1.1 Introduction

The modern domestic chickens are now distributed worldwide and have evolved along with the development of human civilization. Although most of domesticated chickens are used for meat consumption and egg production, the ornamental types have been prevailed worldwide today. Among them, the Silky fowl (*Gallus gallus domesticus*) is well known for its unique fluffy plumage feeling like silk (Zhou *et al*., 2010). It is one of the most popular and ubiquitous ornamental breeds in Japan. Silky fowl is called "Ukokkei" in Japanese (Chang and Huang, 2003). Original Silky fowl are mild, short, with a small and long head but short neck. They can be easily distinguished from other chickens (Koketsu *et al*., 2004). The Silky fowl chicken is noteworthy as a source of phenotypic variations not commonly seen in other domestic breeds of chicken, representing a few of the vast array of morphological differences present across various domesticated poultry species. (Dorshorst *et al*., 2010)

1.2 Origin and domestication of Silky fowl

There is no clear distinction between the Silky fowl and other chicken breeds at the matrilineal component level (Fu *et al*., 2002; Liu *et al*., 2006).The exact scenario regarding where or when Silky fowl with the fur-like plumage were first cultivated was unknown. Silky fowl is thought to originate in China and described by Marco Polo in the 13th century during his explorations of Asia. The Silky fowl is believed to have been established well before the 13th century due to references to the unusual fowl in ancient Chinese writings (Haw, 2006). Most of the existing conventional literatures suggested for an origin in China. Other places in South-east Asia, such as India, have also been proposed as the cultivation

center for the Silky fowl (Arisawa *et al*., 2006). The domestication history of chicken in China could be dated back to 5400 BC (West and Zhou, 1989), but the first detailed description for the Silky fowl was recorded only 700 years ago (Xie, 1995). Nowadays, there are eight Chinese indigenous Silky breeds registered in the Domestic Animal Diversity Information System (DAD-IS, 2007) of the Food and Agriculture Organization (FAO) of the United Nations (http://www.dad.fao.org/). All these breeds could be assorted into white feather type and black feather type based on the feather color (Qiu *et al*., 1988). Among them, the Taihe Silkies and Bairong Silkies, which are distributed in Jiangxi Province and Fujian Province, respectively, are thought to be the ancestor populations of modern Silky fowl in China. Subsequently, these two breeds were diffused into other regions (Qiu *et al*., 1988; Xie, 1995). Unfortunately, there is no strong evidence, especially for molecular data, to support this hypothesis. The Silky fowl was introduced into Japan from China or India early in the 17th century and the modern breeds of the Silky fowl are thought to have been established in China and Japan (Roberts, 1997).

1.3 Characteristics of Silky fowl

Japanese Silky fowl (Figure 1-1) is considered as Natural Monument designated in 1941. This breed is a strange breed having numerous mutant characteristics. Standard body weight in adults is about 1,125 g in male while 900g in female (Tsudzuki, 2003). It is distinct from the other chicken breeds is that the Silky fowl has silky plumage, purple-colored skin and the bluish meat, purple to dark blue colored wattles, beak and comb, the fifth hind toe (Figure 1-4) pointing slightly upwards, beards and feathering (Figure 1-4) in the feet (Wada *et al*., 2004). Silkie fowl has mainly white

and black colored feather along with several other colors. Both types of Silky fowl with white and black colors have fluffy feathers as shown in Figure 1-2.

Figure 1-1: Japanese Silky fowl, white Left: male, Right: female

Figure 1-2: White and black Silky fowls with their crests on head

The Ukokkei or Japanese Silky fowl has no normal feathers (Figure 1-2 and 1-3) on its' body. The feathers of this breed do not have a flat web. The feathers have abnormal barbules and no barbicels, resulting in a silky appearance.

Figure 1-3: Silky fowls' feather; black and white

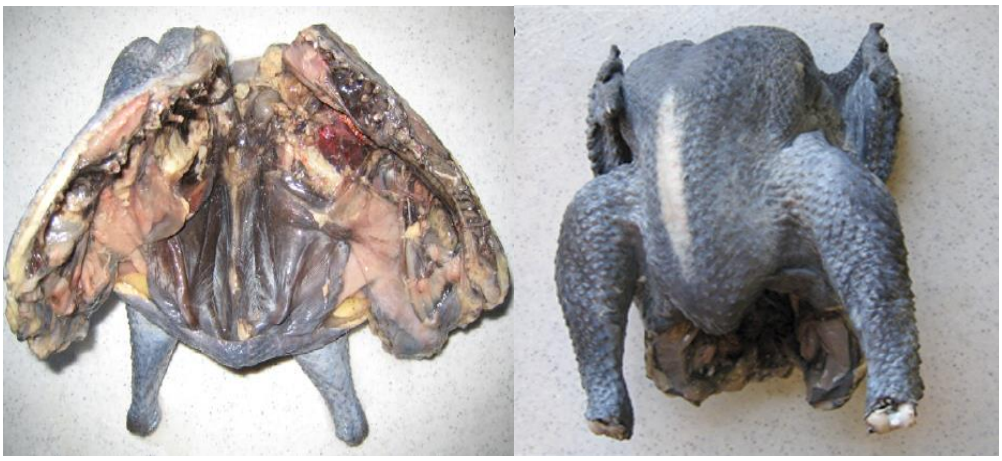
In addition, as compared to the other breeds, the Silky fowl is placid and tamable; and its behavioral characteristic is persistent broodiness, which are often used for "surrogate broody" (Wada *et al*., 2004.) All of these characters may have contributed to the human fascination and subsequent global distribution of this breed seen today. Silky fowl are very popular with exhibition and backyard poultry breeders in the USA and Europe and are also available in many Asian grocery stores within the USA. (Dorshorst *et al*., 2010)

(i) (ii)

Figure 1-4: Rare characteristics of Silky fowl's leg; five toes (i) and feathers in feet (ii) of Silky fowl

1.4 Hyper pigmentation in Silky fowl

Although the Silkie chicken is named for the hair-like appearance of adult feathers, one of the more distinguishing traits of this breed is hyperpigmentation of the dermis (Figure 1-5) and connective tissue throughout the entire body. This presents an opportunity to utilize the Silky fowl as a biological model of hyperpigmentation that can be used to identify genes regulating potentially novel mechanisms of melanoctye migration, proliferation, and differentiation (Dorshorst *et al*., 2010).



(i) (ii)

Figure 1-5: The bluish meat of Silky fowl (i) Internal pigmentation (ii) External pigmentation

In the Silky fowl, pigmentation is found extensively in the dermal layer of skin, sheaths of muscles and nerves, tendons, gut mesenteries, blood vessel walls, trachea, and air sacs (Kuklenski, 1915; Hutt, 1949; Faraco *et al*., 2001).

1.5 Medicinal value of Silky fowl

Silky fowl is a famous black-bone chicken breed with beautiful silky feather. In China, Silky fowl is specially characterized by its unique medical property, which was recorded in Chinese traditional medicine dictionary about 700 years ago (Xie, 1995). Black-Bone Silky fowl (*Gallus gallus domesticus*), a unique breed of chicken native to South China, is a bird with snow-white silky feathers and black-colored bones, meat and skin. As a kind of healthy food and traditional Chinese medicine, it has been well known in the Orient and used to reinforce the immunity of human bodies, to prevent emaciation and feebleness, to treat diabetes and anemia, and to cure women's diseases like menoxenia and postpartum complications for over a thousand years. The farming industry of the breed has recently begun to take shape in China. More and more people have accepted and enjoyed this bird's meat as a health-giving food (Tian *et al*., 2007). It is found that the Silky fowl has many health-giving properties beneficial to humans. In traditional Chinese medicine, peoples not only eat the meat of Silky fowl, but also its bones, organs and eggs is also considered effectively for nutritional fortification and treatment of infertility. Silky fowl has become a source of ingredients for Chinese health and beauty products and in Chinese herbal medicine about 700 years ago (Hu *et al*., 2010).

1.6 Nutrititive value of Silky fowl

The meat of the original Silky fowl is well known in the Orient and for thousands of years has been credited with famous medicinal and health-promoting values. However, a modern scientific approach has only recently been applied to determine its medicinal chemical and biochemical components (Ferrand & L'Hermite, 1985; Sakakibara *et al*., 2000). Silky

fowl eggs are considered to be a chemical storehouse and an excellent source of sialic acid (Koketsu *et al*., 2003), which is an important component for the protection of life (Koketsu *et al*., 1995, 1997). Koketsu *et al* (2003) reported from their research findings that the amount of cholesterol of Silky fowl eggs were found significantly ($P<0.01$) less than white leghorn chicken eggs. Moreover, the amount of vitamins (B2, B6, D and E), calcium and potassium in Silky fowl eggs were found significantly higher than white leghorn chicken eggs. Unsaturated fatty acids in Silky fowl eggs were measured 62.5% among total fatty acids, while the unsaturated fatty acids of white leghorn chicken eggs were found 53.9%. Especially, the contents of arachidonic acid, docosapentaenoic acid and docosahexaenoic acid in Silky fowl eggs were found significantly larger than white leghorn chicken eggs. (Koketsu *et al.*, 2004)

Figure 1-6: Eggs of Japanese Silky fowl

1.7 Review of literature

1.7.1. Candidate gene approach

The candidate gene approach has proven extremely powerful tool for studying the genetic architecture of complex traits. This method studies

the relationship between the trait of interest and known genes that may be associated with the physiological pathways underlying the trait (Andersson, 2001). If the candidate gene is a true causative gene, this approach can be very powerful and can detect loci having even small effects. The implementation of a candidate gene approach consists of the following steps: (1) construction or collection of a resource population, (2) phenotyping of the specific components of the trait(s) (3) selection of functional genetic polymorphisms or related DNA markers that potentially could affect the traits (4) genotyping of the resource population for the selected functional polymorphisms or related DNA markers (5) statistical analysis of the phenotypic and genotypic data (Da, 2003). This is an effective way to find the genes associated with the trait. So far, a number of genes have been investigated. (Felício *et al*., 2013; Ou *et al*., 2009; Dunn *et al*., 2004). Although great progress has been made by using candidate gene approach, the limitation of this approach is obvious.

The candidate gene tests must be interpreted with caution because spurious results can occur due to linkage disequilibria to linked or non-linked ‘‘causative’’ genes, or because the significance thresholds have not been adjusted properly when testing multiple candidate genes (Andersson, 2001). It also requires prior knowledge of the physiology of the specific trait, which is not always available. On the other hand, there are sometimes many candidate genes for the trait; it will take a long time to evaluate all of them. Furthermore, some genes that are not part of the known physiological pathways may contribute to the trait under investigation. The genome sequence, especially the SNP map, would solve a lot of questions on the selection of candidate genes. For example, the chicken SNP map provides abundant and useful SNP information to find the causative mutations among broiler, layer, Silky fowl and red jungle

fowl, which will accelerate the research.

Present research works has also selected some candidate genes on chromosome 2, 24 and Z chromosome derived from the knowledge of reproductive physiology and previous association studies report with reproductive performance in commercial poultry lines. A number of successes have been claimed for the physiological candidate gene approach to explain trait variance (eg Fotouhi *et al.*, 1993; Rothschild *et al.*, 1996; Sourdioux *et al.*, 1996; Urbanek *et al.*, 1999; Oztabak *et al.*, 2009).

1.7.2 Role of DNA Marker in animal selection

Genetic evaluation has usually started by analyzing phenotypes to identify genetic influences, whereas molecular genetics often begins with known alleles or DNA sequences and then examines their influence on phenotypes. Eukaryotic genomes show considerable DNA sequence variations (polymorphisms) between species and among individuals within a species. These genetic polymorphisms are considered as genetic marker. The development of DNA-based genetic markers has had a revolutionary impact on animal genetics. With DNA markers, it is theoretically possible to observe and exploit genetic variation in the entire genome.

The use of DNA markers to define the genetic makeup (genotype) and predict the performance of an animal is a powerful aid to animal breeding. One strategy is known as marker-assisted selection (MAS). MAS facilitate the exploitation of existing genetic diversity in breeding populations and can be used to improve a whole range of desirable traits. DNA markers are, by definition, polymorphic, and the methods used to define DNA markers include restriction fragment length polymorphisms (RFLPs), microsatellites, In/Del polymorphism and single nucleotide polymorphisms (SNPs) (Beuzen *et al*., 2000). This research particularly

focuses on three types of polymorphisms (RFLPs, In/Del, SNPs) and their potential use and characteristics in breeding and selection program of the Oita Silky fowl population.

1.7.2.1 Restriction fragment length polymorphism: (RFLP)

RFLP was among the first techniques to be used for typing DNA polymorphisms. While RFLP typing is a powerful and extensively used technique, its gel-based approach is inconvenient for high throughput screening. Additionally, most mutations do not result in the abolition or creation of restriction endonuclease sites, making such mutations impossible to detect by RFLP analysis.

Nucleotide changes occur in all eukaryotic genomes. If the change results in the creation, or abolition, of a restriction endonuclease recognition site, then the DNA sequence acquires or loses the ability to be cleaved by a particular restriction endonuclease. If a recognition site is absent then digestion with the relevant restriction enzyme will generate a long fragment. If a recognition site is present then digestion with the relevant restriction enzyme will generate two shorter fragments. If the recognition site is in only one of two parental alleles, digestion will produce two different electrophoretic patterns: a long fragment and two shorter fragments. This is the basis of RFLP.

1.7.2.2 Single nucleotide polymorphisms

Single-nucleotide polymorphisms (SNPs) can be broadly defined as any single base substitution/indel in the genome of an individual (Primmer *et al.*, 2002). There are four major reasons for the increasing interest in the use of SNPs as markers for genetic analysis. Firstly, they are prevalent and provide more potential markers near or in any locus of interest than other

types of polymorphism such as microsatellites. For example, in human genomic DNA there appears to be an SNP approximately every 1000 bases (Landegren *et al.*, 1998). Secondly, some SNPs are located in coding regions and directly affect protein function. These SNPs may be directly responsible for some of the variations among individuals in important traits.

