$) are the most common parameters for assessing within breed diversity.

Molecular techniques are now available to detect the genetic diversity of the population. A number of genetic markers have also proved useful in the investigation of the origin and domestication of livestock species, and their subsequent migrations, as well as providing information on evolutionary relationships (phylogenetic tree) and identifying geographical areas of admixture among populations of different origins.

1.2. Phylogenic study

A phylogenetic tree, also known as a phylogeny, is a diagram that depicts the lines of evolutionary descent of different species, organism or genes from common ancestor. Phylogenetic trees are useful for organizing knowledge of biological diversity, for structuring classifications, and for providing insight into events that occurred during evolution. Furthermore, because these trees show descent from common ancestor, and because much of the strongest evidence for evolution comes in the form of common ancestry, one must understand phylogenies in order to be appreciate the overwhelming evidence supporting the theory of evolution. In most cases, researchers draw phylogenetic trees in such a way as to record only those events that are relevant to a set of living taxa. Most commonly, these taxa are species (Baum, 2008).

Phylogenetic relationships of genes or organisms usually are presented in a treelike from with a root, which is called a rooted tree. It is also possible to draw a tree without a root, which is called a unrooted tree. The branching pattern of a tree is called a topology. A clade is a piece of a phylogeny that includes an ancestral lineage and all the descendants of that ancestor.

There are numerous methods for constructing phylogenetic trees from molecular data (Nei and Kumar, 2000). They can be classified into, a distance method, parsimony methods, and likelihood methods. We used distance method to construct a phylogenetic tree in this study. In the distance method, the genetic distances are computed for all pairs of taxa, and a phylogenetic tree is constructed by considering the

relationships among these distance values. There are many methods of constructing the tree from distance data. Currently, neighbor joining (NJ) tree and unweighted pair-group method with arithmetic mean (UPGM) tree are the favored implementation of distance method. Phylogenetic trees provide an efficient structure for organizing knowledge of biodiversity and allow one to develop a conception of evolutionary history.

1.3. Population structure

A population may have substructure or differences in genetic variation among its constituent parts for several different evolutionary reasons (Hedrick, 2005). Exchange of individuals may not have equal probabilities throughout a population, or selection may have different effect in different part of the population. There are several scenarios to cluster individuals or populations of unknown origin as described by Pritchard *et al* (2000). There are broadly two types of clustering methods *i.e.* model-based methods and distance-based methods. The model-based method proceeds by assuming that observation from each cluster are random draws from some parametric model (Pritchard *et al.*, 2000). Inference for the parameters corresponding to each cluster is then done jointly with inference for the cluster membership of each individual, using standard statistical methods e.g. maximum likelihood or Bayesian methods (Pritchard *et al*., 2000). This model can be performed using STRUCTURE program, which assumes that there are *K* populations (where *K* may be unknown). The genetic distance expresses the genetic similarity or dissimilarity of individuals or populations

(Hamilton, 2009). Genetic distance can be estimated by using the standard genetic distance or *Ds* measure developed by Nei (1972, 1978).

1.4. Chicken genome

The chicken genome was announced for the first time in 2004, December. The genome of female Red Jungle fowl (RJF#256) from inbred line (UCD 001) was sequenced to 6 × coverage that serves as the reference genome (Ensembl, 2012). The current updated size of chicken genome is estimated to be 1.05 Gb and approximately 3,000 cM in length (Elferink *et al*., 2010). Therefore, 1 cM is approximately equivalent to 350 kb of DNA in chicken. In contrast, 1 cM in human is about 1,000 kb of DNA, and thus, the chicken genome is about one-third the size of the human genome. However, chicken genome has about the same number of genes as the human genome: 20,000-23,000 versus the human genome's estimated 20,000-25,000 genes. Therefore, the chicken has a compact genome in comparison to mammals.

Compared to mammals, chicken genome contains a larger number of chromosomes. The chicken genome contains 39 chromosome pairs (Burt, 2002). Chicken chromosomes can be divided into autosomes (1-38) and sex chromosomes (Z, W). Unlike mammals, sex chromosomes are Z, W in chicken. Female is heterogametic (ZW) and male is homogameic (ZZ). Chicken chromosomes are also highly variable in size. According to size, chicken chromosomes can be classified into three classes: five macrochromosome (No.1-5), measuring from 50 to 200 Mb in size, five intermediate chromosome (No.6-10) ranging from 20 to 40 Mb, and 28

microchromosomes (No.11-38) on average 12 Mb long (Axelsson *et al*., 2005). According to the data of International Chicken Polymorphism Map Consortium 2004, chicken genome revealed a total of 2.8 million polymorphism and 272,830 length variants.

Chickens are good models for studying the genetic basis of phenotypic traits because of the extensive diversity among domestic chickens (Wong *et al*., 2004). There are about more than 19 millions stocks of chicken are being raised in the world (FAO Statistics, 2010).

1.5. Genetic marker

Genetic marker is a DNA polymorphism that can be easily detected by molecular or phenotypic analysis. The marker can be within a gene or in DNA with no known function. Because DNA segments that lie near each other on a chromosome tend to be inherited together, markers are often used as indirect ways of tracking the inheritance pattern of a gene that has not yet been identified, but whose approximate location is known (FAO, 2007). The ideal genetic markers should be co-dominant expression, high polymorphic, distribute randomly throughout the genome and their assay can be automated. Genetic markers provide information on evolutionary relationships (phylogenetic trees), and identifying geographical areas of admixture among populations of different genetic origin, the relationship between an inherited disease and its genetic cause and for assessment of genetic diversity within and between breeds.

The frequently used markers are microsatellite (a short repetitive DNA sequence), minisatellite (also known as VNTR-variable number of tandem repeat unit), SNP (single nucleotide polymorphism), and STR (short tandem repeat). The existence of these markers are detected by using the technique such as PCR (polymerase chain reaction), RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism) and RAPD (random amplification of polymorphic DNA).

Microsatellite is the most popular markers in livestock genetic characterization studies (Sunnucks, 2001). Their high mutation rate and co-dominant nature permit the estimation of within and between breed genetic diversity, and genetic admixture among breeds even if they are closely related. SNP are most common form of DNA polymorphism (on average one SNP in every 100 to 1000 bp). They are used as an alternative to microsatellites in genetic diversity studies. Several technologies are available to detect and type SNP markers.

The present study introduces the use of insertion and deletion (indels) polymorphisms as genetic markers for studying genetic diversity in genus *Gallus*.

1.6. Insertion and deletion (indels) polymorphisms

1.6.1. What is indels?

Indels is a molecular biology term that has different definitions in different fields: In evolutionnary studies, indels is used to mean an insertion and/or a deletion (Kondrashov and Rogozin, 2004; Ogurtsov *et al*., 2004) and indels simply refers to the mutation class that includes both insertions, deletions, and the combination thereof (William *et al*., 2002; Gregory, 2007; Halangoda *et al.*, 2001), including insertion and deletion events that may be separated by many years (Sachin, 2004).

In germ line and somatic mutation studies, however, indels describes a special mutation class, defined as a mutation resulting in a colocalized insertion and deletion and a net gain or loss in nucleotides, and micro indels is defined as an indels that results in a net gain or loss of 1 to 50 nucleotides (Gonzalez *et al*., 2007). Indels, as defined as either an insertion or deletion, can be used as genetic markers in natural populations, especially in phylogenetic studies (Väli *et al*., 2008; Erixon and Oxelman, 2008).

Figure 1. Example of insertion and deletion in DNA sequence

An indels change of a single DNA base encoding part of an mRNA results in a "frameshift" when translating the mRNA and perhaps reading on to an inappropriate (premature) stop codon in a different frame. Indels are uncommon in coding regions but common in non-coding regions. Indels contrasted with a point mutation; where an indels inserts and deletes nucleotides from a sequence, a point mutation is a form of substitution that replaces one of the nucleotides. Indels also contrasted with Tandem Base Mutations (TBM), which may result from fundamentally different mechanisms.

A TBM is defined as a substitution at adjacent nucleotides (primarily substitutions at two adjacent nucleotides, but substitutions at three adjacent nucleotides have been observed).

Ryan *et al*., (2006) identified five major classes of indels, including (1) insertions and deletions of single-base pairs (2) monomeric base pair expansions (3) multi-base pair expansions of 2-15 bp repeat units (4) transposon insertions and (5) INDELs containing random DNA sequences.

1.6.2. Overall density and distribution of indels events across the chicken genome

Indels density can be given either as the number of indels events per base pair (IDE/bp) or the number of base pairs inserted or deleted (ID/bp) per base- pair-sequence covered by the polymorphism screening. In the study of Brandström and Ellegren (2007) using the data set of 140,484 indels, the mean genome wide, pairwise density of short indels in unique sequence was 1.9×10^{-4} IDE/ bp or 6.7×10^{-4} ID/bp.

There was significant heterogeneity in indels density among chromosomes (ANOVA,

$P<10^{-16}$) with a trend for lower densities in the smaller chromosome, the median density in the large macro chromosomes (chromosome 1-5) was 20% higher (1.89×10^{-4} IDE/ bp) than in the minute micro chromosomes (11-32; 1.50×10^{-4} IDE/ bp, P = 0.030, Mann-Whitney U-test). The Z chromosome showed significantly fewer indels than autosomes, with a density of 1.44×10^{-4} IDE/ bp, 30% lower than that of macro chromosomes (P=0.024, Wilcoxon test). The density of indels in the first 100bp upstream sequence, in the 5′- UTR, and in first introns was significantly lower than in the >100bp upstream sequence, in introns other than the first intron, in the 3′- UTR, and in downstream sequences (Brandström and Ellegren, 2007).

1.6.3. Mechanism of indels mutation

Brandström and Ellegren (2007) found that there is propensity for indels sites to represent deletion mutations in tandemly duplicated sequence. They referred to such mutation events as " unique sequence [CAG] [CAG] unique sequence" change to unique sequence[CAG][---]unique sequence"), or as " unique sequence [CTAG] unique sequence" change to " unique sequence [CTAG][CTAG]unique sequence". With two immediate neighbors of the same sequence motif there is the possibility for out -of-frame reassociation of the two strands as the polymerase traverses the duplicate region during replication. Depending on how far the nascent strand has been synthesized, slippage can give rise to either deletions "[CAG] [CAG] →[CAG][---] "or insertions "[CAG] [CAG] →[CAG] [CAG] [CAG] ". Therefore, replication slippage is an

important mechanism behind the generation of indels polymorphisms.

1.6.4. Objectives

Currently, there are increasing genomic researches using polymorphisms of short insertions and deletions (indels) in humans (Ryan *et al.*, 2006), model species such as *Drosophila melanogaster* (Ometto *et al.*, 2005), in chicken *G. gallus* (Brandström and Ellegren, 2007) and in wolf and dogs (Väli *et al.*, 2008). Natalle *et al*., (2010) evaluated X-linked insertion/deletion polymorphisms in forensic applications. But there is limited of information on the applications of indels polymorphisms as genetic markers in chicken populations. In present study, indels polymorphisms were analysed to fulfill the following objectives:

- To establish a new genetic marker for studying genetic diversity in chicken populations.
- To improve the usefulness of indels polymorphisms as genetic marker.

The development of indel markers for chicken genome including the details of the indel markers design will be comprehensively discuss on the next chapter, chapter 2. In general, the designed indel markers were practically applied to analyze the genetic diversity of native chicken populations in South East Asia. The phylogenetic relationship among native chickens, Jungle fowls and commercial chickens were analyzed. These analyses were divided specifically into four chapters, Chapter 3, Chapter 4, Chapter 5 and Chapter 6.

The genetic diversity of native chicken populations from Myanmar and Indonesia together with Red and Green Jungle fowls will discuss in Chapter 3. The phylogenic relationship between native chicken populations and explore their genetic differentiation to Red and Green Jungle fowls from Java Island will discuss also. In Chapter 4, the genetic diversity in two chicken populations from Satsuma region and other chicken populations including commercial chickens and improved chickens will discuss. The genetic relationship and genetic structure among those chicken populations will examine. In Chapter 5, the genetic variability and population structure among native chickens and crossbred chicken populations from Thai and three subspecies of Red Jungle fowls from Thai and Vietnam will examine. In Chapter 6, about the genetic diversity among native chicken populations from South East Asia countries (Myanmar, Thai, Loas and Indonesia), Satsumadori and Satsuma-jidori chicken populations from Japan, four subspecies of Red Jungle fowl populations and improved chicken populations will discuss. The phylogenetic relationship and population structure in those different types of populations will explore also. The resulting information and data are expected to support the conservation of genetic diversity in native chicken populations form South East Asia.

CHAPTER 2

Designation of indel markers

2.1. Introduction

Although, indels possess only two allele, it was reported that chicken's genome includes 447,388 indels in 2012, October (NCBI database). Indels can be genotyped with simple procedures based on size separation. All of the polymorphisms derive from a single mutation event and they have reduced mutation rates (Natalle *et al.*, 2010). Therefore, indels polymorphisms has been recognized as an abundant source of genetic markers that are widely spread across the genome. Design of indel markers on the chicken genome and development of new indel markers is very essential for the application of indels polymorphisms as genetic markers. Indel markers were developed on the designated loci on each chicken chromosome by using the information on NCBI genome resource or so called as Gene Bank. The NCBI database (http://www.ncbi.nlm.nih.gov/sites/entrez) would be the main data resource for the present study. The new indel markers were validated by using PCR-Electrophoresis method. The development of indel markers for chicken genome will discuss in emphasize.

2.2. Materials and methods

Indels information from NCBI database (http://www.ncbi.nlm.nih.gov/sites/entrez) and DNA from Myanmar native chickens were used as experimental materials.

PCR-Electrophoresis method was used to assess the validity of designed markers. The study composed of three main procedure *i.e.* DNA extraction, designation of indel markers and validation test.

2.2.1. Blood samples and phenol-based DNA extraction

DNA were extracted from blood samples of Myanmar native chickens which were collected in 1998-1999 by Maeda *et al.* They were collected from three locations, i.e. Yangon, Mandalay and Pegu. The number of samples from each location was 27 from Yangon, 40 from Mandalay and 13 from Pegu. The total number of sample for all location was 80.

The genomic DNA was extracted from 10 µl whole blood by Phenol Chloroform method (Sambrook *et al.*, 2002). Generally, the procedure composed of the following steps. At first, washing of the cells and lysing of the erythrocyte by proteinase. Then removing of proteins and contaminants by protein absorbents. Finally, isolation and purification of DNA by ethanol. The detail procedure of Phenol Chloroform method is shown in Figure 2. The quantity of double strand DNA was measured by Gene Quant Calculator. The isolated genomic DNA was diluted in to 10 times with distilled water and stored at 4°C.

