Study for Breeding of Japanese Silkie Fowl

日本の烏骨鶏の育種の研究

JAHAN ROWSHAN

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Abstract

The Silkie fowl (*Gallus gallus* var. *domesticus*) is distinct from other chicken breeds in terms of its appearance and behavioural characteristics. Silkie fowl is well known for its medicinal properties. Now a day it is a rare and expensive breed. The egg of Silkie fowl is also expensive. It is a Japanese native breed and has inhabited Japan since before the Edo era. Although the breed is considered to have originated in India and established in China and Japan, its evolutionary history and genetic relationship with other breeds are not clear. About Silkie fowl, genetic research is very few.

In this study, it is determined the mitochondrial complete D-loop nucleotide sequences of 27 Silkie fowls and three other chicken breeds. In the Silkie fowls, we found 27 sites of single nucleotide polymorphism and 4 sites of single nucleotide insertion. Phylogenetic analysis revealed that the Silkie fowls, nine other chicken breeds, four red jungle fowls and 42 haplotypes in Oka *et al*. (2007) were distributed in five clades. Silkie fowls belonged to five clades (A-E). These results suggest that Japanese Silkie fowls have high genetic divergence. However, all categories except SLSG (Saga Prefectural Livestock Experiment Station, white feathers) were distributed in only one or

two clades, and five individuals with black feathers belonged to clade A. The Silkie fowl's wide distribution in the phylogenetic tree suggests that old Asian breeds crossed with several chicken breeds that had unusual traits to establish the Silkie fowl breed.

The egg production rate of the Silkie fowl is very low because of broodiness. Some researchers have reported that candidate genes located on chromosome 2 of the Silkie fowl, such as the prolactin gene, are related to broodiness, egg production rate, and age at first egg. To investigate the efficiency of marker-assisted selection, the markers of candidate genes (prolactin gene, vasoactive intestinal peptide receptor 1 gene, and neuropeptide Y gene) were genotyped in Silkie fowls of the 5th generation of Oita's selection program. The effect of the father was significant ($P < 0.01$) for all the traits including the egg production rate; however, the genotypic effects of the three candidate gene markers were not significant for all the traits. These results suggested that the genetic variation of Silkie fowls in Oita's selection program is high, and that the egg production rate in the Silkie fowl population can be improved.

Abstract

(Japanese)

烏骨鶏はその外観と行動特性において他の鶏品種と異なっており、現代では、烏骨鶏は希少で高価な品種であり、その卵も高価である。烏骨鶏は日本在来鶏の 1 種であり、江戸時代から日本で飼育されている。この品種はインド起源で、中国で確立されたといわれているが、その成立の過程や他の品種との遺伝的関連性については明らかではない。

本研究では、27 羽の烏骨鶏と他の鶏 3 品種について、ミトコンドリアの D-loop 領域の全長の塩基配列を決定した。その結果、烏骨鶏において 27 か所の 1 塩基置換と 4 か所の 1 塩基挿入が発見された。烏骨鶏と他の 9 品種および 4 羽の赤色野鶏、Oka *et al.*(2007)で示された 42 のハプロタイプを用いて分子系統樹を作成したところ、分子系統樹から 5 つのクレードに分割することができた。烏骨鶏は 5 つのクレードすべてに存在しており、このことは日本の烏骨鶏は高度な遺伝変異を持っていることを示唆している。しかしながら、SLSG（佐賀県畜産試験場、白羽系統）を除く各カテゴリーの烏骨鶏は、それぞれ 1 つか２つのクレードにのみ属しており、5 羽の黒羽の烏骨鶏はクレード A にのみ属していた。分子系統樹上における烏骨鶏の広範囲の分布は、古いアジアの品種に、特異的な形質をもつ数種の品種が交配されて烏骨鶏が形成されたことを示唆している。

就巣性を持っているので烏骨鶏の産卵率は非常に低い。研究者たちはプロラクチンのような烏骨鶏の 2 番染色体に座乗している就巣性や産卵率、初産日齢に関与している候補遺伝子について報告している。そこで、マーカーアシスト選抜の効率を検討するために、候補遺伝子（プロラクチン遺伝子、バソアクティブ腸管ペプチド受容体 1 遺伝子、ニューロペプチド Y 遺伝子）のマーカーについて、大分県の選抜プログラム第 5 世代の烏骨鶏について遺伝子型判定を行った。その結果、すべての形質において３つの候補遺伝子マーカーの効果は有意ではなかったものの、父親の効果は全ての形質で有意（$P<0.01$）であった。これらの結果より、大分県の選抜プログラム第 5 世代の烏骨鶏における遺伝変異は大きく、今回検討した３遺伝子以外の候補遺伝子マーカーを用いた烏骨鶏の産卵率の改良の可能性が示唆された。

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ABBREVIATIONS

bp: Base pair

cDNA: Complementary Deoxyribonucleic Acid

DNA: Deoxyribonucleic acid

DHA : Dococsahexaenoic acid

kb: Kilobase

kDa: Kilodalton

LA-PCR: Long and Accurate PCR

mRNA: Messenger Ribonucleic Acid

mtDNA: Mitochondrial DNA

PCR: Polymerase Chain Reaction

PCR-RFLP: PCR of Restriction Fragment Length Polymorphism

PDB: Protein Data Bank

UTR: Untranslated Region

TCPOBOP: 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene

QTL : Quantitative Trait Loci

CHAPTER 1

INTRODUCTION

1.1History of Silkie fowl

The Silkie fowl (*Gallus gallus var. domesticus*) is a breed of chicken with soft, fluffy plumage and black skin. The breed was recognized in the United States in 1874, and still it is one of the most popular show or ornamental breeds of chicken. Thus, the Silkie fowl is most likely the oldest pure breed of chicken that has existence today. The Silkie fowl is one of the 27 Japanese native breeds which have been inhabited in Japan since before the Edo era (Muramtsu, 1979) and it was designated at national natural treasure of Japan in 1942 (Niasawa *et al.,* 1997). Although the Silkie fowl is considered to have originated in India and later established in China and Japan (Roberts, 1997), its history is not clearly known. These breeds are now bred worldwide and varieties are categorized on the basis of the area of their production. The Silkie fowl in Japan is thus known as the Japanese Silkie (Ukokkei in Japanese). The Japanese Silkie fowl possesses numerous unique characteristics (Tsudzuki, 2003).

Figure 1.1 Silkie fowl, an identical chicken breed

1.2 Characteristics of Silkie Fowl

Silkie fowl, as shown in Fig. 1.1, is distinct from other chicken breeds for its several identical characteristis. The name of Silkie fowl is an English name and it is given due to its special silky feather. It has silky plumage, purple colored skin and bluish meat (Fig. 1.5), purple to dark blue colored wattles, beak and comb and fifth hind toe (Kenjiro *et al.*, 2006) pointing slightly upwards as well as beads and feathering in the feet (Fig. 1.4). They also have sulfur-crested on top of their head as a characteristic feature. It is shaped like

flower petal and can be termed as Silkies' crest as shown in Fig. 1.2. This little tuff of feathers eventually grows out and just barely starts curling around Silkies' face. The Silkies' crest on the top of its head hides a secret though. Below these feathers, Silkie has something called a vaulted skull. Good show chickens and some pet quality Silkies have skulls that are actually arched up at the top of their heads. These leave vulnerable spots on top of their heads though.

Silkie fowl has mainly white and black colored feather along with several other colors. Both types of Silkies with white and black colors have fluffy feathers as shown in Fig. 1.3. The feathers of this chicken breed do not have flat webs like other chickens. They have also abnormal barbules and no barbicels and, therefore, result a silky appearance. Original Silkie fowls are mild, short, along with small and long head but short neck. Thus, for having above mentioned special feature characteristics, Silkie fowls are easily identified among other chicken breeds. The cocks of Silkie fowls weigh from 1.1 kg to 1.8 kg, while the hens weigh from 0.9 kg to 1.1 kg. Ten apparent characteristics that make Silkie fowls identical are shown in Table 1.1 (Chen *et al*., 2008).

Table 1.1 Apparent characteristics of Silkie fowl

1.Marbely crown	Crown is mulberry of hemicycle into the male beak.
2. Hairball in head	It is developed especially on the female and the feather comb stay in the head.
3. Green ear	Ear is green. It changes into the dark purple growing up.
4. Beard	Beard is growing up on under the beak. It is developed to the female.
5.Silk thread feather	The whole body is covered with a beautiful silky feather.
6. The fifth toe	There are five fingers or more of silky fowl's foot.
7. Leg hair	The feather is growing on the feet.
8. Black skin	Silkie fowl's skin is black. It is really different from other chickens.
9.Black meat	Silkie fowl's meat is black. It is a very rare in vertebrate animals.
10.Black bone	Silkie fowl's bone is black.

Figure 1.2 White and black Silkie fowls with their crests on head

Figure 1.3 Silkie fowls' feather; black and white

Figure 1.4 Rare characteristics of Silkie fowl's leg; (i) Silkie's five toes (ii) Silkie's feathers in feet

1.3 Hyperpigmentation

Silkie fowl has internal hyperpigmentation (Smyth *et al.*, 1990). In various organs of Silkie fowl like perimysium of the muscle, the surface of bones, trachea, mesentery, digestive canals, ovary and testis, the internal hyperpigmentation of the surface has been observed. Such visceral pigmentation is extremely rear in higher vertebrates (Muroya *et. al.,* 2000). The mechanism of hyperpigmentation, although, remains unclear, in recent studies it has been shown that the abnormal migration of melanoblast and the

(i) (ii)

Figure 1.5 The bluish meat of Silkie fowl (i) External pigmentation

(ii) Internal pigmentation

absence of environmental barrier molecules are responsible for the hyperpigmentation in Silkie fowl (Li *et al*., 2011). The bluish meat (cause of hyperpigmentation) of Silkie fowl is shown in Fig. 1.5.

1.4 Nutrition of Silkie Fowl Chicken

The eggs and meat of white Silkie fowl are well known in the orient for their abundant contents of unsaturated fatty acids, vitamins, calcium and potassium compared to other chickens (He. *et al.*, 2003; Toyosaki and Koketsu, 2004). It is found that the Silkie fowl has many health-giving properties beneficial to humans. In traditional Chinese medicine, eating not only the meat of Silkie fowl, but also its bones, organs and eggs is considered effective for nutritional fortification and treatment of infertility. Silkie 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).

The meat of Silkie fowl has been credited with medicine and health-promoting benefits. It has very low fat, and includes a lot of collagen, vitamin A, iron and DHA (Kehui. *et al*., 2011). They have a lot of nutrition which modern people find hard to ingest. Generally, the meat of chicken contains

acidic property, as same as the pork and beef contain. However, amazingly, the meat of Silkie fowl contains alkaline property. Alkaline food includes seaweeds and green-vegetables. Acidic food produces blood with high fat and cholesterol. However, alkaline food reduces cholesterol in blood by linoleic acid, EPA, DHA, and lecithin, which are good for activating blood circulation, relieving fatigue and building up immune system. Thus, Silkie fowl has been credited and well evaluated with medicine and health-promoting benefits. The meats and eggs of this type chicken are very good for human body, particularly, for women having pregnancy problem as well as people of having high cholesterol and high blood pressure problems as well. Moreover, the positive effects of Silkie's meats and eggs are found in preventing many adult diseases like diabetes, liver ailment, stroke, neuralgia etc. Several researchers have found that the content of DHA is high in brain, retina and neuro-system (Moriguchi *et al.,* 2004). Some studies on animals have shown that a lack of n-3 fatty acids can lower learning and visual abilities of animals (Narabari, 2001). Because, meat seems to be a major source of fat in the diet and especially of saturated fatty acids, which have been implicated in diseases associated with modern life, especially in developed countries. Therefore, it has become an increasing interest in recent years for having ways to

manipulate the fatty acid composition of meats in the regular diet (Wood *et al*., 2004).

The eggs of the original Silky fowl are well known in the Orient and for thousands of years have 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). The egg of Silkie fowl 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). The ratio of egg yolk weight to whole egg weight of Silkie fowl is significantly larger than that of egg yolk of layer egg. The amount of cholesterol of Silkie fowl egg is significantly ($P < 0.01$) less than that of hen egg. The amount of vitamins (B2, B6, D and E), calcium and potassium in Silkie fowl eggs are significantly higher than those of hen eggs. Unsaturated fatty acids in Silkie fowl eggs are 62.5% among total fatty acids, where the unsaturated fatty acids of hen eggs are 53.9%. Especially, the contents of arachidonic acid, docosapentaenoic acid and docosahexaenoic acid in Silky fowl eggs are significantly higher than that of

layer eggs. Recently, increasing of stable breeders made people easier to get Silkie fowls' eggs and meats at shops and restaurants. Several eggs of Silkie fowl is shown in Fig. 1.6.

Figure 1.6 Eggs of Silkie fowl

1.5 Growth and Egg Production:

As the reproductive capacity of white Silkie fowl is rather low, it is very necessary to investigate the influencing factors of reproduction including hormones, growth factors, cytokines and correlative gene expression. Male and female Silkies become sexually matured at about five months of age. A Silkie fowl chicken will produce 40-50 eggs in an ideal period. From the previous study (Nirasawa *et al.,* 1997) it has been suggested that the egg production rate of Silkie fowl is very low because of its strong low broodiness, where, the broodiness is a phylogenic trait control by some numbers of autosomal genes (Romanov *et al.*, 2000). Moreover, in recent studies it has

been demonstrated that broodiness is not controlled by genes on chromosome Z.

Prolactin (*PRL*), a pituitary hormone that regulates important physiological functions, is thought to be an important candidate gene for broodiness and it plays an important role in incubation behavior of avians (Sharp *et al.*, 1984, El Halawani *et al.*, 1993). A 24-bp deletion in the 5′ flanking region of the prolactin gene has also been detected in Chinese and Japanese Silkie fowl (Jiang *et al*., 2005; Wada *et al*., 2008a, b). Jiang *et al.* (2011) has reported the presence of a linkage disequilibrium block between Silkie fowl and White Leghorn containing the prolactin gene region. Association analysis of polymorphisms has shown that this 24-bp insertion is associated with egg production (Cui *et al.*, 2006), but Wada *et al*. (2008b) reported that the relationship between the genotype of the insertion locus and the performance traits was analyze, however there was no relationship for growth traits and egg production.

1.6 Genetic Relationship:

Genetically, Silkie fowl is very interesting; however, genomic research of this breed is sparse. Wada *et al*. (2004) compared the complete mitochondrial DNA of the Japanese Silkie fowl with that of the White Leghorn and found 99.77% similarity between the two breeds. Oka *et al.* (2007) analyzed the mitochondrial complete D-loop region of Japanese native

chickens to clarify their phylogenetic relationships, possible maternal origin, and routes of introduction into Japan. They identified seven haplogroups and suggested that Chinese and Korean chickens were derived from Southeast Asia, and both non-game and game chickens formed the foundation of Japanese native chickens.