SNPs are generally biallelic systems, which means that there are usually only two alleles in a population. As a consequence, the information content per SNP marker is lower than multiallelic microsatellite markers. Five SNP markers provide similar information to one microsatellite marker, meaning that about 2000 SNPs will be required to cover the equivalent of a 10 cM microsatellite map (http://www.pebio.com/ab/apply/dr/dralbl.html). The number of specific SNPs on the genetic map needs to be greater than the densest microsatellite map currently available. Therefore, high throughput technologies are needed to screen large numbers of SNPs.

There are a number of methods to genotype SNPs. The traditional gel-based approach uses standard molecular techniques, such as sequencing, PCR, restriction digests and various forms of gel electrophoresis, i.e. denaturing gradient gel electrophoresis [DGGE], single-strand conformation polymorphism [SSCP] and cleavase fragment length polymorphism [CFLP] (Parsons & Heflich, 1997; Kwok & Chen, 1998; Nataraj *et al.*, 1999).

1.7.2.3 Insertion/Deletion (In/Del) Polymorphism

An insertion/deletion polymorphism, commonly abbreviated "indel," is a type of genetic variation in which a specific nucleotide sequence is present (insertion) or absent (deletion). While not as common as SNPs, indels are widely spread across the genome. Indels comprise a

total of 3 million of the 15 million known genetic variants (The 1000 Genomes Project Consortium, 2010). An indel in the coding region of a gene that is not a multiple of 3 nucleotides results in a frameshift mutation. Shifting the reading frame and the DNA transcript sequence may now code for an entirely different set of amino acids or result in a premature stop codon, altering the protein structure and function. Indel variants with multiples of 3 nucleotides result in a protein with extra amino acids (insertion) or loss of amino acids (deletion), but the other amino acids are not affected.

Although many studies have been conducted to identify SNPs in humans and animals, still few studies have been conducted to identify alternative forms of natural genetic variation, such as In/Del polymorphisms and its relationship studies for economic trait of farm animals.

1.7.3 Future Developments of molecular marker

Animal genotyping is a powerful aid to animal breeding. The development of genetic markers can be summarized as follows. Polymorphic loci of known chromosomal location are identified, and methods to type these polymorphisms are developed. These polymorphisms are then tested on individuals with relevant phenotypes, and statistical relationships are calculated. The chance of detecting markers depends upon the relative contribution of the marker to the trait, the degree of linkage between the marker and the trait, and the frequency of desirable marker alleles in the population.

Another essential ingredient of marker development is recording accurate and appropriate phenotypic data on the traits of interest. Some

parameters are relatively easy to score, such as litter size or live weight gain, which makes selection relatively straightforward. Other traits, like meat quality and disease resistance, are more demanding to assess. DNA markers are likely to be especially valuable for traits hard to measure (Beuzen *et al*., 2000).

1.8 Objective

The aim of this study was to determine the association between DNA markers on the chromosome 2, 24 and on the Z chromosome and production traits in Oita Silky fowl population.

This research focused on the genotypic effects of In/Del and RFLPs that have significant relation with growth and egg production trait, which could be used for further future selection program of Oita Silky fowl population.

Summary

Silky fowl are very famous in China and Japan; however the egg production rate of the Silky fowl is very low because of broodiness. Chinese researchers have examined the polymorphisms of the 5′-flanking region of the prolactin gene in Taihe Silky fowl and identified a 24-bp In/Del locus but no In/In Silky individuals have been found. In this study, In/In individuals were constructed from an In/Del × In/Del population in order to examine the unique characteristics of Silky fowl. The relationships between production traits and DNA markers of the three candidate genes were studied. To produce In/In Silky individuals, male and female individuals with the In/Del locus in the fourth generation of the Silky fowl selection population were crossed. Genotyping was performed for the prolactin In/Del locus, the DNA marker of neuropeptide Y and the dopamine D2 receptor. In this study, 27 Del/Del, 39 In/Del, and 21 In/In female individuals and 13 Del/Del, 26 In/Del, and 17 In/In male individuals were found. All In/In individuals showed the Silky fowl characteristics. A significant effect of neuropeptide Y was found on eggshell strength ($p<0.05$) in female birds, and a significant effect of prolactin and dopamine D2 receptor gene was found on the body weight at 50 day in male birds ($p< 0.05$).

2.1 Introduction

Silky fowl (Ukokkei), which is a famous black-bone chicken breed with beautiful silky feathers, are one of the most popular and ubiquitous ornamental chicken breeds in Asia. Since about 700 years ago, the meat of this breed has been considered to have unique medicinal properties in Chinese traditional medicine (Zhou *et al.*, 2010). In China and Japan, the meat, bones, organs and eggs are effectively used for nutritional fortification and infertility treatments. Silky fowl eggs are considered to be a chemical storehouse and an excellent source of sialic acid, which is an important component for the protection of life (Koketsu *et al*., 2003). Owing to their medical properties and beautiful appearance, several researchers have attempted to improve the production traits of Silky fowl (He, 2003).

The Silky fowl is noteworthy as a source of phenotypic variations that are not commonly seen in other domestic breeds of chicken and these variations represent a few of the vast arrays of morphological differences that are present across various domesticated poultry species (Dorshorst *et al.,* 2010). Moreover, the Silky fowl commonly exhibits broodiness. Extensive studies on the mechanisms of chicken broodiness have been conducted in order to improve egg production.

It is thought that many of the variations in production traits such as body weight, egg production rate and egg quality are directly or indirectly influenced by some candidate genes such as prolactin(*PRL*), the dopamine D2 receptor (*DRD2*) and Neuropeptide Y (*NPY)*. Xu *et al.* (2010) have reported that mutations in the chicken *DRD2* gene influence the broodiness characteristic and that the *DRD2* gene might have endured strong selection during breeding against the broodiness characteristic. The *NPY* gene has been demonstrated to play an important role in the central control of

voluntary feed intake, which may contribute to differences in performance such as body weight gain and egg production in a variety of species.

Sequencing of the *PRL* gene has revealed a 24-bp insertion in the 5′-flanking region, -377 to 354, of the *PRL* gene in chicken (GenBank accession no. AB011438). Jiang *et al*. (2005) have examined polymorphisms of the 5′-flanking region (2,638 bp) of the chicken *PRL* gene in several populations of Chinese native Yuehuang, Taihe Silky, and imported White Leghorn Layer chicken. They have identified the 24-bp In/Del locus. Three genotypes (In/In, In/Del and Del/Del) have been found in Yuehuang chickens, whereas only two genotypes have been detected in Taihe Silky (In/Del and Del/Del) and White Leghorn chickens (In/In and In/Del). No In/In Taihe Silky individuals have been found. The genotype frequencies were significantly different among the three breeds. They have investigated the potential associations of the 24-bp In/Del locus with *PRL*, plasma prolactin and brooding behaviors and have observed that chickens with the In/Del heterozygous genotype, which have the highest incidence of broodiness, had the highest *PRL* mRNA levels; this finding suggests that this polymorphic site is related to the broodiness characteristic in chickens through modulations in the transcriptional levels of the *PRL* gene.

Cui *et al.* (2006) have produced an F2 population from Nongdahe × Taihe Silkies in order to investigate the relationship between the *PRL* In/Del locus and egg production. A significant association has been found between the In/Del locus and egg production ($p<0.05$) and the H3 haplotype has been identified as the most advantageous trait for egg production. Wada *et al.* (2008) have genotyped the 24-bp In/Del locus in the *PRL* gene in the fourth generation of a Silky fowl selection program in order to study the associations of performance traits. In their study, 282 Del/Del Silkies, 29 In/Del Silkies and no In/In Silky fowl were found. No

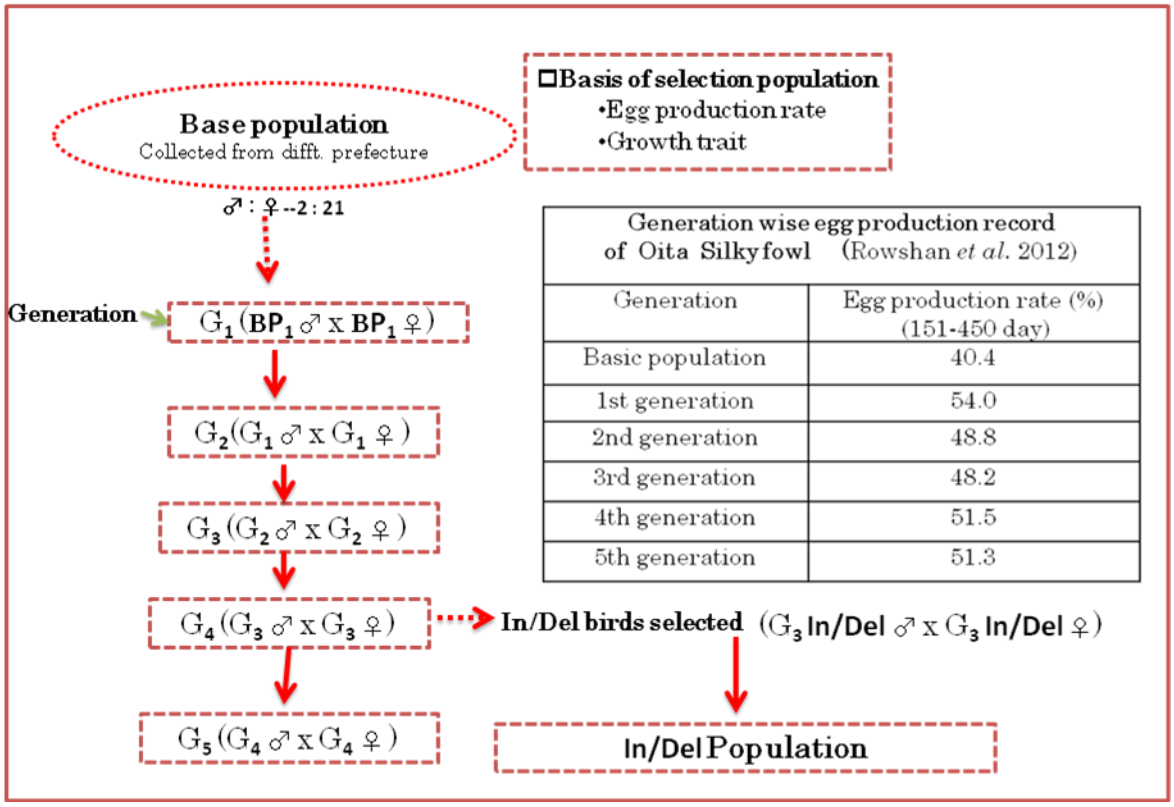
significant relationships between the In/Del genotypes or performance traits were identified. Rowshan *et al.* (2012) have genotyped the markers of candidate genes (*PRL,VIPR1* and *NPY*) in the fifth generation of a Silky fowl selection program in Oita, Japan, in order to investigate the efficiency of marker-assisted selection. The effect of the father was significant for all traits, including the egg production rate but the genotypic effects of the three candidate gene markers were not significant for any of the traits.

In this study, In/In individuals were constructed from an In/Del × In/Del population in order to examine the unique characteristics of Silkies, such as silky feather, hyper pigmentation, etc. The relationships between the genotypes of the DNA markers on the candidate genes and production traits were also investigated.

2.2 Materials and Methods

2.2.1 Resource population

In 2001, Oita's Silky fowl selection program was started from 300 Japanese Silky fowls. The chickens of the base population were introduced from five prefectures (Figure 2-3): Miyazaki, Aomori, Tokyo, Saga and Oita. Parental female birds were selected on the basis of their excellent egg-laying rates, and they were crossed with parental male birds that were selected based on their excellence in vigor. This breeding program (Figure 2-1) was conducted up to the fourth generation (Rowshan *et al.*, 2012). Thus, 2 male and 21 female birds with the In/Del locus in the fourth generation of the Silky fowl selection program were crossed in order to produce In/In individuals, and a total of 145 birds, 58 male and 87 female, were produced (Figure 2-2). For the whole period, commercial mixed feed was supplied *ad libitum*, and the birds were raised in three places and managed in different groups.



Generation wise egg production record of Oita Silky fowl (Rowshan *et al.* 2012)

Generation	Egg production rate (%) (151-450 day)
Basic population	40.4
1st generation	54.0
2nd generation	48.8
3rd generation	48.2
4th generation	51.5
5th generation	51.3

Figure 2-1: Diagram of Oita Silky Fowl Selection Program

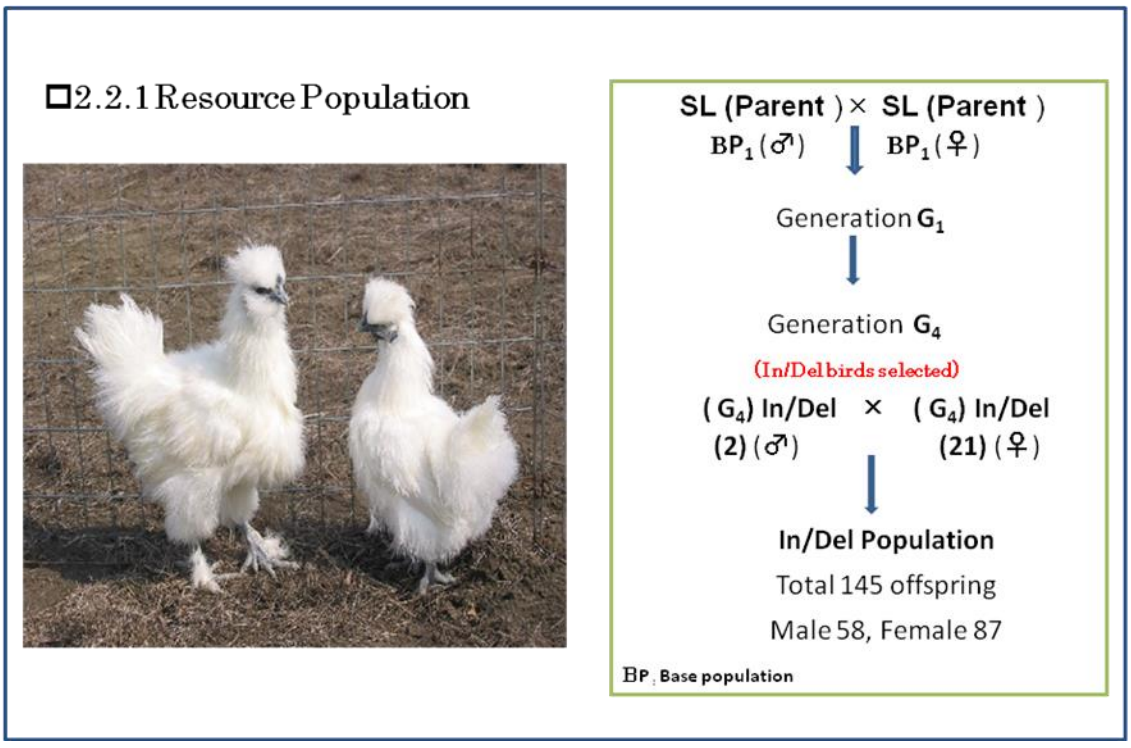


Figure 2-2: Breeding plan of In/Del population of Oita Silky fowl

Figure: 2-3: Prefectural Map of Japan showing the place of chicken sample collection

From day 0–40, chicks were kept in the same house in an electric battery chick brooder. From day 41–80, chicks were kept inside the medium chicken cage. From day 81–120, they were kept in a large chicken cage, and, finally, from day 121–450, the mature birds were kept in a single cage (length, 226 mm; depth, 393 mm; and height, 455 mm).

