# Estimating inbreeding in an inbred line of Japanese quail (*Coturnix Japonica*) using pedigree and microsatellite analyses

by

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## ABSTRACT

Accurately estimating the level of inbreeding in a population is essential because inbreeding reduces fitness, fertility, viability, hatchability, and other production traits in populations. Inbreeding has been estimated by analyses of pedigrees and genetic markers. The objective of this study was to evaluate the accuracy of the two methods in an inbred line of Japanese quail.

The inbred line was derived from a random bred (QO) line and maintained for 17 generations by pedigreed matings of brothers to groups of sisters. Data from analysis of 14 microsatellite markers, GUJ0001, GUJ0024, GUJ0030, GUJ0034, GUJ0040, GUJ0044, GUJ0057, GUJ0059, GUJ0060, GUJ0065, GUJ0068, GUJ0070, GUJ0071, and GUJ0085, were obtained. Pedigree data were used to calculate the inbreeding coefficient ($F_{IT}$), which is the level of inbreeding based on a reference ancestor. From the microsatellite locus data, the population differentiation ($F_{ST}$) of the two lines caused by inbreeding was calculated as $F_{ST} = 1-(H_{E(INB)}/H_{E(RAND)})$, where $H_{E(INB)}$ and $H_{E(RAND)}$ are the expected heterozygosity of the inbred and the random bred lines, respectively. The $F_{IT}$ was then calculated as $F_{IT} = F_{IS}+(1-F_{IS})*F_{ST}$, where $F_{IS}$ is the level of inbreeding within the inbred line. The heterozygosity observed by analysis of the microsatellite markers of the randombred and inbred lines was 0.430 and 0.207, respectively, and the number of alleles was 3.29 and 1.93, respectively, demonstrating that genetic diversity was reduced in the inbred line. The $F_{IT}$ of the inbred line from the pedigree and microsatellite marker analyses was 0.687 (±0.069) and 0.567 (±0.329), respectively.

These results suggest that pedigree analysis seems to be more accurate than

microsatellite marker analyses without multi-generation genotyping for estimating inbreeding in an inbred line of Japanese quail. Further studies will be required to investigate reasons for the high standard deviation of microsatellite marker analyses.

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E. GUJ0068 in the randombred line

F. GUJ0068 in the inbred line

LIST OF ABBREVIATIONS

| | |
|---|---|
| AFLPs | amplified fragment length polymorphism |
| DAD-IS | Domestic Animal Diversity Information System |
| FAO | Food and Agriculture Organization of the United Nations |
| GUJ | Gifu University Japanese quail |
| HWE | Hardy-Weinberg equilibrium |
| IAM | infinite alleles model |
| IBD | identical by descent |
| PCR | polymerase chain reaction |
| QO | Quebec |
| QGRC | Quail Genetic Resource Centre |
| RAPDs | random amplified polymorphic DNAs |
| RFLPs | restriction fragment length polymorphisms |
| SMM | stepwise mutation model |
| SNPs | single nucleotide polymorphisms |
| SSRs | simple sequence repeats |
| STRs | short tandem repeats |

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Co-Authorship Statement

Dr. Fred Silverside, Dr. Kim Cheng, and Dr. Kermit Ritland designed the research program. I performed all the research including the experiment and the data analyses for this thesis, while Dr. Fred Silverside, Dr. Kim Cheng, and Dr. Kermit Ritland, guided me during the research, giving me advice and help with how to deal with the data analyses, and Dr. Carol Ritland helped me with technical problems for the experimental works. Finally, Dr. Cheng contributed to preparing for the manuscript.