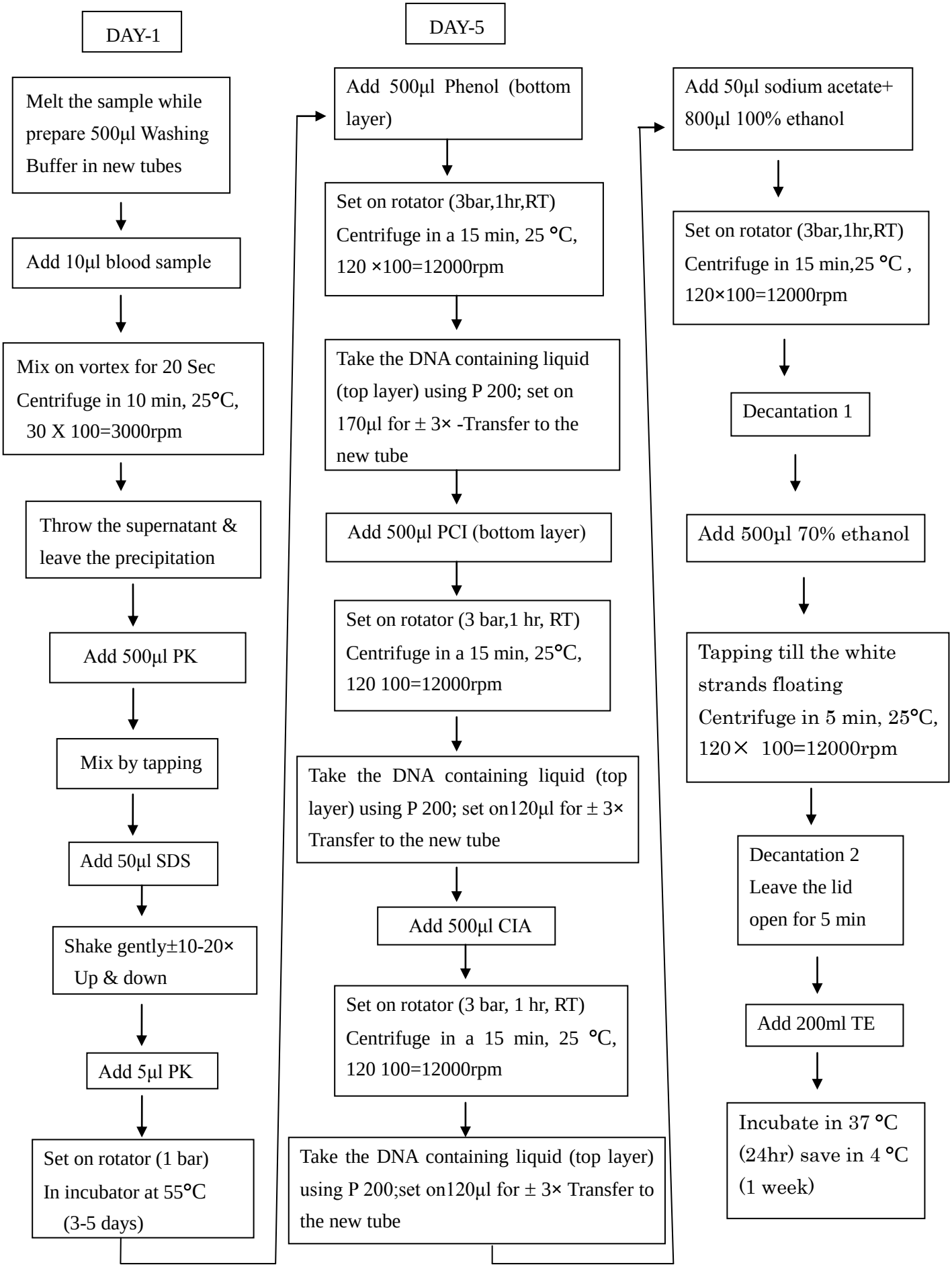

Figure 2. Phenol based DNA extraction procedure

2.2.2. Primer design

The information on the existence of indels polymorphisms along chicken genome was examined in NCBI database. In this experiment, indels length of 20-30 bp was selected as primer candidate. After selecting primer candidate, the detail information of indels such as ID number, the sequence at the forward and reverse part of indels and the position over chromosome were checked at the same NCBI database. We considered as one indels marker on every 10Mbp of chromosome length. The forward and reverse sequence alignment of indels allele was extended from 240bp to 440 bp by Chicken BLAT Search software to set up primer design (http://genome.ucsc.edu/cgi-bin/hgBlat). By regarding indels information from NCBI and BLAT Search software, primers were requested from the program for fragment length between 200bp to 350bp in Primer 3 (http://frodo.wi.mit.edu/). The reason for selecting primer length of 20-30bp is that if the primer is too short (8 bp), they might hybridize to non-target sizes and give undesired amplification products. Because the length of the primer influences the rate, at which it hybridizes to the template DNA, longer primers hybridizing at a slower rate. In practice, primers longer than 30bp are rarely used (Brown, 1998). As the allele sequence is too small (20-30bp), the range of PCR product should be 300-350bp for ease of distinguish between insertion (AA), deletion (BB) and insertion/deletion (AB). To determine the position of designed markers whether they are in coding regions or not were examined at Ensembl (http://asia.ensembl.org/Gallus_gallus/blastview). However, there is limited indels information from chromosome 23-38. Therefore,

indels data from chromosome 1-22 were used to develop marker in present study. The number of designed markers was 110 in total from chromosome 1 to chromosome 22. The size of indels alleles sequences were range from 20-39 bp and their PCR product size were range from 200-370 bp in this study. The outline procedure for development of indel markers was shown in Figure 3.

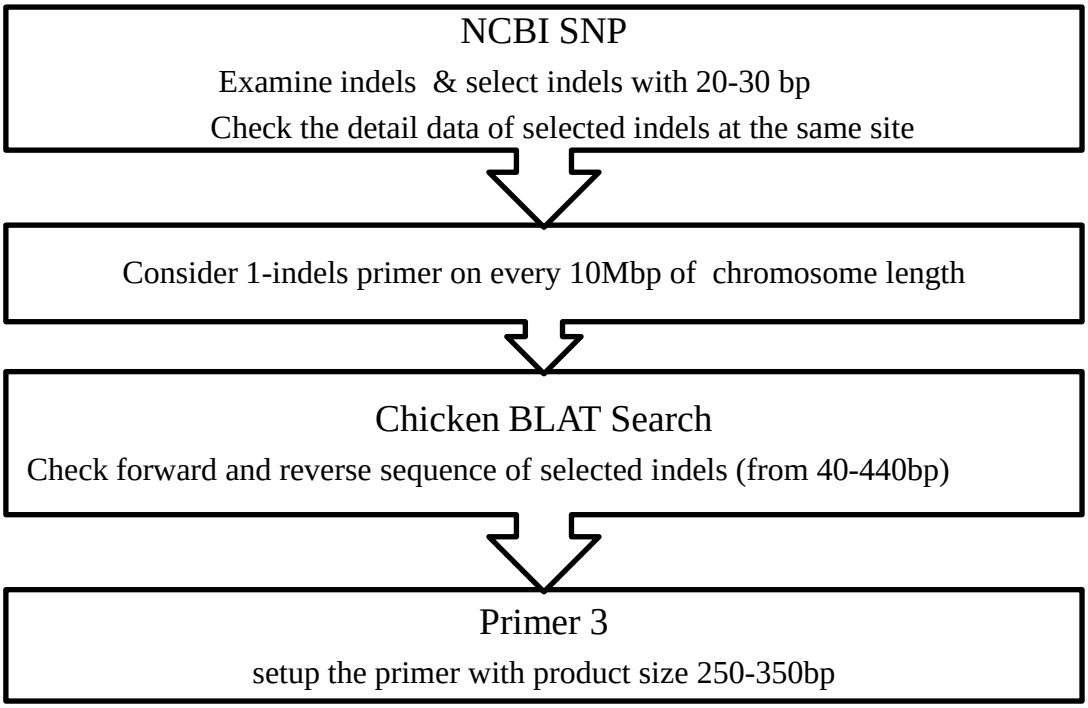

Figure 3. Outline procedure for development of indel markers

2.3. Marker validation

As a validation test for designed markers, we performed PCR (polymerase chain reaction) and electrophoresis. PCR was performed to examine whether the primer sequences were amplified or not. To examine the amplification and polymorphic pattern of indel markers, we performed gel electrophoresis.

2.3.1. PCR and electrophoresis

The PCR amplifications was performed in a 10 µl reaction volume, which included distilled water, 10 X reaction buffer, 2.5mM dNTP , 0.25 units of Ex Taq™ (Takara Bio Inc, Otsu, Japan), 10ng of template DNA, 25µM each of forward and reverse primers. The general PCR mixture for all markers was shown in Table 1 and the detail of PCR mixture to each marker was shown in Appendix 1. The PCR condition was performed by initial denaturation for 2 min at 94 °C followed by 28-35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 48-65 °C and extension for 10 s at 72 °C and final extension for 7 min at 72 °C in Takara PCR thermal cycler - TP650 (Takara Bio Inc). The general PCR condition for all markers was shown in Table 2. The detail of the annealing temperature and number of amplified cycles to each marker were expressed in Table 3. The PCR products and 100 bp DNA ladder (Sib Enzyme Ltd, Russia)were electrophoresed on 1- 2% agarose gel electrophoresis in 1 X TBE. The indels polymorphisms were identified through Toyobo FAS III UV - Transilluminator (Toyobo, Osaka, Japan) after ethidium bromide staining for 15-30 min. The insertion and deletion pattern of indels polymorphisms appear in electrophoresis was demonstrated in Figure 4.

Table 1. General PCR mixture

Reagent	Volume (1x Mix, µl)
DW	4.5- 6.5
10x Buffer	0.8 -1.0
dNTP	0.4 - 0.8
Primer - f	0.4 - 0.5
Primer- r	0.4 - 0.5
Taq DNA Polymerase	0.2 - 0.5
DNA Template	1.0-1.6
∑	7.7-11.4

Table 2. General PCR condition

Step	℃	Time	Cycles
Initial denaturation	94	2 Min	
Denaturation	94	30 Sec	
Annealing	***	30 Sec	***
Extension	72	10 Sec	
Final Extension	72	7 Min	

*** Vary according to the primers

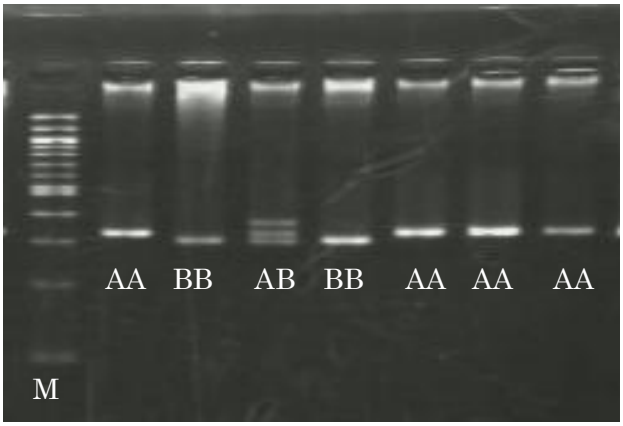

Figure 4. Indertion and deletion pattern found in electrophoresis

M = 100 bp ladder marker, AA = Insertion (327 bp), BB = Deletion (305 bp)

2.4. Results and discussion

In this study, 110 indel markers from chromosome1- chromosome 22 were designed. However, eight markers from chromosome 5 were not amplified and their reference number in NCBI database were: rs 14509588, rs 15666791, rs 16480799, rs 15699594, rs 16496416, rs 14551811, rs 14554385 and rs 15748852. The rest of 102 indel markers were amplified well and showed polymorphism. Therefore, eight indel markers from chromosome 5 were discarded and 102 indel markers were used for further study. The summary of the primer sequences for 102 indel markers were shown in Table 3. The out line of the number of designed marker on each chromosome demonstrates in Appendix 2.

Out of 102 indel markers, 93 showed polymorphism. The mean frequency of 'A' (insertion) allele is 0.65 and 'B '(deletion) allele is 0.35 over the whole population. The mean frequencies of 'A 'and 'B' allele in Yangon, Mandalay and Pegu are (0.626, 0.374), (0.637, 0.363) and (0.639, 0.361) respectively. The detail of allele frequencies data were shown in the Appendix 3. The average minor allele frequency of 93 indel markers was 0.212 and 68% of the polymorphic loci had a minor allele frequency (MAF) of >10% and 47% of > 20%. These results close to the study of Ülo *et al.*, (2008). Their study of indels as genetic markers in wolf showed 74% of the polymorphic loci had MAF of >10% and 49% of >20%. Among 102 markers, 26 markers (25.5%) were located within coding regions of genes and all of these were located in introns. Out of 26 markers, 20 markers are from chromosome 1-10 (macro

chromosome and intermediate chromosome), six markers are from chromosome 11-22 (micro chromosome). The result revealed the number of markers reside in coding region are less frequent in micro chromosome. This may be due to the fact that micro chromosome constitute 30% of all chicken genome (Smith and Burt, 1998) and 60-70% of known chicken genes are associated with micro chromosome (Mc Queen *et al*.,1998) giving them a gene dense structure with shorter intergenic sequences than macro chromosome.

By considering to above results, 102 indel markers developed in this chapter can be apply for further study.

Table 3.1. Summary of primers sequences for 102 indel markers

Loci	Chr: Reference NCBI	Chromosomal Position	Forward Primer (5'-3')	Reverse Primer (3'-5')	Insertion (bp)	Deletion (bp)	TA(C°)	no. of cycles
m 1	1: rs15186483	2380713	agctattcagggaggggatg	ggatgcctgtttctggaaga	311	287	60	28
m 2	1: rs14796695	11865023	caggtagcttggggaatcag	ttcttgtctgggccaaatgt	252	222	60	28
m 3	1: rs14803637	29610732	gggaaagataaaatggcttga	cagatccacacttcagggaaa	292	263	60	28
m 4	1: rs15224689	32491050	aacccatgggggtcttgtat	tagggaacagatggggaaaa	328	300	60	28
m 5	1: rs1524406	41678486	agcatcagcaaggtgctctt	agacaaagcttccctggaca	299	272	60	28
m 6	1: rs15272331	56388055	gccccttcaagcatgatatt	ctcctttcagctgcagatttc	321	297	60	28
m 7	1: rs13879446	67455476	agaatgccctttggattcct	tcagcgggttatacagagca	295	265	60	28
m 8	1: rs14844703	73142545	aggcttacggttggagacaa	aactgcaactccctccagtg	271	241	60	28
m 9	1: rs13893049	86184937	gagagctgggttctccagtg	ttctttttgctggctgcttt	252	223	60	28
m 10	1: rs15340628	92114321	tgttttgccagcatgacatt	gctgtgggagaaacaacaca	289	252	60	28
m 11	1: rs15354680	100316232	tgctcacagcaaacctaacg	acgcaggctagaggacttca	288	265	60	28
m 12	1: rs15381205	112800871	tacagctcaggccacatgaa	gggtccaaaagaaagacatca	297	270	60	28
m 13	1: rs15402998	123160898	ataacgcaaagggagggatg	gtttgcctgtggtgttgtga	255	221	60	28
m 14	1: rs14888652	135638781	atgggctggtgaactgtagc	ccatctggccttagcaaatc	374	349	60	28
m 15	1: rs15443072	143204968	gggaaactctgcattgctgt	ataatgctgccactcctgct	329	298	60	28
m 16	1: rs15477016	159215305	gggatgagcttcaaagtgga	acccaacaaggacgtgtca	296	268	60	28
m 17	1: rs15467491	162349692	cctccttgagctctgacacc	tcctgtgggtcttttccaac	203	174	60	28
m 18	1: rs13968127	170697123	aacagaagcctcctgccttt	aggatggtttggtttgcttg	304	276	60	28
m 19	1: rs13986305	186019671	aaaaagaagtgctgtctgaagaaaa	gaaaacgcgaaatgaatggt	287	257	60	28

3.2

Loci	Chr: Reference NCBI	Chromosomal Position	Forward Primer (5'-3')	Reverse Primer (3'-5')	Insertion (bp)	Deletion (bp)	TA(C°)	no of cycles
m 20	1: rs15550068	196892451	cagaccctacaagccacaca	ttctcagaataattttgccagttg	324	301	60	28
m 21	1: rs15554369	200599560	tgacttccccattcattggt	gcactttcacagcctcccta	326	300	60	28
m 22	2: rs15879191	7098042	attttgcttgacgtgcagtg	ctgcagcctttgggagtatc	291	258	65	25
m 23	2: rs15891921	11463166	tccctttttcaggctgtgatt	ctctaggccaagcatctggt	276	251	60	30
m 24	2: rs14151659	22996352	agggatatttgggacgaagg	gccttttcacacatccaggt	329	298	60	25
m 25	2: rs15953427	37759827	tctatcaggccttgcacctt	ttacttgagggtgcccaatc	282	254	60	30
m 26	2: rs15971881	44143530	tctctgcactctccaaagca	tgtgagcaccgagcaataag	293	254	65	25
m 27	2: rs15102430	57732225	gtagaaggccaaccaaccaa	caaccaaagagggaacatgc	279	253	65	25
m 28	2: rs16011387	61748980	tgcaagctcttgttcattgc	ccgctttgctacatcctctc	291	261	65	25
m 29	2: rs16040761	78554543	tcagaaggatcgcatgaaga	tcattttgagaaaagacaggaactt	262	235	55	30
m 30	2: rs16054197	87816451	aaccacattctggggatgaa	aaccacctcccgtaacagtg	296	272	60	30
m 31	2: rs15132229	99174129	accagcccttaaaccgttg	aatctcaaagcccacccagt	299	276	60	30
m 32	2: rs16088091	106532884	gcacagctatgcccaaaataa	ctctgcctctggtggagact	299	276	60	30
m 33	2: rs14235853	116697385	catgcctgttgcttttacca	ccttgttgaggcatgtcagt	277	257	65	30
m 34	2: rs15147812	123445602	cccctccaacctaagtcattc	gtgcattccccatcatttct	314	285	60	30
m 35	2: rs14250138	131679775	ccagccctgtaggagagagtt	agctgcaggacatgaggtct	255	229	60	30
m 36	2: rs15165839	145558702	aggcttgaggatacgcttca	cagcccaacaggttaccataa	295	265	65	30
m 37	2: rs16144735	152495037	ctgccacagttcaagaagca	ttccaagtaggcgaataccg	272	247	55	30
m 38	3: rs15267007	9421750	caaaatgtgcgacttttcct	gaaactggccgtggtacaat	300	272	60	28