2.2.2 Measurement of phenotype

The body weights of every female bird were measured at 50, 150, and 300 days of age with a digital balance. Male birds were weighed at 50 and 150 days of age. The following egg characteristics were measured: egg production rate (%), average egg weight (g) and average eggshell strength (kg/cm^2). The eggshell intensity of the chicken was measured with the Egg shell strength meter 2 (Fujihira Industry Co. Ltd., Tokyo, Japan). The egg production rate was defined as the number of eggs divided by the number of egg-producing days from day 151–300.

2.2.3 DNA extraction from blood

Blood samples were taken from the ulnar vein of each individual and stored at -20°C. The blood sampling was performed in accordance with the guidelines for animal experimentation of Saga University. Genomic DNA were extracted from Silkie fowl's whole blood using Pure Gene kit (Funakoshi Co. Ltd.,Tokyo, Japan). At first, 15 μℓ whole blood of Silky fowl was filled into the 1.5ml eppendorf tube into which the code was written before. Cell Lysis solution of 600μℓ was added to each tube, inverted the tube gently for proper mixing and preserved it at room temperature. EHB was set at 55^0C. Then, 10μℓ of 1000 x Protease K solution (20mg/ml) were added to the sample which was previously preserved at room temperature. After inverting the tube properly, it was left for three hours incubation into the EHB. The sample was taken out from EHB, light spin down was performed and cooled it at room temperature. The Protein Precipitation Solution (PPS) of 200μl was added into the tube. The tube was then vortex vigorously for 20 seconds to mix the Protein

Precipitation Solution with the sample uniformly until dissolve the cell lysate. It was centrifuged at 4^0C for 3 minutes, @ 15000G. The precipitated protein was found to form in a compact dark brown pellet. Mean while the EHB was set at 65^0C. At this stage, cooler rack was used. Code was written to the new tube. After centrifuge, the top liquid portion i.e. supernatant of the centrifuged tube was carefully transferred to the new tube by pipetting as much as possible. Then, 600 μℓ of 100% isopropanol was added and mixed the sample by inverting 50 times gently. After that, it was centrifuged at 4^0C for 3 minutes @ 15000G. The surrounding isopropanol of the tube was wiped off. At this stage, DNA was found visible as a small white pellet.

Then, 300 μℓ of 70% ethanol was added and inverted the tube several times to mix the DNA. Again it was centrifuged at 4^0C for 1 minute @15000G. After centrifuge, the supernatant fluid was carefully removed, the tube containing DNA was dried in a Micro Vac at 37^0C for 3 minutes, keeping the cover of the tube open. When taking out the tube, the lid was closed inside the Micro Vac. DNA Hydration Solution was added 35μℓ to soloved the DNA. And spin down was performed lightly using vortex. The sample was then kept in incubation at 65^0C for 1 hour.

The sample was taken out from EHB and cooled to room temperature. Again, vortex was done vigorously for 5 seconds at medium speed. At this stage, in order to measure the concentration and quality of extracted DNA, spectrophotometer was used. First, the cuvette of the spectrophotometer was washed by distilled (DW) water and DW was thrown away. DW of 50μℓ was put into the cuvette, run on the spectrometer and the result of the blank was checked. Then, DNA solution

of 45 µℓ was mixed with 5 µℓ distilled water in a new microcentrifuge tube. The solution was transferred to a cuvette and run on spectrophotometer. The concentration of DNA was calculated and data was analyzed before using in downstream reactions. Extracted DNA sample was finally preserved at -20^{0}C.

2.2.4 PCR amplification:

The DNA fragments were amplified using a Takara ExTaq Hot start version. The PCR amplification was performed in a final total volume of 25 µl with extracted genomic DNA (50 ng or more) that was prepared into a template, 0.125µl of ExTaq Hot Start Version (5 U/µL), 2.5µl of Ex Taq buffer (20mM Mg^{2+}), 2µl of dNTP Mixture (2.5mM), 1µl of template DNA, 1 µl of each primer pair (10µM) and 17.375 µl of distilled water that was mixed in a 0.5-mL tube. The primers that were used for PCR are shown in Table 2-1. All fragments that were amplified by primer pairs were electrophoresed on a 3% agarose gel and the PCR products of *NPY1* were digested with the *Kpn*I restriction enzyme. The Tech Gene machine was used to conduct the PCR reaction. The reaction conditions for the PCR were the following: Initial denaturation of 5 min at 95^{0}C; 40 cycles at 95^{0}C for 30 s; each annealing temperature shown in Table 2-1 for 30 s; 60^{0}C for 30 s; 72^{0}C for 1 min; with a final elongation of 10 min at 72^{0}C and the cycle ended at 4^{0}C.

Table 2-1 Information of DNA markers used in this study

Candidate gene	Position of DNA marker[1]		Primer sequences Forward/reverse (5'-3')	Annealing temperature	Restriction enzyme	Size of DNA fragment (bp)
prolactin	GGA2	59724210	GGTGGGTGAAGAGACAAGGA TGCTGAGTATGGCTGGATGT	60^0C		154/130
neuropeptide Y	GGA2	C31394761T	CGTGGCTGCTTTGCTTCCTTTC GGGGTACGAGGCAAGGACATG	65^0C	*KpnI*	TT 202/122 CT 324/202/122 CC 324
dopamine D2 receptor	GGA24	5841629	TGCACTTCAATCCTTCCCAGCTT TTGCGCTGCCCATTGACCA	63^0C		187/165

[1]The site were based on the chicken genome sequences released in May,2006 http://genome.ucsc.edu/cgi-bin/hgGateway

2.2.5 Agarose gel electrophoresis

The PCR products were analyzed in 3% agarose gel electrophoresis stained with ethidium bromide and visualized by using a UV transilluminator. Concentrations of agarose gel were used according to the expectation of expected DNA band

An amount of 100ml 0.5 × TBE stock solution was prepared finally by mixing 10 ml of 5×TBE with 80 ml of distilled water and adding distilled water to make the 100 ml final volume of 0.5 × TBE electrophoresis buffer solution. Agarose gel S (Wako pure medicine Ltd., Tokyo, Japan) of 1.2 g was weighted and poured into 200 ml conical flask. Then, prepared 100 ml 0.5 × TBE buffer solution was added with the conical flask and mixed gently to dissolve the agarose.

To visualize DNA in agarose gel, it was stained by the fluorescent dye ethidium bromide (EtBr). One µl of ethidium bromide was added to the solution. Agarose gel solution was melted in microwave oven. The flask was removed from the microwave oven after several seconds and gently inclined the flask to check any settled powder and gel pieces. The flask was then heated again in the microwave oven until the agarose was dissolved. After dissolving the gel properly, the flask was cooled to 50°C - 60°C. The setting of gel casting platform and gel comb were prepared properly.

Then, prepared agarose gel solution was poured into a gel casting apparatus and allowed to become solid at room temperature (20-30 minutes). If the air bubble was formed, it was quickly removed by using a sterilized micropipette chip. After the gel become solid, the gel comb was carefully removed and the gel was placed in a horizontal electrophoresis tank. Four hundred milliliters of 0.5 × TBE buffer was then poured directly across comb removal place on the top of the solid gel. Five microliters of

100bp DNA marker was loaded to the right side first column. Two microliters of 6 × gel-loading dye (buffer) was mixed with 5µl of sample on the paraffin paper and then the mixture was slowly and carefully loaded from the second column on the top of the solid gel by using a micropipette. The electrophoresis tank was covered with a lid and the DC power supply (100 volts) was connected to the positive and negative electrodes which were attached to the horizontal electrophoresis tank. After the run of electrophoresis for about 30-45 minutes or until the DNA samples or dyes had migrated to a sufficient distance, the power supply was turned off and removed the lid from the gel tank. The gel was then carefully transferred to a clean plastic tray and was visualized and photograph was taken using an ultraviolet bench top transilluminator. At first, the power switch was on. Then, the focus, the size, the position and the brightness etc. were adjusted. Finally, the photograph was taken and the DNA band was confirmed.

2.2.6 Statistical analysis

Genotype judging was performed with the electrophoresis results. The group would have reached Hardy–Weinberg equilibrium with the observed value and the expected value of the genotype. The R statistical language (R version 2.13.2, Becker *et al*., 1988) was used for the assessment of the Chi-square estimates. The significantly differences between genotypes were checked using t-test. For the relevance of a genotype and the production traits, analysis of variance (ANOVA) was performed in order to examine the main effects of the henhouse and the genotype of *PRL*, *NPY* or *DRD2*. The interaction effect was removed in the ANOVA model because interaction effect is not detected at the preliminary analysis.

2.3 Results

2.3.1 Identification of Genotype

The genotype was identified with the electrophoresis results. The restriction enzyme *KpnI* was used for single nucleotide polymorphism (SNP) identification of NPY.

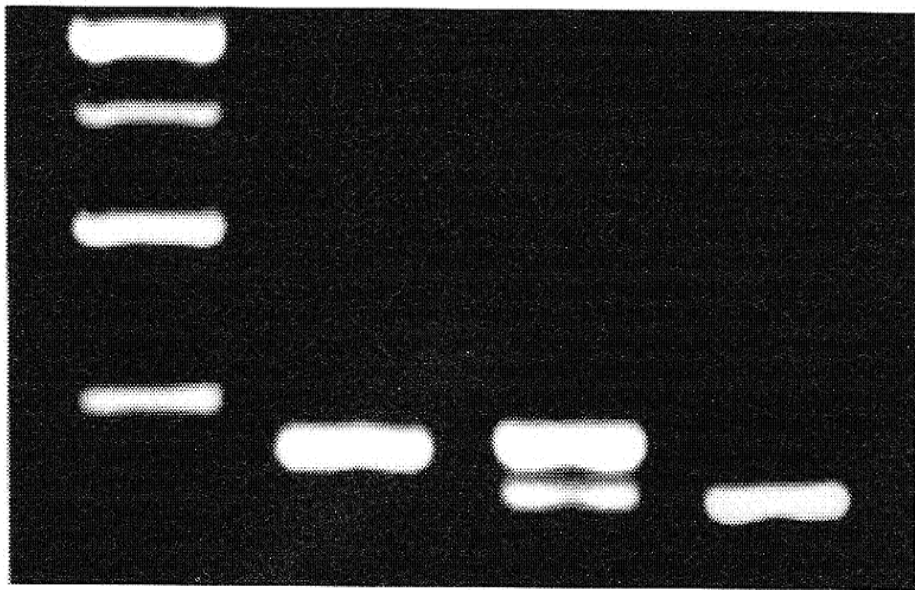


Figure 2-4 The genotype of In/Del locus of *PRL* gene determined by 3.0% agarose gel electrophoresis

Three types of genotypes were identified for each of the *PRL*, *NPY* and *DRD2* genes. For the In/Del locus of the *PRL* and *DRD2* genes, the lengths of the genotypes were considered as In/In-154 bp, In/Del-154/130 bp, Del/Del-130 bp and In/In-187 bp, In/Del-187/165 bp and Del/Del-165 bp, respectively. For the C/T locus of the *NPY* gene, 3 genotypes were identified (Table 2-1 and Figure 2-4 ~2-6).