# CHAPTER 1

## Introduction And Overview

### 1.1 The Japanese quail

#### 1.1.1 Classification and history of Japanese quail

Japanese quail (*Coturnix japonica*) belongs to the order *Galliformes* and the family Phasianidae. The Japanese quail, originally domesticated in the 11th century as a pet song bird (Crawford, 1990), used to be considered a subspecies of the European or common quail (*Coturnix coturnix*) and was designated as *Coturnix coturnix japonica* (Westmore, 1952). However, because of taxonomic evidence in vocalizations, sympatry, sexual isolation and hybrid inviability, the Japanese quail is now considered to be a different species from the common quail and has been given a new designation, *Coturnix japonica* (Howard and Moore, 1984). In spite of the widest natural distribution of any galliform bird and its adaptability to a wide range of ecological conditions (Cheng *et al.*, 1992), because of the loss of breeding habitat, Japanese quail have had extensive population reduction in the last 3 decades (Kimura, 1991). The Japanese quail is originally from Japan, China, Korea and Indochina (Taka-Tsukasa, 1935), and was introduced into Hawaii in 1921 (Schwartz and

Schwartz, 1949). After the Second World War, there was an attempt to rebuild the Japanese quail industry with the few remaining domesticated birds available from Korea, China and Taiwan, and from wild populations (Howes, 1964; Wakasugi, 1984).

### 1.1.2 Characteristics of Japanese quail

The Japanese quail is slightly sexually dimorphic in that females are larger than males, and phylogenetically closely related to the chicken (Stock & Bunch, 1982). Both Japanese quail and chickens have $2n = 78$ chromosomes and a similar genome length of $1.2*10^9$ bp, consisting of morphologically distinct macrochromosomes (1-8 and the ZW sex chromosomes) and microchromosomes. Indeed, Shibusawa *et al.* (2001) found highly conserved chromosome homology between Japanese quail and chickens and very few chromosome rearrangements after divergence of the two species (Shibusawa *et al.*, 2001).

### 1.1.3 Japanese quail as a food source and for research

Japanese quail are kept for their eggs and meat. Egg production is popular in Japan and South-East Asia, whereas meat is the main product in Europe (Minvielle, 1998). The consumption of Japanese quail has been increasing in the United States and in Canada

(Paulson *et al.*, 1989). Because of its small size, inexpensive rearing requirements, rapid maturation compared with other domesticated poultry, and adaptability to a wide range of husbandry conditions, Japanese quail have been used in many studies including behavioural (Mills & Faure, 1991), developmental (Le Douarin *et al.*, 1997), physiological (Balthazart *et al.*, 2003), genetic (Jones *et al.*, 1991) and inbreeding (Mizutani, 2002), and its eggs are frequently used in studies of pathology (Halldin *et al.*, 2003) and virology (Swain *et al.*, 1997).

As to genetic markers for Japanese quail, cross-species amplification has been applied using microsatellites of chicken (Pang *et al.*, 1999), Japanese quail specific microsatellites have been developed (Kayang *et al.*, 2000; Kayang *et al.*, 2002), and linkage maps of amplified fragment length polymorphism (AFLP) markers (Roussot *et al.*, 2003) and that of microsatellite markers (Kayang *et al.*, 2004) of Japanese quail have been published. Some of the studies (Pang *et al.*, 1999; Kayang *et al.*, 2000; Kayang *et al.*, 2002) investigated genetic diversity of Japanese quail.

## 1.2 Genetic diversity

### 1.2.1 Importance of genetic diversity

When many species are being investigated in an attempt to estimate the level of genetic diversity, many studies have shown that we have lost a great deal of genetic diversity. For example, Frankham *et al.* (2002) pointed out that 50% of vertebrate animal species and 12% of all plants are vulnerable to extinction. In poultry, about 50% of the poultry breeds registered in FAO based Domestic Animal Diversity Information System (DAD-IS) were at risk (Hoffmann, 2005). Pisenti *et al.* (1999) reported the elimination of more than 238 poultry research stocks between 1984 and 1998 in the USA and Canada, almost 40% of the US stocks and 60% of the Canadian stocks. Seventy five percent of eliminated stocks were chickens, eleven percent were turkeys, eleven percent were Japanese quail, and four percent were game birds or waterfowl. The University of British Columbia used to hold the largest collection of mutations and unique lines of Japanese quail in the world, which included 12 specialized lines and 27 mutation lines. By 2003, the collection had been reduced to 9 lines and specialized lines, and transferred to Agriculture and Agri-Foods Canada's research station in Agassiz, BC, Canada (Cheng, 2003).