1.7 Objective:

In the present research, the mitochondrial complete D-loop nucleotide sequences are thoroughly investigated for identifying the distribution traits of Japanese Silkie fowls in the phylogenetic tree. Furthermore, the relationships between the production traits and the candidate genes on the chromosome 2 in the 5^{th} generation of selection program of Silkie fowl are also identified. Finally, the dissertation has been arranged as follows; chapter 1: introduction, chapter 2: Japanese Silkie fowls are widely distributed in the phylogenetic tree derived from mitochondrial complete D-loop nucleotide sequences, chapter 3: relationship between the production traits in the 5^{th} generation of selection program of Silkie fowl, chapter 4 conclusions and discussion.

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CHAPTER 2

Japanese Silkie fowls are widely distributed in the phylogenetic tree derived from mitochondrial complete D-loop nucleotide sequences

2.1 Introduction

Animal mitochondrial DNA (mtDNA) is highly polymorphic, almost exclusively maternally inherited and without genetic recombination. The clonal transmission of mtDNA haplotypes allows the discrimination of maternal lineages within species and analysis of sequence of their most variable regions can be used to investigate the genetic origin of animal populations and breeds and thus the domestication processes (Alves *et al.*, 2003). Mitochondrial DNA has been widely employed in phylogenetic studies of various animals because it evolves much more rapidly than nuclear DNA, resulting in the accumulation of differences between closely related species. Mitochondrial DNA has been widely employed in phylogenetic studies of various animals because it evolves much more rapidly than nuclear DNA, resulting in the accumulation of differences. The mitochondrial genome in bird is approximately 17,000 base pairs (bp) in length.

Wada *et al.* (2004) compared the complete mitochondrial DNA of the Japanese Silkie fowl with that of the White Leghorn and found that the similarity between the two breeds was 99.77% (39 nucleotide differences in 16,784 base pairs). Thirty-nine nucleotide substitution have been observed between the Silkie fowl (accession no. AB086102) and White Leghorn (accession no AP003317), and 40 nucleotide substitutions have been noted between the Silkie fowl and the White Plymouth Rock (accession no. AP003318). These substitutions much greater than those (6 substitutions) between the White Leghorn and the White Plymouth Rock (Nishibori *et al.*, 2003), are indicating that the genetic relationship between the Silkie fowl and the current commercial breeds is remoter than that between the commercial breeds.

Several researches have studied the mitochondrial partial D-loop regions of Japanese and Chinese Silkie fowls (Fu *et al*., 2002; Liu *et al*., 2006a; Zhou *et al*., 2010) and Japanese and Chinese native chickens, including Silkie fowls (Fumihito *et al*., 1994, 1996; Komiyama *et al*., 2003, 2004; Liu *et al*., 2006b). Partial D-loop nucleotide sequences provide less information than complete D-loop nucleotide sequences.

Oka *et al.* (2007) analyzed the mitochondrial complete D-loop region of Japanese native chickens to clarify their phylogenetic relationship, possible maternal origin and routes of introduction into Japan. They identified seven haplogroups and suggested that Chinese and Korean chickens were derived from South Asia, and both non-game and game chickens formed the foundation of Japanese native chickens. In the analysis, two Silkie fowls were included in haplogroup B with Satsuma-dori and Aizu-Jidori.

2.2 Motivation

From the above literature review of Silkie fowls, it has been found that the genetic relationship between the Silkie fowls and other chicken breed is not identified clearly yet. Therefore in this study the genetic relationship between the Japanese Silkie fowl and other chicken breeds has to be analyzed by using mitochondrial complete D-loop nucleotide sequences.

In the study, 28 Silkie fowls and several other chicken breeds are to be considered for genetic investigation in order to examine the genetic relationship between Japanese Silkie fowls and other chicken breeds. By using the mitochondrial complete D-loop nucleotide sequence collected from the

DNA database, it will be constructed a phylogenetic tree to analyze the genetic relationships among Silkie fowls and other chicken breeds comparing to the results described by Oka *et al*. (2007).

2.3 Materials and Methods

2.3.1 Samples

In this research twenty seven Silkie fowls (nineteen with white feathers and eight with black feathers), two Nagoya breeds and one F1 individual of Rhode Island Red × White Plymouth Rock in Japan have been investigated. The blood samples of the Silkie fowls were collected from Hiroshima University Experiment Station for Animal Husbandry, Aomori Prefectural Agriculture and Forestry Research Division, Ibaraki Prefectural Livestock Research Center, Livestock Research Division of Mie Prefectural Science and Technology Promotion Center, Saga Prefectural Livestock Experiment Station and Yamanashi Wild Bird Breeding Center. The blood samples of Nagoya breeds and F1 individual of Rhode Island Red × White Plymouth Rock were collected from Saga Prefectural Livestock Experiment Station.

To examine the phylogenetic relationship of each breed and the red jungle fowl, the complete D-loop control region sequences determined in this study were processed together with those of a Silkie fowl in Hiroshima University (S1, accession no. AB086102), White Leghorn (WLCB, accession

no. AP003317; WLP, accession no. AP003580), White Plymouth Rock (WPR, accession no. AP003318), Laos native chicken (Lao11, accession no. AP003319), New Hampshire Red (NHR1, accession no. AY235570; NHR2, accession no. AY235571), and red jungle fowls (*Gallus gallus gallus*, RJFgal, accession no. AP003322; *Gallus gallus spadiceus*, RJFspa, accession no. AP003321; *Gallus gallus bankiva*, RJFban, accession no. AP003323).

White feather Silkie fowl

Black feather Silkie fowl

White Leghorn

New Hampshire

Nagoya

Shamo

White Plymouth Rock

Rhode Red Island

Red jungle fowl

Figure 2.1 Picture of chicken breeds

Table 2.1 Chicken breeds sequenced in this study

Breed	Place	Feather colour	Abbreviation	N
Silkie	Hiroshima University	White	S2	1
	Experiment Station for Animal Husbandry, Aomori Prefectural Agriculture and Forestry Research Center	White	SLKAW1, 4, 5, 6	4
		Black	SLKBW1, 2, 3, 7	4
	Ibaraki Prefectural Livestock Research Center	White	SLKI2, 3, 4, 5, 6	5
	Livestock Research Division, Mie Prefectural Science and Technology Promotion Center	White	SLKWM4, 6, 11, 12	4
		Black	SLKBM3, 4, 5, 6	4
	Saga Prefectural Livestock Experiment Station	White	SLSG1, 2, 4	3
	Yamanashi Wild Birds Breeding Centre	White	VS1, 2	2
Nagoya	Saga Prefectural Livestock Experiment Station		NA1, 3	2
Rhode Island Red × White Plymouth Rock	Saga Prefectural Livestock Experiment Station		GDG1	1

N = number of individuals

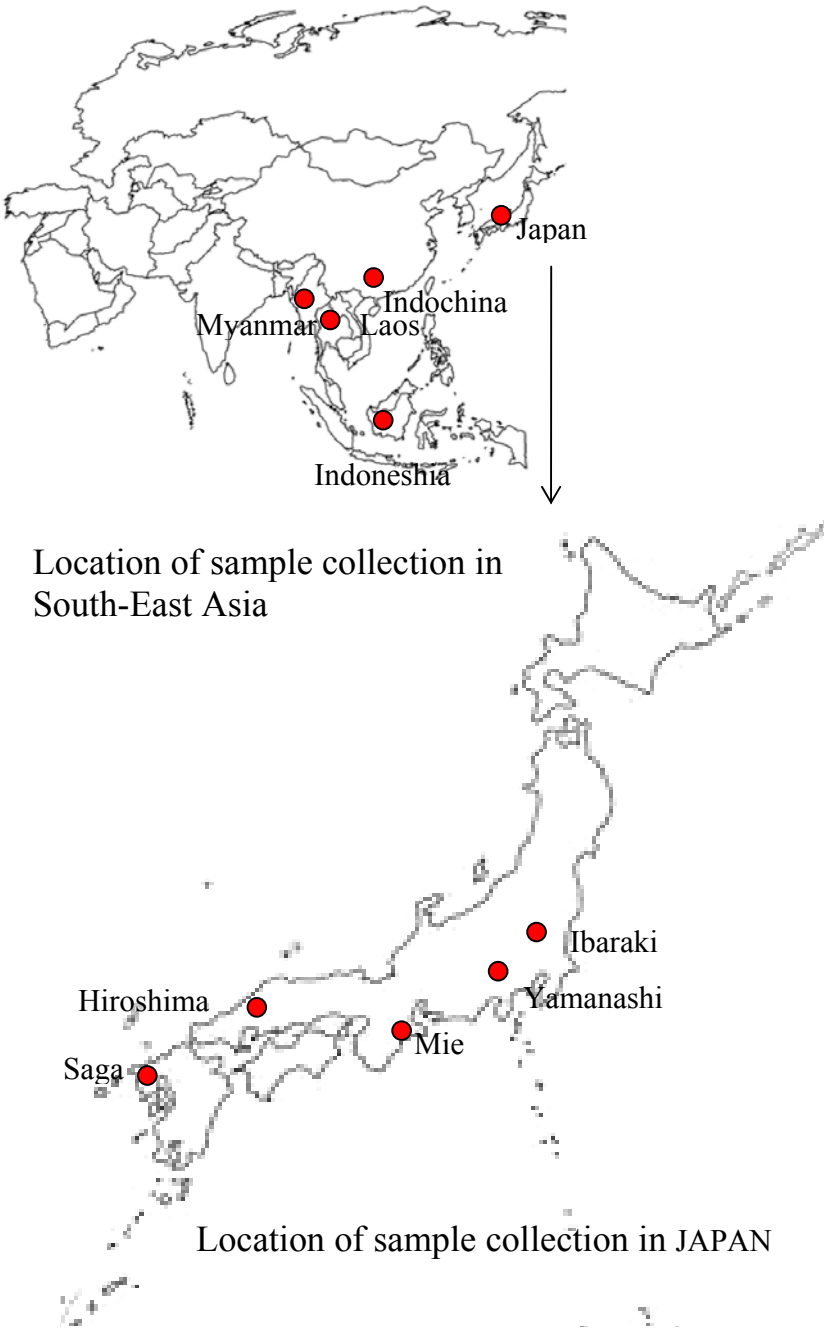

Figure 2.2 Sampling location (red marked) of Japanese Silkie fowl and other chicken breeds

2.3.2 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 was extracted from chickens blood sample and using the standard phenol and chloroform method by Sambrook and Russell (2001).

Mitochondrial DNA fragments were amplified from the mitochondrial genome by using the LA PCR kit and LA PCR primer sets following the procedures described previously (Nishibori *et al.,* 2001).

Amplified 16Kb fragments of the mitochondrial genome were then isolated through agarose gel electrophoresis and used for segmental amplification of the complete D-loop region. Later on, the DNA fragments obtained trough segmental amplifications were sequenced.

DNA has been extracted from Silkie fowl's blood with Pure Gene kit (Funakoshi Co., Ltd, Japan). At first, 15 μℓ of Silkie fowl's blood was filled in the 1.5 ml microcentrifuge tube. Then, the Cell Lysis Solution of 600μℓ and the Protease K solution (20mg/ml) of 2μℓ were added. The tube was inverted at 5 times. It has been incubating for three hours at 55℃.

The sample was cooled at the room temperature. The protein precipitation solution of 100µl was added into the tube. The tube was vortex vigorously at high speeds to 20 seconds for mixing the protein precipitation solution uniformly with the cell lysate. It was centrifuged at 15000g for 3 minutes and 4℃. The precipitated proteins were formed a tight dark brown pellet. The supernatant was carefully transferred to a new 1.5ml microcentrifuge tube by pipetting. Then, 600 µℓ of 100% isopropanol was added and mixed the sample by inverting 50 times gently. After that, it was centrifuged for 3 minutes at 15000g and 4℃. DNA was visible as a small white pellet. Then, 300 µℓ of 70% ethanol was added and inverted the tube several times to wash the DNA. Again it was centrifuged at 15000g for 1 minute and 4℃. The tube containing DNA was dried in a micro vac during 37 ℃ for 3 minutes. Hydration solution of 35 µℓ DNA was added and vortex lightly. The sample was kept in incubation at 65℃ for 1 hour.

The sample was cooled at room temperature. Vortexing was done for 5 seconds in medium speed. The DNA extraction was performed by using spectrophotometer. The cuvette was washed by using distilled water and then added 50 µℓ of distilled water and run on spectrophotometer as a blank control.

DNA solution of 50 µℓ was mixed with 45 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 preserved at -20°C.

2.3.3 Mitochondrial DNA amplification by LA-PCR

Mitochondrial DNA was extracted from the blood of Silkie fowl. Mitochondrial DNA fragment were amplified from the mitochondrial genome by using an LA PCR kit (Takara Bio, Inc., Ohtsu, Japan) with chicken DNA as the template and LA PCR primer set as mentioned in Table 2.2. This primer set was used to amplify a fragment of the chicken complete mitochondrial genome. A code was written to a PCR tube, and then, 24.5 µl distilled water was added in each tube. After that, 0.5 µl LA-Taq, 5 µl TA-Taq Buffer, 5 µl $MgCl_2$, 8 µl dNTP were added in each tube and the tubes were stood to the rack. Next, 2.0µl forward primer (10mM) and 2.0 µl reverse primer were added. Finally, 3.0 µl of DNA sample was added into the PCR tube and slight vortex was done. In negative control, distilled water was put in instead of the DNA sample. The PCR reaction was carried out in a thermal cycler with the

following conditions; an initial denaturation step at 94 °C for 1 minute, followed by 30 cycles of denaturation at 94°C at 10 seconds annealing at 68°C for 16 minutes, elongation at 68°C for 10 minutes and finally holding at 4°C. The PCR products were analyzed in a 0.8 % agarose gel, stained with ethidium bromide, and visualized using an ultraviolet transilluminator (FAS-III- mini FAS-301; TOYOBO co., Ltd., Osaka, Japan).