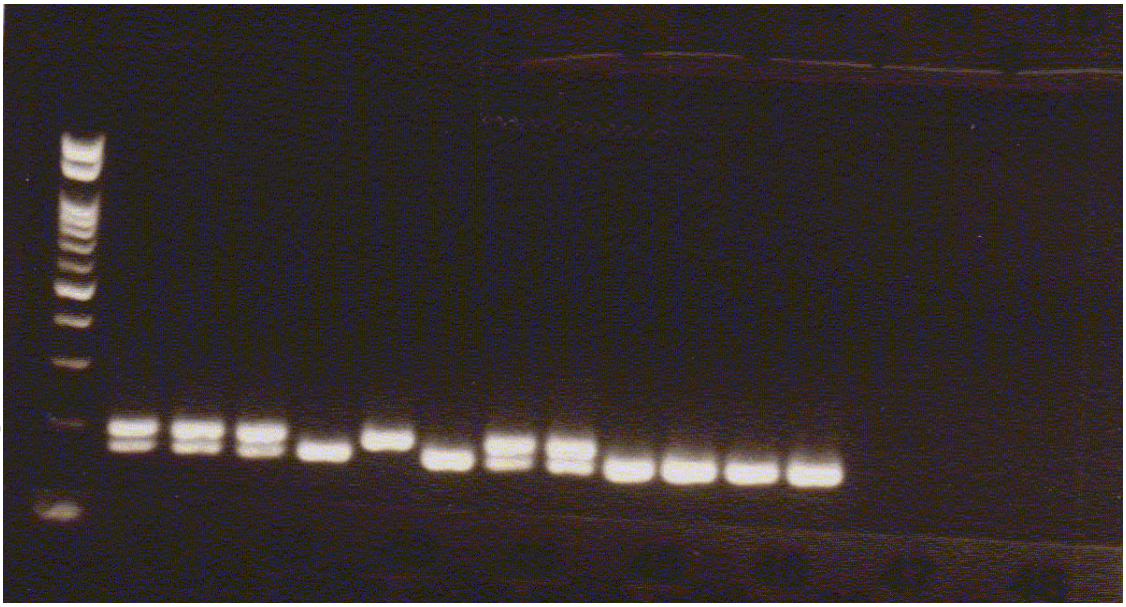


Figure 2-5 The genotype of In/Del locus of *DRD2* gene determined by 3.0% agarose gel electrophoresis

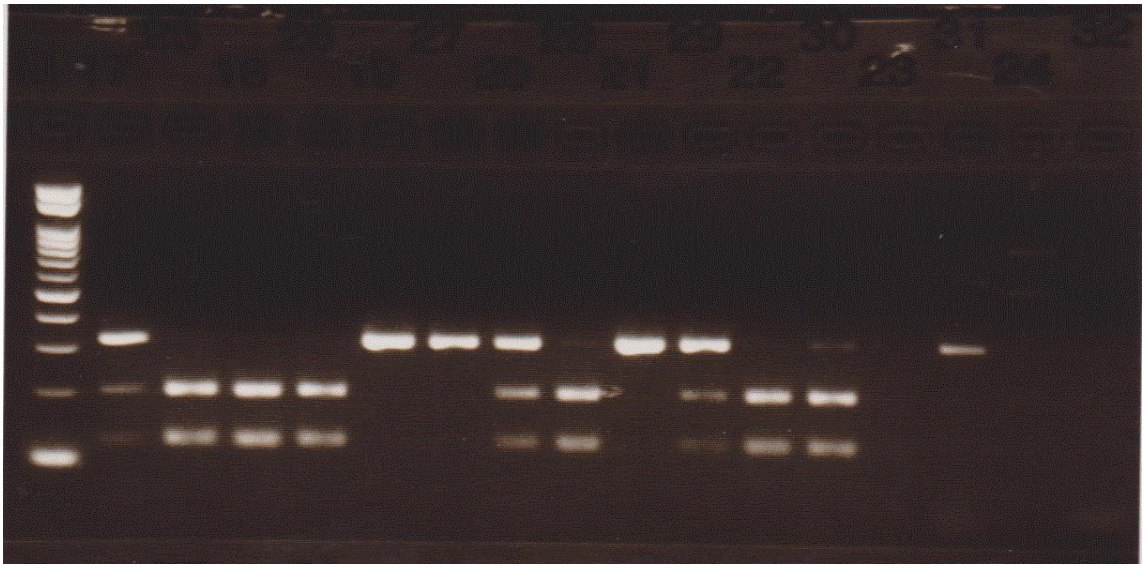


Figure 2-6 The genotype of C/T locus of *NPY* gene determined by 3.0% agarose gel electrophoresis

2.3.2 Estimation of heterozygosity

The chi-square test estimates (Table 2-2) between the observed value and expected value of a genotype from Hardy-Weinberg proportions found no differences so much in case of three genes in female birds and Prolactin (*PRL*) and Dopamine D2 recepter (*DRD2*) in male birds respectively. Hardy-Weighberg Law was established for both the group of candidate genes. But the chi square test estimates between the observed value and the expected value of a genotype from the Hardy–Weinberg Law showed a significant difference (p <0.05) in the *DRD2* gene in female birds (Table 2-2). This might be the reason of mutation of Dopamine D2 recepter (*DRD2*) gene during breeding process against broodiness character.

Table 2-2.a Estimation of heterozygosity of candidate gene locus (Female)

Candidate Gene	Observed Heterozygosity	Expected heterozygosity	χ^2 value of the Hardy-Weinberg Law
prolactin	0.448	0.501	0.96
neuropeptide Y	0.483	0.468	0.09
dopamine D2 receptor	0.218	0.295	5.98*

*p<0.05

Table 2-2.b Estimation of heterozygosity of candidate gene locus (Male)

Candidate gene	Observed Heterozygosity	Expected heterozygosity	χ^2 value of the Hardy-Weinberg Law
prolactin	0.455	0.502	0.499
neuropeptide Y	0.527	0.472	0.774
dopamine D2 receptor	0.327	0.276	1.970

2.3.3 Analysis of the phenotypic variation

In this study, 13 Del/Del, 26 In/Del, and 17 In/In male individuals and 27 Del/Del, 39 In/Del, and 21 In/In female individuals were found in the *PRL* In/Del locus. All *PRL* In/In individuals had all Silky fowl characteristics. With regard to the genotypes of the *NPY* gene, CC female individuals showed significantly (p<0.05) better results than CT individuals for body weight at day 50, whereas CT individuals showed significantly (p < 0.05) better results than TT individuals for average egg weight. TT female individuals showed significantly (p<0.05) better results than CC individuals for average eggshell strength. With regard to the *DRD2* gene, In/In female birds showed better results than Del/Del female birds for body weight at day 300 (Table 2-3). Among the male birds, In/In and In/Del individuals of the *PRL* gene, In/Del individuals of the *DRD2* gene, and CT and TT individuals of the *NPY* gene had a better performance for body weight at day 50 than Del/Del and CC individuals (Table 2-4).

Table 2-3 Genotype and average trait value of In/Del x In/Del population in female birds

Candidate gene	Genotype	No. of birds	Body weight at 50 day (g)	Body weight at 150 day (g)	Body weight at 300 day (g)	Egg production rate (%)	Average Egg weight (g)	Average Egg strength (kg/cm2)
Prolactin	Del/Del	27	288.1 [a] ±47.8 [*]	929.1 [a] ±118.2	1049.8 [a] ±229.1	64.7 [a] ±14.9	41.4 [a] ±2.6	3.87[a] ±0.54
	In/Del	39	301.0 [a] ±37.1	936.5 [a] ±157.6	1133.9 [a] ±174.5	66.6 [a] ±10.3	41.1 [a] ±2.9	3.91[a] ±0.64
	In/In	21	309.4 [a] ±35.9	945.1 [a] ±128.5	1135.9 [a] ±208.5	66.6 [a] ±9.9	41.4 [a] ±2.4	3.65[a] ±.061
Neuropeptide Y	CC	11	311.8 [b] ±36.9	957.7 [a] ±97.5	1127.6 [a] ±201.1	67.0 [a] ±12.2	41.0 [ab] ±3.1	3.93 [b] ±0.48
	CT	42	290.8 [a] ±40.6	909.5 [a] ±170.3	1080.2 [a] ±213.4	65.0 [a] ±12.5	41.8 [b] ±2.2	3.73 [ab] ±0.65
	TT	34	293.5 [ab] ±45.4	971.8 [a] ± 93.0	1157.5 [a] ±154.2	66.7 [a] ±7.4	40.0 [a] ±2.1	3.47 [a] ±0.74
Dopamine D2 receptor	Del/Del	62	297.2 [a] ±40.0	931.0 [a] ± 151.7	1109.2 [a] ±207.6	65.4 [a] ±8.1	41.4 [a] ±2.7	3.74 [a] ± 0.64
	In/Del	19	300.6 [a] ±40.4	963.9 [a] ± 101.9	1172.1 [b] ±168.3	68.6 [a] ±10.8	40.9 [a] ±2.4	3.92 [a] ± 0.47
	In/In	6	317.6[a] ± 62.8	901.3 [a] ± 91.1	902.3 [b] ±91.1	64.4 [a] ±13.4	40.9 [a] ±2.4	3.72 [a] ± 0.73

a,b means in the same column within each candidate gene having different superscripts are significantly different (p<0.05).*;standard deviation

Table 2-4 Genotype and average trait value of In/Del x In/Del population in male birds

Candidate gene	Genotype	No. of birds	Body weight at 50 day (g)	Body weight at 150 day (g)
Prolactin	Del/Del	13	312.3[a] ±51.6*	1193.5 [a] ±78.5
	In/Del	26	349.2[b] ±36.7	1213.2 [a] ±111.7
	In/In	17	336.0[b] ± 46.6	1218.3 [a] ±93.2
Neuropeptide Y	CC	6	323.3 [a] ± 57.6	1221.7 [a] ±156.7
	CT	29	329.8 [a] ± 43.9	1207.5 [a] ± 90.3
	TT	21	356.8 [b] ± 40.	1214.5 [a] ±100.5
Dopamine D2 receptor	Del/Del	38	328.1 [a] ±48.5	1208.8 [a] ± 98.3
	In/Del	19	357.5 [b] ± 33.2	1223.2 [a] ± 107.3

a,b means in the same column within each candidate gene having different superscripts are significantly different (p<0.05). *;standard deviation

Table 2-5 Results of analysis of variance

Female						
Factor	Body weight At 50 day (g)	Body weight at 150 day (g)	Body weight at 300 day (g)	Egg production rate (%)	Average Egg weight (g)	Average Egg strength (kg/cm^2)
Henhouse	4.495*	3.316*	102.465***	1.537	0.969	0.199
Prolactin	0.998	0.0096	0.058	0.057	0.044	1.630
Neuropeptide Y	3.079	1.738	0.272	0.142	1.997	3.957*
Dopamine D2 receptor	1.153	1.415	0.2249	0.211	0.385	1.459
Male						
Henhouse	0.320	1.368				
Prolactin	3.857*	0.133				
Neuropeptide Y	1.158	0.083				
Dopamine D2 receptor	4.458*	0.130				

†Numerical value represented the F value *p<0.05; **p<0.01;***p<0.001

2.3.4 ANOVA for the production traits of Silky fowl

ANOVA was performed to examine the relationships between the production traits and the genotypes of the candidate genes (Table 2-5). *NPY* had a significant effect on eggshell strength ($p<0.05$) in female birds. Significant effects of the *PRL* and *DRD2* gene were found on body weight at day 50 in male birds ($p < 0.05$). The henhouse had a significant effect ($p < 0.05$) on the body weight in female birds. No significant effects of body weight or the egg production rate of female birds was found for the three markers.

2.4 Discussion

Wada *et al.* (2008) have genotyped the 24-bp In/Del locus in the region of the *PRL* gene in the fourth generation of the Silky fowl selection program in order to study the association of production traits. They found 282 Del/Del Silkies and 29 In/Del Silkies, but no In/In Silky fowl. In this study, we tried to produce In/In individuals of the *PRL* In/Del locus in Silky fowls. All In/In individuals had all Silky fowl characteristics. No relationship was demonstrated between Silky fowl characteristics and the *PRL* In/Del locus.

Physiologically, it has been well established that *PRL* is the main candidate gene for broodiness owing to its critical roles in the onset of broodiness (Sharp *et al*., 1988; March *et al*., 1994; Youngren *et al*., 1998). Increased plasma *PRL* concentrations have been associated with the occurrence of broodiness in female turkeys (Burke and Dennison, 1980). During incubation, *PRL* mRNA reaches its highest level (Talbot *et al*., 1991; Karatzas *et al*., 1997), which infers that prolactin is important in the

maintenance of broodiness. Broodiness characteristics are commonly observed in Silky fowl. Owing to the medicinal properties and beautiful appearance of Silky fowl, several researchers have attempted to improve the egg production levels of Silky fowls (He, 2003). Previous studies have suggested that a 24-bp In/Del locus in the 5′-flanking region of the chicken *PRL* gene is a genetic marker in breeding against broodiness (Jiang *et al*., 2005; Cui *et al*., 2006).

Li *et al*. (2009) examined the polymorphisms of *NPY* genes by developing PCR-restriction fragment length polymorphism methods in Wenchang chickens, and they found a significant association, as well as an additive effect, between *NPY* polymorphisms and the total number of eggs at day 300 ($p < 0.05$). Kahtane *et al*. (2003) have reported that, in chickens, the broodiness trait was related to the activity of the dopaminergic system. In avian breeds, dopamine has been demonstrated to be involved in both stimulating and inhibiting prolactin secretion in the brain (Youngren *et al*., 1995). In addition, the inhibitory effects of dopamine on prolactin secretion have been shown to be mediated through the DRD2 receptor at the pituitary level (Youngren *et al.*, 1998; Kahtane *et al*., 2003). Hens that were treated with dopamine receptor antagonists or receptor blocking agents exhibited a termination in the maintenance of broodiness due to the inhibition of prolactin secretion (Hall *et al.*, 1984). In addition, Sartsoongnoen *et al*. (2008) have found an association between dopamine neurons and the regulation of the reproductive system in Thai chickens. Xu *et al.* (2010) studied 644 female Ningdu Sanhuang chickens and identified 2 variations, A-16105G and T+619C, in the chicken *DRD2* gene. They reported the

association between the variations and the broodiness trait. The results of all these studies have suggested that the DRD2 receptor is involved in the regulation of avian reproductive behavior.

In Oita Silky fowls, Wada *et al.* (2008) did not identify any relationship between In/Del genotypes in the *PRL* gene and production traits. In this study, we examined the relationships between production traits and the genotypes of DNA markers of candidate genes such as *PRL*, *DRD2* and *NPY* in the In/Del × In/Del population of Silky fowl. No significant associations were found among the genotypes of the 3 candidate genes and the egg production rate. The differences between the previous Chinese results and the results of our study may be caused the genetic divergence of Chinese and Japanese Silky population. Rowshan *et al.* (2012) suggested that the genetic variations of Silky fowls in the Oita selection program are high and that the egg production rate in the Silky fowl population could be improved. We can therefore study other candidate genes in order to improve the egg production rate in Oita Silky fowl.

Prolactin has a wide variety of functions, including the growth, behavior, and ovarian activities, in chickens. Bhattacharya *et al*. (2011) have reported that the *PRL* promoter was highly polymorphic and had significant associations with growth traits in White Leghorn chickens. Several scientists have reported that dopamine, which is an abundant neurotransmitter in the central nervous system and periphery, has important roles in cognition, emotion, endocrine function, and hyperprolactinemia in mammals (Missale *et al.,* 1998; Hansen *et al.,* 2005). Its physiological effects are exerted through the activation of dopamine receptors. These studies have suggested possible

roles of the *PRL*, *NPY* and *DRD2* genes on the body weight of avian species. In the present study, we found significant effects of the *PRL*, *NPY* and *DRD2* genes on male body weight at day 50. Our results therefore suggested that these genes may be considered candidate genes for improving the growth traits of Oita Silky fowl and other Japanese traditional breeds.