3.3

Loci	Chr:Reference NCBI	Chromosomal Position	Forward Primer (5'-3')	Reverse Primer (3'-5')	Insertion (bp)	Deletion (bp)	TA(C°)	no of cycles
m 39	3: rs16229905	18459587	tgacagaatcaggggaaaatg	gccttctatcaaagccagca	293	274	55	30
m 40	3: rs15299757	26141512	aagaaggagctaccgcactg	actgcctggcaagtgaagat	261	236	55	28
m 41	3: rs16248030	32579565	ggtttccagcaacaggagaa	ggatgagccaaaattggaga	286	261	55	28
m 42	3: rs15336669	44685823	ccaggttgtgtacgcagaga	tttgccttccacgtttgtct	265	236	60	28
m 43	3: rs15364542	58211486	gccatttctgccactgtctt	ggaaatggatcctgcaaaaa	360	337	60	28
m 44	3: rs15372369	65825652	gctttaaaagaagccgagca	ccagaattcccaatttttcaa	326	297	60	30
m 45	3: rs15380325	70288536	aggatccttggtcaatgtgg	cagttcaggcagatccatca	297	273	60	28
m 46	3: rs15409517	84493460	tccttctaagatgcggcaat	cagtttggggttgggtaaaa	281	250	60	28
m 47	3: rs15427052	94441494	tgacaacgcatcacagcata	gcttccgtattacccagcag	289	260	60	28
m 48	3: rs16338959	108511888	atattgggaactcgctgtgg	atgtcacacaaagcctgctg	276	250	60	28
m 49	3: rs14413503	111873254	acatggcacttgatgaagca	aattggcttttggacacctg	284	263	60	28
m 50	4: rs16359295	9542599	tataaatggggtgggtgtgg	caccaaaagcagaaatgcaa	305	281	60	28
m 51	4: rs14432370	14488517	atttttgatctgggcacgaa	gaggcaggaggtggaagag	252	227	60	28
m 52	4: rs14438548	24411304	aggcagacagatgtggaaga	caggaatacaaagccgcagt	282	253	60	28
m 53	4: rs16384349	32708827	gcaaccctgaagaaaaccaa	gcaaagcagagtttggaacc	287	258	60	28
m 54	4: rs16399900	46455122	ttgcagcaaaagggaagatt	tggaggaatgcagctgacta	306	280	65	28
m 55	4: rs16414997	56511101	tctgtgcagattcggtatgg	gtcactgcccttcagcaaat	272	243	60	28
m 56	4: rs16429149	68586191	catcagcttccctttgtga	tctcattgctcattgtacagctc	250	222	60	28
m 57	4: rs16431940	70693757	ttagcttcccccaaacactg	aacagcggctcattcattct	314	289	60	28

3.4

Loci	Chr:Reference NCBI	Chromosomal Position	Forward Primer (5'-3')	Reverse Primer (3'-5')	Insertion (bp)	Deletion (bp)	TA(C°)	no of cycles
m 58	4: rs16449188	89179812	agcatctcagccttccttca	ctgggctcatacccatgtct	289	260	60	28
m 59	4: rs15642550	90357511	gctcatgcatggaatttgtg	tcctgtgctctccatctatgc	290	264	60	28
m 60	5: rs14508864	1531872	tgtcacaaatgcaggaggtg	cagacctcaaagcatcacaca	311	281	60	28
m 61	5: rs16463699	10884770	aggctccaagtgctgtgatt	ccaaaataaagtcccgacaa	276	242	60	28
m 62	5: rs16478463	26549515	ccttgcatctcctccttcag	ggagggaaagggtcaatgat	266	238	60	28
m 63	5: rs14529374	31922437	tggtcatgatggtttggaga	gtgcaggacattttgcttga	297	268	60	28
m 64	5: rs14542642	48345965	tcctaatgtcggtcatgctg	caagtctgtggccaggaagt	299	270	60	28
m 65	5: rs16508335	51981730	ggcagaggagagcagaaatg	tgctgtttgtccgaagtttg	291	261	60	28
m 66	5: rs15745605	60173479	ccacccgagtcctaagtctg	tcttcatggggaaggaagtg	271	242	60	28
m 67	6: rs14570404	9359709	gctgttcacttggtcttgc	cgaggactgaaggaaatgaca	349	326	60	28
m 68	6: rs14580218	19952173	gctctgcttccctcctttct	ttgtgatccacacctgcatt	305	279	60	28
m 69	6: rs15811157	29943028	ttttgttaaccaggggcaat	gtagcatctgcagcccaaat	363	334	60	28
m 70	6: rs15823004	36962704	tgcctcagtctggtctgttg	tgcatgagggttcagaagtg	308	279	60	28
m 71	7: rs14604441	7009917	agcatcacaccaactgcaag	cattcctccagagcttcctc	314	294	60	28
m 72	7: rs16591682	18682408	gctgctataagctgcccatc	ggcaagcaggaaatgaagag	306	284	60	28
m 73	7: rs14622212	29604888	ttaaagccagcacacaatgc	catccagcagtcagcctttt	360	331	60	28
m 74	7: rs16615778	37651290	gaggatatgggcaagtctgg	tccccttgtcctgctgttat	350	324	60	28
m 75	8: rs15908922	9253123	ttttcatgggtagttcattagaga	atgctgccttccataactgc	327	307	60	28
m 76	8: rs16636129	19782818	gcgtcagagtgtgaaatgct	agcacgctgttcctctgaat	324	302	60	28

3.5

Loci	Chr:Reference NCBI	Chromosomal Position	Forward Primer (5'-3')	Reverse Primer (3'-5')	Insertion (bp)	Deletion (bp)	TA(C°)	no of cycles
m 77	8: rs16649376	29327443	catttggggcagcagtattc	cacctcccaaacttgcatct	363	339	60	28
m 78	9: rs16664917	4736922	tcctcctggaacttcctcct	ttcagtttgccttggtcctc	345	320	60	28
m 79	9: rs14683671	24883034	gtcgcagcttcagaaaggac	ctgtacacaaacggcgatgt	311	286	60	32
m 80	9: rs14672349	13452188	attgaaagcgaccattccag	gccttctgaaacctaccaagtt	302	277	60	32
m 81	10: rs15572293	8045518	tgcacaaaactaatcttgctgtct	ggtgttgtcaatcctgtttgc	330	309	60	30
m 82	10: rs14012832	20119574	ggccaggatctcaaaacaga	tccccttgtatgtcctctgc	346	319	60	30
m 83	11: rs15611781	9281820	ctgcctccaggcctttctat	gcacaagaatcaccagcaag	348	319	60	28
m 84	11: rs14018578	1883678	ccagggctatggaatgctta	cactggctgactgcagatgt	347	319	60	28
m 85	11: rs14693330	20791178	tttgagcaccaccgtgagta	cccagctcaagagtcgaaac	315	290	60	32
m 86	12: rs15648972	9133627	cggtcctcatgttgtcaaag	gacaatgcacagctgcataaa	327	305	62	35
m 87	12: rs15672210	19136567	aagggcagagaactgttcca	tgggttggagggtatcttca	364	334	60	28
m 88	13: rs15695194	9834883	ggtgggtaatccagtctctcc	cttcaggctcaacaggaacc	326	293	60	28
m 89	13: rs15706498	15621848	agcgcacacatttgcattag	aggctgaggaaggttgtcct	325	299	60	28
m 90	14: rs14077825	9856807	tgtttggcatacctgtgcat	taggaagaaagggctctgct	336	315	60	30
m 91	14: rs15740439	11675614	aggcatgccagaacattcat	ggtcttttccagcctgagtg	301	278	60	35
m 92	15: rs14094135	9649390	tagttcccagtggtgtgtgg	agggtgtctcttcagcctca	334	310	60	35
m 93	15: rs15783434	10844586	taattgattcagcgcagagc	ccagccagcttcattgagat	315	291	60	34
m 94	16: rs15026709	133330	actcattgggaatggactcg	cacgtccctctccatgtttt	346	331	60	28
m 95	17: rs15790503	9512181	ctcagcccttgctttctttg	ttggattctccctcatttgc	311	282	48	28

3.6

Loci	Chr:Reference NCBI	Chromosomal Position	Forward Primer (5'-3')	Reverse Primer (3'-5')	Insertion (bp)	Deletion (bp)	TA(C°)	no of cycles
m 96	17: rs15027282	10043385	ccacaacgactcggttaagaa	gtcattgctgggaacctcat	315	287	60	28
m 97	18: rs15818344	3551368	ttcagttttggtgtcgctca	ttctctgagcctgccagaat	323	300	60	32
m 98	19: rs14121581	6252496	cgccacacataaatcagtcg	ccttgttgctacctggctgt	376	349	60	28
m 99	20: rs14277689	9478707	cgaggatgacctgtggtgta	tcctgaaagcttttgtgtgc	355	326	65	28
m 100	20: rs16174629	11941342	accatgggctgttctttgaa	ggcaggttgtgaaggatagc	347	323	65	28
m 101	21: rs16179814	3159840	acaaccgctcgacagaaagt	agttgacctcccctggaaat	310	284	65	30
m 102	22: rs16183765	3842053	tcagggacatcccagaagac	gcaccagaaatgctctctcc	368	339	65	30

CHAPTER 3

Genetic diversity of Myanmar and Indonesia native chicken together with two Jungle fowls species

3.1. Introduction

Nowadays, it increasingly recognizes that insertion and deletion (indels) polymorphisms are an important source of genetic as well as phenotypic diversity (Brandström and Ellegren, 2007). The analysis of indels as genetic markers can be carried out using relatively simple and inexpensive. Indel markers have many genetic advantages for analytical use: they are widely spread throughout the genome, all of the polymorphisms derive from a single mutation event and they have reduced mutation rates (Natalle *et al*., 2010).

Native chickens, as the most adaptable and geographically widespread livestock species, form an integral part of the Myanmar and Indonesian ecosystem. Native chickens possess unique adaptive traits that permit them to survive and reproduce under harsh climatic, nutritional and management conditions typically associated with low input - output production systems (Mwacharo *et al.*, 2006). Although these native chickens were not available for commercial use, they were raised as a dual- purpose for meat and egg, providing cash income in times of need.

Myanmar is the largest country on the main land South East Asia with a total land area of 676,577 sq Km. Myanmar possesses tropical and sub tropical climates and rich with diverse species of animal and wild relatives of livestock. Latest estimates

indicate that there are 105 million birds raised in Myanmar, out of which 94 million are chicken, mostly 78.7 million birds (84%) kept under backyard production system (Burgos et al., 2009). Some of local chicken breeds are Hle Pyaung, Tanyin, Taik Kyet, Sittagaung, Inbinwa and kyet linda. All are dual- purpose breed; their average egg production is 40-60 eggs per year with the weight of 47 gm. About 60-70 percent of eggs are used for hatching and the survival rate is only 40-60 percent of chick hatched.

The land area of Indonesia covers 1,919,445 sq Km, spreading from Aceh Province in the north west of Sumatra to the Western part of Papua. It is a large tropical Island in South East Asia, which shelters a large number and variety of wild as well as domesticated animals. According to livestock statistic from 2007, Indonesia has an estimated standing population of 620 million chickens which include 317 million native/village chicken (51%) kept by rural farmers (Ministry of Agriculture -2007 cited by Bambang *et al.*, 2009). Some of the local chicken varieties are Kedu, Kampung and Ayam Arab. Kedu chickens are a dual-purpose breed, in that they produce good quality meat and eggs (Johari *et al.*, 2009). Kampung, is a local ecotype that is raised free range in most rural areas of Java. Ayam Arab breed is also known to produce good quality eggs.

The Green Jungle fowl (*Gallus varius)*, which is a local species confined to the Java Island and adjacent islands. These birds are bred with domestic chickens by many people, producing a hybrid known as Bekisar. Their genetic diversity is disappearing

because of the forest erosion narrow their inhabitant area. The Green Jungle fowl is evaluated as Least Concern on the IUCN (International Union for Conservation of Nature) Red List of threatened species. *Gallus gallus bankiva* is one of the subspecies of *Gallus gallus*. They inhabit in Java Island. They are a very distinct entity of *Gallus gallus* and clearly separated from *Gallus gallus gallus* as well as *Gallus gallus spadiceus* (Akishinonomiya *et al.*,1994).

In this chapter, by using indels polymorphisms as genetic markers the genetic variability among native chicken from two south East Asia countries will examine. The goals of the study were: to evaluate the indels polymorphisms as genetic markers to characterize the genetic variability of the native chicken populations from Myanmar and Indonesia and to determine the genetic relationship among Myanmar and Indonesia native chicken populations and two jungle fowl species.

3.2. Materials and methods

Totally 11 populations of Myanmar and Indonesian native chickens and two Jungle fowl species were used. In Myanmar native chickens, three populations from Yangon (Yan) and Pegu (Peg) of lower Myanmar and from Mandalay (Man) of central Myanmar were examined. In Indonesian native chicken, four populations of local chicken and four populations of local varieties were examined. The local chicken populations were collected from Semarang (Sem), Kendal (Ken), Yogyakarta region (Yog) of the central Java, and Karawang region (Kar) of west Java. The populations of local varieties were Ayam Kedu (AK) from Temanggun (AK-Tem) and Solo

(AK-Sol) of the central Java, Black Kedu (BK) from Semarang (BK-Sem) and Ayam Alab (AA) from Temanggun (AA-Tem). Two Jungle fowls species from Java, Red Jungle fowl (*Gallus gallus bankiva*, RJF) and Green Jungle fowl (*Gallus varius*, GJF) were also examined. Blood samples were obtained from Yan (n = 27), Peg (n = 13), Man (n = 40), Sem (n = 24), Ken (n = 40), Yog (n = 57), Kar (n = 15), AK-Tem (n = 10), AK-Sol (n = 19), BK-Sem (n = 23), AA-Tem (n = 20), RJF (n = 3), GJF (n = 3) respectively. The sampling locations in Myanmar and Indonesia are shown in Figure 5 and Figure 6.

Genomic DNA was extracted from blood samples using standard phenol-chloroform extraction protocols (Sambrook, 1989). Here, 102 indel markers developed in Chapter 2 will used. The PCR amplifications, PCR condition and electrophoresis condition was reported in Chapter 2. Genotypes of indels polymorphisms were determined by size difference between the PCR fragments.

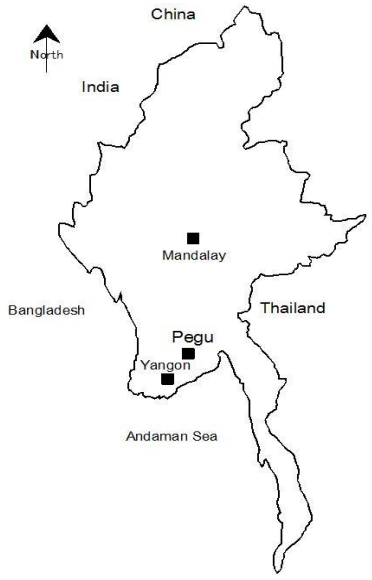

Figure 5. Map of Myanmar showing sampling locations

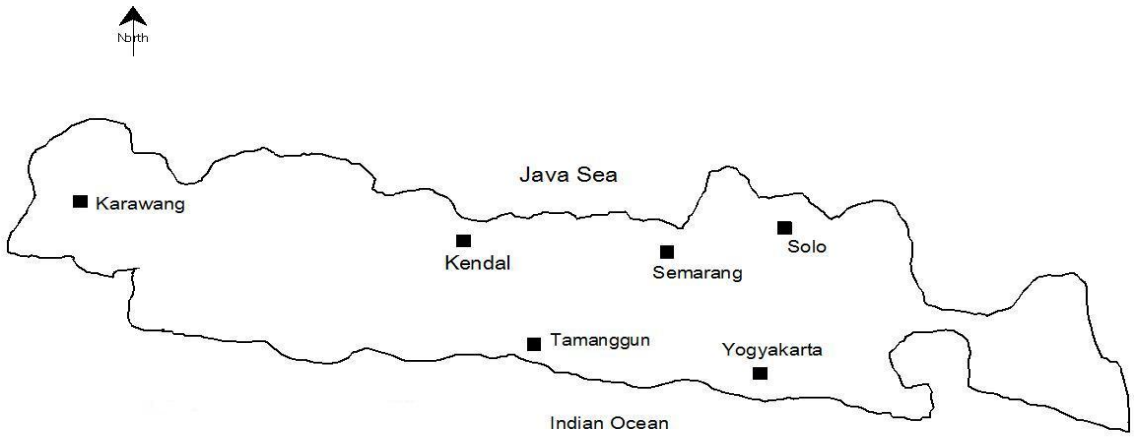

Figure 6. Map of Indonesia showing sampling location

3.2.1. Statistical analysis

Allele frequencies were calculated from genotyping data by direct counting. The proportion of polymorphic loci (*Ppoly*) was calculated as the ratio of polymorphic loci to the total number of loci analyzed. Chi square (X^2) approximation was used to test Hardy-Weinberg Equilibrium (HWE) at each locus (Weir, 1996). To estimate the genetic variability, we calculated the average observed heterozygosity ($\overline{H}_O$), the average expected heterozygosity ($\overline{H}_E$) of each population and degree of genetic differentiation (*GST*) (Nei, 1973). $\overline{H}_E$ was estimated by Nei's formula (Nei, 1978). To assess the genetic relationships between 13 populations, pair wise standard genetic distance (Ds) (Nei, 1972) was computed using PHYLIP ver. 3.69 (Felsenstein, 2009). From the data of genetic distance matrix, constructed phylogenetic tree by using neighbor- joining (NJ) method (Saitou and Nei, 1987) implemented by MEGA software ver.4.1 (Tamura *et al*., 2007).

3.3. Results

3.3.1. Indels polymorphisms

The mean (± S E) *Ppoly* value of each population was listed in Table 4. Out of 102 indel markers, 97 markers (*Ppoly* = 95%) were polymorphic in native chicken populations, all of which were in HWE. The *Ppoly* was higher in Indonesian native chicken populations (*Ppoly* = 94%) than Myanmar native chicken populations (*Ppoly* = 92%). One marker (m 66) was monomorphic in Indonesian native chicken populations whereas three markers (m 11, m 33 and m 92) were monomorphic in Myanmar native chicken populations.