2.3. 4 Agarose gel electrophoresis

The PCR products were analyzed in 0.8% and 1.2% agarose gel electrophoresis stained with ethidium bromide and visualized by using a UV transilluminator. Concentrations of agarose gel were used according to expected DNA bands. One point two grams (g) of agarose gel S (Wako pure medicine Ltd., Tokyo, Japan) was weighted (for 1.2%) and dissolved in 200 ml Erlenmeyer flask, which contained 100 ml of 0.5×TBE electrophoresis buffer. One hundred of 0.5× TBE electrophoresis buffer was diluted from 5 × TBE stock (as shown in Table 2.3) solutions by mixing 10 ml of 5× TBE with 80 ml of distilled water (DW) and adding DW to make up 100 ml final volume of 0.5× TBE electrophoresis buffer. To visualize DNA in agarose gel, it was stained by the fluorescent dye ethidium bromide (EtBr). One microliter

of 10 mg/ml EtBr was then added to solution. Agarose gel solution was melted in a microwave oven until bubbles were appeared. The flask was removed from the microwave oven and gently swirled the flask to resuspend any settled powder and gel pieces. The flask was then heated again in the microwave oven until the agarose was dissolved. After gel properly dissolved, put the flask in room temperature and let it cooled to 50-60 ℃. The gel casting platform and gel comb were setting in order to make the sample wells. Warm agar rose gel solution was then poured into a gel casting apparatus and allowed to harden at room temperature (20-30 min). If the air bubbles were formed, it was removed by using a sterilized micropipette tip. After the gel had hardened, 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 on to the top of the harden gel. Five microliters of DNA size marker was loaded to a far right well. Two microliters of 6 × gel-loading buffer was mixed with 5 µl of sample on the paraffin paper and then the mixture was slowly and carefully loaded to a gel well by using a micropipette. The electrophoresis tank was covered by a lid and the DC power supply (100 volts) was connected to the positive and negative electrodes that attached to the horizontal electrophoresis tank. After, the electrophoresis was

run about 40-50 min or until the DNA samples or dyes had migrated a sufficient distance, turned off the power supply. The gel was then carefully transferred to a clean plastic tray and visualized and photographed by using an ultraviolet benchtop transluminator (FAS-III-mini FAS-301; TOYOBO Co., Ltd., Osaka, JAPAN).

Table 2.2 Primer for LA-PCR

Primer name	Sequence
LA16SF	5′- CCTACGTGATCTGAGTTCAGACCGGAGCAATCCAG-3′
LA16SR	5′- TGCACCATTAGGTTGTCCTGATCCAACATCGAGGT-3′
LACytbF	5′- TACACGAATCAGGCTCAAACAACCCCCTAGGCATC-3′
LACytbF	5′- AGATACAGATGAAGAAGAATGAGGCGCCGTTTGCG- 3 ′

.

Table 2.3 PCR Primers for D-loop region

Primer name	Sequence
AV1F	5′-CCAAGGACTACGGCTTGAAAAGCCAT-3′
AV1R	5′-GCTGAGTACCCGTGGGGGTGTGGCT-3′

Table: 2.4 Formulation of agarose gel electrophoresis buffer

5 × TBE

Reagent	Grade	The final density	Amount of use
Trisma base	Reagent high grade	0.445M	27g
Boric acid	Reagent high grade	0.445M	13.75g
0.5M EDTA(PH8.0)	Sterilization is unnecessary.	0.01M	10ml
Distilled water	Sterilization is unnecessary.	—	400ml
Distilled water	Sterilization is unnecessary.	—	Plus α
Total			500ml

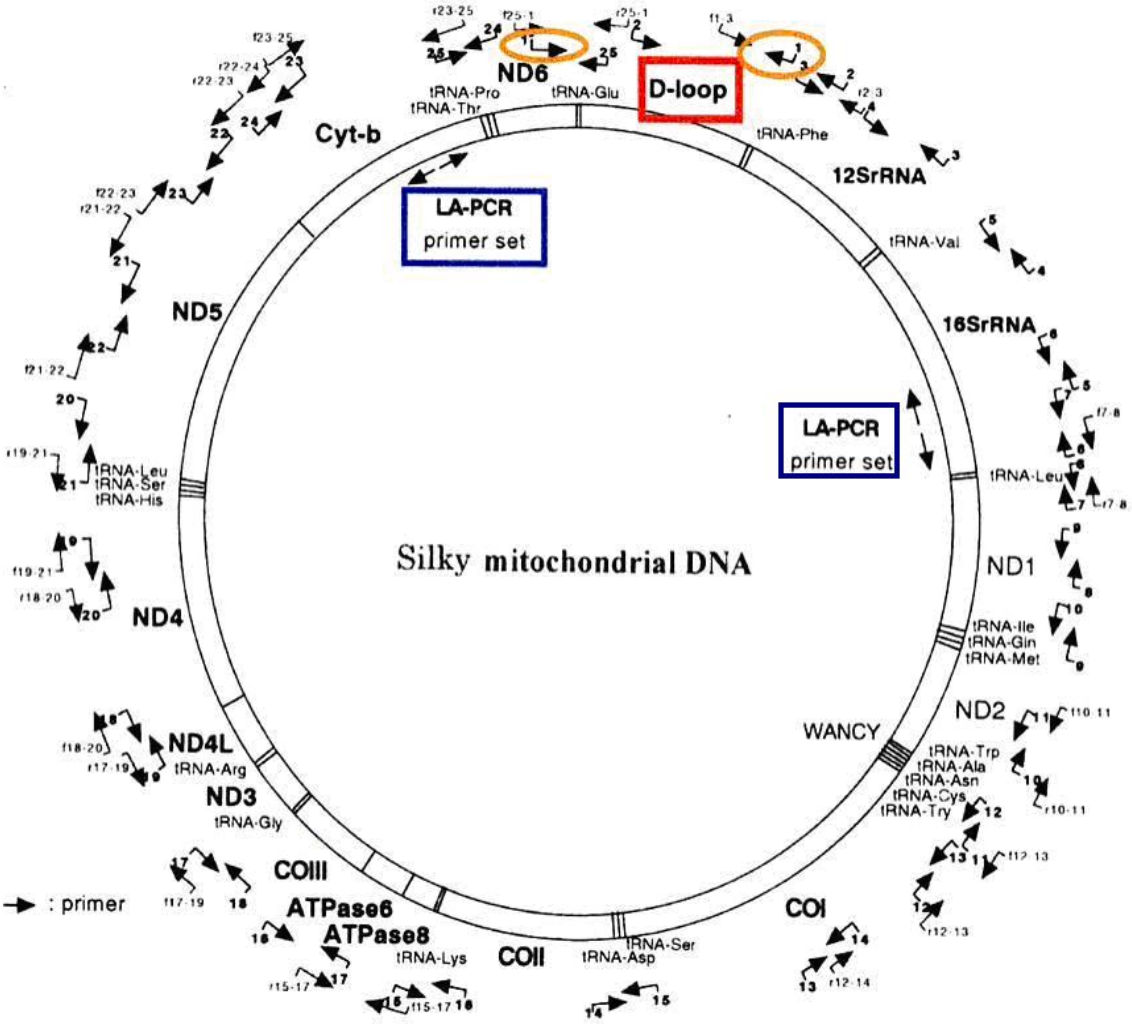

Figure 2.3 Genetic structure and primer position of Silkie fowl's mitochondria DNA

The Fig. 2.3 shows the genetic structure and the position of the primer of mitochondrial DNA of a Silkie fowl. In this research the control region is shown in red, the joining position of each LA-PCR primer set is shown in blue and the position of the primer set for direct sequencing is shown in yellow.

2.3.5 Mitochondrial DNA purification by RECOCHIP

The targeted mitochondrial DNA bands were recovered by using RECOCHIP (TaKaRa Bio Inc. Shiga, Japan).

The electrophoresis of PCR products were carried out using the SeaKem GTG agarose gel (Cambrex Bio Science Roak land, Inc., Rockland, ME, USA). The procedures of electrophoresis were the same as mentioned above and the expected electrophoresis buffer was the TBE buffer (Table 2.3). The agarose gel of 0.8% was used. DNA samples were migrated to a sufficient distance, the DC power supply was turned off and the EtBr stained agarose gel was visualized by using UV transilluminator.

The targeted band was confirmed and the gap was made at nonside nearby DNA band using gel cutter. The polestar RECOCHIP was pushed into the gap by facing the black surface of RECOCHIP towards the DNA band and the white surface towards the anode side. The gel was returned to the electrophoresis chamber and the electrophoresis was performed again. The loading time for this second electrophoresis was about 10 minutes corresponding to the protocol's guide. The electrophoresis was terminated, visualized and photographed in an UV transilluminator again to confirm the mobility and remaining amount of DNA band after recovery. The RECOCHIP

was brought back into a 2.0 ml tube again. The ethanol precipitation method was used to precipitate DNA solution. The sample tube contained RECOCHIP was centrifuged at 5,000 rpm for 10 seconds. The RECOCHIP was discarded using micro forceps. Five micro liters of 3 M sodium acetate (Na0Ac, pH 5.2), 1 µl of glycogen (20 mg/µl), and 1 ml of cold ethanol (99% ethanol) were successively added to the tube. The tube was gently inverted to 20 times and was incubated on ice-cooled box for 10 minutes. The tube was centrifuged at 15,000 rpm in a refrigerated microcentrifuge for 20 minutes at 4° C. The supernatant in the upper part of the tube was carefully discarded without disturbing the pellet by using the micro pipette. The tube was placed on ice-box and 500ml of 75% cold ethanol was added; followed by, RECOCHIP tube was gently inverted 20 times. The sample tube was centrifuged at 15,000 rpm at 4°C for 5 minutes. The supernatant was slowly and carefully decanted without disturbing the pellet. The tube was dried by uncap at room temperature for 10 minutes. The DNA pellet was resuspended using 20µl of distilled water, followed by briefly inverted and bench top centrifuged. The DNA solution was then transferred to properly labeled collection tube. Quantitation of DNA was performed using UV visible spectrophotometer. The cuvette was washed by using distilled water and then added 50 µl of distilled

water, and was run on spectrophotometer as a blank control. Five microliters of DNA solution was mixed with 45 µl distilled water in a new microcentrifuge tube. The solution was transferred to a cuvette, run on spectrophotometer and measured it. The refine DNA sample was preserved at -20°C.

2.3.6 Amplification of mitochondrial DNA in control region

The PCR pre-mix (Table 2.5), primer set, and mitochondrial DNA sample were taken out from the -20°C freezer and kept it few minutes in room temperature until completely melt. Then centrifuge slightly. Taken new PCR tubes, then added 17.375 µl distilled water, 4.625 µl PCR pre-mix, 1.0µl forward primer 5′-CCAAGGACTACGGCTTGAAAAGCCAT-3′ and 1.0 µl reverse primer 5′-GCTGAGTACCCGTGGGGGTGTGGCT-3′ and the tube was stood to the cool rack in each tube.

Table 2.5 PCR pre-mix composition

ExTaq(5U/µl)	**0.125** µL
ExTaq buffer	2.5 µL
dNTP (2.5 mM)	2 µL
Total	4.625

Finally 1.5 µl of mitochondrial DNA sample was added into the PCR tube and slight vortex was done. In negative control, 1.5µl distilled water was put instead of the DNA sample. The PCR reaction was carried out in a thermal cycler with the following conditions; an initial denaturation step at 95 °C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 1 minute, annealing at 58°C for 1 minutes, elongation at 72°C for 10 minutes and a final hold at 4°C. Sample of 5 µl and loading dye of 2 µl were used in the 1.2% gel electrophoresis. The PCR products were analyzed in a 1.2 % agarose gel, stained with ethidium bromide, and DNA band were visualized by using UV transilluminator.

2.3.7 Base sequence determination in complete D-loop region of mtDNA

Purification and direct sequence reaction in PCR product

The DNA fragments obtained through segmental amplification were sequenced using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit and ABI PRISM 310 (Applied Biosystems, Japan). The PCR products for sequencing reactions were purified by using ExoSAP-IT (USB Corporation, Cleveland, OH, USA). The ExoSAP-IT treatment was

used for removal of unincorporated dNTPs and primers from PCR products prior to sequencing reactions. Twenty microliters of PCR product was mixed with 3 μl ExoSAP-IT mixture, containing exonuclease I and shrimp alkaline phosphatase, in a 0.5 ml PCR tube. The reaction was carried out in a thermal cycler (Techne) with the following conditions: 37 ℃ for 30 minutes (incubation) followed by 80 ℃ for 15 minutes (inactivation of ExoSAP-IT). The purified PCR product was then used in the sequencing reactions. The sequencing reaction (Terminator sequencing kit; ABI Big Dye Ver. 3.1) was composed of 1.8 l of BigDye, 6.2 l of 2.5X sequencing buffer, 1.6 l of primer, 2 l of purified template DNA, and 8.4 l of sterilized distilled water. The reaction conditions were as follows: 25 cycles of 96 ℃ for 30 seconds, 50 ℃ for 15 seconds, and 60 ℃ for 4 minutes followed by the final holding at 4 ℃.

Purification of sequence reaction solution

Sequencing reactions were purified using the AutoSeq G-50 (Amersham Biosciences UK Ltd, UK). The AutoSeq G-50 column was used for removal of excess dye-labeled dideoxynucleotides from sequencing reactions. One hundred microliters of 10.5 mM EDTA (pH 8.0) was added to the AutoSeq G-50 column. The spin column was gently vortexed in order to resuspend the

resin. Loosen the top cap of spin column and placed the tube into a new 1.5 ml screw-cap microcentrifuge tube in which the top cap had been cut off. The AutoSeq G-50 spin column was pre-spun for 1 minute at 2000 g. The spin column was then placed in a new 1.5 ml microcentrifuge tube, followed by adding 20 μl of sample to the center of the angled surface of the compacted resin bed, being careful not to disturb the resin. The spin column was centrifuged at 2000 g for 1 min. The purified sample was collected in the bottom of the 1.5 ml microcentrifuge tube and was used in the automated sequencing at the Saga University.

2.3.8 Sequencing using ABI 320

After the purification of the reaction solution the decision for the nucleotide sequence was determined. The determination of the nucleotide sequence was carried out by using the ABI310 DNA sequencer (Saga University plant virus study laboratory) and the ABI377DNA sequencer (Hiroshima University livestock breeding study laboratory). The nucleotide sequence of each PCR product was assembled by using Vector NTI Suite7, and the nucleotide sequence of the mitochondria control region of individual object was determined.

2.3.9 Making of alignment and phylogenetic tree

To examine the phylogenetic relationship of each chicken breed and the Red Jungle Fowl, the complete D-loop control region sequences determined in this study were processed in Hiroshima University together with those of a Silkie fowl (S1, accession no. AB086102), White Leghorn (WLCB, accession no. AP003317; WLP, accession no. AP003580), White Plymouth Rock (WPR, accession no. AP003318), Laos native chicken (Lao 11, accession no. AP003319), New Hampshire Red (NHR 1, accession no. AY235570; NHR2, accession no. AY235571), and Red Jungle Fowl (*Gallus gallus gallus,* RJFgal, accession no. AP003322; *Gallus gallus spaduceus*; RJFspa, accession no. AP003321 *Gallus gallus bankiva*; RJFban, accession no. AP 003323). The 42 haplotypes of the complete D-loop control region (A01-A10, B01-B08, C01-C08, D01-D09, E01-E04, F01-F02, G01; accession nos. AB268506-AB26854) described in Table 2.1 by Oka *et al.* (2007) were also included in the analysis. The sample size of each breed is in the range of 2 (Silkie fowl) to 31 (Shamo).