In this study, *NPY* showed a significant effect on eggshell strength ($p < 0.05$). NPY is an abundant and widespread neuropeptide in the nervous system of avian species (Kuenzel and McMurtry, 1988), but we did not find any reports on the relationship between eggshell strength and the *NPY* gene. Tuiskula-Haavisto *et al.* (2010) have reported that quantitative trait loci (QTLs) that affect the deformation and breaking force of eggs are found on chromosome 2. However, the position of the QTLs was distinct from the position of the *NPY* gene on chromosome 2. Thus, in the future, we can study the relationship between eggshell strength and the genes on chromosome 2 for the genetic improvement of commercial layer.

Rowshan *et al.* (2012) reported no significant relationship between production traits and the DNA marker of *PRL* and *NPY* in female birds of the Oita's 5th generation, but some relationships were detected in this study. These differences suggests the detection efficiency of the relationship in our In/Del × In/Del population is more than the 5th generation. In the female results of this study, a significant difference was detected at *NPY* gene on body weight at 50 day and average egg weight, but *NPY* didn't showed a significant effect on these traits in the results of ANOVA. The disagreement suggests that QTL analysis is required for the determination of candidate gene for these traits in our In/Del × In/Del population.

Summary

The Silky fowl is a famous black-bone chicken breed. Adult Silky fowl are characterized by low body weight and produces less number of eggs. Current own research efforts have mainly focused on the improvement in egg production. Previous studies have indicated a relationship between egg production and DNA markers and a high genetic variation in the Oita's selection population. We have embarked on the improvement in the rate of egg production in this specific population. Some valuable markers related with egg production include the prolactin receptor and the growth hormone receptor, which were localized to the Z chromosome. In this study, five DNA markers on the Z chromosome were analyzed in the 5th generation of the Oita's Silky fowl population. Genotyping of DNA markers was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to study the relationship between DNA marker and production trait. The relationship was analyzed by linear mixed model using SAS Mixed procedure. Hatching group and genotype effects of five DNA markers were included as fixed effect in the model. Sire and dam effect was included as random effect. Genotypes and trait values were obtained for 202 female and 58 male individuals of the 5th generation. In the females, *rs16773406* showed significance for egg production rate (p<0.05) and for body weight at 150 day (p<0.001). Marker *rs15991083* showed significance for average egg weight (p<0.05). In the males, *embigin* and *rs15991083* showed significance for body weight at 150 days of age (p<0.05), whereas *rs16763148* and *rs16773406* showed significance for body weight at 300 days of age (p<0.05).

3.1 Introduction

The Silky fowl is one of twenty-seven native breeds that were introduced to Japan before the beginning of Edo era and was appointed as Japanese natural treasure in 1942 (Tsudzuki, 2003). It is a famous black-boned chicken breed with beautiful Silky feathers. In Chinese traditional medicine, about 700 years ago, the meat of the breed was considered to have unique medicinal properties (Zhou *et al*., 2010). The body weight of a male adult is approximately 1125g, whereas the female weight approximately 900g. The Silky fowl generally produces 30–40 eggs per year but through research, the national and prefectural poultry experimental stations of Japan have succeeded in raising Silky fowl that can produce as many as 120–150 eggs per year, sometimes over 200 (Sugiyama and Washimi, 2010). Moreover, these Silky fowl eggs are considered excellent material for food processing (Koketsu and Toyosaki, 2004; Toyosaki and Koketsu, 2004).

The Silky fowl has limited yearly egg production and low adult body weight compared to that in the commercial chicken such as White Leghorn and extensive research efforts have aided in increasing its egg production and growth trait. Rowshan *et al*. (2012) genotyped three candidate gene markers on the chromosome 2 in the 5^{th} generation Silky fowl in Oita's selection population to improve the egg production rates. The results showed that these markers did not impart any significant genotypic effects on all traits. However, they suggested that the genetic variation of the population was high and that the egg production rate in the Silky fowl population can be improved.

In chickens, the male sex chromosomes are homogametic (ZZ) while those of the female are heterogametic (ZW). Briggs and Nordskog (1973) segregated a population from Fayoumi and another heavy breed synthetic population and reported that both sex chromosomes in chickens influenced the age of production of the first egg, with the Z chromosome playing a more important role. They found that pullets with the heavy breed matured 17 days earlier and produced 5% more eggs than those with the Fayoumi for the Z chromosome, whereas no significant effect on egg production was observed with the heavy breed for the W chromosome.

It has been earlier reported that genes related to egg production, such as the prolactin receptor (*PRLR*) and growth hormone receptor (*GHR*), are localized close to each other on the short arm of the Z chromosome (Suzuki *et al*., 1999). The *PRLR* in chicken was located to 10.5Mb on the Z chromosome, adjacent to the *GHR* at position 13.3Mb (International Chicken Genome Sequencing Consortium, 2004). Both *PRLR* and *GHR* are single chain proteins with a single transmembrane domain. The action of the prolactin and growth hormones is mediated by their respective receptors, which belong to the cytokine receptor superfamily that has the ability to activate JAK2 and STATs (Fleenor *et al*., 2006). As the *PRLR* gene is located on the Z chromosome, the hypothesis of sex-linked inheritance of reproductive traits may be associated with the *PRLR* gene. One SNP (G1836C), which was identified on the exon 6 of the *PRLR* gene in the Chinese Erlang Mountainous chicken, has been shown to impart a highly significant ($p<0.01$) effect on the CC genotype for the age of first egg production (Li *et al*., 2012).

Embigin is a developmentally expressed protein that is a member of the immunoglobulin superfamily (IgSF) class of cell adhesion molecules (Guenette *et al*., 1997). The *embigin* gene was detected using fluorescence *in situ* hybridization, as a cDNA clone from the cDNA library of the left ovary of 1–3-day-old chickens and was located in the middle of the short arm of the Z chromosome (Saitoh *et al*., 1993). The *embigin* gene is expressed during tissue regression in rat prostate and lactating mammary gland following hormonal ablation. *Embigin* is also expressed in a variety of adult tissues, including the heart, liver, lung, and brain (Guenette *et al*., 1997). Thus, it is evident that the *embigin* gene possesses diversified functions in various species, including the chicken, and may play a vital role in production performance.

On the basis of the findings of previous studies, we considered that the *embigin* gene might play a vital role in the production traits of the Silky fowl. Other SNP markers were randomly selected. In this study, five DNA markers (*PRLR, rs16763148, Embigin, rs16773406, and rs15991083*) on the Z chromosome were analyzed in the 5th generation Oita's Silky fowl population to study the relationship between the genotypes of markers and production traits.

3.2 Materials and Methods

3.2.1 Chicken population

The parental Silky females were selected on the basis of their excellent egg production rate, and they were crossed with the parental Silky males that were selected on the basis of their excellence in vigorous forms and shapes at each generation.

Figure 3-1: Prefectural Map of Japan showing the place of chicken sample collection

This breeding program was conducted up to the 5th generation (Rowshan *et al.*, 2012).

A total of 321 chickens (106 males and 215 females) were produced in the 5th generation. These chickens were hatched three times (Oct.25 2009, Dec.25 2009, Jan27 2010) and each hatching group was raised in the

different henhouse. For the whole period, commercial mixed feed was supplied as *ad libitum* for the growth. The chicks were raised in battery cage until 40 days of age and were raised in small cage until 80 days of age, in large cage until 120 days of age and in a single cage for adult chicken (226 x 393 x 455mm) until 450 days of age.

3.2.2 Measurement of phenotype

Body weight of every female and male chicken was measured at 150 and 300 days of age by using a digital balance. Three egg characteristics were measured: average egg weight (g), egg production rate (%) and average egg strength (kg/cm^2). The eggshell intensity of the chicken was measured using an eggshell strength meter 2 (Fujihira Industry Co. Ltd., Tokyo, Japan). Egg production rate was defined as the number of eggs divided by the number of egg-producing days, which ranged from 151 to 300 days. Total 260 chickens (58 males and 202 females) having all traits data and DNA samples were analyzed in this study.

3.2.3 DNA extraction from blood

Blood samples were collected from 260 Silky fowls and DNA was extracted from whole blood of Silky fowl using the Pure Gene kit (Funakoshi Co. Ltd., Tokyo, Japan). The methodology of DNA extraction has already been described in details in chapter 2.

3.2.4 PCR amplification and genotyping

Five DNA markers (*PRLR, rs16763148, Embigin, rs16773406, and rs15991083*) on the Z chromosome were selected in this study (Table 3-1). Polymerase chain reaction (PCR) was performed in a total volume of 25 μL that consisted of the following: ≥50 ng of extracted genomic DNA as template, 0.125 μL of Ex Taq Hot Start Version (5 U/mol), 2.5 μL of Ex Taq buffer (20 mM Mg), 2 μL of dNTP Mixture (2.5 mM), 1 μL of template DNA, 1 μL of each primer pair (10 μM), and 17.375 μL of purified water, all mixed in a 0.2-mL PCR-plate. The primer used for the PCR is shown in Table 3-1. The PCR conditions were as follows: initial denaturation at 95°C for 5 min, 40 cycles at 95°C for 30 s, each annealing temperature for 30 s, 72°C for 1 min, a final elongation at 72°C for 10 min, and final hold at 4°C. Genotyping of DNA markers was performed using PCR-restriction fragment length polymorphism (PCR-RFLP). The PCR products were digested using each restriction enzyme (Table 3-1). The genotypes were examined through 3% agarose gel electrophoresis analysis.

3.2.5 Agarose Gel Electrophoresis

The methodology of Agarose Gel Electrophoresis has already been illustrated in details in chapter 2.

Table 3-1 Information of DNA markers used in this study

Candidate gene/SNP	Position of DNA marker[2]	Primer sequences Forward/reverse (5'-3')	Annealing temperature	Restriction enzyme	Size of DNA fragment	Reference
Prolactin receptor	NW_001488830.2 5632507	GGACAGGTAAGAAGCGGTGA TTTTACCCCATTCTGCAAGC	61℃	*EcoNI*	345 388	This study
rs16763148	NW_001488830.2 9613587	AAGCGGATTCCATACTCTTTGA TTCCAGTTGATCTTTGTGTTGAA	59℃	*EcoRI*	755 398 / 541 398 214	Riztyan *et al.* (2011)
Embigin	NW_001488830.2| 9759776	TTCTGCTTGCCCTGATCTTT TGTTTTCTTGGGCTTCCTTG	59℃	*BsmI*	185 329	This study
rs16773406	NW_003764321.1 2133453	GTTTTCCTGATGCCTGCAAT CCCATAGCAGAATGCCTGTC	60℃	*EcoRI*	175 164 / 164 130 45	This study
rs15991083	NW_003764324.1 3475743	GCACCTTCCCTATGAGGACA TTGACTTTGCGTGACACCTT	59℃	*EcoRI*	993 212 / 606 387 212	Riztyan *et al.* (2011)

[2]The sites were based on the chicken genome sequences released in May, 2006 http://genome.ucsc.edu/cgi-bin/hgGateway

3.2.6 Statistical analysis

The R statistical language (R version 2.13.2; Becker *et al*. 1988) was used for genotype assessment. Differences among genotypes were examined using the *t*-test. The relationship between DNA marker and production trait was analyzed by linear mixed model using SAS Mixed procedure (SAS Institute Inc. 1999).

The Classical Linear Mixed Model is written as

$$Y = X\beta + Z\gamma + \varepsilon$$
$$\gamma \sim N(0, G)$$
$$\varepsilon \sim N(0, \sigma^2 I_n)$$

Here,

β is a p x 1 vector of unknown fixed effects with design matrix X

Z is the n x s design matrix for the random effects

γ is an s x 1 vector of unknown random effects with design matrix Z

ε is an n x 1 vector of unknown random errors

G is an s x s diagonal matrix with identical entries for each fixed effect

In is an n x n identity matrix

γ and ε are independent

Here, everything is the same as in the general linear model except for the addition of the known design matrix, Z and the vector of unknown random-effects parameters, γ. The matrix Z can contain either continuous or

dummy variables, just like X. The name mixed model comes from the fact that the model contains fixed effects parameters, β and random-effects parameters, γ. In this study, G is assumed to σ_g^2A where A is a numerator relationship matrix among the tested birds, sire, dam, grandsire and granddam birds. The fixed effect is hatching group and DNA markers. The random effect is the animal's effect. The variance components were estimated using REML method. The interaction effect was removed in the model because the interaction effect was not detected at the preliminary analysis.

3.3 Results

3.3.1 Genotype Identification

The genotype was identified with the electrophoresis results. The restriction enzyme *EcoNI, BsmI and EcoRI* was used for single nucleotide polymorphism (SNP) identification. Two types of genotypes were identified in the females and three types in the male. The lengths of the genotypes were shown in Table 3-1 & Figure 3-2 to 3-6.