In two Jungle fowl species, out of 102 indel markers RJF showed polymorphic in 22 loci whereas GJF showed polymorphic in four loci. RJF showed more polymorphic loci (*Ppoly* = 21%) than GJF (*Ppoly* = 3.9%). In addition, indels polymorphisms in RJF and GJF were much fewer than native chicken populations.

3.3.2. Genetic variability

The $\overline{H}_O$ and $\overline{H}_E$ values were ranged from 0.206 to 0.268 and from 0.229 to 0.284 in 11 native chicken populations (Table 4). In the Jungle fowls, RJF showed higher $\overline{H}_O$ and $\overline{H}_E$ values (0.101 and 0.078) than GJF (0.003 and 0.012). Among the Myanmar and Indonesian native chickens, the *GST* value was 0.041 for Myanmar and 0.098 for Indonesia (Table 5). The two Jungle fowl species showed highest genetic differentiation between them with *GST* value of 0.436. The *GST* observed between two

Jungle fowl species and Myanmar native chicken (0.213 to RJF and 0.264 to GJF) was higher than the G_{ST} between two Jungle fowl species and Indonesian native chicken (0.162 to RJF and 0.186 to GJF). The G_{ST} among 13 populations was calculated as 0.227.

Table 4. The genetic variability from 13 populations of native chickens and two Jungle fowl species

Population	No. of Samples	P_{Poly}	±SE	$\overline{H}_O$	±SE	$\overline{H}_E$	±SE
Yan	27	0.901	0.034	0.216	0.004	0.266	0.004
Peg	13	0.713	0.045	0.218	0.005	0.239	0.004
Man	40	0.871	0.034	0.229	0.005	0.263	0.005
Sem	24	0.861	0.035	0.258	0.004	0.269	0.004
Ken	40	0.901	0.029	0.242	0.004	0.276	0.004
Yog	57	0.901	0.029	0.268	0.005	0.284	0.004
Kar	15	0.792	0.041	0.231	0.004	0.263	0.004
AK-Tem	10	0.772	0.041	0.239	0.005	0.255	0.004
AK-Sol	19	0.881	0.032	0.250	0.004	0.278	0.004
BK-Sem	23	0.743	0.044	0.207	0.004	0.242	0.004
AA-Tem	20	0.812	0.039	0.206	0.004	0.229	0.004
R J F	6	0.218	0.041	0.101	0.005	0.078	0.004
G J F	3	0.039	0.019	0.003	0.002	0.012	0.003

Yan = Yangon; Peg = Pegu; Man = Mandalay; Sem=Semarang; Ken= Kendal; Yog = Yogyakarta; Kar = Karawang; AK-Tem = Ayam Kedu from Temmanggun; AK-Sol = Ayam Kedu from Solo; BK-Sem = Black Kedu from Semarang; AA-Tem = Ayam Alab from Temmanggun; RJF = Red Jungle fowl; GJF = Green Jungle fowl

Table 5. Coefficient of genetic differentiation (G_{ST}) in various subsets of 13 populations of native chickens and two Jungle fowl species estimated from 102 indels loci

Subset	G_{ST}
Among Myanmar native chickens	0.041
Among Indonesia native chickens	0.098
Among Myanmar and Indonesia native chickens	0.119
Between 2 Jungle fowl species	0.436
Between Myanmar native chicken and RJF	0.213
Between Myanmar native chicken and GJF	0.264
Between Indonesia native chicken and RJF	0.162
Between Indonesia native chicken and GJF	0.186
Among 13 subpopulations	0.227

3.3.3. Genetic distance and phylogenic analysis

The *Ds* distances was shown in Table 6. The *Ds* distances between the native chicken populations were ranged from 0.034 to 0.078 among Myanmar, from 0.009 to 0.147 within Indonesia and from 0.063 to 0.200 between the two countries. The *Ds* distances between the two countries, the smallest *Ds* (0.063) was observed between the Ken and Yan population and between the Yan and Yog populations. The largest *Ds* (0.200) was obtained from between BK-Sem and Peg populations. The genetic distances between native chicken populations and two Jungle fowl species were ranged from 0.247 to 0.405.

The NJ tree constructed from the *Ds* distances between the 13 populations gave two major clades as shown in Figure 7. The first clade was composed of Myanmar and Indonesian native chicken populations. The second clade was composed of RJF and GJF, which was located outside the first clade.

Table 6. Pair wise genetic distance (*Ds*) between 11 populations of native chickens and two Jungle fowl species

	Yan	Peg	Man	Sem	Ken	Yog	Kar	AK-Tem	AK-Sol	BK-Sem	AA-Tem	RJF	GJF
Yan		0.078	0.034	0.082	0.063	0.063	0.099	0.087	0.072	0.139	0.102	0.405	0.367
Peg			0.062	0.156	0.133	0.136	0.146	0.165	0.143	0.200	0.150	0.342	0.333
Man				0.093	0.065	0.067	0.110	0.088	0.075	0.174	0.118	0.337	0.347
Sem					0.040	0.049	0.088	0.048	0.054	0.129	0.057	0.326	0.344
Ken						0.012	0.046	0.017	0.009	0.092	0.086	0.288	0.289
Yog							0.055	0.026	0.019	0.103	0.089	0.287	0.294
Kar								0.076	0.049	0.093	0.124	0.282	0.247
AK-Tem									0.023	0.114	0.093	0.308	0.337
AK-Sol										0.100	0.099	0.318	0.335
BK-Sem											0.147	0.304	0.263
AA-Tem												0.342	0.334
RJF													0.251
GJF													

Yan = Yangon; Peg = Pegu; Man = Mandalay; Sem = Semarang; Ken = Kendal; Yog = Yogyakarta; Kar = Karawang; AK-Tem = Ayam Kedu from Temmanggun; AK-Sol = Ayam Kedu from Solo; BK-Semarang = Black Kedu from Semarang; AA-Tem = Ayam Alab from Temmanggun; RJF = Red Jungle fowl; GJF = Green Jungle fowl

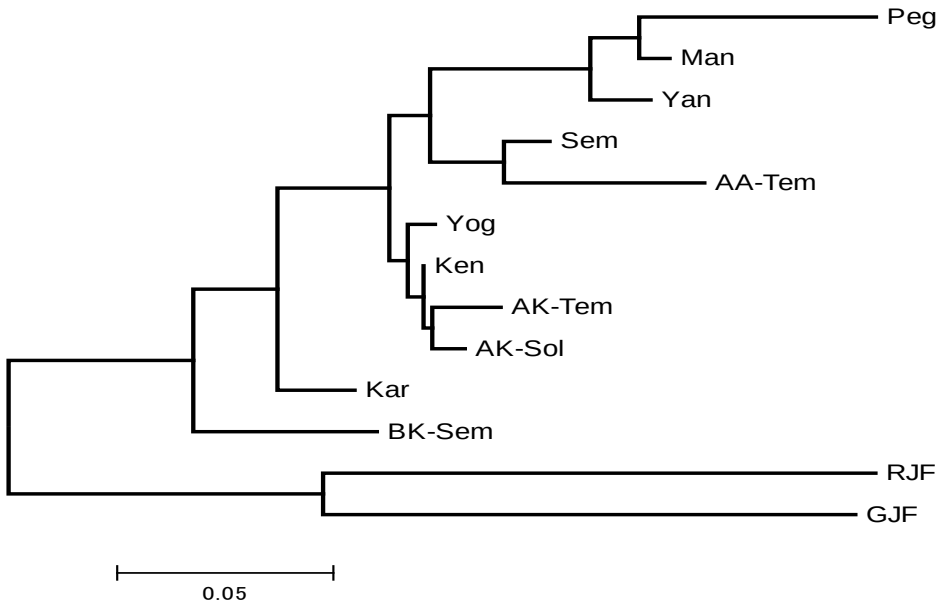

Figure 7. Neighbor-joining (NJ) tree constructed by genetic distance matrix from 13 populations of native chickens and two Jungle fowl species

Yan = Yangon; Peg = Pegu; Man = Mandalay; Sem = Semarang; Ken = Kendal; Yog = Yogyakarta; Kar = Karawang; AK- Tem = Ayam Kedu from Temmanggun; AK-Sol = Ayam Kedu from Solo; BK-Sem = Black Kedu from Semarang; AA- Tem = Ayam Alab from Temmanggun; RJF = Red Jungle fowl; GJF = Green Jungle fowl

3.4. Discussion

3.4.1. Indels polymorphisms

The results revealed both of the native chicken populations showed polymorphic in most of the indels loci (greater than 90%). It is larger than the previous study of Väli *et al.*, (2008) and their study stated that 81 and 76 out of 94 indel markers (86.2% and 80.9%) could be validated as polymorphic loci in dogs (n = 7) and wolves (n = 18). Jungle fowl populations especially in GJF showed much fewer polymorphic indels loci (4%) than native chicken populations. These results suggest that the indels

polymorphisms examined here may be acquired in the chickens after the separation of ancestral species of the GJF and chickens.

3.4.2. Genetic variability

The $\overline{H}_O$ and $\overline{H}_E$ values were 0.206-0.268 and 0.229-0.284 in native chicken populations and 0.003-0.101 and 0.012-0.078 in two Jungle fowl species respectively. These $\overline{H}_E$ values were closed to those of the gene constitution of blood group in Myanmar and Indonesian native chickens (0.299 and 0.279) (Yamamoto e*t al.*, 2010). The $\overline{H}_E$ values were higher when compared to the range of egg white protein polymorphisms estimated from native fowl populations in Asia ($\overline{H}_E$ = 0.089 - 0.170, Kinoshita *et al.*, 2004) and blood protein variation in Myanmar native chicken ($\overline{H}_E$ = 0.198, Okamoto *et al.*, 2004). However the $\overline{H}_E$ values in this chapter were lower when compared to the heterozygosity of four calpain gene polymorphisms in Myanmar and Indonesian native chickens revealed 0.388 and 0.389 respectively (Okumura *et al.*, 2006). These differences may be arisen from difference in the sample size, sample population and source of genetic marker in each study.

In the present chapter, the heterozygosities of native chickens were higher than the heterozygosities of ancestral species. This finding was similar to the earlier report of Väli *et al.*, (2008) where the heterozygosities in dogs were higher than the heterozygosities of their ancestral "wolf". They reported that the values of $\overline{H}_O$ and $\overline{H}_E$ were 0.268 and 0.355 in dogs and 0.194 and 0.261 in wolf by using indel markers. The *GST* values of Myanmar and Indonesian native chicken populations were 0.041 and

0.098, indicating that the degree of genetic differentiation is higher in Indonesian native chicken than Myanmar native chicken. It might be related to the sample population of Indonesian native chicken, which contained local chicken varieties like BK, AA, and AK, and there may be some degree of genetic differentiation between them.

The G_{ST} of present chapter was higher than those of Myanmar and Indonesian native chicken (0.024 and 0.020) estimated in genetic constitution of four calpain gene polymorphisms (Okumura *et al.*, 2006). However G_{ST} of Myanmar native chicken was closed to the range of 0.001-0.039 (Kinoshita *et al.*, 2004) estimated from egg white protein polymorphism of local populations in Asian countries (Myanmar, Indonesia, China, Nepal, Vietnam and Laos). The G_{ST} of Indonesian native chicken was closed to blood protein polymorphism of four chicken breeds from Yunnan Province of China (G_{ST} = 0.075, Okamoto *et al.*, 2003) and Nepal (G_{ST} = 0.093, Maeda *et al.*, 1992).

The G_{ST} between Myanmar and Indonesian native chickens was 0.119, indicating that the genetic differentiation between them was not large. Therefore, Myanmar and Indonesia native chickens can be regarded as genetically close populations. The G_{ST} values between Myanmar native chicken populations and two Jungle fowl species (0.213 to RJF and 0.264 to GJF) are greater than between Indonesian native chicken populations and two Jungle fowl species (0.162 to RJF and 0.186 to GJF). It may be the fact that small G_{ST} was obtained between populations of closed inhabitant area. However, highest G_{ST} (0.436) was observed between RJF and GJF from Java Island.

The G_{ST} value in this chapter may be higher than the value of other studies. According to the review of Theresa *et al*., (2002), a wide range of G_{ST} values usually resulted from uneven allele frequency distributions across populations at some loci.

In this chapter, 87 indels loci showed the same tendency in major allele frequency in all populations. Whereas uneven allele frequency distributions were observed in the remaining 15 loci: The major allele in most populations was minor in some populations, which may contribute to high G_{ST} value.

3.4.3. Genetic distance and phylogenic analysis

The low genetic distance observed among native chicken populations (0.009 0.200), reflecting the fact that these populations are not much genetically isolated from each other. In addition, the average genetic distances among native chicken populations (0.088) observed in present study is close to the statement of Yamashita *et al.*, (1994) by DNA fingerprinting analysis among the stock of domestic fowls (0.104). The larger genetic distances were found between native chicken populations and two Jungle fowl species from Java Island.

The topology of NJ tree showed that Myanma**r** native chickens and Indonesian native chickens form a respective cluster in one clade whereas RJF and GJF from Java Island formed another clade, suggesting that native chickens are genetically closely related to each other and remote from Jungle fowls of Java Island.

In the previous studies of Yamashita *et al*., (1994) and Okumura *et al.*, (2006), the GJF was located far away from native chicken populations and it is consistent to the

hypothesis that domestication of the chicken might start from RJF. However, RJF from Java Island comprises as a different clade and it was far away from native chicken populations, which agreed to the un-rooted neighbor joining (NJ) population tree of Niu *et al.,* (2002). In their NJ tree, domestic fowls belonged to the same cluster as *Gallus gallus gallus* and *Gallus gallus spadiceus* in Thailand and its adjacent areas, whereas *Gallus gallus bankiva* from Java Island formed a separate cluster. Furthermore Akishinonomiya *et al.,* (1994) stated that the domestic fowl from Indonesian Island had large genetic differences compared with *Gallus gallus bankiva* from the same place.

The present chapter examined the genetic characteristics of the Myanmar and Indonesia native chickens and two Jungle fowl species from Java Island by using indels polymorphisms as genetic marker. The genetic variability is higher among native chicken populations and lower in two Jungle fowl species. The high genetic differentiation occurred between native chicken populations and two Jungle fowl species from Java Island. The native chickens from two countries were genetically close to each other and remote from Jungle fowls of Java Island. Although the indel markers showed low heterozygosity compared to microsatellite markers, it can demonstrate close genetic variability and phylogenic topology to other studies stated as above. Therefore, indels polymorphisms are efficient for studying genetic diversity of population.

CHAPTER 4

The Genetic diversity of eight chicken populations assessed by 102 indel markers

4.1. Introduction

Satsumadori is a well-known Japanese indigenous breed native to Satsuma region. Satsuma used to be the former name of the Kagoshima Prefecture in Southern Kyushu. Satsumadori was a breed of game chicken developed by crossing with Shamo and Shokoku (Catalogue of Asian native chicken, 1991). As the appearance of Satsumadori was beautiful with abundant long tail feather, the breed was designated a natural monument in 1943 (Catalogue of Asian native chicken, 1991). In the year 1990, the Kagoshima Prefectural Institute for Agricultural Development (KIAD) began to develop a new variety of the local Satsuma chickens by inter-breeding between the male Satsumadori and the female Rhode Island Red. In the year 2000, the project was completed and resulted in the Satsuma-jidori. This new variety was distinguished for its fine muscle fiber, a light crunchy texture, good meat color and lean meat. Since they are more resistant to disease and heat compared to ordinary broilers, Satsuma-jidori chicken has set a new benchmark for the industry. Today Satsuma-jidori meat is well-known brand in Japan. Due to these excellent genetic characteristics of Satsumadori and Satsuma-jidori, it is special interest to assess the genetic variation between these two chicken populations from Satsuma region and other chicken populations by utilizing modern molecular tools.

The genetic variability and relationship of Japanese and foreign chicken has been assessed by microsatellite DNA profiling (Osman *et al.*, 2006). The genetic relationship between the Japanese native chicken (Satsumadori and Ingie) and the commercial chickens has been evaluated using 70 chicken autosomal SNP genotypes by the DigiTag 2 assay (Shimogiri *et al.*, 2011).

In this study, we emphasized on 102 indels markers developed in Chapter 2 as genetic markers to assess the genetic diversity and to determine the genetic relationship of two chicken populations from Satsuma region and other chicken populations.

4.2. Materials and methods

4.2.1.Chicken samples

In total, 474 birds of two chicken populations from Satsuma region and six chicken populations representing three improved and three commercial chicken populations were examined. Two chicken populations from Satsuma region were Satsumadori (SD, n = 20) and Satsuma-jidori (SJ, n = 40). Three improved chicken populations were Rhode Island Red (RIR, n = 55), White Leghorn (WL, n = 60) and Barred Plymouth Rock (BPR, n =119). Three commercial chicken populations were Ross (RS, n = 60), Cobb (CB, n = 60) and Boris Brown (BB, n = 60).