The nucleotide sequence data was aligned by using clustal X (Thompson *et al*., 1997). The evolutionary history was inferred using the neighbour-

joining method (Saitou and Nei, 1987). The optimal tree was possessed a sum of branch lengths of 0.056. The percentage of replicate trees in which the associated taxa clustered together with the bootstrap test (1000 replicates) has been shown adjacent to the branches (Felsenstein, 1985). The tree has been drawn to a scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura's two-parameter method (Kimura, 1980), and results have been expressed as the numbers of base substitutions per site. The analysis was involved 80 nucleotide sequences. All positions containing gaps and missing data were eliminated. In total, 1231 positions were included in the final dataset. The evolutionary analysis was conducted with MEGA5 (Tamura *et al*., 2007).

2.4 Results

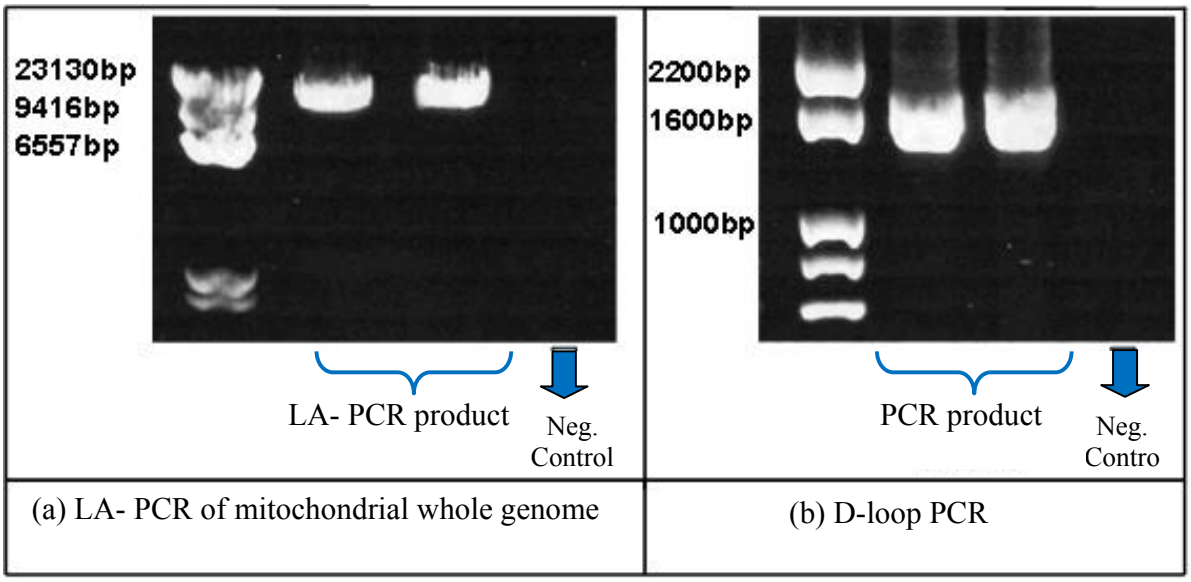

Figure 2.4 The photograph of Electrophoresis of PCR

The LA-PCR as mentioned above has been done by 0.8% agarose gel electrophoresis. Amplified 16-kb fragments of the mitochondrial genome were solated in this electrophoresis process. The LA-PCR (Fig. 2.4 (a)) fragment is located between 23,130bp and 9416bp (1Kb ladder). The D-loop PCR has been performed by using the PCR primer AV1F, AV1R for the purified LA-PCR products. This primer set amplified a 1,231bp fragment of mtDNA

control region (Fig. 2.3). The D-loop PCR product is isolated by 1.2% agarose gel electrophoresis. The DNA band is found between 1,600bp and 1,000bp (Fig 2.4 (b)).

The nucleotide sequences of the miotrocondrial complete D-loop regions of 27 Silkie fowls and three other chicken breeds (Nagoya, Rhode Island Red and white Plymouth Rock) were determined in the present research. The accession numbers of 27 Silkie fowls are AB263947- AB263970 and for other chickens are AB263971- AB263973. The total DNA sequences are shown as appendix B.

In the Silkie fowls, it has been found that 27 sites of single nucleotide polymorphism, which were transitioned except at 2 positions and 4 sites of single nucleotide insertion. Domain I of the D-loop region has 22 nucleotide substitutions (positions from 21 to 447). The results of this research are quite consistent with the previous reports where the Domains I and III were largely varied compared with the Domain II (Baker and Marshall, 1997; Nishibori et *al* 2004).

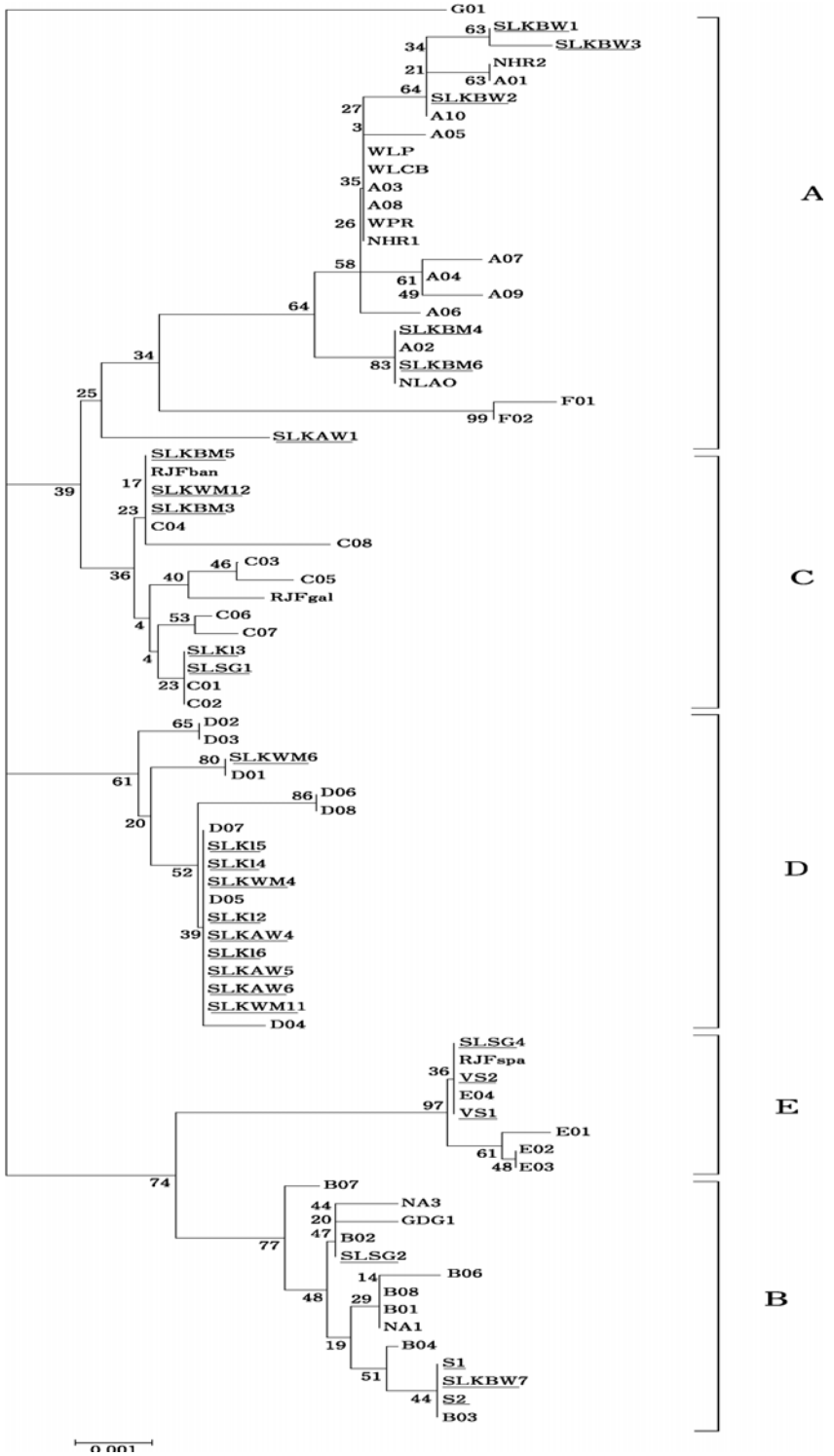

Figure 2.5 Phylogenetic tree derive from complete D-loop nucleotide sequences.

Phylogenetic trees (Fig. 2.5) were constructed from the nucleotide sequence of complete D-loop region of the 28 Silkie fowls, 9 other chicken breeds, 4 Red Jungle Fowls and 42 haplotypes as described in Table 2 by Oka *et al*. (2007). Total 83 D-loop sequences have been used in this research in which 41 numbers are from the current research and 42 numbers are from Oka *et al*. (2007). The Silkie fowls were distributed in five clades which is indicated in A-E clades. The number of Silkie fowl individuals in each clade is shown in the Table 2.6. The phylogenetic distributions of the Red Jungle Fowl and other chicken breeds in this study and Oka *et al*. (2007) are shown in Table 2.7.

Table 2.6 Distribution of the examined Silkie fowls in each clade

Clade[1]	S1,S2[2]	SLKAW	SLKBW	SLKI	SLKWM	SLKBM	SLSG	VS	Oka *et al.* (2007)
A		1	3			2			
B	2		1				1		2
C				1	1	2	1		
D		3		4	3				
E							1	2	

[1]According to Oka *et al.* (2007).

[2]Abbreviations, which represent the population of Silkie fowls in Japan as explained in Table 2.1.

Above results show that the Silkie fowls are distributed in all five clades (A-E). The number of Silkie fowl individuals in each clade is shown in Table 2.6. It is mentioned in the table that six Silkie fowls are located in clade A, among which one white feather type Silkie fowl (SLKAW) and 3 black feather type Silkie fowls (SLKBW) are located in Experiment Station for Animal Husbandry, Aomori Prefectural Agriculture and Forestry Research Center, and 2 black feather type Silkie fowls (SLKBM) in Livestock Research Division, Mie Prefectural Science and Technology Promotion Center

respectively. Six Silkie fowls are located in clade B, among which there are two white feather type Silkie fowls (S1 and S2) in Hiroshima University. Five Silkie fowls are located in clade C among which one white feather type Silkie fowl (SLKI) in Ibaraki Prefectural Livestock Research Center, one white feather type Silkie fowl(SLKWM) in Livestock Research Division, Mie Prefectural Science and Technology Promotion Center and two black feather type Silkie fowls (SLKBM) in Livestock Research Division, Mie Prefectural Science and Technology Promotion Center, and one white feather type Silkie fowl (SLSG) in Saga Prefectural Livestock Experiment Station are located. Ten Silkie fowls are located in clade D, in which there are three white feather type Silkie fowl (SLKAW) in Experiment Station for Animal Husbandry, Aomori Prefectural Agriculture and Forestry Research Center, and four white feather type Silkie fowls (SLKI) in Ibaraki Prefectural Livestock Research Center, three white feather type Silkie fowl (SLKWM) in Livestock Research Division, Mie Prefectural Science and Technology Promotion Center. Three Silkie fowls are located in clade E, among which there are one white feather type Silkie fowl (SLSG) in Saga Prefectural Livestock Experiment Station and two white feather Silkie fowls (VS) in Yamanashi Wild Birds Breeding Centre. Thus, it is seen that Silkie fowls have five clades (A-E). However, in all

categories except SLSG (Saga Prefectural Livestock Experiment Station, white feathers) only two clades (B and C) are distributed. Moreover, five individuals with black feathers are belonged to clade A. Therefore, these results suggest that Japanese Silkie fowls have high genetic divergence.

Table 2.7 Distribution of examined Red Jungle Fowl and chicken breeds in each clade

Breed	Clade[1]				
	A	B	C	D	E
Red Jungle fowl			*		*
Silkie fowl	*	*	*	*	*
Gifu-Jidori	*				
Tosa-Jidori			*		
Aizu-Jidori	*	*			
Shokoku	*	*			
Shamo	*	*		*	*
Ko-Shamo		*		*	
Koeyoshi	*			*	
Tomaru	*			*	
Totenko	*	*		*	
Onaga-dori	*	*			
Hinai-dori	*				
Satsuma-dori		*		*	
Minohiki	*		*		
Minohiki-Chabo		*	*		
Uzura-Chabo			*		
Kurokashiwa				*	
Jitokko		*			
Kawachi-Yakko		*			
Chabo	*	*	*	*	
Nagoya		*			
White Leghorn	*	*			
White Plymouth Rock	*				
Rhode Island Red	*	*			
New Hampshire Red	*				
Indonesian fighting cock			*		*
Indonesian Bantam	*		*		
Laos native chicken	*				

[1]According to Oka *et al.* (2007).
*Individuals of a breed in the clades.

The phylogenetic distribution of the Red Jungle Fowl and other chicken breeds according to the results of present study and Oka *et al.* (2007) has shown in Table 2.7. The Red Jungle Fowl is distributed only in clade C. Gifu-Jidori, Hinai-dori, White Plymouth Rock, New Hampshire Red and Laos native chicken are distributed in clade A. Tosa-Jidori and Uzura-Chabo are also distributed in clade C. Aizu-Jidori and Shokoku are distributed in clades A and B. Similarly, Shamo is distributed in clades A, B, D and E. Ko-Shamo is distributed in clades B and C. Koeyoshi and Tomaru breeds are distributed in clades A and D. Totenko is distributed in clades A, B and D. Onaga-dori is distributed in clades A and B. Satsuma-dori is distributed in clades B and D. Minohiki is distributed in clades A and D. Minohiki- Chabo is distributed in clades B and C. Kurokashiwa is distributed in clade A. Jitokko and Kawachi-Yakko are distributed in clade B. Chabo is distributed in clades A, B, C and D. Nagoya breed is distributed in clade B. White Leghorn and Rhode Inland Red are distributed in clades A and B. Indonesian fighting cock is distributed in clades C and E. Indonesian bantam chicken is distributed in clades A and D. Silkie fowl is distributed in clades A-E. Thus, it is found many chicken breeds are distributed in free clades or less. Chabo and Shamo are distributed in four

clades. Only the Silkie fowl is distributed in all five clades. Thus, these results also suggested that the Japanese Silkie fowls are widely distributade in the phylogenetic tree.

The evolutionary history was inferred using the Neighbor-Joining method (N. Saitou and Nei M (1987)). The optimal tree with the sum of branch lengths of 0.08177153 is shown. The evolutionary tree is drawn in scale. With branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura K, Nei M and Kumar S, 2004). All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1229 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura K, Dudley J, Nei M and Kumar S, 2007).

The evolutionary tree shows the relationship of 28 Silkie fowls, 9 other chicken breeds, 4 Red Jungle Fowl and 42 breeds' haplotype from Oka *et al*. (2007). Red Jungle fowl *(Galus gallus bnnkiva)* shows a big evolutionary distance and it is fair compared to other breeds.