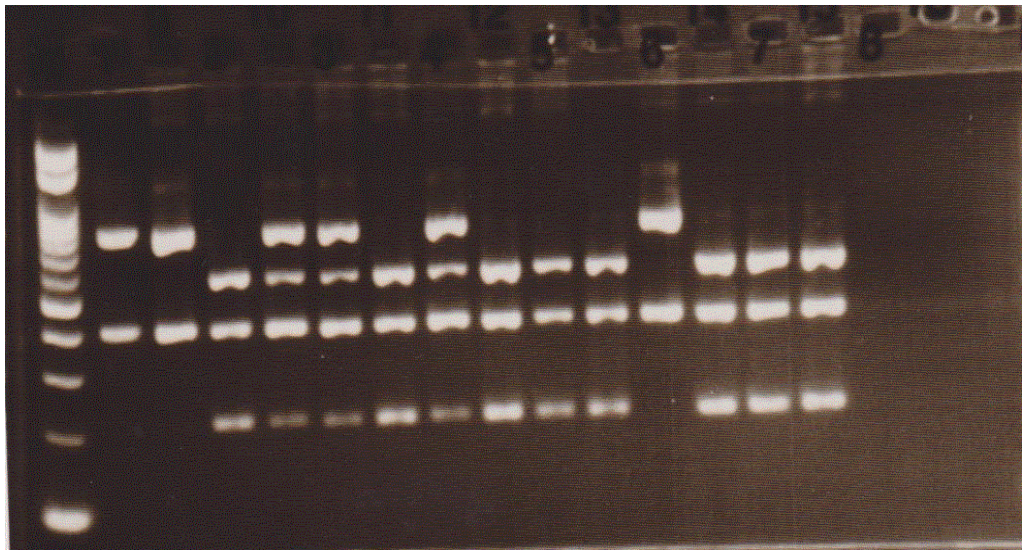


Figure 3-2 The genotype judging of *rs16763148* marker determined by 3.0% agarose gel electrophoresis

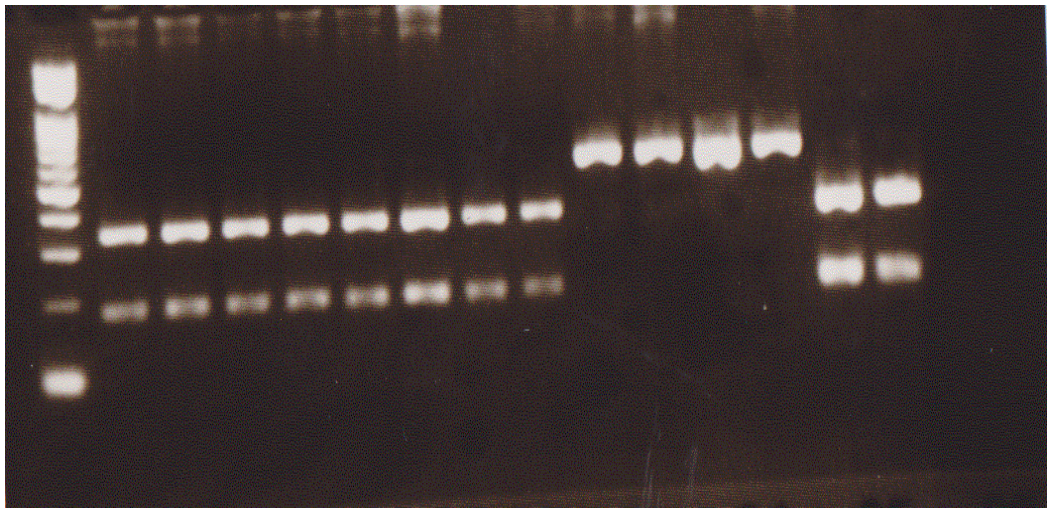


Figure 3-3 The genotype judging of *Embigin* gene determined by 3.0% agarose gel electrophoresis

Figure 3-4 The genotype judging of In/Del locus of *Prolactin receptor* determined by 3.0% agarose gel electrophoresis

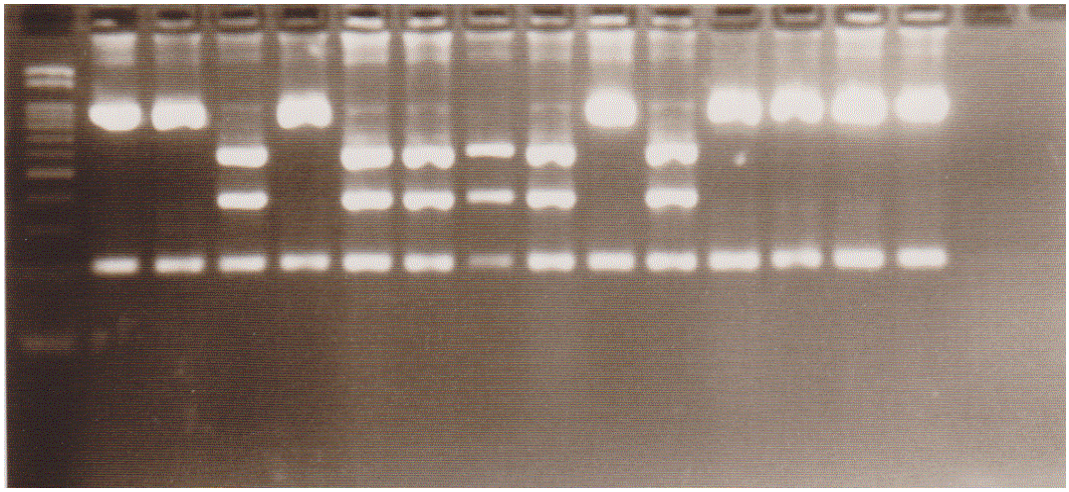


Figure 3-5 The genotype judging of *rs15991083* marker determined by 3.0% agarose gel electrophoresis

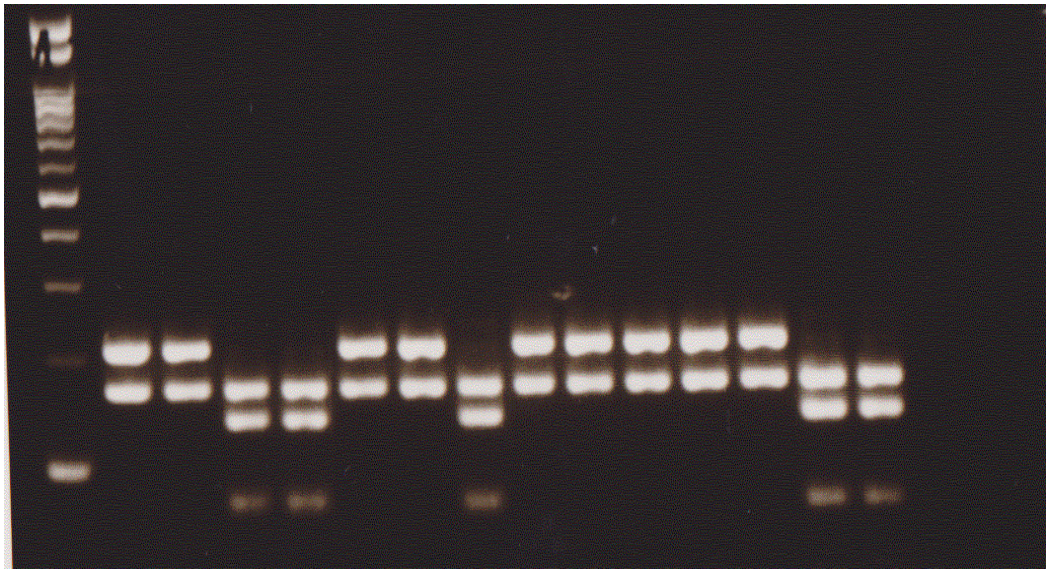


Figure 3-6 The genotype judging of *rs16773406* determined by 3.0% agarose gel electrophoresis

3.3.2 Analysis of the phenotypic variation

In this study, 202 females and 58 males were analyzed. In the females (Table 3-2), in terms of the genotypes related to the marker *rs16773406*, C individuals showed significantly (p<0.05) better performance than that by the A individuals for egg production rate and body weight at 150 days of age. Regarding the *embigin* gene, C individuals showed significantly (p<0.05) better results than that by the T individuals for average eggshell strength. For the rs15991083 marker, G individuals showed significantly (p<0.05) better performance than that by the A individuals for average egg weight. No significant differences of the genotypes of the *PRLR* and *rs16763148* were observed for any other trait in female birds.

Table 3-2 Genotype and average trait value in female birds

Candidate Gene/SNP	Genotype	No. of birds	Body weight at 150 day (g)	Body weight at 300 day (g)	Egg production rate (%)	Average Egg weight (g)	Average Eggshell strength (kg/cm2)
Prolactin receptor	A	172	1027.0±122.9 †	1204.9±151.1	56.5±17.8	38.7±2.6	3.89±0.65
	G	30	1017.9±128.5	1170.7±143.3	55.7±21.7	39.2±2.5	4.11±0.68
rs16763148	G	74	1034.3±128.0	1220.0±147.1	56.2±18.1	38.7±2.2	3.85±0.62
	A	119	1017.9±121.5	1185.8±154.1	55.9±19.1	38.7±2.8	3.97±0.69
Embigin	C	67	1019.3±123.1	1193.7±137.4	54.7±20.8	38.6±2.5	4.10^{b}±0.66
	T	135	1028.8±124.0	1202.9±156.4	57.2±17.1	38.8±2.6	3.84^{a}±0.65
rs16773406	C	118	1047.2^{b}±124.2	1214.2±145.4	58.9^{b}±16.7	38.8±2.7	3.90±0.65
	A	83	994.2^{a}±116.8	1177.9±155.2	52.6^{a}±20.1	38.6±2.4	3.96±0.68
rs15991083	G	116	1030.7±128.7	1208.1±158.1	58.1±16.1	39.2^{b}±2.7	3.93±0.65
	A	86	1018.8±116.4	1188.7±138.7	53.9±21.1	38.2^{a}±2.2	3.92±0.68

†; average ± s.d. a,b means in the same column within each candidate gene having different superscripts are significantly different (p<0.05)

Table 3-3 Genotype and average trait value in male birds

Candidate Gene/SNP	Genotype	No. of birds	Body weight at 150 day (g)	Body weight at 300 day (g)
Prolactin receptor	AA	34	1331.9±111.9 †	1601.6±142.2
	AG	15	1292.8±135.4	1576.9±118.3
	GG	9	1328.7± 78.2	1562.2±149.7
rs16763148	GG	11	1286.7±140.8	1633.5±114.2
	AG	30	1343.7±109.5	1586.9±155.0
	AA	17	1304.0±106.7	1564.5±129.8
Embigin	CC	3	1389.3^{ab}±170.0	1680.0±171.0
	CT	18	1375.8^{b}±102.0	1628.2±142.1
	TT	35	1285.7^{a}±106.7	1561.1±130.0
rs16773406	CC	26	1340.4± 86.4	1618.5^{b}±113.0
	AC	18	1308.8±152.6	1573.4^{ab}±173.5
	AA	13	1306.0±104.8	1540.9^{a}±110.6
rs15991083	GG	28	1288.9^{a}±104.4	1555.9±135.1
	AG	20	1328.5^{ab}± 95.4	1614.6±118.1
	AA	10	1397.6^{b}±141.2	1631.2±162.3

†; average ± s.d. a,b means in the same column within each candidate gene having different superscripts are significantly different (p<0.05).

In the males (Table 3-3), the CT individuals of the *embigin* and CC individuals of the *rs16773406* showed significantly ($p< 0.05$) better results for body weight at 150 and 300 days of age, respectively. Regarding the *rs15991083* marker, the AA individuals showed significance ($p< 0.05$) for body weight at 150 days of age compared to the other individuals. The genotypes of the *PRLR* and *rs16763148* markers showed no significance for two growth traits in male birds.

Table 3-4 Results of analysis of type 3 test in female birds

Factor	Body weight at 150 day(g)	Body weight at 300 day (g)	Egg production rate （%）	Average Egg weight (g)	Average Eggshell strength （kg/cm^2）
Hatching group	2.51†	1.14	0.50	13.04***	2.38
Prolactin receptor	0.24	0.34	0.00	0.10	0.42
rs16763148	1.35	2.59	0.01	0.58	0.83
Embigin	1.16	0.37	0.10	0.32	2.47
rs16773406	9.16***	0.19	6.60*	0.31	0.66
rs15991083	1.48	2.01	1.60	4.55*	0.34

Numerical value represented the F value *p<0.05; **p<0.01; ***p<0.001

3.3.3 Analysis for the relationship between production trait and the DNA markers

The relationship between production traits and the Z chromosome markers was analyzed by linear mixed model using SAS Mixed procedure. In the females (Table 3-4), *rs16773406* showed significance for egg production rate ($p < 0.05$) and a high significance for body weight at 150 days of age ($p < 0.001$). Marker *rs15991083* showed significance for average egg weight ($P < 0.05$). Hatching group showed high significance ($p< 0.001$) for average egg weight. In the females, *PRLR*, *rs16763148* and *embigin* showed no significance for any other traits. Furthermore, no other marker showed any significance for average eggshell strength. In the males (Table 3-5), *embigin* and *rs15991083* marker showed significance for body weight at 150 days of age ($p < 0.05$). Marker *rs16763148* and *rs16773406* showed significance for body weight at 300 days of age in males ($p<0.05$). *PRLR* and hatching group showed no significance for growth traits in the males.

Table 3-5 Results of type 3 test in male birds

Factor	Body weight at 150 day (g)	Body weight at 300 day(g)
Hatching group	0.31[†]	2.01
Prolactin receptor	1.87	0.33
rs16763148	0.85	4.85*
Embigin	3.97*	0.65
rs16773406	1.88	3.37*
rs15991083	4.62*	2.36

[†]Numerical value represented the F value *$p<0.05$; **$p<0.01$; ***$p<0.001$

3.4 Discussion

Prolactin and growth hormone are secreted by the pituitary and exert a wide spectrum of biological effects that are mainly related to reproduction, lactation, growth, and metabolism. These bioactivities are initiated by the interaction of the hormones with specific membrane receptors found in their numerous target tissues (Fleenor *et al*., 2006). The available data suggest that *GH* and *PRL* arose early in vertebrate evolution through the duplication of a common ancestral gene, followed by divergent evolution (Forsyth *et al*., 2002). The *PRLR* and *GHR* genes show limited overall sequence identity (~30%), whereas their tertiary structures are highly related. On the basis of their structural features, PRLR and GHR were initially believed to form a distinct receptor family (Goffin and Kelly, 1997). PRLR is the specific receptor for prolactin, involved in different physiological activities and is essential for reproductive success (Bole-Feysot *et al*., 1998 ; Kelly *et al*., 2001).

Rahman *et al*. (2014) genotyped the prolactin In/Del locus and DNA markers of neuropeptide Y and dopamine D2 receptor in In/Del × In/Del population of the Oita's Silky fowl to study the relationship among specific production traits and the three candidate genes. The neuropeptide Y was found to have a significant effect on egg strength ($p< 0.05$). The prolactin and dopamine D2 receptor genes were found to impart significant effects on body weight in 50-day-old male birds. By using the three markers, no significant effects on body weight and egg production rate were observed in female birds.