Satsumadori and Satsuma-jidori (both of them were referred to as Satsuma's chickens) samples were obtained from KIAD. Genomic DNA was extracted from blood samples using standard phenol- chloroform extraction protocols (Sambrook *et*

al., 1989). Information and experiment method of the 102 indel markers used in this study were the same method in Chapter 2. Genotypes of indels polymorphisms were determined by size difference between the PCR fragments. As for genotypes, *A* and *B* denoted insertion and deletion alleles respectively.

4.2.2. Statistical analysis

The genetic variability of each population was assessed by calculating the minor allele frequency (MAF), proportion of polymorphic loci (*Ppoly*: Lewontin and Hubby, 1966), and average observed and expected heterozygosity (H_O, H_E: Nei, 1978). Chi square (χ^2) approximation was used to test Hardy-Weinberg Equilibrium (HWE) (Weir, 1996) and calculate the degree of genetic differentiation (G_{ST}) (Nei, 1973). The pair wise standard genetic distance (*Ds*) (Nei, 1972) was computed using PHYLIP ver. 3.69 (Felsenstein, 2009). Then we constructed phylogenetic tree by using unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal, 1973) implemented by MEGA software ver.4.1 (Tamura *et al.*, 2007). To examine population substructure, the individual genotype for 102 indels were subjected to a principal component analysis (PCA) using EIGENSOFT version 3.0 (Patterson *et al*., 2006).

4.3 Results

4.3.1 Indels polymorphisms

Out of the 102 indel markers, 98 were polymorphic in the 474 chickens and the remaining four were monomorphic. Of the 98 loci, 36 loci were fixed in SJ whereas 47 loci were fixed in SD excluding four monomorphic markers for all populations. Then 35 loci in RIR, 37 loci in WL, 41 loci in BPR, 20 loci in RS, 16 loci in CB and 24 loci in BB were fixed. One monomorphic (m31; *BB* = 1) was observed in the SJ whereas six (m6; *BB* = 1, m9; *AA* = 1, m22; *AA* = 1, m45; *BB* = 1, m47; *AA* = 1, m94; *BB* = 1) in the SD. In addition, two was monomrphic only in RIR (m10; *BB* = 1, m91; *AA* = 1), five in WL (m5; *AA* = 1, m 16; *AA* = 1, m71; *BB* = 1 , m77; *AA* = 1, m92; *BB* = 1), and in BPR (m4; *BB* = 1, m25; *BB* = 1, m46; *AA* = 1, m50; *BB* = 1, m82; *BB* = 1). Then two was monomorphic in RS (m1; *BB* = 1, m14; *AA* = 1).

In this study, 80% of the polymorphic loci had a minor allele frequency (MAF) of >10% and 49% of >20%. The average MAF by 98 polymorphic markers was estimated as 0.227. Tests of Hardy-Weinberg equilibrium (HWE) over all experimental populations and within populations indicated that all of the populations were in HWE except SJ at m6.

4.3.2 Genetic variability

The genetic variability of eight chicken populations was demonstrated in Table 7. The *Ppoly* value of each population was ranged from 0.500 to 0.814. The RS, CB and BB populations showed higher values than other populations. The SJ population showed higher value (0.608±0.048) than SD (0.500±0.049) but lower than RIR (0.618±0.048).

The $\overline{H}_O$ and $\overline{H}_E$ values were ranged from 0.147 in SD to 0.257 in BB and from 0.175 in SD to 0.247 in RS. The RS, CB and BB populations showed higher values than other populations. The SJ population showed higher $\overline{H}_O$ and $\overline{H}_E$ values (0.196 and 0.212) than SD (0.147 and 0.175) and RIR (0.153 and 0.177). The $\overline{H}_E$ values of CB, WL and RIR (0.245, 0.192 and 0.177, respectively) were consistent to the previous study of Riztyan *et al.*, (2012) using 98 autosomal SNP markers.

The degree of genetic differentiation (G_{ST}) value was calculated as 0.124 in Satsuma's chickens, 0.289 among improved chickens and 0.305 among commercial chicken populations. The G_{ST} value for over all populations was 0.329.

Table 7. Genetic variability of eight chicken populations

Populations	n	P_{poly}	± SE	$\overline{H}_O$	± SE	$\overline{H}_E$	± SE	G_{ST}
Satsuma-Jidori (SJ)	40	0.608	0.048	0.196	0.004	0.212	0.004	
Satsumadori (SD)	20	0.500	0.049	0.147	0.004	0.175	0.004	
Rhode Island Red(RIR)	55	0.618	0.048	0.153	0.004	0.177	0.004	
White Leghorn (WL)	60	0.598	0.048	0.203	0.005	0.192	0.004	
Barred Plymouth Rock(BPR)	119	0.549	0.049	0.160	0.004	0.176	0.004	
Ross (RS)	60	0.765	0.042	0.243	0.005	0.247	0.004	
Cobb (CB)	60	0.814	0.038	0.237	0.005	0.245	0.004	
Boris Brown (BB)	60	0.716	0.045	0.257	0.005	0.225	0.004	
Satsuma's chickens	60							0.124
Improved chickens	234							0.289
Commercial chickens	180							0.305
Total Population	474							0.329

Satsuma's chickens: SJ and SD

Improved chickens: RIR, WL and BPR

Commercial chickens: RS, CB and BB

4.3.3 Phylogenetic analysis

The UPGMA tree constructed from the *Ds* distances matrixes between eight chicken populations calculated from 102 indel markers in Figure 8. Satsuma's chicken populations were genetically related with each other with the smallest *Ds* value of 0.072. The UPGMA tree divides 8 chicken populations to two major clades. The first clade was composed of the improved and commercial chicken populations and the second clade was composed of Satsuma's chicken populations.

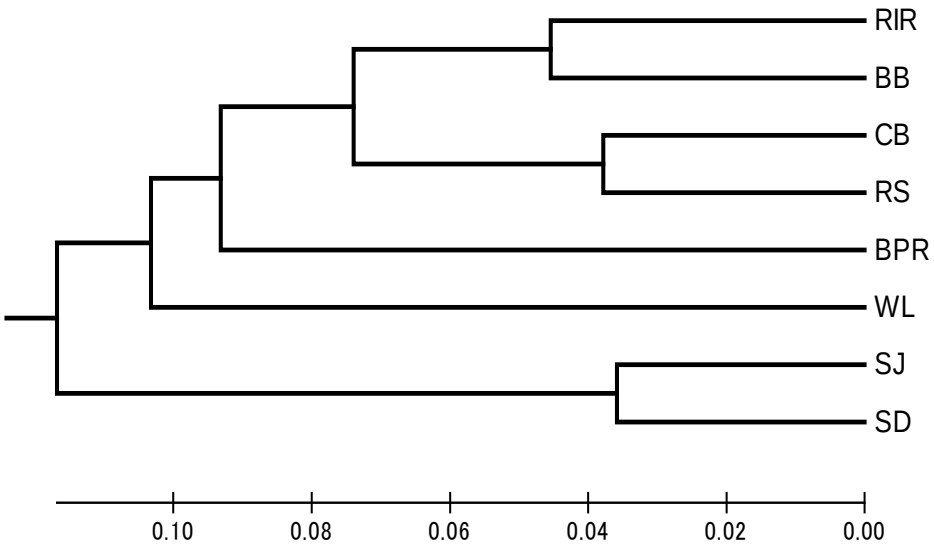

Figure 8. UPGMA tree constructed by genetic distance matrices from eight chicken populations by using 102 indel markers

SJ = Satsuma-Jidori; SD = Satsumadori; RIR = Rhode Island Red; WL = White Leghorn; BPR = Barred Plymouth Rock; RS = Ross; CB = Cobb; BB = Boris Brown

4.3.4 Population substructure by using PCA

The 2-D scatter plot of the first two principal components (PCs) was given in Figure 9. Contribution ratios of PC1 and PC2 were 13.8 and 10.7 %, respectively.

By considering PC1 and PC2 cumulatively, individuals were grouped, corresponding to their respective populations. In addition, Satsuma's chicken populations showed admixture whereas RIR did not show admixture to SJ. The two commercial chickens (RS and CB) were closely positioned near each other, which was consistent to UPGMA tree in Figure 8.

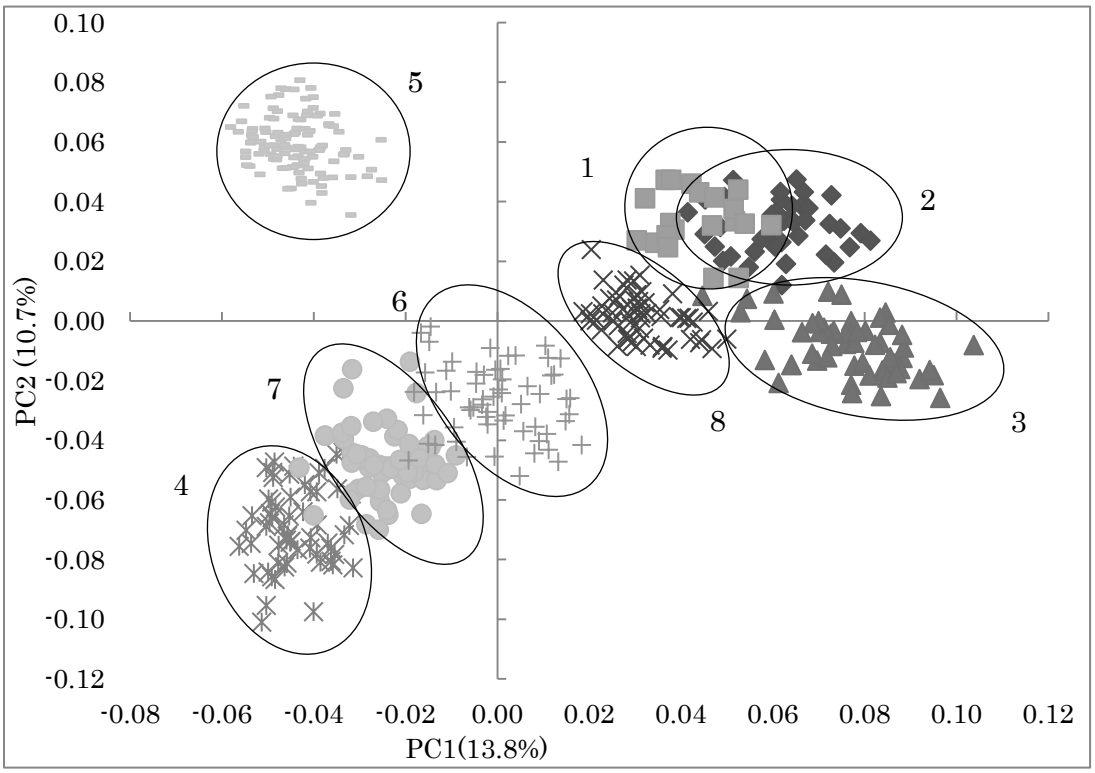

Figure 9. 2 - D scatter plot of the first two principal components (PC1 and PC 2) for 474 individuals of eight chicken populations based on 102 indels genotypes.
1 = Satsuma-Jidori; 2 = Satsumadori; 3 = Rhode Island Red; 4 = White Leghorn;
5 = Barred Plymouth Rock; 6 = Ross; 7 = Cobb; 8 = Boris Brown

4.4. Discussion

The present chapter examined the genetic diversity of eight chicken populations by using 102 indels polymorphisms as genetic markers. Out of 102 indels loci, 98 were polymorphic over the whole populations. This was almost the same number to the

previous study (Maw *et al*., 2012). The MAF percentage in present study was close to the previous study of Väli *et al.* (2008); using indels as genetic markers in wolf populations showed 74% of the polymorphic loci had MAF of >10% and 49% of >20%.

The genetic variability based on *Ppoly* and heterozygosities was higher in commercial (RS, CB and BB) and SJ than improved (RIR, WL and BPR) and SD chickens populations. These results support hybridization of breeds when establishing commercial chickens and SJ by interbreeding between SD and RIR. In addition, the layers (WL and BB) showed lesser genetic variability than the broilers (CB and RS). These results were consistent with those obtained by microsatellites and SNPs in earlier studies (Crooijmans *et al*., 1996; Vanhala *et al*.,1998; Hillel *et al*., 2003; Shimogiri *et al.*, 2011).

For genetic relationships, the UPGMA dendrogram showed that the Satsuma's chickens and the group of chickens representing improved and commercial chicken populations were genetically distant with each other (Figure 8). The highest G_{ST} value (0.329) supported their genetic relationship. The G_{ST} value (0.289 and 0.305) was also high among the improved and commercial chicken populations. These results might be the result of intensive selection to fix desirable traits during the process of improvement in commercial chickens. Satsuma's chickens were shown as closely related to each other in the dendrogram. However, RIR, one of the parental breeds of SJ, was genetically distant from the Satsuma's chickens in the dendrogram

(Figure 8). The similar results were obtained from the 2-D plot of PCA analysis (Figure 9) where individuals from RIR population were not showed admixture to SJ. These may be resulted from differences in sample collection: The SD samples were taken from a parental stock of SJ in KIAD while RIR were not.

In the Japanese chicken market, some brand chickens are directly associated with pure breeds or particular hybrids. Therefore, DNA test for breed or brand identification is realistic and desirable for types of brand chicken. In the report of Takahashi *et al*., (2007) five microsatellite markers which have fixed allele in Nagoya chicken were used to discriminate between Nagoya breed and all other chicken. Rikimaru and Takahashi (2007) reported the use of 14 microsatellite markers, which were fixed in Hinai-jidori to distinguish Hinai-jidori chicken (Hinai-dori X Rhode Island Red) from other chicken breeds. Nakamura *et al*. (2010) stated that the utilization of one fixed fragment size in five microsatellite markers to check the authenticity of Nagoya breed-labeled chicken on the market. Currently, there is no application on indel markers for breed identification. In this chapter, 36 of the 102 markers were fixed in the SJ chickens, some of which were not fixed in other chickens. This finding may provide a possibility for distinguishing the SJ from others.

In summary, the results reported here were in good reflection to the characteristics of populations and agreement to previous reports. Therefore, indels polymorphisms were convenient and useful markers to assess the genetic diversity and genetic relationships of chicken populations.

CHAPTER 5

The genetic diversity and population structure in Thai native chickens, crossbred chickens and Red Junglefowls by using 102 indel markers

5.1. Introduction

Domestication of chickens was believed to originate from Red Jungle fowl in South East Asia (Nishibori *et al*., 2005, Sawai *et al*., 2010). Thai native chickens may be regarded as much diversified populations due to long-term adaptation with respond to varied agro-ecological zones. Thailand is situated in the mainland Southeast Asia, lying between 5° to 20° North and 97° to 105° East. The country's area is 514,000 km^2; about 70 percent of which is used for agriculture. The climate is tropical with relatively high temperatures (24 - 36 °C) and high humidity (66–83 percent). Currently, about six million households, or 50 percent of Thai keep indigenous chicken at home. Each family produces 30-50 birds of marketable size annually, which represents 100-120 million birds for country as a whole (Choprakarn, 2007). Thai native chicken strains are classified by feather colour into black, yellow, red, white, green, grey, bronze and stripe types. The more common rooster strains in all regions of Thailand are the black and yellow types.

Jungle fowl are the bird from the genus *Gallus* in the *Gallinaceous* bird order. There are four species of Jungle fowl. They are Red Jungle fowl, *Gallus gallus*, Ceylonese Jungle fowl, *Gallus lafayetii*, Grey Jungle fowl, *Gallus sonneratii* and Green Jungle fowl, *Gallus varius*. The Red Jungle fowl is well known as the most important ancestor of domestic chicken in four

species of Jungle fowls (Hashiguchi *et al*., 1981, 1993, Okada *et al*., 1984, Yamashita *et al.*,1994, Akishinonomiya *et al*., 1996). They often inhabit the sparse forest near a cultivated land. Since early 20 century, the habitat of Red Jungle fowl has been reported in the Indian sub-continent, the Southeast Asia, China, Nepal, Bhutan, Maley archipelago, Indonesian Island of Sumatra, Java, Bali, Sulawei, Philippines and South Pacific Islands (Nishida, 1974). The Red Jungle fowl was further divided into five subspecies, *Gallus gallus gallus, Gallus gallus murgi, Gallus gallus spadiceus, Gallus gallus bankiva, Gallus gallus jabouillei,* based on morphological features and geographical distribution (Johnsgard, 1999). Ceylonese and the Green Jungle fowl do not usually produce fertile hybrids with the Red Jungle fowl, suggesting that it is the sole ancestor of the domestic chicken. However, recent research has revealed the absence of the yellow skin gene in the wild Red Jungle fowl found in domestic birds, which suggests hybridisation with the Grey Jungle fowl during the domestication of the species (Eriksson *et al.,* 2008). Purebred Red Jungle fowl are thought to be facing a serious threat of extinction because of hybridization at the edge of forests where domesticated free ranging chickens are common.

In Thai and Philippine, the captured Red Jungle fowls originated in wild have been generally crossed to native chicken in order to use for cock fighting and as a source of game bird for enjoyment (Nishida *et al.*, 2000). Because free scavenging chickens can easily be reproduce with wild ones and which may lead to increase genetic diversity in Thai native chicken populations.