Keeping a high level of genetic diversity, which will be a reservoir of genetic material in the future is very important because a low level of genetic diversity in a population may reduce fitness and viability by increasing inbreeding and thus increasing homozygosity and could even lead species to extinction. Moreover, a low level of genetic diversity can make species too vulnerable to the outbreak of certain diseases. For example, when a disease swept through several captive cheetah colonies, it caused 50%-60% mortality over a 3-year period (Avise, 2004). In this respect, for endangered and captive species, good management will be required to reduce the loss of genetic diversity, inbreeding depression, or even extinction.

### 1.2.2 Inbreeding and heterozygosity

Inbreeding is the mating of closely related individuals. Inbreeding changes genotypes by increasing the frequency of homozygotes and decreasing that of heterozygotes compared to random mating, leading to a reduction in fitness. The negative effect of inbreeding on fitness has made inbreeding a concern in genetic conservation because many populations of endangered or captive species may be small. In large random mating, populations with no mutation, migration or selection, allele and genotype frequencies reach

an equilibrium, which is called the Hardy-Weinberg equilibrium (HWE). In mathematical model, the HWE can be referred as $p2 + 2pq + q2 = 1$, where p and q are the relative frequency for alleles A1 and A2, respectively. If mating occurs between two related parents, the proportions of the equilibrium change by the degree of inbreeding (inbreeding coefficient).

Not just does parental relatedness cause inbreeding, but genetic drift can also do so in small populations. Genetic drift, which is allelic fluctuation from generation to generation, has major impacts on small populations. The allelic frequency can be large in small populations, and can even result in fixation or loss of an allele, leading to a high level of homozygosity. On the other hand, if the population is large enough, for example if the size is 500 or more (Hartl and Clark, 1997), random genetic drift hardly has any impact on the population. However, migration or gene flow between populations will reduce the chance of genetic drift and inbreeding (Barton and Whitlock, 1997). Previous studies have shown that inbreeding is common both in captive and in wild populations (Wright, 1977; Ralls *et al.*, 1986; Charlesworth and Charlesworth, 1987; Thornhill, 1993; Keller, 1998). Briefly, there are two ways to measure inbreeding, calculating inbreeding coefficient (F) from pedigree analysis (Hartl and Clark, 1997), and estimating F from heterozygosity by using genetic

markers (Galeuchet *et al.*, 2005).

### 1.2.3 Inbreeding depression

Because inbreeding increases homozygosity and decreases heterozygosity, it causes inbreeding depression along with genetic drift, which reduces fitness (Keller and Waller, 2002; Frankham *et al.*, 2002) and because deleterious mutations occur frequently, increased homozygosity will increase the genetic load in the population (Keller and Waller, 2002). Inbreeding causes inbreeding depression via two different mechanisms: homozygotes of deleterious recessive or partially recessive alleles reduce fitness, and overdominance, where the fitness of heterozygotes is higher than that of homozygotes. These mechanisms produce different outcomes and inbreeding depression resulting from homozygosity of deleterious alleles can be more easily removed by selection than from that caused by the elimination of overdominance (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987; Holsinger, 1991). There are studies supporting both, dominance-based (Moll *et al.*, 1964; Hallauer and Miranda, 1985) and overdominance-based (Apirion and Zohary, 1961; Hallauer and Miranda, 1985) inbreeding depression. Various studies have reported that inbreeding depression is widely spread (Bulmer, 1973; Gibbs and Grant, 1989; Rosenfield and

Bielefeldt, 1992; Coltman *et al.*, 1998). Frankel and Soule (1981) pointed out that an increase in inbreeding by 10% accounts for a reduction of up to 5 to 10% in fitness components. Miglior *et al.* (1992) found regression coefficients of -9.84 kg, -0.55 kg, and -0.0011% for milk, fat, and fat percentage per 1% increase of inbreeding in Jersey cattle. In 3 White Leghorn lines selected for different egg production traits (egg weight, egg number, and egg number and egg weight combination), Sewalem *et al.* (1999) showed that an increase in inbreeding of the hen and embryo resulted in decreasing reproductive fitness such as egg number, egg fertility, and mean hatchability. In other studies with the Great Tit, mortality was, on average, 27.7% among inbreeders and 16.2% among outbreeders (Greenwood *et al.*, 1978), and van Noordwijk and Scharloo (1981) showed that a 10% increase in inbreeding coefficient decreased the viability of eggs by 7.5%.