In this study, we examined the relationship between the five markers on the Z chromosome and the production traits in the 5th generation of Oita's Silky fowl's selection program. One SNP (A/G) was identified on the *PRLR* gene, although no significant effects of the genotypes of the *PRLR* marker were observed on the production trait in females and males. Rowshan *et al*. (2012) genotyped the prolactin In/Del marker in the 5th generation Silky fowl in Oita's selection population but did not observe any significant effect on egg production rate. Tuiskula-Haavisto *et al*. (2002) genotyped 99 microsatellite loci for QTL analysis of an F_2 population consisting of 307 hens resulting from a line cross between two egg layer lines. They identified the QTLs on the Z chromosome for the traits of age at the first egg production and the number of eggs produced. Sasaki *et al*. (2004) genotyped 123 microsatellite markers for detecting QTLs in an F_2 population consisting of 265 hens generated from a cross between White Leghorn and Rhode Island Red breeds. They detected a significant QTL for age at the first egg production in the Z chromosome. In this study, in the females, *rs16773406* showed significance for egg production rate ($p< 0.05$), which suggested that this may be a pioneer marker on the Z chromosome for the genetic improvement of egg production in Oita's Silky fowl.

Tuiskula-Haavisto *et al*. (2002) reported QTLs for egg weight traits on the chicken Z chromosome. In this study, *rs15991083* on the Z chromosome showed significance for average egg weight. Individuals with the genotype G showed significantly better egg weight than that by the A individuals. Our study suggests that markers on the Z chromosome might be considered for the genetic improvement of egg weight traits in Oita's Silky fowl.

Abasht *et al*. (2006) published a detailed review report on QTLs identified for the egg production and growth traits in chickens, including those for body weight traits on the chicken Z chromosome. In our study, the high significance ($p < 0.01$) of *rs16773406* on body weight at 150 days of age in females suggest that we can consider this marker for the improvement of body weight in Oita's Silky females.

Embigin is produced predominantly in the embryonic gonads and steroid-producing cells in the ovarian follicles. It has been previously suggested that embigin plays a role in the differentiation or maintenance of steroidogenic cells in ovarian follicles (Kunita *et al*., 1997). In this study, the *embigin* gene was selected based on the possibility of the improvement for egg production rate. The DNA marker of *embigin* showed significance ($P < 0.05$) for body weight at 150 days of in the males in this study. Similarly, on the basis of the *t*-test results, CT individuals showed better body weight at 150 days of age. The *embigin* gene homologs exist in human, chimpanzee, Rhesus monkey, dog, cow, mouse, rat, and fruitfly and are expressed in a variety of adult tissues (Guenette *et al*,. 1997). Embigin may be related to the body growth of vertebrates. The results of this study involving the embigin marker suggest that this marker might serve for improving the body weight traits of Oita's Silky fowl.

4.1 General Discussion

To date, advances in molecular genetics have led to the identification of valuable genes or markers associated with genes that affect the phenotypic trait in domestic and commercial chicken. This study tried to find out the relationship of some potential markers with production traits of Japanese Silky fowl to enhance genetic improvement program in domestic chicken. In this study, In/In individuals were constructed from an In/Del × In/Del population in order to examine the unique characteristics of Silkies. To know the relationships between body weight, egg production rate, average egg weight and egg strength with DNA markers, three candidate genes markers from chromosome 2, 24 and five markers on the Z chromosome were studied.

To produce In/In Silky individuals, male and female individuals with the In/Del locus in the fourth generation of the Silky fowl selection population were crossed. Based on previous research report, no significant association was identified between production traits of Silky fowl and the *PRL* In/Del locus. (Wada *et al.*, 2008; Rowshan *et al*., 2012). In this study, All In/In individuals had found Silky fowl characteristics. In the male population, a significant effect of prolactin and dopamine D2 receptor gene was found on the body weight ($p < 0.05$). No significant effects of body weight of female birds were found for the three markers on chromosome 2 and 24. But, most of the markers on the Z chromosome in male Silky and one marker in the female showed significant result for body weight trait. This result suggests that these markers may be

considered to improve the growth traits of Oita's Silky fowl.

For the relationship study of egg production rate with In/Del locus of *PRL* gene, no significant associations were found among the genotypes of the 3 candidate genes on chromosome 2 and 24 and the egg production rate in this study. Rowshan *et al*. (2012) genotyped the prolactin In/Del marker in the 5th generation Silky fowl in Oita's selection population but also did not observe any significant effect on egg production rate. Moreover, most of the markers on the Z chromosome did not showed any significant effect on egg production rate in this study except marker *rs16773406* showed significance for egg production rate ($p < 0.05$). This result suggested that *rs16773406* may be considered as a potential marker on the Z chromosome for future improvement of egg production trait in Oita's Silky fowl.

From the result of ANOVA to examine the relationship between the egg weight and the genotypes of the candidate genes on chromosome 2 and 24, no significant effect was found of the three markers for egg weight. But, in another study, QTLs for egg weight traits on the chicken Z chromosome were reported by Tuiskula-Haavisto *et al*. (2002). In this study, marker *rs15991083* on the Z chromosome showed significance result for average egg weight. Individuals with the genotype G showed significantly better egg weight than that of A individuals. Our study suggests that markers on the Z chromosome might be considered for the genetic improvement of egg weight traits in Oita's Silky fowl. In this study, no significant effects were found between most of the markers on chromosome 2, 24 with the eggshell strength. Only, *NPY* showed a significant effect on eggshell strength ($p< 0.05$). This study suggests that, we can study the

relationship between eggshell strength and the genes on chromosome 2 for the genetic improvement of commercial layer. Tuiskula-Haavisto *et al*. (2002) reported significant QTLs for eggshell strength on the chicken Z chromosome. But in this study, no significant result for eggshell strength on the chicken Z chromosome was identified.

In this study, most of the markers of chromosome 2, 24 and Z chromosome were not significant for all the egg production traits. We need to look for other markers in another location and can use different population of Silky fowl, may be good results can be achieved.

Moreover, QTL study was not done in this study. Previous QTL studies showed significant results for the Z chromosome markers on egg production traits. The results of this study and previous QTL mapping for the chicken Z chromosome suggest that we can select other markers on the adjacent location of the QTLs and perform fine QTL mapping of the Z chromosome in the 5^{th} generation of Oita's Silky fowl.

In Bangladesh, approximately 135 million chickens throughout the country are mostly of indigenous type (BBS, 2008) and show high level of morphological and phenotypic variability under natural condition (Maeda *et al.*, 1988). About 89 % of the rural households (HH) keep chicken with an average flock size of 5.33 per house hold under backyard scavenging system (BBS, 2008) which mirrored the significance of indigenous chicken for Bangladesh perspective.

Among indigenous chicken genetic resources, non-descript Deshi (ND) and Naked Neck (NN) chickens are noteworthy (Bhuiyan *et al.,* 2005). Islam (2000) found that Bangladeshi indigenous chickens are well adapted to tropical climate, and naked neck chicken is especially a promising and probable

genetic resource in terms of productive adaptability. The annual egg production per hen was 50-55 in Naked Neck and was 45-50 in Deshi chickens under scavenging conditions. In general the eggs of indigenous chicken are much smaller than those of exotic breeds/strains. Egg weight varied from 35-39 g in Deshi to 42 g in Naked Neck chickens. (FAO, 2004)。

Faruque *et al.*, (2013) reported that naked neck is genetically superior to non-descript desi in terms of reproductive parameters like age at sexual maturity and fertility and productive parameters like egg weight and body weight. Crossbred of indigenous naked neck with exotic chicken can perform even better than exotic chicken in terms of productive and reproductive traits (Islam and Nishibori, 2009; Islam and Nishibori, 2010). Faruque *et al.*, (2013) suggested that genetic selection for body weight may be effective to improve growth trait.

Still now in Bangladesh, genetic selection of chicken using DNA markers is in primary stage. Based on our research findings, for future development of native chicken stock of Bangladesh, we can plan cross breeding program between White leghorn breed or Rhode Island Red with naked neck type chicken and genotype the segregating population for relationship study of egg production trait and growth rate with respective markers where we have found significant result in our study.

4.2 Conclusion

This study focused on selecting suitable genetic markers in Japanese Silky fowl which are directly or indirectly related with growth and egg production trait may be of great importance in the poultry industry particularly in marker based selection program of Japanese Silky and other domestic chickens.

Current research works found seven potential genetic markers on chromosome 2, 24 and on the Z chromosome in Japanese Silky Chicken that could be effective to improve body weight trait such as rs16773406 in female and *PRL, DRD2, rs16763148, embigin, rs16773406* and *rs15991083* in male; rs16773406 for egg production rate, *NPY* for egg shell strength and *rs15991083* for average egg weight trait.

References

Abasht B, Dekkers J CM and Lamont SJ. 2006. Review of quantitative trait loci identified in the chicken. Poultry Science, 85: 2079–2096.

Andersson, L. 2001. Genetic dissection of phenotypic diversity in farm animals. Nature reviews. Genetics, 2(2)：130–138.

Arisawa K, Yazawa S, Atsumi Y, Kagami H and Ono T. 2006. Skeletal analysis and characterization of gene expression related to pattern formation in developing limbs of Japanese silkie fowl. Journal of Poultry Science, 43: 126-134.

Bangladesh Bureau of Statistics. 2008. Statistical yearbook. Bangladesh Bureau of Statistics, Dhaka

Becker RA, Chambers JM and Wilks AR. 1988. The New S Language. Chapman & Hall, London, pp.702.

Beuzen ND, Stear MJ and Chang KC. 2000. Molecular markers and their use in animal breeding.The Veterinary Journal, 160: 42–52.

Bhattacharya TK, Chatterjee RN, Sharma RP, Niranjan M, Rajkumar U and Reddy BLN. 2011. Polymorphism in the prolactin promoter and its association with growth traits in chickens. Biochemical Genetics, 49: 385-394.

Bhuiyan AKFH, Bhuiyan MSA, Deba GK. 2005. Indigenous chicken genetic resources of Bangladesh—current status and future outlook. Animal Genetic Resources Information, 36: 73–84.

Bole-Feysot C, Goffin V, Edery M, Binart N and Kelly PA. 1998. Prolactin (PRL) and its receptor: actions signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocrine Reviews, 19: 225–268.

Briggs DM and Nordskog AW. 1973. Influence of sex chromosomes on laying-house performance of chickens. British poultry science, 14: 403-412.

Burke WH and Dennison PT. 1980. Prolactin and luteinizing hormone levels in female turkeys (*Meleagris gallopavo*) during a photoinduced reproductive cycle and broodiness. General and Comparative Endocrinology, 41: 92-100.

Chang HL and Huang YC. 2003. The relationship between indigenous animals and humans in APEC region. The Chinese Society of Animal Science, Taiwan.

Cui JX. Du HL, Liang Y, Deng XM, Li N and Zhang XQ. 2006. Association of Polymorphisms in the Promoter Region of Chicken Prolactin with Egg Production. Poultry Science, 85: 26-31.

Da, Y. 2003. Statistical analysis and experimental design for mapping genes of

complex traits in domestic animals. Yi Chuan Xue Bao (Acta genetic Sinica), 3012: 1183–1192.

Dorshorst B, Okimoto R and Ashwell C. 2010. Genomic Regions Associated with Dermal Hyperpigmentation, Polydactyly and Other Morphological Traits in the Silkie Chicken. Journal of Heredity, 101: 339-350.

Dunn IC, Miao YW, Morris A, Romanov MN, Wilson PW and Waddington D. 2004. A study of association between genetic markers in candidate genes and reproductive traits in one generation of a commercial broiler breeder hen Population. Heredity, 92: 128–134.

FAO,2004.

Faraco CD, Vaz SA, Pastor MV, Erickson CA. 2001. Hyperpigmentation in the Silkie fowl correlates with abnormal migration of fate-restricted melanoblasts and loss of environmental barrier molecules. Developmental Dynamics, 220: 212-225.

Faruque S, Siddiquee NU, Afroz M A and Islam MS. 2010. Phenotypic characterization of Native Chicken reared under intensive management system.Journal of the Bangladesh Agricultural University, 8 : 79–82.

Felício AM, Boschiero C, Balieiro JCC, Ledur MC, Ferraz JBS, Filho TM, Moura AS and Coutinho LL. 2013. Identification and association of

polymorphisms in *CAPN1* and *CAPN3* candidate genes related to performance and meat quality traits in chickens. Genetics and Molecular Research, 12 (1): 472-482.

Ferrand R, L'Hermite A. 1985. Experimental analysis of the extensive pigmentation in the Silkie fowl embryo: evidence for an environmental regulatory process. Experientia, 41: 512–514.

Fleenor D, Arumugam R and Freemark M. 2006. Growth hormone and prolactin receptors in adipogenesis: STAT-5 activation, suppressors of cytokine signaling and regulation of insulin-like growth factor I. Hormone Research, 66: 101–110.

Forsyth IA and Wallis M. 2002. Growth Hormone and Prolactin-Molecular and Functional Evolution. Journal of Mammary Gland Biology and Neoplasia, 7: 291-312.

Fotouhi N, Karatzas CN, Kuhnlein U, Zadworny D. 1993. Identification of growth hormone DNA polymorphisms which respond to divergent selection for abdominal fat content in chickens. Theoritical and Applied Genetics 85: 931–936.

Fu Y, Niu D, Ruan H, Luo J and Zhang YP. 2002. Studies on genetic relationship between silky chicken and other breeds. Chinese Journal of Animal

Science, 35: 5–7.

Goffin V and Kelly PA. 1997. The Prolactin/Growth Hormone Receptor Fam ily: Structure/ Function Relationships. Journal of Mammary Gland Biology and Neoplasia, 2: 7-17.

Guenette RS, Sridhar S, Herley M, Mooibroek M, Wong P and Tenniswood M. 1997. Embigin, A Developmentally Expressed Member of the Immunog lobulin Super Family, Is Also Expressed During Regression of Prostate and Mammary Gland. Developmental genetics, 21: 268–278.