Molecular tools offer a new approach to investigate both the genetic diversity and phylogenetic relationship among the subspecies of *Gallus gallus* and domestic chicken. The genetic characterization of Thai indigenous chickens and commercial chicken has been assessed by 20 microsatellite loci (Dorji *et al.*, 2010). The genetic variation and phylogeographic analysis of native chicken populations from Myanmar and Thailand has been studied by using 98 autosomal SNP markers (Riztyan *et al*., 2012).

The goals of this chapter were to assess the genetic diversity and to clarify the population structure in native chickens and crossbred chickens from Thai and Red Jungle fowl from Thai and Vietnam. Analysis of indels polymorphisms will perform by using102 indel markers developed in Chapter 2 .

5.2. Materials and methods

A total number of 185 blood samples were collected from nine experimental populations, including three populations each of native chickens and crossbred chickens populations and one population each of three Red Jungle fowl's subspecies. Crossbred chickens were assumed as hybrid chicken obtained by hybridization between native chicken and Jungle fowls. Three subspecies of Red Jungle fowl were *Gallus gallus spadiceus*, *Gallus gallus jabouilei* and *Gallus gallus gallus*. The blood samples for native and hybrid chicken populations were collected from Trat and Chantaburi (Eastern area), Kanchahaburi (Western area) and from Nan, Chiang Rai, Lampang (Northern area). The sampling area, abbreviation and number of sample in each population are presented in Table 8. A map of Thai showing sampling area was given in

Figure 10. Genomic DNA was extracted from blood samples using standard phenol- chloroform extraction protocols (Sambrook *et al.*, 1989). The genotyping method for 102 indel markers was the same to Chapter 2.

Table 8. Sampling areas, populations and their abbreviation

Area	Population	Abbreviation	No. of Sample
Eastern Thailand	Native Chicken	EN	34
Western Thailand	Native Chicken	WN	5
Northern Thailand	Native Chicken	NN	34
Eastern Thailand	Crossbred Chicken	EC	15
Western Thailand	Crossbred Chicken	WC	35
Northern Thailand	Crossbred Chicken	NC	19
Northern Thailand Hanoi-Vietnam	*Gallus* gallus spadiceus	GS	21
Eastern Thailand Hanoi-Vietnam	*Gallus gallus gallus*	GG	7
Hanoi-Vietnam	*Gallus gallus jabouilei*	GJ	15
	Total		185

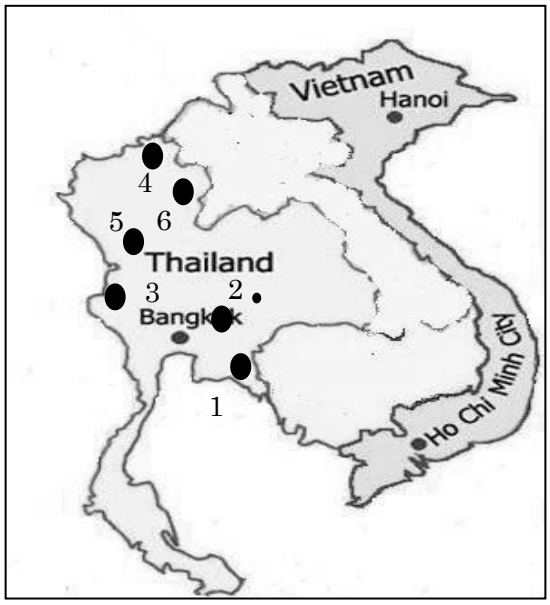

Figure 10. A map of Thai showing sampling area
1: Chanthaburi, 2: Trat, 3: Kanchanaburi, 4: Chiang Rai, 5: Lampang, 6: Nan

5.2.1. Statistical analysis

The genetic variability of each population was assessed by calculating the proportion of polymorphic loci (*Ppoly*: Lewontin and Hubby, 1966), and average observed ($\overline{H}_O$) and expected heterozygosity ($\overline{H}$ E) (Nei, 1978). Chi square (χ^2) approximation was used to test Hardy-Weinberg Equilibrium (HWE) (Weir, 1996) and calculate the degree of genetic differentiation (G_{ST}) (Nei, 1973). The gene flow between populations was expressed as the number of migrants per generation (*Nm*), estimated by ' *n* ' Island model of population structure (Slatkin, 1995). Using *Fstat* version 2.9.3.2, pair wise distance matrix (F_{ST}) values (Weir and Cockerham, 1984) was estimated . To construct neighbor joining (NJ) tree implemented by MEGA 4 (Tamura et al., 2007), computed the pair wise standard genetic distance (*Ds*) (Nei, 1972) using PHYLIP ver. 3.69 (Felsenstein, 2009). R^2 was calculated to determine whether a tree's topology and branch lengths accurately reflect the genetic relationship among nine experimental populations (Klinowski, 2009).

Population structure was investigated by using Bayesian clustering algorithm implemented in the STRUCTURE 2.3.3 application (Pritchard *et al.*, 2000). A burn-in period of 20,000 was applied and followed by 20,000 iterations of Markov chain Monte Carlo (MCMC) inference (Pritchard *et al*., 2000). The probable number of genetic clusters (*K*) was set to the range of $1 \leq K \leq 15$, with 20 independent run for each value of *K*. For the inference of *K* value, the estimated likelihood of the probability of data Ln Pr (X/ *K*) and the second order rate of change of the likelihood function with respect to *K* (ΔK) was used. The ΔK value was calculated using Structure Harvester 0.56.3 application (Riztyan *et al*., 2011). The optimal *K* value was

determined by the greatest value of ΔK value as described in *Evanno et al*. (2005). The DISTRUCT 1.1 application (Rosenberg, 2004) was used to display the probabilities for population subdivisions. Subdivisions were represented as colors and individuals were depicted as bar portioned in to color segments corresponding to the membership coefficients for the populations.

5.3. Results

5.3.1. Indels polymorphisms and genetic variability

The mean (± S E) *Ppoly*, $\overline{H}_O$ and $\overline{H}_E$ value of each population were listed in Table 9. All of 102 indels loci (*Ppoly* = 100%) were polymorphic as total populations. The *Ppoly* was almost the same in all experimental populations (0.913±0.029 to 0.989±0.011) except GG population which showed the lowest *Ppoly* (0.685±0.048). The $\overline{H}_O$ and $\overline{H}_E$ were ranged from 0.197±0.004 to 0.254±0.005 and 0.340±0.004 to 0.392±0.004. WC showed the highest H_O (0.254±0.005) and EC and NC showed the highest H_E (0.392±0.004). GG showed the lowest $\overline{H}_O$ and $\overline{H}_E$ (0.154 and 0.259) among all experimental populations. Tests of Hardy-Weinberg equilibrium (HWE) over all experimental populations and within populations indicated that all of the populations were in HWE.

The degree of genetic differentiation (G_{ST}) in crossbred chicken populations was very low (0.039) whereas native chickens and three Red Jungle fowl's subspecies showed moderate differentiation (0.088 and 0.091) in Table 9. The G_{ST} observed between native chickens and crossbred chickens (0.089) was lower than the G_{ST} between crossbred chickens and Red Jungle fowl (0.147). The highest genetic differentiation (0.152) occurred between native chicken

populations and Red Jungle fowl's subspecies. The *GST* among nine populations was calculated as 0.139.

Table 9. Genetic variability of native chicken, crossbred chicken and three Red Jungle fowl's subspecies

Population	No of Sample	*Ppoly*	± SE	$\overline{H}_O$	± SE	$\overline{H}_E$	± SE	*GST*
EN	34	0.935	0.026	0.221	0.005	0.369	0.004	
WN	5	0.967	0.018	0.212	0.004	0.381	0.004	0.139
NN	34	0.946	0.024	0.205	0.005	0.340	0.004	
EC	15	0.967	0.018	0.197	0.004	0.392	0.004	
WC	35	0.913	0.029	0.254	0.005	0.374	0.005	
NC	19	0.989	0.011	0.211	0.004	0.392	0.004	
GS	21	0.946	0.024	0.231	0.005	0.366	0.004	
GJ	15	0.924	0.027	0.218	0.005	0.342	0.004	
GG	7	0.685	0.048	0.154	0.005	0.259	0.005	
Among native chicken populations								0.088
Among crossbred chicken populations								0.039
Among Red Jungle fowl populations								0.091
Between native chickens and crossbred chickens								0.089
Between native chickens and Red Jungle fowls								0.152
Between crossbred chickens and Red Jungle fowls								0.142

EN = Native chicken from East; WN = Native chicken from West; NN = Native chicken from North; EC = Crossbred chicken from East; WC = Crossbred chicken from West;
NC= Crossbred chicken from North; GS= *Gallus gallus spadiceus*; GJ= *Gallus gallus jabouilei*; GG= *Gallus gallus gallus*

5.3.2. Gene flow and phylogenic analysis

Estimated gene flow (*Nm*) and pair wise distance matrix (*FST*) between each populations pair are presented in Table 10. The *Nm* values ranged from 1.9 to 3.51 among native chicken populations, from 2.5 to 5.7 among crossbred chicken populations and from 4.1 to 22.1 among Red Jungle fowl's subspecies. The *FST* values ranged from 0.066 to 0.115 among native

chicken populations, from 0.042 to 0.088 among crossbred chicken populations and from 0.0112 to 0.091 among Red Jungle fowl's subspecies. The *FST* distance matrices between native chicken and crossbred chicken populations (0.066 to 0.185) were smaller than the matrices between native chicken populations (0.102 to 0.269) and Red Jungle fowl's subspecies. In addition, *FST* distance matrices between crossbred chicken and Red Jungle fowl's subspecies was 0.139 to 0.245. Among Red Jungle fowl's subspecies, GS showed the largest *Nm* (1.265 to 2.199) and smallest *FST* (0.102 to 0.169) to native chicken and crossbred chicken populations.

The NJ tree constructed from the Nei's genetic distance matrix between the nine populations gave two major clades as shown in Figure 11. The first clade was composed of native chickens, crossbred chickens and GS populations in which GS population inserts between NC and WC populations. The second clade was composed of GJ and GG populations and these two Red Jungle fowl's subspecies are separated from native and crossbred chicken populations. The R^2 value (0.926) was near one and represented a good summary of the genetic relationships shown in NJ tree.

Table 10. Estimated gene flow (*Nm*) and pair wise *FST* among native chicken, crossbred chicken and three Red Jungle fowl's subspecies

	EN	WN	NN	EC	WC	NC	GS	GJ	GG
EN		3.509	2.035	3.038	1.627	2.923	1.929	1.531	0.793
WN	0.066		1.923	3.532	2.371	3.110	2.199	1.759	0.930
NN	0.109	0.115		1.823	1.101	2.817	1.265	1.224	0.712
EC	0.076	0.066	0.121		2.581	5.702	1.539	1.342	0.820
WC	0.133	0.095	0.185	0.088		3.327	1.233	1.085	0.769
NC	0.079	0.074	0.082	0.042	0.069		1.503	1.259	0.835
GS	0.115	0.102	0.165	0.139	0.169	0.143		22.071	2.485
GJ	0.140	0.124	0.169	0.157	0.187	0.166	0.011		4.083
GG	0.239	0.212	0.260	0.234	0.245	0.231	0.091	0.058	

The data above diagonal are estimated gene flow (*Nm*) and lower diagonal are pair wise *FST* among nine experimental populations

EN = Native chicken from East; WN = Native chicken from West; NN = Native chicken from North; EC = Crossbred chicken from East; WC = Crossbred chicken from West; NC = Crossbred chicken from North; GS = *Gallus gallus spadiceus;* GJ = *Gallus gallus jabouilei;* GG = *Gallus gallus gallus*

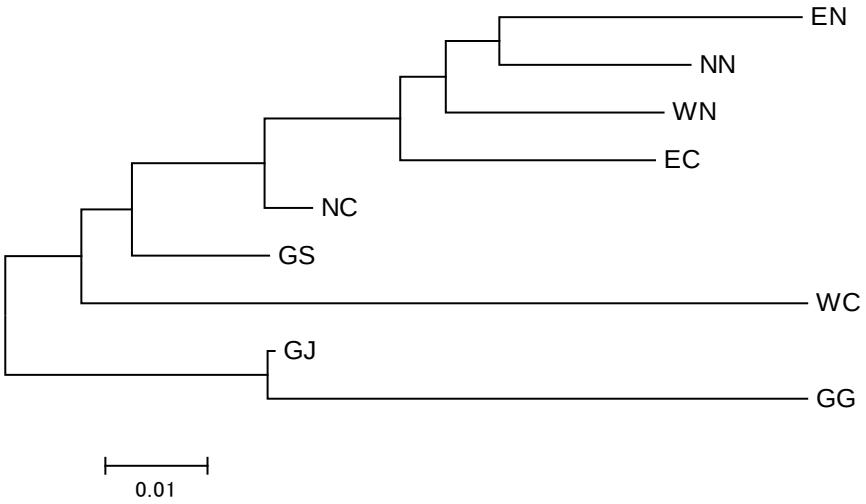

Figure 11. NJ tree constructed by genetic distance matrices from nine experimental populations

EN = Native chicken from East; WN = Native chicken from West; NN = Native chicken from North; EC = Crossbred chicken from East; WC = Crossbred chicken from West; NC = Crossbred chicken from North; GS = *Gallus gallus spadiceus;* GJ = *Gallus gallus jabouilei;* GG = *Gallus gallus gallus*

5.3.3. Population substructure

The STRUCTURE analysis revealed that the Ln Pr (X/ *K*) increased sharply at *K*= 2 and *K*=5 with the maximum likelihood value of – 19843.48 and – 18387.14. This results indicated that the appropriate value of *K* would be between *K*=2 and *K*= 5. According to the method of Evanno *et al*., (2005) for the inference of optimal *K* values, the *K* value was calculated to be two and five. Their maximum value for the *ad hoc* statistic Δ*K* were 31.8 and 22.97, this values were greater than Δ*K at K=3* and *K*= 4 by 15.76 and 0.317, respectively (Figure 12). Therefore the probable number of clusters (*K*) was thought to be two or five. The contribution of two and five ancestral populations (*K*= 2 and *K*= 5) to the nine populations is presented in Figure 2. At *K*=2, the proportion of membership which suggested a common origin were detected in native chicken and crossbred chicken populations. Except GG, GS and GJ populations share the origin with native and crossbred chicken. At *K*=5, native chicken, crossbred chicken and Red Jungle fowls were seem to be different origins in which NN population may be different origin to EN and WN. Population substructure occurred in EC and present of admixture among native chickens, crossbred chickens and Red Jungle fowls populations. However, GG showed very low level of admixture to other populations.

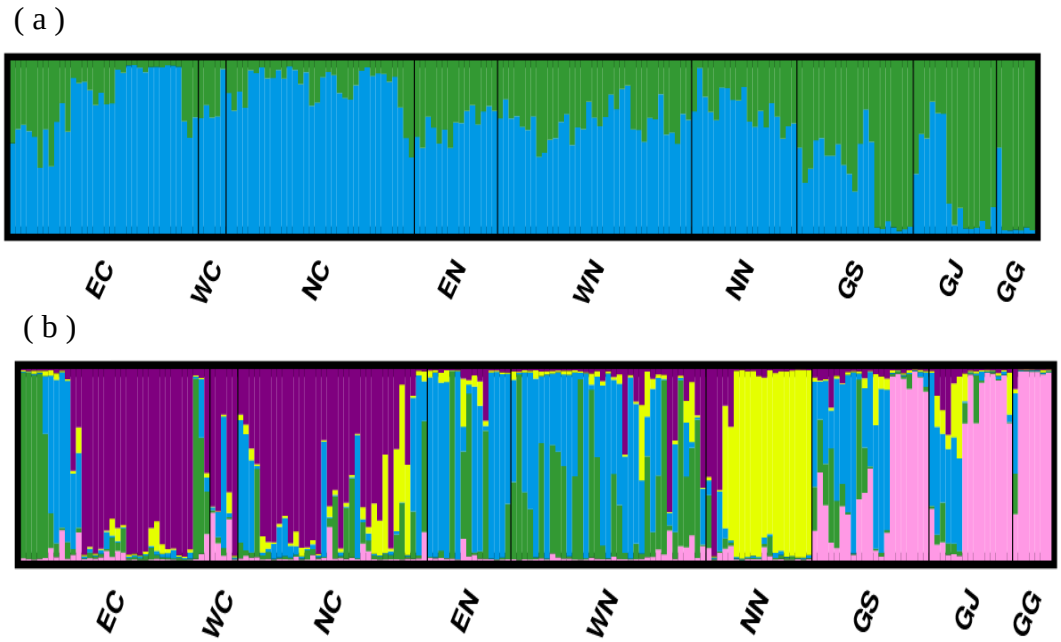

Figure 12. Individual assignment according to the estimated membership coefficient at *K*=2 and *K*=5; (a) *K*= 2, Δ *K* = 31.8, (b) *K*= 5, Δ *K* = 22.97
(Abbreviations for populations are the same as in Table 8).