However, several mechanisms have evolved to avoid inbreeding in animals, such as, extra pair copulation (Byrne *et al.*, 2003; Zeh and Zeh, 2006), and purging (Kirkpatrick and Jarne, 2000; Boakes and Wang, 2005). However, previous studies have reported that the severity of inbreeding depression is affected by environmental factors (Keller *et al.*, 2002; Vermeulen and Bijlsma, 2004; Gallardo and Neira, 2005). Therefore, inbreeding depression can be underestimated in captive or laboratory animals because normal environment for these

animals is not as harsh as in nature (Hedrick and Kalinowski, 2000).

### 1.2.4 Inbreeding and purging

Because most inbreeding depression is caused by many partially recessive and deleterious alleles, it is possible that those alleles with less fitness can be purged by natural selection (Templeton and Read, 1984; Crow, 1993; Hedrick, 1994). With overdominance partially recessive and deleterious alleles will remain in a heterozygote, leaving themselves less chances to be purged (Wang, 2000).

However, even though a lot of studies have supported that purging decreases inbreeding depression in various species, it seems that purging reacts in each species differently, because various studies showed diverse results regarding purging (Kirkpatrick and Jarne, 2000; van Oosterhout *et al.*, 2000; Crnokrak and Barrett, 2002). For example, even though Crnokrak and Barrett (2002) found frequent and substantial purging in various species using three measures: (1) changes in inbreeding depression, (2) changes in fitness components of inbred lines relative to original outbred line, and (3) purged population (outcrossed inbred lines) trait means as a function of ancestral out bred trait means, they mentioned that adaptation to laboratory conditions may have caused rebounds in fitness, not

purging. In another case, although van Oosterhout *et al.* (2000) found considerable purging during inbreeding, they found out that the genetic load still remained higher than that of many outbreeding species.

### 1.2.5 Inbreeding and F statistics

A population has a hierarchical population structure. To investigate the effect of population substructure, Wright (1921) presented F statistics, $F_{IS}$, $F_{ST}$, and $F_{IT}$. In small populations, the rates of inbreeding will be relatively high, because inbreeding is the mating of closely related individuals. Therefore, the genetic differentiation among subpopulations estimated by F statistics can be related to the inbreeding effect resulting from population substructure (Hartl and Clark, 1997), because the smaller population within a subpopulation will have a greater chance to mate to their relatives. $F_{IS}$ is measure of genetic variance inbreeding coefficient of an individual (I) relative to the subpopulation (S), $F_{ST}$ is the effect of the subpopulation (S) compared to the total population (T), and $F_{IT}$ is the inbreeding coefficient of an individual (I) relative to the total population (T) (Wikipedia, 2006). The followings are the equations:

$F_{IS} = 1-(H_I/H_S)$

$F_{ST} = 1-(H_S/H_T)$

$F_{IT} = 1-(H_I/H_T)$,

where $H_I$ is the observed heterozygosity averaged across all population fragments, $H_S$ is the expected Hardy-Weinberg heterozygosity averaged across all population fragments, and $H_T$ is the expected Hardy-Weinberg heterozygosity for the total population (Frankham *et al.*, 2004).

The inbreeding coefficient $F_{IS}$ or F is the probability that an individual inherits both alleles at a locus that are identical by descent. The effects of inbreeding can be explained using F by the general formulation of P = p2 + Fpq, H = 2pq(1-F), and Q = q2 + Fpq, where P is the frequency of A1A1, Q that of A2A2, and H that of A1A2 in the next generation and where p and q are the relative frequency for alleles A1 and A2, respectively (Wright, 1931). Therefore, F can be calculated as F=1-H/2pq or F=1-Ho/He, where Ho is observed heterozygosity and He expected heterozygosity, when expected heterozygosity is calculated by $He = 1-\sum pi$, where pi is the frequency of the ith allele (Frankham *et al.*, 2004).