2.5 Discussion

The relationships among red jungle fowls, Laos native chicken, and commercial breeds in this phylogenetic tree are similar to those in the tree from chicken repeat 1 (CR1) elements (Figure 3 in Nishibori *et al.* 2005). Domain I of the mitochondrial D-loop region and CR1 elements (Chen *et al.* 1991) are considered to have not been under evolutionary selective pressure. The relationships might be associated with the true evolutionary event.

The wide distribution of Japanese Silkie fowl shows that the present Japanese Silkie fowl has high genetic divergence. However, each category of Silkie fowls except SLSG (Saga Prefecture Livestock Experimental Station, white feather) are distributed in only one or two clades and five Silkie fowls with black feathers belong to clade A (Table 2.6).

Ten Silkie fowls are located in clade D; Shamo, Ko-Shamo, Koeyoshi, Tomaru, Totenko, Satsuma-dori, Kurokashiwa, and Chabo are also located in this clade. These breeds are native to Japan. The results suggest the possibility of crossbreeding of the ten Silkie fowls with the Japanese native chicken. However, the evolutionary origin of this clade remains unclear.

Six Silkie fowls are located in clade C, which also includes two Red Jungle Fowls (*Gallus gallus gallus* and *Gallus gallus bankiva*) and the Tosa-

Jidori, Minohiki, Minohiki-Chabo, Uzura-Chabo, Chab, Indonesian fighting cock, and Indonesian Bantam breeds. *Gallus gallus gallus* is found in Indochina, and *Gallus gallus bankiva* is found in Java, suggesting that this clade originated in Indochina and Java.

Five Silkie fowls with black feathers, two White Leghorn, one White Plymouth Rock, two New Hampshire Red, and one Laos native chicken are located in clade A. Oka *et al*. (2007) reported that the Gifu-Jidori, Aizu-Jidori, Shokoku, Shamo, Koeyoshi, Tomaru, Totenko, Onaga-dori, Hinai-dori, Minohiki, Chabo, White Leghorn, Rhode Island Red, and Indonesian Bantam breeds are classifiable under clade A. Many commercial breeds and Japanese native chickens were located in this clade. The Silkie fowls with black feathers and some Japanese native chickens could have crossed with the commercial breeds.

Six Silkie fowls are located in clade B, which also includes two Nagoya breeds and a Rhode Island Red × White Plymouth Rock F1 chicken. In Oka *et al.* (2007), the Aizu-Jidori, Shokoku, Shamo, Ko-Shamo, Totenko, Onaga-dori, Satsuma-dori, Minohiki-Chabo, Jitokko, Kawachi-Yakko, Chabo, White Leghorn, and Rhode Island Red chickens are classified under clade B. The White Leghorn and Rhode Island Red are distributed in clades A and B;

however, the genetic distance between these clades is large. The possibility of multiple origins of the commercial chicken breeds should be considered.

Three Silkie fowls and a Red Jungle Fowl are located in clade E. Oka *et al*. (2007) classified seven Shamo chickens and an Indonesian Fighting individual under this clade. Seven Red Jungle Fowls are found in Burma. The results suggest that clade E is originated in the center of the Indochina peninsula.

Nishibori *et al*. (2005) provided molecular evidence for hybridization of species in the genus *Gallus*. The phylogenetic tree in this study shows hybridization between Silkie fowls and other chicken breeds or red jungle fowls at the old age. The Silkie fowl's wide distribution in the phylogenetic tree suggests that old Asian breeds crossed with several chicken breeds having special and unusual traits to establish the Silkie fowl breed.

In the present research the nucleotide sequence of mitochondrial complete D-loop region on Silkie fowl and other chicken breeds have been determined, and the phylogenetic tree for their genetic relationship has been constructed. In this result it is suggested that the Silkie fowl has high genetic divergence and therefore, it has been crossed with many other chicken breeds.

Further research has been suggested in the present study to examine the detail genetic status of Silkie fowl with other chicken breeds using the sequences obtained in the current research.

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CHAPTER 3

Relationship between the production traits and the candidate genes on chromosome 2 in the 5th generation of selection program of Silkie fowl

3.1 Introduction:

Silkie fowl is well known for its medical properties. The eggs of Silkie fowls are very expensive in Japan. As reproductive capacity of Silky fowl is rather low, it is necessary to investigate the influencing factors of reproduction including hormones, growth factors, cytokines, correlative gene etc. Broodiness is often observed and production ratio is very small in Silkie fowl chicken. Broodiness is also observed in other most breeds of domestic fowl with exception of the White Leghorn, which has undergone long-term artificial selection to minimize phenotypic expression of this behavior. Broodiness basically means a hen site on her egg for the purpose of hatching the embryos.

(i) (ii)

Figure 3.1: (i) Broody Silkie (ii) Hatched Silkie Chick

Physiologically, it has been well established that prolactin (*PRL*) plays an important role in the onset of incubation of hens. Avian prolactin hormone gene is located in chromosome 2 (Masoud *et al.,* 2011). It has been reported that most of sequence polymorphisms in the chicken *PRL* gene occur in 5' flanking region, 3' flanking region, and coding region of the signal peptide (Wong *et al*., 1991; Zhou *et al*., 2001; *Cui et al*., 2005). Previous studies showed that a 24-bp In/Del in the chicken *PRL* promoter region is associated with broodiness (Jiang *et al*., 2005; Liang *et al*., 2006). Jiang *et al*., (2005) examined the polymorphisoms of 5' flanking region of chicken prolactin gene in several population of Chinese native chicken, Thai Silkie and White Leghorn chicken and identified a 24bp in In/Del locus. They found three

genotypes (In/In, In/Del, Del/Del) in Thai native chicken when only two genotypes (In/Del, Del/Del) were found in Thai Silkie chicken but no In/ In genotype was not found in Silkie fowl. In their research they concluded that the chicken with In/Del locus might be related to the broodiness characters in chicken breed.

Wada *et al*. (2008 a,b) genotyped the 24- bp In/Del locus in the region of prolactin gene in the fourth generation of Silky fowl's selection program to study the association of performance traits. They found 282 Del/Del Silkies, 29 In/Del Silkies but no In/In in Silkie.

Jiang *et al.* (2011), has also reported that the presence of a linkage disequilibrium block between Silkie fowl and White Leghorn containing the prolactin gene region. Association analysis has also shown that this 24-bp insertion is associated with egg production (Cui *et al*., 2006). Therefore, the present study the *Prolactin* has been investigated as a candidate gene for broodiness.

Researchers of the capital part have claimed that broodiness is heredity of autosomal chromosome nature (Goodale *et al.,* 1920), although, the sex-linked inheritance is carried out in a Chabo kind. The crossing report of a Red Jungle Fowl and a White Leghorn shows that the gene of broodiness is in the

Z chromosome (Saeki and Inoue, 1979). The studies of Romanov *et al*. (1999, 2002) and Jiang *et al*. (2005) about the relation of prolactin gene and broodiness have showed that the SNP of the prolactin receptor gene region on Z chromosome. Broodiness concludes the relevance when it does not accept.

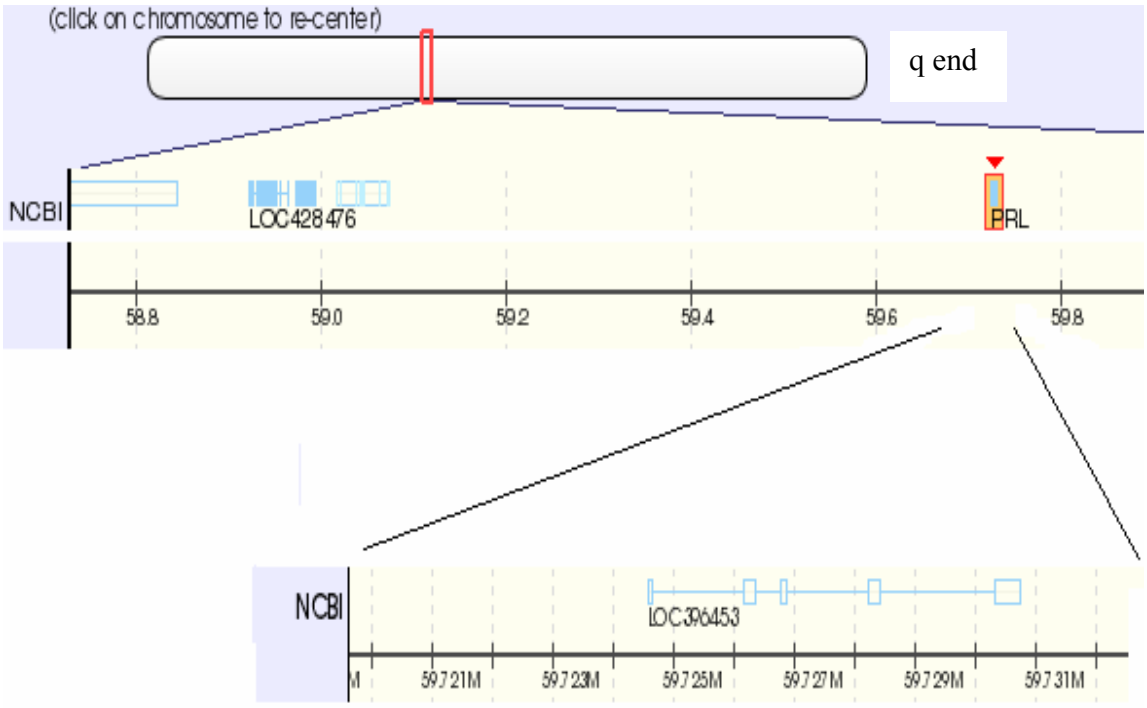

Figure 3.2 The position of exon and intron structure in a prolactin gene on the chicken chromosome 2.

Some researchers have been reported that candidate genes are located on chromosome 2 of the Silkie fowl. It is thought that many of the variation of population characteristics such as body weight, egg production rate, egg quality and age at first egg are directly or indirectly influenced by some candidate gene such as the prolactin (*PRL*), vasoactive intestinal peptide receptor-1 (*VIPR-1*), Neuropeptide Y *(NPY)* gene etc. Prolactin (*PRL*) are also related to broodiness, egg production rate and age at first egg.

Neuropeptide Y *(NPY)* is a 36 amino acid neuropeptide that is widely distributed in both the central and peripheral nervous systems. In physiology, neuropeptide Y (NPY) is a peptide chemical messenger which is secreted by the hypothalamus. This portion of the brain controls hunger, thirst, fatigue, body temperature etc. *NPY* plays a role in various basic processes in the brain, including energy regulation, memory formation, and seizure activity. Neuropeptide Y (*NPY*) is found to be involved in the regulation of GnRH secretion via its receptor. The injection of NPY can induce precocious puberty in chicks (Xu *et al*., 2011). Li (2009) has found strong evidence of significant and simultaneous beneficial effects of *NPY* SNP associated with chicken reproductive traits.

Xu *et al*. (2011) reported that candidate genes *PRL, VIPR-1* and *NPY* are located in chromosome 2 and they are related to broodiness, egg production rate as well as the age at first egg. Therefore, in the present study *NPY* gene has been chosen as a candidate gene.

Vasoactive intestinal peptide (*VIP*) is a *PRL* releasing factor in birds (El Halawani *et al.,* 1990, 1997; Sharp *et al*., 1998). In avian, *VIP* is involved in regulation of reproductive activity and appears to act at the level of hypothalamus and pituitary (Mauro *et al*., 1989; Chaiseha *et al*., 1999). Zhou *et al*. (2008) reported that VIPR-*1* gene was associated with broodiness traits. From the study of Zhou *et al.* (2010) it has been identified that *VIP* gene is a candidate gene for chicken egg production and broody traits.

In the study, vasoactive intestinal peptide receptor-1 (*VIPR-1*) gene has been taken as a candidate gene of chicken broodiness, and its genomic variations and genetic effects on chicken broodiness traits have been analyzed as well.

SNP marker of prolactin gene, vasoactive intestinal peptide receptor 1 gene, and neuropeptide Y gene will be genotyped to find selection marker for egg production.

3.2 Motivations

In the present research it is aimed to improve the egg production rate according to the removal of broodiness and to improve the productivity of a Silkie fowl. The DNA markers of candidate genes (prolactin gene, vasoactive intestinal peptide receptor 1 gene, and neuropeptide Y gene) were genotyped in Silkie fowls of the 5th generation of Oita's selection program; and the relationship between the genotypes and egg production traits was investigated using ANOVA.

3.3 Materials and Methods

3.3.1 Samples of Silkie Fowls

Figure 3.3 A Silkie fowl

In the present research 200 females of Silkie fowls have been investigated from the fifth generation of the Oita's selection program. The samples were collected from Oita Agriculture Research Center of Animal production Division. The age of the first egg and the rate of egg production have been recorded for each hen. These populations were fed commercial combination feed. It considered as whole period *ad libitum*. Chick age at 0 – 40 days has been given an electric heat by battery. The chickens have been developed age at 40 – 80 days in medium chick cage and age at 80- 120 days in big chick cage. The single cage for matured chickens at the age of 120-450

days with a frontage of 226 mm (frontage of 226 mm, length of 393 mm, and height of 455 mm) for individual assay.

The chickens in the first poultry house were stayed from November 25, 2009 to February 18, 2011, the chickens in the second poultry house were stayed from December 25, 2009 to March 20, 2011 and the chickens in the third poultry house were stayed from January 27, 2010 to April 22, 2011.

Relevance with 50 day age weight, 100 day age weight, 150 day age weight, 300 day age weight, a laying-eggs rate, and average egg shell intensity were analyzed. Details of Silkie fowl generation of the Oita's selection program is shown in Table 3.1.

Table 3.1 Results according to Silkie fowl generation of the Oita's selection population

Generation	At the time of 150-day age Number of chickens		Egg production rate (%) (151- 450 day)	Average egg weight(g) (294-300 day)	Average eggshell intensity (kg/cm^2) (294-300 Day)	Body weight (g) at 450 day	
	Male	Female				Male	Female
Basic chicken	53	135	40.5	39.7	3.52	1638	1227
Basic population	50	281	40.4	39.6	3.59	1635	1190
1st generation	168	354	54.0	41.0	3.97	1696	1212
2nd generation	125	262	48.8	40.2	3.99	1679	1251
3rd generation	113	253	48.2	39.3	3.77	1711	1310
4th generation	97	309	51.5	39.0	3.73	1710	1217
5th generation	106	215	51.3	38.1	3.93	1735	1269

Figure 3.4 Location of Oita Prefecture; a sample collection center for Japanese Silkie fowl

3.3.2 DNA extraction from blood

Blood samples were taken from the ulnar vein of each individual and stored at -20°C. Genomic DNA was extracted from chicken blood sample. The blood sampling was performed in accordance with guidelines for animal experimentation of Saga University.

DNA has been extracted from the whole blood of the Silkie fowls with Pure Gene kit (Funakoshi Co. Ltd., Tokyo). The blood of 15 μℓ was filled in the 1.5 ml microcentrifuge tube. The cell lysis solution of 600μℓ and protease K solution of 20 μℓ were added. The tube was inverted for five times. It has been incubated for three hours and put in EHB at 55℃.