5.4. Discussion

In this chapter, the genetic diversity of native chicken, cross and Red Jungle fowl populations was examined. All of 102 indels loci were polymorphic over the whole populations. This was a little higher than our previous chapter in Myanmar and Indonesia native chickens demonstrated 97 loci (in Chapter 3). The *Ppoly* and $\overline{H}_E$ of native and crossbred chicken populations exceeded the previous value stated in Ritzyan *et al.*, 2012. According to the $\overline{H}_O$ and $\overline{H}_E$ values, crossbred chickens are more diversified populations than other populations. The genetic variability in three Red Jungle fowl's subspecies was much higher than our previous study of indels polymorphisms in *Gallus gallus bankiva* and *Gallus gallus varius* (*Ppoly* = 0.218 and 0.039 ; $\overline{H}_E$ = 0.078 and 0.012) (Maw *et al.*, 2012) . In addition, the Red

Jungle fowls populations (except GG) demonstrated similar *Ppoly*, $\overline{H}_O$ and $\overline{H}_E$ values to native and crossbred chicken populations. These may suggest that the indels polymorphisms examined here have been occurred in three Red Jungle fowl's subspecies and they may be the closer ancestor of domestic chicken than *Gallus gallus bankiva* and *Gallus gallus varius*. The genetic differentiation between native chicken and crossbred chicken populations was (G_{ST} = 0.089) lower than between crossbred chicken and Red Junglefowls (G_{ST} =0.142). This was indicating that the crossbred chicken populations were genetically closer to native chicken populations than Red Junglefowls.

In NJ tree, the genetic make up of native chicken and crossbred chicken populations were closely related and GS may shared the genetic make up with domestic chickens (Figure 11). The lower F_{ST} and high *Nm* values between native and crossbred chicken populations provide their close genetic relationship. The GS demonstrate low F_{ST} and high *Nm* values to native and crossbred chickens than GJ and GG populations. The Phylogenetic relation in present study was similar to the NJ tree of Bao *et al* ., 2008 . In their NJ tree, *Gallus gallus spadiceus* showed closer phylogenetic relationship with Chinese domestic chicken breeds than *Gallus gallus gallus*. Also in STRUCTURE analysis, both at *K*=2 and *K*=5, GG was not share the origin to native and crossbred chickens whereas GS and GJ share the origin and present of genetic admixture to native and crossbred chicken populations (Figure 12). This may be due to the very wide inhabitant area of GS provides the greater opportunity to be domestication by human. Because GS habitat in southern, northern and western area of Thai. GG habitat only in eastern and central area of Thai where is not inhabitant area for GJ (Nishida *et al*., 2000).

The analysis of 102 indels polymorphisms in present chapter showed that native chicken, crossbred chicken and three Red Jungle fowl's subspecies have great quantity of genetic diversity.

The genetic variability is higher among crossbred chicken populations than other populations. The highest genetic differentiation occurred between native chicken populations and three Red Jungle fowl's subspecies. Native chicken and crossbred chicken showed close genetic relation and GS showed closer genetic relation to native and crossbred chickens than GJ and GG. It was found that the present of common origin and genetic admixture occurred among native chickens, crossbred chickens, GS and GJ populations.

CHAPTER 6

Genetic diversity and phylogenetic relation of various chicken populations

6.1. Background and previous study

It was reported that chickens became domesticated from Red Jungle fowl in South East Asia (Nishibori *et al*., 2005, Sawai *et al*., 2010). The domestication of chicken has been continued by the extensive breeding programs which results in many breeds of chickens. These chicken breeds can be typed into four distinct types i.e. Egg-type, game type, meat-type and bantam type (Moiseyeva *et al* ., 2003).

In Chapter 3, the genetic diversity of Myanmar and Indonesia native chickens together with two jungle fowl species was analysed. It was found that the native chickens from two countries were genetically close to each other and remote from Red and Green jungle fowl from Java Island. In Chapter 4, the genetic diversity of eight chicken populations representing chickens from Satsuma region, commercial and improved chickens was examined. The genetic variability based on *Ppoly* and heterozygosities was higher in commercial chickens and hybrid chickens than improved chicken breeds and local variety chickens. The layers showed lesser genetic variability than the broilers. In Chapter 5, the genetic variability and population structure of native chickens and crossbred chickens from Thai as well as Jungle fowl from Thai and Vietnam was examined. The genetic variability is higher in crossbred chicken populations than other populations. The study revealed that the native chickens and crossbred chicken populations was genetically close and they share the genetic make up to *Gallus gallus*

*spadiceus p*opulation.

However, it was important to explore the over all genetic variability and genetic relationship among these various chicken populations of native chickens, local variety, improved chicken and Jungle fowls stated as above. In this Chapter, to summarize the genetic differentiation and phylogenetic relationship of various types of chicken populations, the indels polymorphisms in various native chicken populations from South East Asia, two chicken populations from Japan, improved chicken breed, four subspecies of Red Jungle fowl and Green Jungle fowl will analyze.

6.2. Materials and methods

In this analysis, the indels data from Chapter 3, 4 and 5 was summarized and then the indels data of Lao's native chicken populations was added. Totally 14 chicken populations were used as materials in which four native chicken populations, two chicken populations from Japan, three breeds of improved chickens, four subspecies of Red Jungle fowls and Green Jungle fowl. Four native chicken populations were native chickens from Myanmar, Thai, Laos and Indonesia. Satsumadori and Satsuma-jidori chickens from Japan and they are referred to as Satsuma's chicken later. Three improved chicken breeds were Rhode Island Red, White Leghorn and Barred Plymouth Rock. Four subspecies of Red Jungle fowls were *Gallus gallus spadiceus, Gallus gallus gallus, Gallus gallus jabouilei and Gallus gallus bankiva.*

The abbreviation for each population and the number of birds in each population were presented in Table 11. The same procedures of statistical analysis in Chapter 3 were used.

Phylogenetic tree was constructed by using unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal, 1973) implemented by MEGA software ver.4.1 (Tamura *et al.*, 2007).

6.3. Results

6.3.1. Indels polymorphisms and genetic variability

The genetic variability of each population were listed in Table 11. The *Ppoly* in native chickens, Satsuma's chickens and improved chicken breeds were ranged from 0.5± 0.049 to 0.978± 0.015. The average $\overline{H}_O$ and $\overline{H}_E$ were ranged from 0.147±0.004 to 0.295±0.004 and 0.175±0.004 to 0.416±0.004. Thai native chicken population showed the highest *Ppoly,* $\overline{H}_O$ and $\overline{H}_E$ out of all populations. The *Ppoly,* $\overline{H}_O$ and $\overline{H}_E$ were higher in native chicken populations from four countries than Satsuma's chicken and improved chicken breeds. In Jungle fowl population, GS, GJ and GG showed similar *Ppoly,* $\overline{H}_O$ and $\overline{H}_E$ values to native chicken populations and it is very much higher than those of GB and GV (Table 11). GB and GV showed the lowest *Ppoly,* $\overline{H}_O$ and $\overline{H}_E$ among all experimental populations.

The *GST* for total populations was calculated as 0.101and the highest *GST* was observed among improved chicken breeds (0.332) and among Jungel fowl populations (0.417). The *GST* among native chicken populations was 0.045. The *GST* in native chicken populations and Satsuma's chickens were ranged from 0.024 to 0.124. Laos native chicken populations revealed the lowest *GST* (0.024) and Satsuma's chicken populations revealed the highest *GST* (0.124).

Table 11. The genetic variability from 14 populations of various chickens and Jungle fowls

Population	Abbrevi-ation	No. of Birds	*P poly*	± *SE*	$\overline{H}_O$	± *SE*	$\overline{H}_E$	± *SE*	*GST*
Myanmar	MN	80	0.921	0.027	0.230	0.008	0.268	0.008	0.041
Thai	TH	73	0.978	0.015	0.295	0.004	0.416	0.004	0.088
Laos	LO	136	0.905	0.029	0.257	0.008	0.310	0.004	0.024
Indonesia	ID	136	0.941	0.024	0.229	0.004	0.291	0.005	0.081
Satsumadori	SD	20	0.500	0.049	0.147	0.004	0.175	0.004	
Satsuma-jidori	SJ	40	0.608	0.048	0.196	0.004	0.212	0.004	
White Leghorn	WL	60	0.598	0.048	0.203	0.005	0.192	0.004	
Rhode Isalnd Red	RIR	55	0.618	0.048	0.153	0.004	0.177	0.004	
Barred Plymouth Rock	BPR	119	0.549	0.049	0.160	0.004	0.176	0.004	
G.g. spadiceus	GS	21	0.946	0.024	0.231	0.005	0.366	0.004	
G.g.jabouilei	GJ	15	0.924	0.027	0.218	0.005	0.342	0.004	
G.g.gallus	GG	7	0.685	0.048	0.154	0.005	0.259	0.005	
G.g.bankiva	GB	3	0.218	0.041	0.101	0.005	0.078	0.004	
G.varius	GV	6	0.039	0.019	0.003	0.002	0.012	0.003	
Native chickens									0.045
Native and Satsuma's									0.120
Improved chickens									0.289
Satsuma chickens									0.124
Jungle fowls									0.417
Total									0.101

6.3.2. Phylogenic analysis

The UPJMA tree constructed from the *Ds* distances divided 14 populations into two major clades (Figure 13). The first clade was composed of native chickens, Satsuma's chickens, improved chickens and three subspecies of Red Jungle fowls (GS, GJ and GG). The second clade was composed of another subspecies of Red Jungle fowl (GB) and Green Jungle fowl (GV), which was located outside the first clade. In first clade, native chicken populations

from Myanmar, Thai, Laos and Indonesia as well as GS, GJ and GG were formed a large cluster. Whereas Satsuma's chickens and improved chicken breeds were formed another cluster and they were remote from Asian's native chickens and Red Jungle fowl populations (GS, GJ and GG).

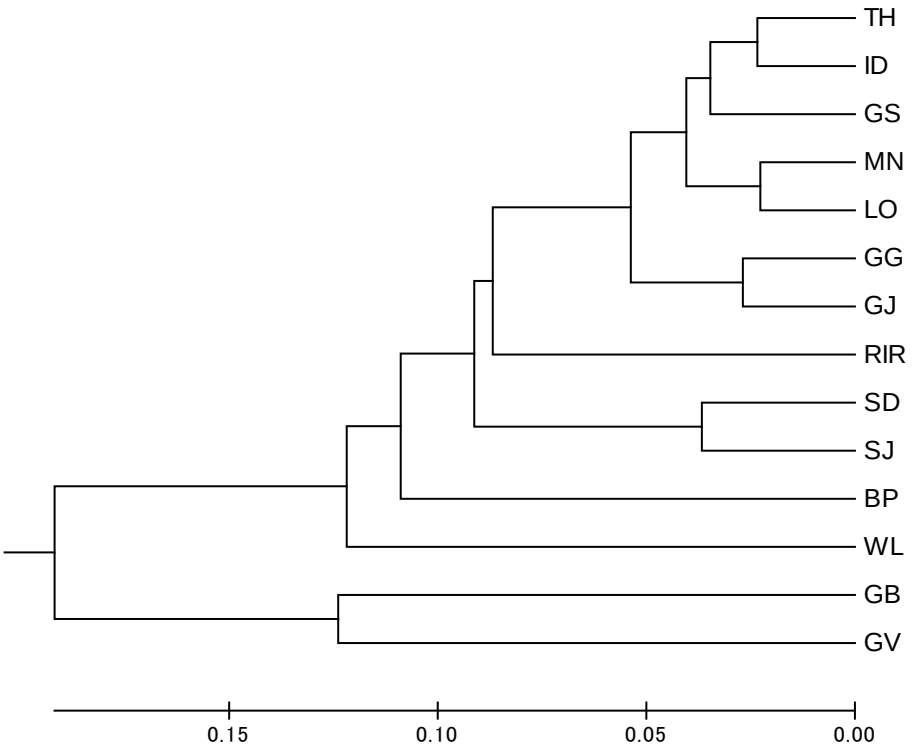

Figure 13. UPGMA tree constructed by genetic distance matrices from14 populations of chickens and Jungle fowl

TH = Thai native chickens; ID = Indonesia native chickens; MN = Myanmar native chickens; LO = Laos native chickens; SD = Satsumadori ; SJ = Satsuma-jidori; RIR = Rhode Island Red; BP = Barred Plymouth Rock; WL = White Leghorn; GS = *Gallus gallus spadiceus*; GJ = *Gallus gallus jabouilei*; GG = *Gallus gallus gallus*; GB = *Gallus gallus bankiva*; GV = *Gallus varius*

6.4. Discussions

6.4.1. Genetic variability

This chapter examined the genetic diversity of various populations by using 102 indels polymorphisms as genetic markers. The results revealed that the *Ppoly*, $\overline{H}_O$ and $\overline{H}_E$ of native chicken populations from South East Asia were very close to each other. It was suggest that the indels polymorphisms in South East Asia's native chicken populations were very similar and the degree of genetic differentiation (G_{ST}) among them was small enough (0.045) to indicated that they are genetically close populations. However, the indels polymorphisms in Satsuma's chickens and improved chickens were different from that of native chicken populations. Because Satsuma's chickens and improved chickens showed lower *Ppoly*, $\overline{H}_O$ and $\overline{H}_E$ than native chicken populations. This was indicated that the genetic variability in native chicken populations was higher than Satsuma's chickens and improved chicken populations and it was agreed to Kinoshita *et al.*, 2004 stated that the heterozygosity values for native chicken tend to higher than for improved breeds in their study of egg white protein polymorphisms estimated from native fowl populations in Asia. Furthermore, the G_{ST} estimated from native chicken and Satsuma's chicken was 0.120, which referred that they are moderately different populations. The high G_{ST} was observed in improved chicken populations (0.332) and it might be concerning to the intensive selection to fix desirable traits during the process of improvement in domestic chicken.

Among Jungle fowl, except GG population, GS and GJ demonstrated high *Ppoly*, $\overline{H}_O$ and $\overline{H}_E$ that was close to those of native chicken populations. Therefore, the genetic variability of

GS and GJ was generally similar to that of native chicken populations and these three subspecies of Red Jungle fowls were not much genetically different populations (G_{ST} = 0.091).

Whereas the indels polymorphisms in GB and GS were very different to native chicken populations as they showed the lowest genetic variability among 14 populations. In addition, GB and GS were genetically different from the rest of Jungle fowl populations (GS, GJ and GG) because the G_{ST} among five Jungle fowl populations was very high (0.417).

6.4.2. Phylogenetic analysis

The topology of UPGMA tree demonstrated the genetic relationship of various populations of native chicken, local variety, improved chicken and Jungle fowl. The UPGMA tree in present analysis reflected that the native chicken populations were genetically close populations and they were closely related to GS, GJ and GG. Therefore, GS, GJ and GG, especially GS were concerning to the domestication of chickens. Satsuma's chickens and improved chickens were remote from native chickens and GS, GJ and GG, telling that their genetic relationship to native chickens and GS, GJ and GG were not close. On the other hand, GB and GS were not only separate away from other Red Jungle fowl populations but also away from native and improved chicken breeds. This was consistent to the previous studies of Yamashita *et al.* (1994) and Okumura *et al.* (2006). In NJ tree of Niu *et al.* (2002), domestic fowls belonged to the same cluster as *Gallus gallus gallus* and *Gallus gallus spadiceus* in Thailand and its adjacent areas, whereas *Gallus gallus bankiva* from Indonesian Island formed a separate cluster. Furthermore Akishinonomiya *et al* (1994) stated that the domestic fowl from Indonesian Island had large genetic differences compared with *Gallus gallus bankiva* from the same place.

The present analysis examined the genetic diversity of various chicken and Red Jungle fowl population by using indels polymorphisms as genetic marker. The results revealed that native chicken populations from South East Asia and three subspecies of Red Jungle fowl (*Gallus gallus spadiceus*, *Gallus gallus jabouilei*, *Gallus gallus gallus*) were genetically rich diversified populations and they were genetically close to each other. According to the results of present analysis, domestication of the chicken might start from Red Jungle fowl and *Gallus gallus bankiva* and *Gallus varius* may not contribute to domestication. However, one subspecies of Red Jungle fowl (*Gallus gallus murghi*) not included in present analysis. Therefore, it is suggest that phylogenetic relationship of all Red Jungle fowl subspecies including *Gallus gallus murghi* and various chicken populations should be examine.