$F_{ST}$, the fixation index, is the probability that two alleles drawn randomly from a subpopulation are identical by descent (Frankham *et al.*, 2002) and measures the type of inbreeding where there is a reduction in average heterozygosity among the subpopulations

(Hartl and Clark, 1997). Allele frequencies among subpopulations can be affected by a lot of factors, such as random genetic drift, gene flow among subpopulations, and natural selection. Frankham *et al.* (2002) mentioned that high rates of gene flow among subpopulations decrease $F_{ST}$, but low rates of gene flow among subpopulations will diverge the gene pools of the subpopulations and lead to inbreeding, increasing $F_{ST}$. According to Wright (1978), $F_{ST}$ of 0 to 0.05 of may be considered little genetic differentiation, 0.05 to 0.15 is moderate genetic differentiation, 0.15 to 0.25 is high genetic differentiation, and $F_{ST}$ above 0.25 shows very great genetic differentiation. $F_{IT}$, the heterozygosity of the inbred organisms relative to the total population, is the most inclusive measure of all inbreeding measures (Hartl and Clark, 1997). Hartl and Clark (1997) pointed out that $F_{IT}$ shows both the effects of mating between close relatives within a subpopulation and the accumulated inbreeding resulting from mating between remote relatives at all levels of the population hierarchy. With $F_{IS} = 1-(H_I/H_S)$, $F_{ST} = 1-(H_S/H_T)$, and $F_{IT} = 1-(H_I/H_T)$, the following equation can be derived:

$$1- F_{IT} = (1- F_{IS})(1- F_{ST})$$

(Hartl and Clark, 1997).

## 1.3 Genetic markers

### 1.3.1 Microsatellite markers

Microsatellite markers, which are referred to as short tandem repeats (STRs) or simple sequence repeats (SSRs) are 1 to 6 non-coding nucleotide repeats, with Mono-, di- and trinucleotide repeats being the most common. Microsatellites, which are one of the most common and variable types of DNA sequence and are regarded as neutral markers, are found in either intergenic regions or introns (Toth *et al.*, 2000). With the advent of the polymerase chain reaction, their high mutation rate (Jarne & Lagoda, 1996) and polymorphism have made microsatellites one of the most important markers in genetic research.

### 1.3.2 Genetics of microsatellites

Microsatellites are said to account for 3% of the human genome (International Human Genome Sequencing Consortium, 2001), and it has been reported that microsatellite density tends to be positively related to genome size (Primmer *et al.*, 1997; Toth *et al.*, 2000). Bats, which have considerably smaller genomes than other mammals appear to have fewer microsatellites than other mammals (Van Den Bussche *et al.*, 1995). Mice were shown to

have two or three times more microsatellites than humans (review: Ellegren, 2004). Primmer *et al.* (1997) found that more di-, tri-, and tetranucleotide repeats were observed in mammals than in birds, and the density of microsatellites in mammals was higher than in birds, which is attributed to the fact that genome size of birds is about one third of that of humans in terms of total base pairs (Bloom *et al.*, 1993; Wachtel and Tiersch, 1993).

### 1.3.3 Microsatellite mutation

Most microsatellites show high mutation rates, ranging from $10^{-2}$ to $10^{-5}$ per generation (Edwards *et al.,* 1992), with the average being approximately $10^{-3}$ (Weber & Wong 1993; Jarne & Lagoda 1996), but mutation rates may vary among species (Ellegren, 2000), microsatellite length or allele size (Schug *et al.,* 1997; Brinkmann *et al.,* 1998; Brohede *et al.,* 2002), with long alleles being generally more mutation prone than shorter ones, repeat types, with dinucleotide repeats having higher mutation rates than tri- and tetranucleotide repeats (Kimmel & Chakraborty, 1996; Schug *et al.*, 1998), base composition of the repeat (Bachtrog *et al.*, 2000), and flanking sequences (Glenn *et al.,* 1996; Bachtrog *et al.,* 2000).