The sample was cooled at room temperature. One hundred microliters of protein precipitation solution was added into the tube. The tube was vigorously vortexed at high speed for 20 seconds to mix the protein precipitation solution uniformly with the cell lysate. Centrifuged 15000g at 4℃ for 3 minutes. The precipitated proteins were formed a tight brown pellet. Supernatant was carefully transferred to a new 1.5ml microcentrifuge tube by pipetting. Six hundred microliters of 100% isopropanol was added and mixed

the sample by inverting gently for 50 times. Centrifuged for 3 minutes at 15000g and 4℃. DNA was visible as a small white pellet. Three hundred microliters of 70% Ethanol was added and inverted the tube several times to wash the DNA. Again centrifuged it for 1 minute at 15000g and 4℃. Air-dried the tube containing DNA in a Micro Vacuum at 37 ℃ for 3 minutes. Then 35 µℓ DNA Hydration solution was added and vortexed lightly. A sample was kept in incubation at 65℃ for 1 hour. The sample was cooled at room temperature. Vortexing was done vigorously for five seconds in medium speed. By using the spectrophotometer the A260, A280 of measured. The cuvette was washed by using distilled water. Then 50 µℓ of distilled water was added again and run on spectrophotometer as a blank control. Five micro liters DNA solution was mixed with 45 µℓ distilled water in a new microcentrifuge tube. The solution was transferred to a cuvette and was run on the spectrophotometer. The concentration of DNA was calculated and the data was analyzed before using the downstream reactions. Extracted DNA sample was preserved at -20°C.

3.3.3 PCR amplification:

The primer was designed for targeting the homologous region of the chicken whole genome sequence. The PCR was performed by using the primer set which is shown in Table 3.2. The PCR amplification was carried out in a final volume of 25 µl with 50 ng genomic DNA template, 1 µl (10µM) of each primer, 2µl dNTP Mixture (2.5mM), 2.5µl Takara Ex Taq buffer (20mM Mg^{2+}), and 17.375 µl distilled water. The PCR amplification cycle was carried out as follows: an initial denaturation step at 95 ℃ for 5 minutes, followed by 40 cycles of denaturation at 95 ℃ for 30 seconds, annealing at 60 ℃ *(PRL)*, 62℃ (*VIPRI*), and 65 ℃ (*NPY*) for 30 seconds, elongation at 72 ℃ for 1 minute, final elongation at 72 ℃ for 10 minutes, and a final hold at 4 ℃.

Table 3.2 PCR primer used by this research

Gene	Forward primer	Reverse primer	Length of PCR products	Anneling temperature
PRL	GGTGGGTGAAGA GAC AAG GA	TGCTGAGTATGGCTGGAT GT	130/154	60℃
VIPR1	TGAAAGCCCCCAGGATCT	AGCAAAACAAAACCCAAATCA	364	62℃
NPY	CGTGGCTGCTTTGCTTCCTTTC	GGGGTACGAGGCAAGGACATG	324	65℃

3.3.4 Agarose Gel Electrophoresis

The PCR products were analyzed by the 3.0% agarose gel electrophoresis. The methodology of agarose gel electrophoresis has already been illustrated in details in chapter 2.

3.3.5 Genotype

Genotypes were determined by the PCR-PFLP method for *VIPR1* and *NPY* gene. The restriction enzyme *Hha* and *KpnI* were used for single nucleotide polymorphism of *VIPR1* and *NPY*. The reaction was processed at about 37 ℃ 3 hours. The genotypes were determined by 3.0% agarose gel electrophoresis. The genotypes of In/Del locus in prolactin gene were determined from the length of PCR products. The band of the In allele is 154bp, and Del allele is 130bp.

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Table 3.3 PCR- RFLP amplification

Resent	Volume
Hha & KpnI (Restriction enzyme)	0.5 µl
Buffer	2.0 µl
PCR product	8.0 µl
Distilled water	9.5 µl
Total	20 µl

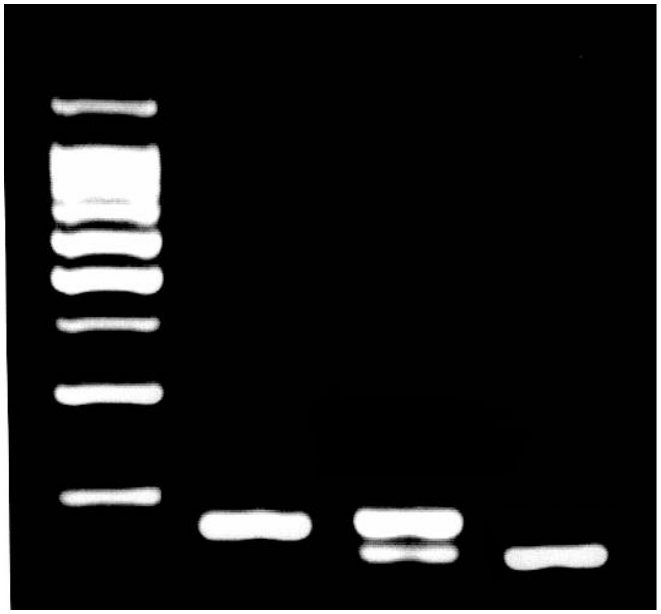
3.3.6 Statistical Analysis

POPGEN version1.32 (http://www.ualberta.ca/~fyeh/) was used for a genotypic analysis. Expected genotypic frequencies under random mating using the algorithm by Levene (1949), and perform chi-square (χ^2) and likelihood ratio (G2) tests for Hardy-Weinberg equilibrium at each locus only for co-dominant markers.

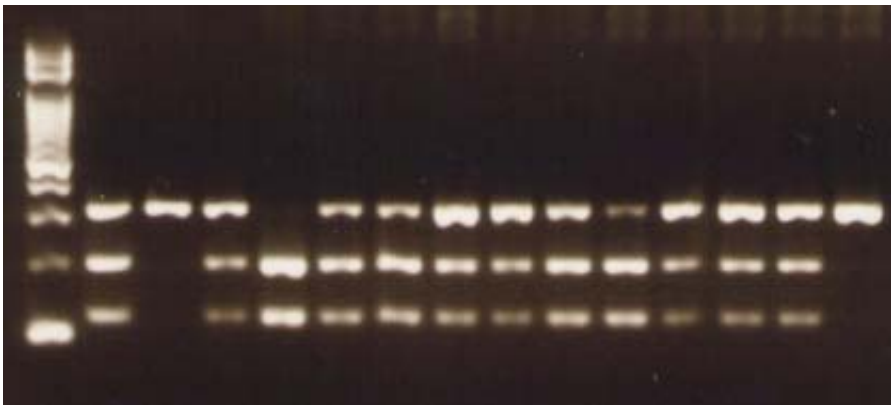
The relationship between the genotypes and egg production traits were analyzed by using ANOVA. The analysis was computed by lm and ANOVA function of the statistical language R.

3.4 Results

The results of genotyping of the In/Del locus of *PRL*, Del/Del type was individually 189, In/Del type was individually 11, In/In type was individually 0. The results of SNP of the *NPY* gene, CC type were individually 21, CT types were individually 78 and TT types were individually 0.1. The results of SNP of the *VIPR1* gene, CC genotype were individually 198, genotype CT were individually 2 and TT genotype did not exist at all.

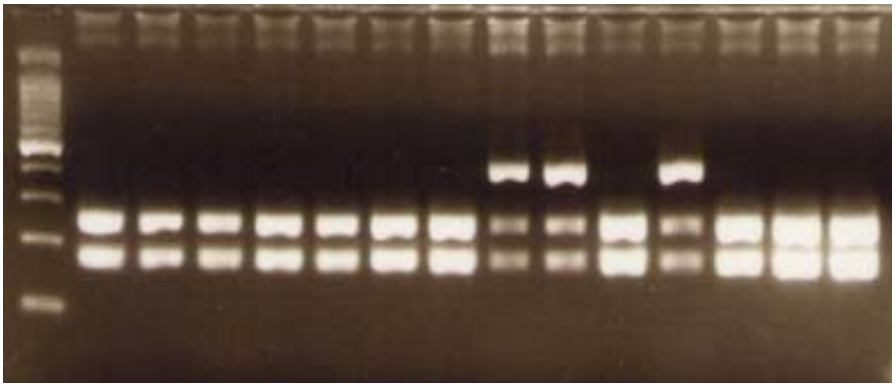


Finger 3.5 The genotype of In/Dal locus of *PRL* gene determination by 3.0% agarose gel electrophoresis



Finger 3.6 The genotype of C/T locus of *NPY* gene determination by 3.0% agarose gel electrophoresis (*KpnI* restriction enzyme)

This figure (Figure 3.6) shows the genotype of CC, CT and TT.



100bp ladder CC CC CC CC CC CC CC CT CT CC CT CC CC CC

Figure 3.7 The genotype of C/T locus of *VIPR-1* gene determination by 3.0% agarose gel electrophoresis *(Hha* restriction enzyme)

This figure (Figure 3.7) shows the genotype of CC and CT locus in *VIPR-1* gene. TT genotype is not found in this analysis.

Table 3.4 Heterozygote frequency, expected heterozygosity and χ^2 value

Candidate gene	Heterozygote frequency	Expected heterozygosity	χ^2 value in Hardy- Weinberg Equilibrium
PRL	0.055	0.0536	0.1450 ^{NS}
NPY	0.390	0.4211	1.0954 ^{NS}
VIPR1	0.010	0.0100	0.0025 ^{NS}

NS : Not significant

Expected heterozygosity (Levene 1949) and χ^2 value in Hardy- Weinberg Equilibrium are shown in Table 3.4. The expected heterozygote frequency for PRL and *VIPRI* was less 0.1. Chi- square value over a Hardy- Weinberg Equilibrium is small. It is thought that three gene loci have been reached the Hardy Weinberg Equilibrium in the 5th population.

Table 3.5 Results of analysis of variance

	Degree of freedom	50 day age	100 day age	150 day age	300 day age	450 day age	Egg production rate (151-300 day)	Average eggshell intensity
Father	19	2.13†**	3.21**	2.21**	5.17**	2.93†**	2.67**	3.14**
Mother	27	0.88	1.56	1.19	0.91	0.96	1.47	0.88
Poultry house	2	3.33*	1.71	3.04	0.91	0.14	0.86	2.43
PRL	1	0.77	0.00	0.21	0.18	0.00	0.49	0.08
VIPR1	1	0.50	0.66	1.48	0.40	2.49	0.45	0.26
NPY	2	1.32	0.14	0.39	0.08	0.13	0.11	0.05
Error	147							

† ; figure is F value **; $P < 0.01$ *; $P < 0.05$

The result of the analysis of variance is shown in Table 3.5. The effect of the father is significant ($P < 0.01$) for all the traits including the egg production rate; however, the genotypic effects of the three candidate genes are not significant for all the traits. The effect of poultry house is significant ($P < 0.05$) for only the 50 day age, weight traits.

These results suggest that the genetic variation of Silkie fowls in Oita's selection program is high and therefore, the egg production rate in Silkie fowls population can be improved.

3.5 Discussion

Wada *et al*. (2008b) has reported that the relationship between egg production rate and genotype of the PRL IN/Del locus is not statistically significant. But the difference in the distribution between In/Del individuals and Del/Del individuals are found for the egg production rate.

The White Leghorn is an excellent layer breed without broodiness and produces more than 300 eggs per year. However, Silkie fowl lays eggs only about 50 ~ 200 pieces per year and therefore, the production efficiency of Silkie fowl is very low due to broodiness. Because, broodiness is a main factor leading to low egg production. Broodiness is observed in most avian species, and the average duration of broodiness in indigenous breeds of chickens is approximately 20 d (Jiang *et al*., 2005). As a result, researchers have associated broodiness with reduced egg production because of the cessation of lay during the broody period (Yami, 1995; Chen and Li, 2007). Thus it has been found from the above data that the rate of laying egg of Silkie fowl is very low because having strong broodiness in it.

PRL secretion is controlled by *VIP* at the hypothalamus level and in part by *VIP* receptors at the pituitary level throughout the avian reproductive cycle (Chaiseha *et al*., 2004). Recent studies have demonstrated that broodiness is

not controlled by genes on the Z chromosome (Romanov *et al*., 2002; Jiang *et al*., 2005). Zhou *et al*. (2008) has suggested that the genotype of *VIPR1* located at chromosome 2 might be candidate gene associated with chicken broodiness traits. Moreover, it is also valid in spite of adding the selection to an egg production rate. It is reported that candidate genes *PRL, VIPR-1* and *NPY* are located in chromosome 2 and they are related to broodiness, egg production rate as well as the age at first egg.

To investigate the efficiency of marker-assisted selection, the markers of candidate genes (prolactin gene, vasoactive intestinal peptide receptor 1 gene, and neuropeptide Y gene) are genotyped in Silkie fowls of the 5th generation of Oita's selection program in this study. Wada *et al*. (2008 a) has also reported about the significant differences of the genotype frequencies for the In/Del locus of the promoter region of prolactin gene between Silkie fowl and other chicken breeds.

In the present study, both the *VIPR1* and *NPY* locus have been reached the Hardy Weinberg Equilibrium. The effect of the father was significant ($P < 0.01$) for all the traits in age weight, egg production rate, and average eggshell intensity. However, the genotypic effects of the three candidate gene markers have not been significant for all the traits.

As a further study, the obtained DNA markers of the candidate gene will be improved to be significant in future as effective tools for the future selection program of Silkie fowls and other chicken breeds. Moreover, the findings in the current study will be utilized as a valuable support in the future analysis of gene expressions aiming to identify the responsible genes for the specific traits of the Silkie fowls. Further improvements in egg production rate will be conducted by the other genetic markers in the 5th generation of selection programs of Silkie fowls.

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CHAPTER 4

General Discussion and Conclusions

The genomic research of Silkie fowls is sparse. Wada *et al.* (2004) compared the complete mitochondrial DNA of the Japanese Silkie fowl with that of the White Leghorn and found that the similarity between the two breeds was 99.77% (39 nucleotide differences in 16,784 base pairs). Several researches have studied the mitochondrial partial D-loop regions of Japanese and Chinese Silkie fowls (Fu *et al*., 2002; Liu *et al*., 2006a; Zhou *et al*., 2010) and Japanese and Chinese native chickens, including Silkie fowls (Fumihito *et al*., 1994,1996; Komiyama *et al*., 2003, 2004; Liu *et al*., 2006b) , Partial D-loop nucleotide sequences provide less information than complete nucleotide sequences; therefore, the genetic relationship between the Silkie fowl and other native chickens is not clear. The study of Oka *et al*. (2007) was analyzed the mitochondrial complete D-loop region of Japanese native chickens to clarify their phylogenetic relationships, maternal origin, and routes of introduction into Japan. They identified seven haplogroups and suggested

that Chinese and Korean chickens were derived from Southeast Asia, and both non-game and game chickens formed the foundation of Japanese native chickens. In the analysis, two Silkie fowls were included in haplogroup B with Satsuma-dori and Aizu-Jidori.