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Appendix 1. PCR mixture for each marker

Marker No	D/W µl	Buffer µl	dNTP µl	Primer-f µl	Primer-r µl	Taq µl	DNA µl
m 1	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 2	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 3	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 4	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 5	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 6	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 7	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 8	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 9	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 10	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 11	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 12	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 13	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 14	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 15	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 16	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 17	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 18	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 19	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 20	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 21	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 22	4.2	0.8	0.4	0.4	0.4	0.4	1.6
m 23	4.5	0.8	0.4	0.4	0.4	1.0	1.5
m 24	4.2	0.8	0.4	0.4	0.4	0.5	1.6
m 25	4.5	0.8	0.4	0.4	0.4	1.0	1.6
m 26	5.0	1.0	0.5	0.5	0.5	1.0	2.0
m 27	4.2	0.8	0.4	0.4	0.4	0.5	1.6
m 28	5.0	1.0	0.5	0.5	0.5	1.0	2.0
m 29	6.15	1.0	0.8	0.5	0.5	1.0	1.6
m 30	4.5	0.8	0.4	0.4	0.4	1.0	1.6
m 31	5.2	0.8	0.4	0.4	0.4	0.6	1.6
m 32	4.5	0.8	0.4	0.4	0.4	1.0	1.6
m 33	4.2	0.8	0.4	0.4	0.4	0.4	1.6
m 34	4.2	0.8	0.4	0.4	0.4	0.4	1.6
m 35	4.5	0.8	0.4	0.4	0.4	1.0	1.0
m 36	4.1	0.8	0.4	0.4	0.4	0.6	1.6
m 37	6.15	1.0	0.8	0.5	0.5	1.0	1.0

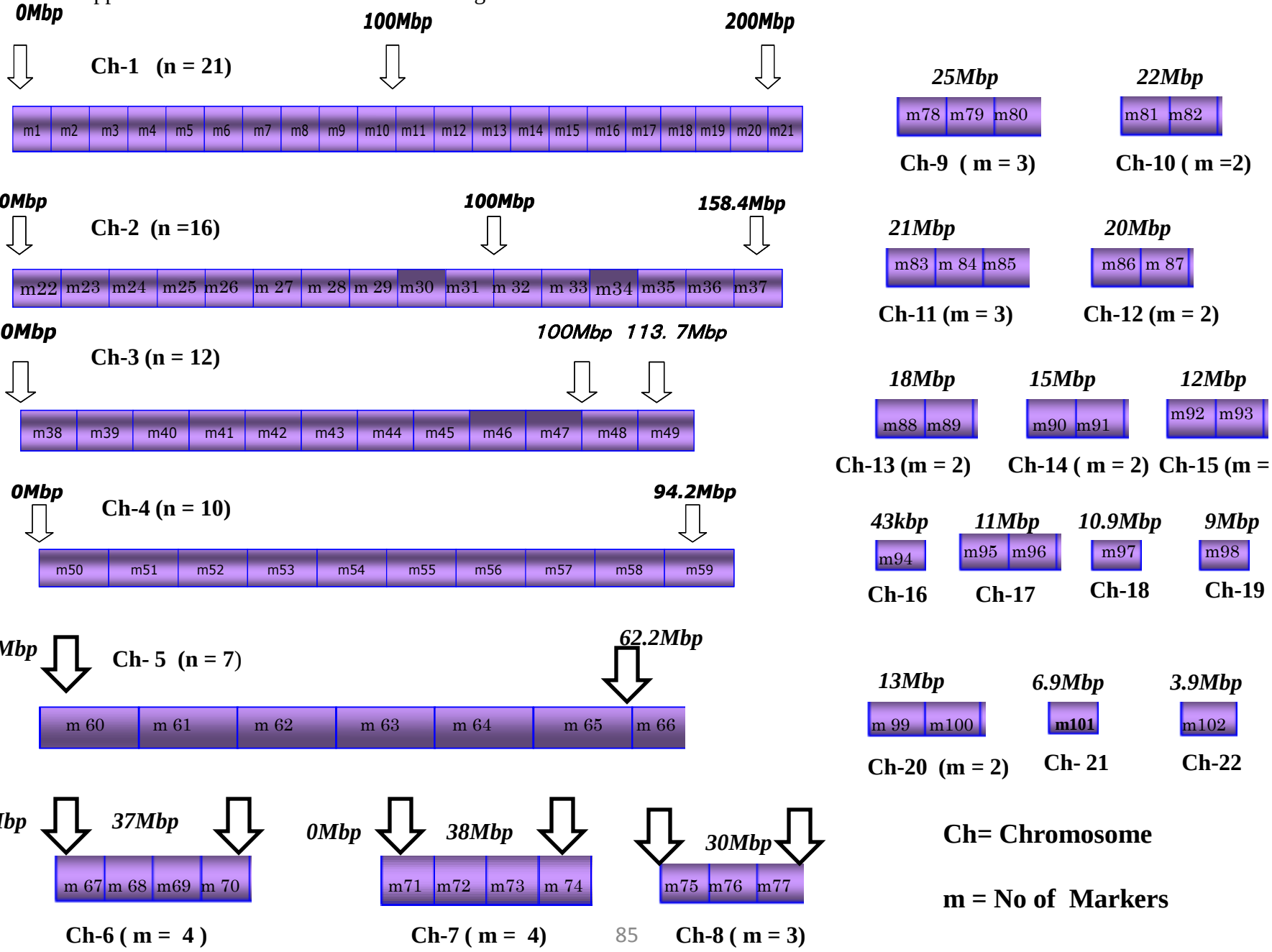
Appendix 1. Continue

Marker No	D/ W µl	Buffer µl	dNTP µl	Primer-f µl	Primer-r µl	Taq µl	DNA µl
m 38	4.0	0.8	0.4	0.4	0.4	1.0	1.6
m 39	4.0	0.8	0.4	0.4	0.4	0.8	1.6
m 40	4.0	0.8	0.4	0.4	0.4	0.8	1.6
m 41	4.2	0.8	0.4	0.4	0.4	0.5	1.6
m 42	4.2	0.8	0.4	0.4	0.4	0.5	1.6
m 43	5.0	0.8	0.4	0.4	0.4	0.5	1.6
m 44	4.0	0.8	0.4	0.4	0.4	1.0	1.6
m 45	4.5	0.8	0.4	0.4	0.4	1.0	1.6
m 46	4.5	0.8	0.4	0.4	0.4	1.0	1.6
m 47	4.5	0.8	0.4	0.4	0.4	1.0	1.6
m 48	4.5	0.8	0.4	0.4	0.4	1.0	1.6
m 49	4.5	0.8	0.4	0.4	0.4	1.0	1.6
m 50	4.5	0.8	0.4	0.4	0.4	1.0	1.5
m 51	4.5	0.8	0.4	0.4	0.4	1.0	1.5
m 52	4.5	0.8	0.4	0.4	0.4	1.0	1.5
m 53	4.5	0.8	0.4	0.4	0.4	1.0	15
m 54	4.5	0.8	0.4	0.4	0.4	1.0	1.5
m 55	4.5	0.8	0.4	0.4	0.4	1.0	1.5
m 56	4.5	0.8	0.4	0.4	0.4	1.0	1.5
m 57	4.5	0.8	0.4	0.4	0.4	1.0	1.5
m 58	4.5	0.8	0.4	0.4	0.4	1.0	1.5
m 59	4.5	0.8	0.4	0.4	0.4	1.0	1.5
m 60	4.5	0.8	0.4	0.4	0.4	0.5	1.5
m 61	4.5	0.8	0.4	0.4	0.4	0.5	1.5
m 62	4.5	0.8	0.4	0.4	0.4	0.5	1.5
m 63	4.5	0.8	0.4	0.4	0.4	0.5	1.5
m 64	4.5	0.8	0.4	0.4	0.4	0.5	1.5
m 65	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 66	4.5	0.8	0.4	0.4	0.4	0.5	1.0
m 67	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 68	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 69	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 70	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 71	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 72	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 73	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 74	5.0	0.8	0.4	0.4	0.4	0.5	1.0

Appendix 1. Continue

Marker No	D/W µl	Buffer µl	dNTP µl	Primer-f µl	Primer-r µl	Taq µl	DNA µl
m 75	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 76	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 77	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 78	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 79	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 80	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 81	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 82	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 83	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 84	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 85	5.0	0.8	0.4	0.4	0.4	0.3	1.0
m 86	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 87	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 88	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 89	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 90	5.0	0.8	0.4	0.4	0.4	0.3	1.0
m 91	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 92	5.0	0.8	0.4	0.4	0.4	0.5	1.0
m 93	5.0	0.8	0.4	0.4	0.4	0.3	0.7
m 94	5.0	0.8	0.4	0.4	0.4	0.3	0.7
m 95	5.0	0.8	0.4	0.4	0.4	0.3	0.7
m 96	5.0	0.8	0.4	0.4	0.4	0.3	0.7
m 97	5.0	0.8	0.4	0.4	0.4	0.3	0.7
m 98	5.0	0.8	0.4	0.4	0.4	0.3	0.7
m 99	5.0	0.8	0.4	0.4	0.4	0.3	0.7
m 100	5.0	0.8	0.4	0.4	0.4	0.3	0.7
m101	5.0	0.8	0.4	0.4	0.4	0.3	0.7
m102	5.0	0.8	0.4	0.4	0.4	0.3	0.7

Appendix 2. Outline of the number of designed markers on each chromosome



Appendix 3. Allele frequencies at 102 indels loci of Myanmar native chickens

Marker	Populations							
	Yangon		Mandalay		Pegu		Total	
	n = 27		n = 40		n = 13		n = 80	
	A	B	A	B	A	B	A	B
m 1	0.037	0.963	0.063	0.937	0.077	0.923	0.056	0.944
m 2	0.056	0.944	0.225	0.775	0.346	0.654	0.187	0.813
m 3	0.778	0.222	0.788	0.212	0.731	0.269	0.775	0.225
m 4	0.685	0.315	0.563	0.437	0.577	0.423	0.606	0.394
m 5	0.852	0.148	0.875	0.125	0.692	0.308	0.838	0.162
m 6	0.352	0.648	0.262	0.738	0.000	1.000	0.250	0.750
m 7	0.667	0.333	0.663	0.337	0.615	0.385	0.656	0.344
m 8	0.907	0.093	0.813	0.187	0.885	0.115	0.856	0.144
m 9	0.982	0.018	1.000	0.000	0.962	0.038	0.988	0.012
m 10	0.241	0.759	0.188	0.812	0.115	0.885	0.194	0.806
m 12	0.518	0.482	0.725	0.275	0.500	0.500	0.619	0.381
m 13	0.389	0.611	0.313	0.687	0.154	0.846	0.312	0.688
m 14	0.796	0.204	0.863	0.137	0.692	0.308	0.812	0.188
m 15	0.111	0.889	0.038	0.962	0.077	0.923	0.070	0.930
m 16	0.815	0.185	0.888	0.112	0.654	0.346	0.825	0.175
m 17	0.796	0.204	0.775	0.225	0.846	0.154	0.794	0.206
m 18	0.926	0.074	0.963	0.037	1.000	0.000	0.960	0.040
m 19	0.111	0.889	0.525	0.475	1.000	0.000	0.463	0.537
m 20	0.574	0.426	0.500	0.500	0.577	0.423	0.540	0.460
m 21	0.370	0.630	0.275	0.725	0.539	0.461	0.350	0.650
m 22	0.741	0.259	0.825	0.175	1.000	0.000	0.825	0.175
m 23	1.000	0.000	0.975	0.025	1.000	0.000	0.987	0.013
m 24	0.333	0.667	0.675	0.325	0.423	0.577	0.520	0.480
m 25	0.481	0.519	0.450	0.550	0.154	0.846	0.413	0.587
m 26	0.500	0.500	0.225	0.775	1.000	0.000	0.394	0.606
m 28	0.037	0.963	0.050	0.950	0.115	0.885	0.056	0.944
m 29	0.167	0.833	0.450	0.550	0.154	0.846	0.306	0.694
m 30	0.778	0.222	0.512	0.488	0.808	0.192	0.650	0.350
m 31	0.778	0.222	0.475	0.525	0.346	0.654	0.556	0.444
m 32	0.815	0.185	0.763	0.237	0.962	0.038	0.812	0.188
m 34	0.519	0.481	0.363	0.637	0.269	0.731	0.400	0.600

Marker	Populations							
	Yangon		Mandalay		Pegu		Total	
	27		40		13		80	
	A	B	A	B	A	B	A	B
m35	0.815	0.185	0.700	0.300	0.577	0.423	0.720	0.280
m 36	0.352	0.648	0.538	0.462	0.577	0.423	0.481	0.519
m 37	0.944	0.056	0.925	0.075	1.000	0.000	0.944	0.056
m 38	0.222	0.778	0.175	0.825	0.615	0.385	0.263	0.737
m 39	0.759	0.241	0.700	0.300	1.000	0.000	0.769	0.231
m 41	0.574	0.426	0.713	0.287	0.962	0.038	0.706	0.294
m 42	0.889	0.111	0.850	0.150	1.000	0.000	0.888	0.112
m 44	0.889	0.111	0.950	0.050	0.923	0.077	0.925	0.075
m 45	0.056	0.944	0.050	0.950	0.038	0.962	0.050	0.950
m 46	0.370	0.630	0.425	0.575	0.308	0.692	0.388	0.612
m 47	0.722	0.278	0.563	0.437	0.654	0.346	0.631	0.369
m 48	0.907	0.093	0.863	0.137	0.962	0.038	0.894	0.106
m 49	0.407	0.593	0.862	0.138	0.808	0.192	0.700	0.300
m 50	0.352	0.648	0.438	0.562	0.577	0.423	0.430	0.570
m 51	1.000	0.000	0.988	0.012	0.846	0.154	0.969	0.031
m 52	0.389	0.611	0.650	0.350	0.539	0.461	0.544	0.456
m 53	0.500	0.500	0.638	0.362	0.846	0.154	0.625	0.375
m 54	0.185	0.815	0.312	0.688	0.154	0.846	0.244	0.756
m 55	0.870	0.130	0.775	0.225	1.000	0.000	0.844	0.156
m 56	0.000	1.000	0.025	0.975	0.000	1.000	0.013	0.987
m 57	0.907	0.093	0.988	0.012	0.962	0.038	0.956	0.044
m 58	0.685	0.315	0.688	0.312	0.731	0.269	0.694	0.306
m 59	0.204	0.796	0.300	0.700	0.077	0.923	0.231	0.769
m 60	0.426	0.574	0.600	0.400	0.500	0.500	0.525	0.475
m 61	1.000	0.000	0.962	0.038	0.846	0.154	0.956	0.044
m 62	0.796	0.204	0.950	0.050	0.769	0.231	0.869	0.131
m 63	0.037	0.963	0.000	1.000	0.000	1.000	0.988	0.012
m 64	0.796	0.204	0.838	0.162	0.769	0.231	0.813	0.187
m 65	0.000	1.000	0.088	0.912	0.000	1.000	0.044	0.956
m 66	0.982	0.018	0.975	0.025	0.000	1.000	0.981	0.019
m 67	0.482	0.518	0.400	0.600	0.154	0.846	0.388	0.612
m 68	0.685	0.315	0.688	0.312	0.462	0.538	0.650	0.350

Marker	Populations							
	Yangon		Mandalay		Pegu		Total	
	27		40		13		80	
	A	B	A	B	A	B	A	B
m 69	0.815	0.185	0.525	0.475	0.654	0.346	0.640	0.360
m 70	0.889	0.111	0.812	0.188	0.615	0.385	0.810	0.190
m 71	0.982	0.018	1.000	0.000	1.000	0.000	0.994	0.006
m 72	0.982	0.018	0.937	0.063	0.962	0.038	0.956	0.044
m 73	0.926	0.074	0.912	0.088	1.000	0.000	0.930	0.070
m 74	0.926	0.074	0.925	0.075	0.885	0.115	0.920	0.080
m 75	0.056	0.944	0.088	0.912	0.077	0.923	0.075	0.925
m 76	0.018	0.982	0.013	0.987	0.231	0.769	0.050	0.950
m 77	0.778	0.222	0.775	0.225	0.731	0.269	0.769	0.231
m 78	0.907	0.093	0.925	0.075	0.731	0.269	0.888	0.112
m 79	0.093	0.907	0.000	1.000	0.000	1.000	0.031	0.969
m 80	0.870	0.130	0.912	0.088	0.923	0.077	0.900	0.100
m 81	0.889	0.111	0.963	0.037	1.000	0.000	0.944	0.056
m 82	0.889	0.111	0.800	0.200	1.000	0.000	0.862	0.138
m 83	0.889	0.111	0.950	0.050	0.808	0.192	0.906	0.094
m 84	0.926	0.074	0.988	0.012	1.000	0.000	0.969	0.031
m 85	0.870	0.130	0.888	0.112	0.731	0.269	0.860	0.140
m 86	0.611	0.389	0.638	0.362	0.731	0.269	0.644	0.356
m 87	0.778	0.222	0.625	0.375	0.885	0.115	0.719	0.281
m 88	0.815	0.185	1.000	0.000	1.000	0.000	0.937	0.063
m 89	0.889	0.111	0.850	0.150	0.846	0.154	0.863	0.137
m 90	0.444	0.556	0.738	0.262	0.923	0.077	0.669	0.331
m 91	0.667	0.333	0.863	0.137	0.462	0.538	0.897	0.103
m 93	0.574	0.426	0.488	0.512	0.462	0.538	0.512	0.488
m 94	0.630	0.370	0.563	0.437	0.654	0.346	0.600	0.400
m 96	0.982	0.018	0.775	0.225	0.885	0.115	0.881	0.119
m 97	0.648	0.352	0.663	0.337	0.923	0.077	0.700	0.300
m 99	0.852	0.148	0.888	0.112	1.000	0.000	0.894	0.106
m 100	0.056	0.944	0.075	0.925	0.077	0.923	0.069	0.931
m 101	0.926	0.074	0.925	0.075	0.808	0.192	0.906	0.094
m 102	0.352	0.648	0.238	0.762	0.500	0.500	0.319	0.681