### 1.3.4 The mutation models of microsatellites

There are thought to be two possible mutation mechanisms for length changes of microsatellites, the replication slippage model (Levinson & Gutman, 1987), which occurs when two strands dissociate and then realign out of register and new replication will cause insertion or deletion of repeat units relative to the template strand, and the unequal crossing-over model which occurs during recombination in meiosis. However, it is most likely that replication slippage is the major mechanism for microsatellite mutations, because it has been shown in yeast that the primary mechanism of mutation is DNA slippage (Strand *et al.*, 1993), even though occasional unequal crossing-over may affect the allele length (Wierdl *et al.*, 1997). Schug *et al.* (1998) found no significant correlation between microsatellite mutation rate and recombination rate, which indicates that unequal crossing-over accounts for little microsatellite mutation.

For the mutation models, the infinite alleles model (IAM; Kimura & Crow, 1964) and the stepwise mutation model (SMM; Kimura & Otha 1978) have been presented. In the IAM model, each mutation creates a novel allele, and in SMM, the more size difference, the more distantly related, because it is presumed that each mutation produces a novel allele either by adding or deleting a single repeat at a fixed rate. However, many changes have been

added to the simple SMM, because SMM is of limited help in explaining the observation that microsatellites tend to show an upper size limit (Review: Ellegren, 2004), and replication slippage can make more than one repeat mutation. An upper limit on allele sizes (Feldman *et al.*, 1997; Stefanini & Feldman, 2000), and a mutational bias that favors large alleles mutating to smaller ones (Zhivotovsky, 1999; Calabrese & Durrett, 2003) have been introduced to the SMM. Another model, in which two mutational forces, length mutations and point mutations, work together in creating microsatellite repeat length differences (Bell & Jurka, 1997; Kruglyak *et al.*, 1998) has been suggested to overcome the weaknesses of SMM. In this model, length mutations tend to increase repeats whereas point mutations break long repeats into smaller ones.

### 1.3.5 Microsatellites and other markers

Allozyme or isozyme markers are allelic variants of enzymes which are composed of amino acids. Because enzymes are electrically charged, allelic variation due to mutation of any amino acids in the enzyme can be detected through electrophoresis. Before other genetic markers using DNA were popularized, allozyme markers were applied to investigate population structure (Spieth, 1975; Beckwitt and Chakraborty, 1980; Ochman *et al.*, 1983).

Although, these markers require no DNA, DNA probes, or primers, their low abundance and low level of polymorphism have made DNA based markers more popular (Nybom, 2004).

RAPD (Random amplified polymorphic DNA), AFLP (Amplified fragment length polymorphism) and microsatellite markers are PCR-based markers. RAPDs and AFLPs both use random DNA sequences as primers to detect the variation in flanking sites, but they are different in that AFLPs utilize RFLPs (Restriction fragment length polymorphisms) along with RAPDs. RAPDs and AFLPs are abundant in the genome and no sequence data are required to develop primers. The DNA based but non PCR based markers are RFLPs and SNPs (Single nucleotide polymorphisms). RFLPs are DNA fragments cut by restriction enzymes, followed by elctrophoresis and Southern blotting. RFLP is a good way to investigate point mutations (Nishimukai *et al.*, 1996), which can be base exchanges, base deletions or insertions. SNPs are DNA sequence variations to nucleotide (A, G, C, and T) and are abundant and widespread in the genomes of many species. SNPs are used to investigate the relationship between common genetic variation and common heritable diseases (Pritchard and Cox, 2002; Klein *et al.*, 2005).

Although DNA based markers, such as RAPDs, AFLPs, RFLPs, and SNPs are more abundant and randomly distributed throughout genome than allozyme markers,

microsatellite markers have been used in many fields since they were developed for several reasons. First of all, microsatellites are widely distributed throughout the genome (Kashi *et al.*, 1990; Weber, 1990), which makes them easy to isolate. Microsatellites are highly polymorphic due to different numbers of repeats compared to other markers (Rowe *et al.*, 1997). While randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphisms (AFLPs) are dominant markers, and SNP loci have a maximum of two alleles, microsatellites are codominant and can have many alleles, which means microsatellite markers are more informative.