The objective was in this research, to determined the mitochondrial complete D–loop nucleotide sequence of the 28 Silkie fowls and 3 other chicken breeds. Twenty seven sites of single nucleotide polymorphism and 4 sites of single nucleotide insertion were found in the Silkie fowls breed. The phylogenetic tree were constructed from the nucleotide sequences of the complete D-loop region of the 28 Silkie fowls, 9 other chicken breeds, 4 red jungle fowls, and the 42 haplotypes by Oka *et al.* (2007). In the study of Oka *et al*. (2007) were distributed in 5 clade (A-E) with G01 (Indonesian Fighting) as an out group. The Silkie fowls were distributed in all 5 clades. The number of Silkie fowl individuals in each clade is shown in Table 2.6 and this result suggest that Japanese Silkie fowls have high genetic divergence. The phylogenetic distribution of the red jungle fowl and chicken breeds in this study and Oka *et al.* (2007) is shown in Table 2.7. The red jungle fowl and many chicken breeds are distributed in 2 or 3 clades. Only the Silkie fowl is distributed in all five clades.

The phylogenetic analysis shows that five Silkie fowl are located in the clades A with the 2 White Leghorn (A03), 1 White Plymouth Rock (WPR), 2 New Hampshire Red (NHR), Gifu-Jidori (A07), Aizu-Jidori (A06), Shokoku (A03), Shamo(A04,F01,F02), Koeyoshi (A06), Tomaru (A03), Totenko(A03), Onaga-dori (A03), Hinai-dori (A01,A02) Minohiki (A07), Chabo (A05), Indonesian Fighting (G01) and 1 Laos native chicken. Six Silkie fowls (are located in clade B, which also includes 2 Nagoya breeds (NA1), 1 Rhode Island Red × White Plymouth Rock F1 Shokoku (B02), Shamo (B01), Ko-Shamo(B01), Totenko(B03), Onaga-dori (B04), Satsuma-dori (B01), Minohiki-Chabo(B04), Jitokko(B08), Kawachi-Yakko(B01), Chabo (B01). Six Silkie fowls (SLKBM, SLKWM, SLKI, SLSG) are located in clade C, which also includes 2 red jungle fowls *(Gallus gallus gallus* and *Gallus gallus bankiva*) and the Tosa-Jidori (C01), Minohiki(C01), Minohiki-Chabo(C01), Uzura-Chabo C08), Chabo (C03), Indonesian fighting cock(C06,C07) and Indonesian Bantam breeds(C04, C04*). Gallus gallus gallus* is found in Indochina, and *Gallus gallus bankiva* is found in Java. This result suggested that this clade originated in Indochina and Java. Ten Silkie fowls (SLKI, SLKWM, SLKWM) are located in clade D; Shamo (D05-9), Ko-Shamo (D03), Koeyoshi (D06), Tomaru (D07), Totenko (D07), Satsuma-dori (D06),

Kurokashiwa (D06), and Chabo (D01) are also located in this clade. These breeds are native to Japan. The results suggest the possibility of crossbreeding of the 10 Silkie fowls with the Japanese native chicken. Three Silkie fowls (SLGS, VS1, and VS2), 1 Indonesian fighting (E02), 7 Shamo (E01, E03, E04) and 1 red jungle fowl (*Gallus gallus spadiceus*) are located in clade E. This *Gallus gallus spadiceus* is found in Barma. The results suggest that clade E originated in the centre of the Indochina peninsula.

However, all categories except SLSG (Saga Prefectural Livestock Experiment Station, white feathers) were distributed in only 1 or 2 clades, and 5 individuals with black feathers belonged to clade A. The Silkie fowl's wide distribution in the phylogenetic tree suggests that old Asian breeds crossed with several chicken breeds that had unusual traits to establish the Silkie fowl breed.

Silkie fowl (*Gallus gallus* var. *Domesticus)* is distinct from other chicken breeds in terms of its appearance and behavioural characteristics. Silky fowl is well known for its medicinal properties. Now a day it is a very rear and expensive breed. It is a Japanese native breed and has inhabited Japan since before the Edo era. Although the breed is considered to have originated in India and established in China and Japan, its evolutionary history and

genetic relationship with other breeds are not clear. About silkie fowl's Genetic research about silkie fowl's is very few.

The Silkie fowls egg has low cholesterol and good for human's health. However, the egg production rate of the Silky fowl is very low because of broodiness. It is thought that prolactin gene is an essential for the start and maintain of broodiness in hens. It is difficult to improve the egg production ratio of this breed by selection methods (Wada *et al*., 2008b). Some researchers have reported that candidate genes located on chromosome 2 of the Silkie fowl, such as the prolactin gene, are related to broodiness, egg production rate, and age at first egg. One objective of this research was also to improve the egg production rate by the candidate genes. The markers of candidate genes (prolactin gene, vasoactive intestinal peptide receptor 1 gene, and neuropeptide Y gene) were genotyped in Silkie fowls of the 5th generation of Oita's selection program; and the relationship between the genotypes and egg production traits have been investigated as well.

In the present research 200 females of the Silkie fowls have been investigated from the fifth generation of the Oita's selection program. In poultry breeding programs, egg number at 300 days of age is usually used as a

valuable indicator for total egg production. Relevance with 50 day age weight, 100 day age weight, 150 day age weight, and 300 day age weight, a laying-eggs rate, and average eggshell intensity have been analyzed in this study.

Expected heterozygosis (Levene, 1949) and χ^2 value in Hardy-Weinberg Equilibrium are shown in Table 3.4. The expected heterozygote frequency for *PRL* and *VIPRI* was less 0.1. It was shown that it is in the state currently mostly fixed about the In/Del locus of *PRL* and SNP of *VIPR1*. Chi-square value over a Hardy- Weinberg Equilibrium is small. The hypothesis of Hardy- Weinberg Equilibrium was not significant in three genes. The result of the analysis of variance is shown in Table 3.5. The effect of the father was significant ($P < 0.01$) for all the traits including the egg production rate; however, the genotypic effects of the three candidate gene markers were not significant for all the traits. The effect of poultry house was significant ($P <0.05$) for only the 50 day age, weight traits. These results suggested that the genetic variation of Silky fowls in Oita's selection program is high and the egg production rate in Silkie fowl population can be improved.

Many researches on QTL (quantitive trait) analysis have been conducted for egg production of the chicken breeds, where QTL analysis is used in chicken to identify the chromosomal regions for contributing to

variation in traits related to growth, egg production, behaviour and disease resistance. By using QTL mapping Abasht *et al.* (2006) confirmed a sex interaction for fatness QTL which was identified in an F2 population. Some receptors also can be developed for the improvement of chicken breeds. For example, in the study of Georginaf *et al.*, (2006), it has been found that the neuronal nicotinic acetylcholine receptor (nAChR) is located at a QTL region for abdominal fat. This study has identified 197 putative candidate genes as well in probable QTL regions of chicken in the Z chromosome. Therefore, as a rare existing chicken breed QTL analysis can play an important role in the further development of the breeding characteristics of Japanese Silkie fowl.

APPENDIX A: AMINO ACID CODES

One-letter code	Three-letter code	Amino acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

APPENDIX B: DNA Sequence of mitochondrial complete D-loop

S1 (Accession no. AB086102)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCCAGGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC
AATGTCCATTCTATGCATGATCCAGGACACACTCGTTCACCCTCCCCATAGACAGCTCCAAA
CCACTACCAAGTCACCTAACTATGAATGGTTACAGGACATAAATCTCACTCTCATGTTCTTC
CCCCAACAAGTCACCTAATTATGAATGGTTACAGGACATACATTTAACTACCATGTTCTAAC
CCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG
CAACCCCTGCTTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCCT
CGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACTC
CTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGATC
GCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTGC
CCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCGT
CCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTGA
CACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAATGGTGCTA
TTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAAAA
CTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAACA
TTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAACG
TTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACCA
AAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATTT
ATATTGTTAATTAGCAAACACAAAACCTGCCTTCTACCACTATAAA

S2 (Accession no. AB263947)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCCAGGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC
AATGTCCATTCTATGCATGATCCAGGACACACTCATTCACCCTCCCCATAGACAGCTCCAAA
CCACTACCAAGTCACCTAACTATGAATGGTTACAGGACATAAATCTCACTCTCATGTTCTTC
CCCCAACAAGTCACCTAACTATGAATGGTTGCAGGACATACATTTAACTACCATGTTCTAAC
CCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG

CAACCCCTGCTTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCCT
CGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACTC
CTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGATC
GCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTAC
CCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCGT
CCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTGA
CACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAATGGTGCTA
TTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAAAA
CTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAACA
TTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAACG
TTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACCA
AAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATTT
ATATTGTTAATTAGCAAACACAAAACCTGCCTTCTACCACTATAAA

SLKAW1 (Accession no. AB263948)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGT
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CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC
AATGTCCATTCTATGCATGATCCAGGACACACTCATTCACCCTCCCCATAGACAGCTCCAAA
CCACTACCAAGTCACCTAACTATGAATGGTTACAGGACATAAATCTCACTCTCATGTTCTTC
CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATTTAACTACCATGTTCTAAC
CCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG
CAACCCCTGCTTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCCT
CGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACTC
CTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGATC
GCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTGC
CCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCGT
CCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTGA
CACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAATGGTGCTA
TTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAAAA
CTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAACA
TTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAACG
TTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACCA

AAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATTT
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SLKAW4 (Accession no. AB263959)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCCAGGGGGGGT
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CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC
AATGTCCATTCCATGCATGATCCAGGACACACTCATTCACCCTCCCCATAGACAGCTCCAAA
CCACTACCAAGTCACCTAACTATGAATGGTTACAGGACATAAATCTCACTCTCATGTTCTTC
CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATTTAACTACCATGTTCTAAC
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CAACCCCTGCTTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCCT
CGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACTC
CTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGATC
GCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTGC
CCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCGT
CCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTGA
CACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAATGGTGCTA
TTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAAAA
CTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAACA
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TTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACCA
AAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATTT
ATATTGTTAATTAGCAAACACAAAACCTGCCTTCTACCACTATAAA

SLKAW6 (Accession no. AB2639450)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCCAGGGGGGGT
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CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC
AATGTCCATTCTATGCATGATCCAGGACACACTCATTCACCCTCCCCATAGACAGCTCCAAA
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CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATTTAACTACCATGTTCTAAC

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CGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACTC
CTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGATC
GCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTGC
CCTTCACAGTGCGGGTGCGGAGTACTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCGT
CCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTGA
CACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCCGGTAAATGGTGCT
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GTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACC
AAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATT
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SLKBW1 (Accession no. AB263951)

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CCCCAACAAGTCACTAACTATGAATGGTTACAGGACATACATTTAACTACCATGTTCTAACC
CATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAGC
AACCCCTGCTTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCCTC
GCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACTCC
TGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGATCG
CGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTGCC
CTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCGTC
CTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTGAC
ACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAATGGTGCTAT
TTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAAAAC
TAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAACAT
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TTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACCAA
AACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATTTA
TATTGTTAATTAGCAAACACAAAACCTGCCTTCTACCACTATAAA

SLKBW2 (Accession no. AB263952)

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CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC
AATGTCCATTCCATGCATGATCCAGGACACACTCATTCACCCTCCCCATAGACAGCTCCAAA
CCACTATCAAGTCACCTAACTATGAATGGTTACAGGACATAAATCTCACTCTCATGTTCTTC
CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATTTAACTACCATGTTCTAAC
CCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG
CAACCCCTGCTTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCCT
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CCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCGT
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CACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAATGGTGCTA
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CTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAACA
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TTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACCA
AAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATTT
ATATTGTTAATTAGCAAACACAAAACCTGCCTTCTACCACTATAAA

SLKBW3 (Accession no. AB263953)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCCAGGGGGGGT
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CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC
AATGTCCATTCTATGCATGATCCAGGACACACTCATTCACCCTCCCCATAGACAGCTCCAAA
CCACTACCAAGTCACCTAACTATGAATGGTTACAGGACATAAATCTCACTCTCATGTTCTTC

CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATTTAACTACCATGTTCTAAC
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CAACCCCTGCTTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCCT
CGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACTC
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GCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTGC
CCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCGT
CCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTGA
CACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCCGGTAAATGGTGCT
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ACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAAC
ATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAAC
GTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACC
AAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATT
TATATTGTTAATTAGCAAACACAAAACCTGCCTTCTACCACTATAAA

SLKBW7 (Accession no. AB263954)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC
AATGTCCATTCCATGCATGATCCAGGACACACTCATTCACCCTCCCCATAGACAGCTCCAAA
CCACTACCAAGTCACCTAACTATGAATGGTTACAGGACATAAATCTCACTCTCATGTTCTTC
CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATTTAACTACCATGTTCTAAC
CCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG
CAACCCCTGCTTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCCT
CGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACTC
CTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGATC
GCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTGC
CCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCGT
CCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTGA
CACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCCGGTAAATGGTGCTA
TTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAAAA
CTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAACA

TTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAACG
TTTATCGTATAACATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACCA
AAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATTT
ATATTGTTAATTAGCAAACACAAAACCTGCCTTCTACCACTATAAA

SLK12 (Accession no. AB263955)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC
AATGTCCATTCTATGCATGATCCAGGACACACTCATTCACCCTCCCCATAGACAGCTCCAAA
CCACTACCAAGTCACCTAACTATGAATGGTTACAGGACATAAATCTCACTCTCATGTTCTTC
CCCCAACAAGTCACCTAATTATGAATGGTTACAGGACATACATTTAACTACCATGTTCTAAC
CCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG
CAACCCCTGCTTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCCT
CGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACTC
CTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGATC
GCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTGC
CCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCGT
CCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTGA
CACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCCGGTAAATGGTGCT
ATTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAAA
ACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTATTTTTTAAAAAC
ATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAAC
GTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACC
AAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATT
TATATTGTTAATTAGCAAACACAAAACCTGCCTTCTACCACTATAAA

SLK13 (Accession no. AB263956)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAACCTATATTCCACATTTCTCCC
AATGTCCATTCTATGCATGATCCAGGACATACTCATTTACCCTCCCCATAGACAGTTCCAAA

CCACTATCAAGCCACCTAACTATGAATGGTTACAGGACATAAATCTCACTCTCATGTTCTCC
CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATTTAACTACCATGTTCTAAC
CCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG
CAACCCCTGCCTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCC
TCGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACT
CCTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGAT
CGCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTA
CCCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCG
TCCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTG
ACACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAATGGTGCT
ATTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAAA
ACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAAC
ATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAAC
GTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACC
AAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATT
TATATTGTTAATTAGCAAACACAAAACCCGCCTTCTACCACTATAAA