Hall TR and Chadwick A. 1984. Dopaminergic inhibition of prolactin release from pituitary glands of the domestic fowl incubated *in vitro*. Journal of Endocrinology, 103: 63-69.

Hansen KA, Zhang Y, Colver R, Tho SP, Plouffe L Jr and McDonough PG. 2005.The dopamine receptor D2 genotype is associated with hyperprolactinemia. Fertility and Sterility, 84: 711-718.

Haw SG. 2006. Marco Polo's China: a Venetian in the realm of Khubilai Khan. Routledge studies in the early history of Asia 3. London (NY): Routledge.

He YC. 2003. Black-bone chicken and Chinese Taihe chicken and their medical property. Review of China Agricultural Science and Technology, 5: 64-66.

Hu YD, Zhou Y, Liu XH and Zhu Q, 2010. Correlations between Microsatellite Loci and Growth and Carcass Traits in Chinese Silkies. Research Journal of Poultry Sciences, 3: 5-10.

Hutt FB. 1949. Genetics of the fowl. New York: McGraw-Hill. Faraco CD, Vaz SA, Pastor MV, Erickson CA. 2001.

International Chicken Genome Sequencing Consortium. Sequence and compara tive analysis of the chicken genome provide unique perspectives on vertebrate evolution. 2004. Nature, 432: 695-716.

Islam MA. 2000. Effect of local and exotic strains of chicken for broiler production at hot-humid climate. Ph. D. Thesis. Institute of Animal Science, Faculty of Agriculture and Horticulture, Humboldt University of Berlin, Germany.

Islam MA and Nishibori M. 2009. Indigenous naked neck chicken: a valuable genetic resource for Bangladesh. World's Poultry Science Journal, 65: 125-138.

Islam MA and Nishibori M. 2010. Crossbred chicken for poultry production in the tropics. Journal of Poultry Science, 47: 271-279.

Jiang RS, Xu GY, Zhang XQ and Yang N. 2005. Association of polymorphisms for prolactin and prolactin receptor genes with broody traits in chickens. Poultry Science, 84: 839-845.

Kahtane Al-A, Chaiseha Y and EI Halawani M. 2003. Dopaminergic regulation of avian prolactin gene transcription. Journal of Molecular Endocrinology, 31: 185-196.

Karatzas CN, Guemene D, Zadworny D and Kuhnlein U. 1997.Changes in expression of the prolactin and growth hormone gene during different reproductive stages in the pituitary gland of turkeys. Reproduction Nutrition Development, 37: 69-79.

Kelly PA, Binart N, Freemark M, Lucas B, Goffin V and Bouchard B. 2001. Prolactin receptor signal transduction pathways and actions determined in prolactin receptor knockout mice. Biochemical Society Transactions, 29: 48–52.

Koketsu M and Toyosaki T. 2004. Nutritive constituents of silky fowl eggs: Comparison with hen eggs of White Leghorn origin. Animal Science Journal, 75: 67–69.

Koketsu M, Sakuragawa E, Linhardt RJ and Ishihara H. 2003. Distribution of

N-acetylneuraminic acid and sialylglycan in eggs of the Silky fowl. British Poultry Science, 44: 145-148.

Koketsu M, Nitoda T, Sugino H, Juneja LR, Kim M, Yamamoto T, Abe N, Kajimoto T, Wong CH. 1997. Synthesis of a novel sialic acid derivative (sialylphospholipid) as an antirotaviral agent. Journal of Medicinal Chemistry, 40: 3332–3335.

Koketsu M, Nitoda T, Juneja LR, Kim M, Kashimura N, Yamamoto T. 1995. Sialyloligosaccharides from egg yolk as an inhibitor of rotaviral infection. Journal of Agricultural Food Chemistry, 43: 858–861.

Kuenzel WJ and McMurtry J. 1988. Neuropeptide Y: brain localization and central effects on plasma insulin levels in chicks. Physiology & Behavior, 44: 669-678.

Kuklenski J. 1915. Uber das vorkommen und die verteilung des pigmentes in den organen und geweben bei japanischen seiden huhnern. Arch Micro Anat Entwickl, 87: 1–37.

Kunita R, Nakabayashi O, Kikuchi T and Mizuno S. 1997. Predominant expres sion of a Z-chromosome-linked immunoglobulin superfamily gene, ZOV3, in

steroidogenic cells of ovarian follicles and in embryonic gonads of chickens. Differentiation, 62: 63–70.

Kwok, PY and Chen X. 1998. Detection of single nucleotide variations. Genetic Engineering, 20: 125–134.

Landegren, U, nilsson, M. and Kwok, PY. 1998. Reading bits of genetic information: Methods for single-nucleotide polymorphism analysis. Genome Research, 8: 769–76.

Li HF, Zhu WQ, Chen KW, Wu X, Tang QP, Gao YS, Song WT and Xu HL. 2009. Polymorphism in NPY and IGF-I genes associate with reproductive traits in Wenchang chicken. African Journal of Biotechnology, 8: 4744-4748

Li L-B, Li D-Y, Zhao X-L, Liu Y-P, Wang Y and Zhu Q. 2012. Polymorphism of Prolactin Receptor Gene and its Association with Egg Production Traits in Erlang Mountainous Chicken. Asian Journal of Animal and Veterinary Advanc es, 7: 1183-1190.

Liu YP, Wu GS, Yao YG, Miao YW, Luikart G, Baig M, Beja-Pereira A, Ding ZL, Palanichamy MG and Zhang Y-P. 2006. Multiple maternal origins of chickens: out of the Asian jungles. Molecular Phylogenetics and Evolution, 38: 12-19.

Maeda Y, Okada I, Hashiguchi T, Hasnath MA (1988) Blood protein polymorphism of native fowl and Red Jungle Fowl in Bangladesh. Report of the Society for Researches on Native Livestock, 12 : 233–250.

March JB, Sharp PJ, Wilson PW and Sang HM. 1994.Effect of active immunization against recombinant-derived chicken prolactin fusion protein on the onset of broodiness and photoinduced egg laying in bantam hens. Journal of Reproduction and Fertility, 101: 227-233.

Missale C, Nash SR, Robinson SW, Jaber M and Caron MG. 1998. Dopamine receptors: from structure to function. Physiological Reviews, 78: 189-225.

Nataraj AJ, Olivos-Glander I, Kusukawa N and Highsmith WE. 1999. Single-strand conformation and heteroduplex analysis for gel-based mutation detection. Electrophoresis, 20: 1177–1185.

Oztabak K, Ozkan E, Soysal I, Paya I, Un C. 2009. Detection of prion gene promoter and intron1 indel polymorphisms in Anatolian water buffalo (Bubalus bubalis). Journal of Animal Breeding and Genetics, 126(6): 463-7

Parsons BL and Heflich RH. 1997. Genotypic selection methods for direct analysis of point mutations. Mutation Research, 387: 97–121.

Primmer CR, Borge T, lindell J and Sætre GP. 2002. Single-nucleotide polymorphism characterization in species with limited available sequence information: high nucleotide diversity revealed in the avian genome. Molecular Ecology, 11 : 603-612.

Qiu XP, Chen E and Chen YX. 1988. Poultry breeds in China. Shanghai Scientific & Technical Publishers, Shanghai.

Ou JT, Tang SQ, Sun DX and Zhang Y. 2009. Polymorphisms of three neuroendocrine-correlated genes associated with growth and reproductive traits in the chicken. Poultry Science, 88: 722–727.

Rahman MM , Matsuda R, Matsuda T, Anann K and Wada Y. 2014. Relation ship between the Production Traits and the Candidate Genes in the Prolactin's In/Del x In/Del Population of Silky Fowl. Journal of Poultry Science, 52: 138-143.

Roberts V. 1997. British Poultry Standards. 5th ed. Blackwell Publishing Ltd. Malden. MA. USA.

Rothschild M, Jacobson C, Vaske D, Tuggle C,Wang LZ and Short T. 1996. The estrogen receptor locus is associated with a major gene influencing litter size in pigs. The Proceedings of the National Academy of Sciences of the United

States of America, 93: 201–205.

Rowshan J, Matsuda T, Matsuda R, Sunagawa C, Rahman MM, Kohara E, Anann K and Wada Y. 2012. Relationship between the production traits and the candidate genes on chromosome 2 in the 5th generation of selection pro gram of silky fowl. Journal of Warm Regional Society of Animal Science, Ja pan. 55: 115-120.

Saitoh Y, Ogawa A, Hori T, Kunita R and Mizuno S. 1993. Identification and localization of two genes on the chicken Z chromosome: implication of evolu tionary conservation of the Z chromosome among avian species. Chromosome Research, 1: 239–251.

Sakakibara K, Tabata S, Shiba N, Gotoh T, Nishimura S, Iwamoto H. 2000. Myofibre composition and total collagen content in *M. iliotibialis lateralis* and *M. pectoralis* of Silkie and White Leghorn chickens. British Poultry Science, 41:570–574.

Sartsoongnoen N, Kosonsiriluk S, Prakobsaeng N, Songserm T, Rozenboim I, Halawani ME and Chaiseha Y. 2008. The dopaminergic system in the brain of the native Thai chicken, Gallus domesticus: localization and differential expression across the reproductive cycle. General and Comparative

Endocrinology, 159: 107-115.

SAS Institute Inc. 1999. SAS/STAT® User's Guide, Version 8. Chapter 41 The MIXED Procedure.

Sasaki O, Odawara S, Takahashi H, Nirasawa K, Oyamada Y, Yamamoto R, Ishii K, Nagamine Y, Takeda H, Kobayashi E and Furukawa T. 2004. Genet ic mapping of quantitative trait loci affecting body weight, egg character and egg production in F2 intercross chickens. Animal Genetics, 35: 188-194.

Sharp PJ, McNamee MC, Sterling RJ, Lea RW and Pederson HC. 1988. Relationships between prolactin, LH and broody behavior in bantam hens. Journal of Endocrinology, 118: 279-286.

Sourdioux M, Douaire M, Delabrosse Y. 1996. DNA polymorphisms of lipogenesis genes and analysis of linkage with fatness in turkeys. Poultry Science 75: 1018–1026.

Sugiyama M and Washimi T. 2010. Food system of domestic fowls in Japan: A research on how the food culture of silky fowls occurred and it will develop in the future in Japan. The bulletin of Tokai Gakuin University, 4: 77-82.

Suzuki T, Kansaku N, Kurosaki T, Shimada K, Zadworny D, Koide M, Mano T, Namikawa T and Matsuda Y. 1999. Comparative FISH mapping on Z chromosomes of chicken and Japanese quail. Cytogenetics and Cell Genetics, 87: 22-26.

Talbot RT, Hanks MC, Sterling RJ, Sang HM and Sharp PJ. 1991. Pituitary prolactin messenger ribonucleic acid levels in incubating and laying hens: effects of manipulating plasma levels of vasoactive intestinal polypeptide. Endocrinology, 129: 496-502.

Tian Y, Xie M, Wang W, Wu H, Fu Z, Lin L. 2007. Determination of carnosine in Black-Bone Silky Fowl (*Gallus gallus domesticus* Brisson) and common chicken by HPLC. European Food Research and Technology, 226: 311-314

Toyosaki T and Koketsu M. 2004. Oxidative stability of silky fowl eggs. Com parison with hen eggs. Journal of Agriculture and Food Chemistry, 52: 1328–1330.

Tsudzuki, M. 2003. Japanese native chickens. In The Relationship between Indigenous Animals and Humans in APEC Region. (Ed. Chang and Huang). The Chinese Society of Animal Science, Taiwan, Tainan. pp. 91-116.

Tuiskula-Haavisto M, Honkatukia M, Preisinger R, Shmutz M, de Koning DJ, Wei WH and Vilkki J. 2010. Quantitative trait loci affecting eggshell traits in an F_2 population. Animal Genetics, 42: 293-299.

Tuiskula-Haavisto M, Honkatukia M, Vilkki J, de Koning DJ, Schulman NF and Mäki-Tanila. 2002. A Mapping of quantitative trait loci affecting quality and production traits in egg layers**.** Poultry Science, 81: 919-927.

Urbanek M, Legro RS, Driscoll DA, Azziz R, Ehrmann DA and Norman RJ. 1999. Thirty-seven candidate genes for polycystic ovary syndrome: strongest evidence for linkage is with follistatin. Proceedings of the National Academy of Sciences of the United States of America, 96: 8573–8578.

Wada Y, Nakamuta Y, Jiang Y, Matsuda R, Abe S and Anan K. 2008. Relationship between 24-bp insertion of prolactin promoter region and performance traits in the selected population of Silkie chicken. Journal of Poultry Science, 45: 82-86.

Wada Y, Yamada Y, Nishibori M and Yasue H. 2004. Complete nucleotide sequence of mitochondrial genome in Silkie fowl (*Gallus gallus var. domesticus*). Journal of Poultry Science, 41:76-82.

West B and Zhou BX. Did chickens go north. 1989. New evidence for

domestication. World's Poultry Science Journal, 45: 205-209.

Xie CX. 1995. The breeding history of poultry in China. Chinese Agriculture Publishers, Beijing.

Xu HP, Shen X, Zhou M, Luo CL, Kang L, Liang Y, Zeng H, Nie QH, Zhang DX and Zhang XQ. 2010. The dopamine D2 receptor gene polymorphisms associated with chicken broodiness. Poultry Science, 89: 428-438.

Youngren OM, Pitts GR, Phillips RE and El Halawani ME. 1995. The stimulatory and inhibitory effects of dopamine on prolactin secretion in the turkey. General and Comparative Endocrinology, 98: 111-117.

Youngren OM, Chaiseha Y and El Halawani ME. 1998. Regulation of prolactin secretion by dopamine and vasoactive intestinal peptide at the level of the pituitary in the turkey. Neuroendocrinology, 68: 319-325.

Zhou B, Chen SY, Zhu Q, Yao Y-G and Liu Y-P. 2010. Matrilineal components and genetic relationship of Silkies from China and Japan. Journal of Poultry Science, 47: 22-27.