Studying inbreeding in a population is greatly enhanced by neutrality. Because neutral markers will not be subjected to selection, they make it easier to look at the effect of inbreeding. In this respect, microsatellite markers have advantage over allozyme markers, which may not be neutral (Van Oosterhout *et al.*, 2004). Codon degeneracy contributes too little variation to make allozyme markers very useful. In addition, PCR-based microsatellite markers require only a small amount of DNA, unlike RFLP. For parentage assignment, microsatellites are the most extensively used (Parker *et al.*, 1998) as well because a high level of polymorphism is required. Gerber *et al.* (2000) showed that from 100 to 200 dominant loci are similar to 10 highly polymorphic codominant markers for parentage assignment. In

comparing genetic variation among populations, fewer microsatellite loci are required than SNPs because microsatellite loci normally have many alleles, whereas two are normal for SNPs (Leberg, 1992). All in all, thanks to their advantages over other markers, microsatellites are one of the most suitable and well used markers to investigate population structures such as inbreeding coefficients, F, or $F_{ST}$. Bouzat and Johnson (2004) investigated the genetic structure of four closely spaced leks in lesser prairie-chickens with six microsatellite loci and found $F_{ST}$=0.036 (P=0.002) and $F_{IS}$=0.190-0.307 for the leks. Yang *et al.* (2003) reported $F_{IT}$=0.330, $F_{IS}$=0.274, and $F_{ST}$=0.077 on everage with 26 microsatellite loci in 18 Chinese indigenous pig breeds.

## 1.4 Objectives of thesis

The objectives of this research were (1) to investigate the level of inbreeding of the inbred line derived from QO compared to the random bred line (QO) in Japanese quail, and (2) to evaluate the accuracy of the two methods, the pedigree and microsatellite markers for estimating the level of inbreeding in the inbred line.

## LITERATURE CITED


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CHAPTER 2

# Estimating inbreeding in an inbred line of Japanese quail (*Coturnix Japonica*) using pedigree and microsatellite analyses.

## 2.1 Introduction

Because inbreeding increases homozygosity and exposes the deleterious recessive mutations carried by the population (Keller and Waller, 2002), it causes inbreeding depression which reduces the fitness of the population (Keller and Waller, 2002; Frankham *et al.*, 2002). Frankel and Soule (1981) pointed out that an increase in inbreeding of 10% accounts for a reduction of up to 5 to 10% in fitness components. In wild song sparrows, Keller (1998) reported a 17.5% reduction in annual survival rate of offspring of a full-sib mating.

Japanese quail (*Coturnix japonica*) are very susceptible to inbreeding depression (Sittmann *et al.*, 1966) probably because of the heavy genetic load that they carry. None of the inbred lines tha[1]t Sittmann *et al.* (1966) started by consecutive full-sib matings lasted beyond the third generation. Of the 17 full-sib mating inbred lines that Kulenkamp *et al.* (1973) started, only five lasted to the 5$^{th}$ generation. There has been no report of any inbred

[1] A version of this chapter will be submitted for publication.

Japanese quail line surviving more than 8 generations of consecutive full-sib matings (Okimoto, R., University of Arkansas, pers comm; Wada, M., Tokyo Medical and Dental University, pers comm). However, highly inbred lines are very useful in genome analysis and gene mapping (e.g. Hoti and Sillanpää, 2006). At the Quail Genetic Resource Centre (QGRC) at the University of British Columbia, a less intensive inbreeding scheme, adapted from the one used for the development of the I-420 inbred White Leghorn chicken line (Shoffnor *et al.*, 1953), was applied to the development of an inbred Japanese quail line. This quail inbred line has gone through 17 generations of such inbreeding and still appears to be strong. It is therefore of interest to estimate the inbreeding coefficient of this population and compare that with what 8 generations of full-sib mating would have accomplished.

There are several measures available to describe the genetic variability of populations. The average inbreeding coefficient estimated from pedigree information is a frequently chosen option. Due to the development of molecular genetics, it is also possible to estimate inbreeding coefficient based on genetic markers information (Keller and Waller, 2002). Genetic markers have been used extensively for estimating relatedness and level of inbreeding in populations in the absence of pedigree information (e.g. Vargo *et al.*, 2003). Baumung and Sölkner (2003) used a simulated study to compare measures of inbreeding

based on pedigree or microsatellite marker information. I found no empirical study of this kind.