SLK14 (Accession no. AB263957)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAACCTATATTCCACATTTCTCCC
AATGTCCATTCTATGCATGATCCAGGACATACTCATTTACCCTCCCCATAGACAGTTCCAAA
CCACTATCAAGCCACCTAACTATGAATGGTTACAGGACATAAATCTCACTCTCATGTTCTCC
CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATTTAACTACCATG-
TTCTAACCCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAG
AGATCAGCAACCCCTGCCTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCT
ACACCCCTCGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATG
CATAACTCCTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGT
CCGTGATCGCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCA
CAGGTTGCCCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTG
CGTTGCGTCCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAAT
CATCTTGACACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAA
TGGTGCTATTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTT

CACAAAACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTT
AAAAACATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTAT
ACAAACGTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTC
CACTACCAAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAA
TCGTTATTTATATTGTTAATTAGCAAACACAAAACCCGCCTTCTACCACTATAAA

SLK15 (Accession no. AB263958)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAACCTATATTCCACATTTCTCCC
AATGTCCATTCTATGCATGATCCAGGACATACTCATTTACCCTCCCCATAGACAGTTCCAAA
CCACTATCAAGCCACCTAACTATGAATGGTTACAGGACATAAATCTCACTCTCATGTTCTCC
CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATCTAACTACCATGTTCTAA
CCCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCA
GCAACCCCTGCCTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCC
CTCGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAAC
TCCTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGA
TCGCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTT
ACCCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGC
GTCCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTT
GACACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAATGGTGC
TATTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAA
AACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAA
CATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAA
CGTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTAC
CAAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTA
TTTATATTGTTAATTAGCAAACACAAAACCCGCCTTCTACCACTATAAA

SLK16 (Accession no. AB263959)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAACCTATATTCCACATTTCTCCC

AATGTCCATTCTATGCATGATCCAGGACATACTCATTTACCCTCCCCATAGACAGTTCCAAA
CCACTATCAAGCCACCTAACTATGAATGGTTACAGGACATAAATCTCACTCTCATGTTCTCC
CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATCTAACTACCATGTTCTAA
CCCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCA
GCAACCCCTGCCTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCC
CTCGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAAC
TCCTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGA
TCGCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTT
GCCCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGC
GTCCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTT
GACACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAATGGTGC
TATTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAA
AACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAA
CATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAA
CGTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTAC
CAAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTA
TTTATATTGTTAATTAGCAAACACAAAACCCGCCTTCTACCACTATAAA

SLKWM11 (Accession no. AB263960)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAACCTATATTCCACATTTCTCCC
AATGTCCATTCTATGCATGATCTAGGACATACTCATTTACCCTCCCCATAGACAGTTCCAAA
CCACTATCAAGCCACCTAACTATGAATGGTTACAGGACATAAATCTCACTCTCATGTTCTCC
CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATTTAACTACCATG-
TTCTAACCCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAG
AGATCAGCAACCCCTGCCTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCT
ACACCCCTCGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATG
CATAACTCCTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGT
CCGTGATCGCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCA
CAGGTTACCCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTG
CGTTGCGTCCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAAT
CATCTTGACACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAA

TGGTGCTATTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTT
CACAAAACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTT
AAAAACATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTAT
ACAAACGTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTC
CACTACCAAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAA
TCGTTATTTATATTGTTAATTAGCAAACACAAAACCCGCCTTCTACCACTATAAA

SLKWM12 (Accession no. AB263961)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAACCTATATTCCACATTTCTCCC
AATGTCCATTCTATGCATGATCCAGGACATACTCATTTACCCTCCCCATAGACAGTTCCAAA
CCACTACCAAGCCACCTAACTATGAATGGTTACAGGACATAAATCTCACTCTCATGTTCTCC
CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATTTAACTACCATGTTCTAAC
CCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG
CAACCCCTGCCTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCC
TCGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACT
CCTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGAT
CGCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTA
CCCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCG
TCCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTG
ACACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAATGGTGCT
ATTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAAA
ACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAAC
ATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAAC
GTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACC
AAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATT
TATATTGTTAATTAGCAAACACAAAACCCGCCTTCTACCACTATAAA

SLKBM3 (Accession no. AB263962)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC

AATGTCCATTCTATGCATGATCCAGGACATACTCATTCACCCTCCCCATAGACAGCTCCAAA
CCACTACCAAGTCACCTAACTATGAATGGTTGCAGGACATAAATCTCACTCTCATGCTCTTC
CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATTTAACTACCATGTTCTAAC
CCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG
CAACCCCTGCCTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCC
TCGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACT
CCTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGAT
CGCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTG
CCCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCG
TCCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTG
ACACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAATGGTGCT
ATTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAAA
ACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAAC
ATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAAC
GTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACC
AAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATT
TATATTGTTAATTAGCAAACACAAAACCCGCCTTCTACCACTATAAA

SLKBM4 (Accession no. AB263963)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC
AATGTCCATTCTATGCATGATCCAGGACATACTCATTCACCCTCCCCATAGACAGCTCCAAA
CCACTACCAAGTCACCTAACTATGAATGGTTGCAGGACATAAATCTCACTCTCATGCTCTTC
CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATTTAACTACCATGTTCTAAC
CCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG
CAACCCCTGCCTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCC
TCGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACT
CCTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGAT
CGCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTG
CCCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCG
TCCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTG
ACACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCCGGTAAATGGTGC

TATTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAA
AACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAA
CATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAA
CGTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTAC
CAAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTA
TTTATATTGTTAATTAGCAAACACAAAACCCGCCTTCTACCACTATAAA

SLKBM5 (Accession no. AB263964)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC
AATGTCCATTCTATGCATGATCCAGGACATACTCATTCACCCTCCCCATAGACAGCTCCAAA
CCACTACCAAGTCACCTAACTATGAATGGTTGCAGGACATAAATCTTACTCTCATGCTCTTC
CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATTTAACTACCATGTTCTAAC
CCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG
CAACCCCTGCCTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCC
TCGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACT
CCTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGAT
CGCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTG
CCCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCG
TCCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTG
ACACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAATGGTGCT
ATTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAAA
ACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAAC
ATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAAC
GTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACC
AAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATT
TATATTGTTAATTAGCAAACACAAAACCCGCCTTCTACCACTATAAA

SLKBM6 (Accession no. AB263965)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC

AATGTCCATTCTATGCATGATCCAGGACATACTCATTCACCCTCCCCATAGACAGCTCCAAA
CCACTACCAAGTCACCTAACTATGAATGGTTGCAGGACATAAATCTCACTCTCATGCTCTTC
CCCCAACAAGTCACCTAACTATGAATGGTTGCAGGACATACATTTAACTACCATGTTCTAAC
CCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG
CAACCCCTGCCTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCC
TCGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACT
CCTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGAT
CGCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTG
CCCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCG
TCCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTG
ACACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAATGGTGCT
ATTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAAA
ACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAAC
ATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAAC
GTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACC
AAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATT
TATATTGTTAATTAGCAAACACAAAACCCGCCTTCTACCACTATAAA

VS1 (Accession no. AB263966)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC
AATGTCCATTCTATGCATGATCCAGGACATACTCATTCACCCTCCCCATAGACAGCTCCAAA
CCACTACCAAGTCACCTAACTATGAATGGTTGCAGGACATAAATCTTACTCTCATGCTCTTC
CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATTTAACTACCATGTTCTAAC
CCATTTGGTTATGCTCGTCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG
CAACCCCTGCCTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCC
TCGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACT
CCTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGAT
CGCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTG
CCCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCG
TCCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTG
ACACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCCGGTAAATGGTGC

TATTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAA
AACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAA
CATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAA
CGTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTAC
CAAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTA
TTTATATTGTTAATTAGCAAACACAAAACCCGCCTTCTACCACTATAAA

VS2 (Accession no. AB263967)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC
AATGTCCATTCTATGCATGATCCAGGACATACTCATTCACCCTCCCCATAGACAGCTCCAAA
CCACTACCAAGTCACCTAACTATGAATGGTTGCAGGACATAAATCTCACTCTCATGCTCCTC
CCCCAACAAGTCACCTAACTATGAATGGTTGCAGGACATACATTTAACTACCATGTTCTAAC
CCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG
CAACCCCTGCCTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCC
TCGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACT
CCTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGAT
CGCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTG
CCCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCG
TCCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTG
ACACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAATGGTGCT
ATTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAAA
ACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAAC
ATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAAC
GTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACC
AAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATT
TATATTGTTAATTAGCAAACACAAAACCCGCCTTCTACCACTATAAA

SLSG1 (Accession no. AB263968)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC

AATGTCCATTCTATGCATGATCCAGGACATACTCATTCACCCTCCCCATAGACAGCTCCAAA
CCACTACCAAGTCACCTAACTATGAATGGTTGCAGGACATAAATCTCACTCTCATGCTCCTC
CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATTTAACTACCATGTTCTAAC
CCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG
CAACCCCTGCCTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCC
TCGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACT
CCTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGAT
CGCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTG
CCCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCG
TCCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTG
ACACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAATGGTGCT
ATTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAAA
ACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAAC
ATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAAC
GTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACC
AAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATT
TATATTGTTAATTAGCAAACACAAAACCCGCCTTCTACCACTATAAA

SLSG2 (Accession no. AB263969)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTACATTCCACATTTCTCCC
AATGTCCATTCTATGCATGATCCAGGACATACTCATTCACCCTCCCCATAGACAGCTCCTAA
CCACTACCAAGTCACCTAACTATGAATGGTTGCAGGACATAAATCTCACTCTCATGCTCTCC
CCCCAACAAGTCACCTAACTATGAATGGTTGCAGGACATACATTTAACTACCATGTTCTAAC
CCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG
CAACCCCTGCCTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCC
TCGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACT
CCTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGAT
CGCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTG
CCCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCG
TCCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTG
ACACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAATGGTGCT

ATTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAAA
ACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAAC
ATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAAC
GTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACC
AAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATT
TATATTGTTAATTAGCAAACACAAAACCCGCCTTCTACCACTATAAA

SLSG4 (Accession no. AB263970)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC
AATGTCCATTCTATGCATGATCCAGGACATACTCATTCACCCTCCCCATAGACAACTCCAAA
CCACTACCAAGCCACCTAACTATGAATGGTTGCAGGACATAAATCTCACTCTCATGTTTTTC
CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATTTAACTACTATGCTCTAAC
CCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG
CAACCCCTGCCTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCC
TCGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACT
CCTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGAT
CGCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTG
CCCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCG
TCCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTG
ACACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAATGGTGCT
ATTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAAA
ACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAAC
ATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAAC
GTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACC
AAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATT
TATATTGTTAATTAGCAAACACAAAACCCGCCTTCTACCACTATAAA

NA1 (Accession no. AB263971)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC

AATGTCCATTCTATGCATGATCCAAGACATACTCATTCACCCTCCCCATAGACAACTCCAAA
CCACTACCAAGCCACCTAACTATGAATGGTTGCAGGACATAAATCTCACTCTCATGTTCTTC
CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATTTAACTACTATGCTCTAAC
CCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG
CAACCCCTGCCTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCC
TCGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACT
CCTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGAT
CGCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTG
CCCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCG
TCCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTG
ACACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCGGTAAATGGTGCT
ATTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAAA
ACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAAC
ATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAAC
GTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTACC
AAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTATT
TATATTGTTAATTAGCAAACACAAAACCCGCCTTCTACCACTATAAA

NA3 (Accession no. AB263972)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC
AATGTCCATTCTATGCATGATCCAAGACATACTCATTCACCCTCCCCATAGACAACTCCAAA
CCACTACCAAGCCACCTAACTATGAATGGTTGCAGGACATAAATCTCACTCTCATGTTCTTC
CCCCAACAAGTCACCTAACTATGAATGGTTACAGGACATACATTTAACTACTATGCTCTAAC
CCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG
CAACCCCTGCCTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCC
TCGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAGGCACATCCCATGCATAACT
CCTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGAT
CGCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTG
CCCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCG
TCCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTG

ACACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCCGGTAAATGGTGC
TATTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAA
AACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAA
CATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAA
CGTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTAC
CAAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTA
TTTATATTGTTAATTAGCAAACACAAAACCCGCCTTCTACCACTATAAA

GDG1 (Accession no. AB263973)

AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGT
ATACTATGCATAATCGTGCATACATTTATATACCACATATATTATGGTACCGGTAATATATA
CTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCC
AATGTCCATTCTATGCATGATCCAGGACATACTCATTCACCCTCCCCATAGACAACTCCAAA
CCACTACCAAGCCACCTAACTATGAATGGTTGCAGGACATAAATCTCACTCTCATGTTCTTC
CCCCAACAAGTCACCTAACTATGAATGGTTGCAGGACATACATTTAACTACTATGCTCTAAC
CCATTTGGTTATGCTCGCCGTATCAGATGGATTTATTGATCGTCCACCTCACGAGAGATCAG
CAACCCCTGCCTGTAATGTACTTCATGACCAGTCTCAGGCCCATTCTTTCCCCCTACACCCC
TCGCCCTACTTGCCTTCCACCGTACCTCTGGTTCCTCGGTCAAGCACATCCCATGCATAACT
CCTGAACTTTCTCACTTTTCACGAAGTCATCTGTGGATTATCTTCCCCTCTTTAGTCCGTGAT
CGCGGCATCTTCTCTCTTCTATTGCTGTTGGTTCCTTCTCTTTTTGGGGCTTCTTCACAGGTTG
CCCTTCACAGTGCGGGTGCGGAGTGCTATTCAAGTGAAGCCTGGACTACACCTGCGTTGCG
TCCTATCCTAGTCCTCTCGTGTCCCTCGATGAGACGGTTTGCGTGTATGGGGAATCATCTTG
ACACTGATGCACTTTGGATCGCATTTGGTTATGGTTCTTCCACCCCCCCCGGTAAATGGTGC
TATTTAGTGAATGCTTGTCGGACATATTTTTATCAATTTTCACTTCCTCTATTTTCTTCACAA
AACTAGGAAATTCACCACAATTTTTTCTTTGTTATTTTTTAATTTTTTTTTTTATTTTTTAAAAA
CATTTTTTAAAAAACTAAATTACATACAAACTACCGCATAAAATCCCTCAAACTATACAAA
CGTTTATCGTATAATATATATACATTATTGTTTATTCTATCATTATTAGAGAAACTCCACTAC
CAAAACCATCATTAAAACAAAAATTTACATGCCACTTAACTCCCCTCACAAACAATCGTTA
TTTATATTGTTAATTAGCAAACACAAAACCCGCCTTCTACCACTATAAA