The aim of this study was (1) to compare genetic diversity between the inbred quail line and the randombred line from which the inbred line was derived, and (2) compare microsatellite markers with pedigree analyses for calculating the level of inbreeding.

## 2.2 Methods

### 2.2.1 $F_{IT}$ calculation

To compare the two methods, pedigree and microsatellite marker analyses, for calculating the level of inbreeding in the inbred line of Japanese quail, $F_{IT}$, the inbreeding coefficient of an individual relative to the total population, was calculated. Because the inbred line was derived from the randombred line (QO) in this study, the randombred line is the total population. Therefore, calculating inbreeding of an individual of the inbred line based on the randombred line is calculating $F_{IT}$ of an individual of the inbred line based on the randombred line. $F_{IT}$ can be estimated by $(1-F_{IS})(1-F_{ST})=1-F_{IT}$ (Hartl and Clark, 1997), where $F_{IS}$ is the level of inbreeding within the population, which in this case is the inbred line. $F_{ST}$ can be derived from the equation of $Ft=1-(Ht/Ho)$, where Ft is the accumulated

affect of inbreeding over time, Ht is the heterozygosity at generation t, and Ho is the initial heterozygosity (Frankham et al., 2004), as $F_{ST} = 1-(H_{E(INB)}/H_{E(RAND)})$, where $H_{E(INB)}$ and $H_{E(RAND)}$ are the expected heterozygosity of the inbred and randombred strains, respectively.

### 2.2.2 Experimental populations

In 1990, a Japanese quail line (QO) that has been previously selected for 45 days body weight (Caron *et al.,* 1990) was acquired from the Deschambault Agricultural Research Station, Quebec, Canada. At the QGRC, the QO line was maintained as a randombred population with 48 males and 96 females per generation. In 1992, an inbred line was started from this population (Aggrey, 1994) using the breeding scheme described in Figure 2.1. While efforts were made to adhere to this scheme, in some generations there were less than four females per cage. At the time of this study, the inbred line has gone through its $17^{th}$ generation.

71 individuals were sampled from these two lines of Japanese quail (40 samples from QO and 31 samples from the inbred strain).

### 2.2.3 Pedigree analysis

Two individuals, as less related as possible from each other, of the last generation of the inbred line were chosen and two pedigrees per each of them representing the maximum and the minimum possible coancestry matings were used (Table 2.5, and Figure 2.2). With the four pedigrees, $F_{IT}$ was calculated by ENDOG (v3.0: Gutiérrez and Goyache, 2005) and the mean and sampled standard deviation were calculated from the 4 $F_{IT}$ values by following:

$$s = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \overline{x})^2}$$

, where N is the number of chosen pedigrees, which is 4 in this study, $x_i$ is the $F_{IT}$ of each pedigree, and $\overline{x}$ is the mean of 4 $F_{IT}$ values of the pedigrees.

### 2.2.4 DNA extraction

Fifty μl of blood was taken from each individual through the brachial vein. Genomic DNA was extracted following protocol used by J. Fulton (J. E. Fulton, Hy-Line International, pers comm). After centrifugation at 1500 rpm for 2 minutes, approximately 40μl of packed red blood cells from each sample were resuspended into 3 ml of lysis buffer (1M Tris-HCL pH 8.0, 5M NaCl, 0.5M disodium EDTA, $dH_2O$) and approximately 200 μl of SDS was added and the tube was inverted immediately. The solution was digested with 16 μl

of 20 mg/ml proteinase K (Fisher Scientific, https://www1.fishersci.com/) and shaken at 180 rpm for 1 hour and 60 rpm overnight at 37□. The next day, approximately 2 ml of 5 M NaCl was added to each sample, which was vigorously shaken by hand, and centrifuged at 3500 rpm for 30 minutes. Approximately 4 ml of the supernatant from each sample was added to 8 ml of room temperature 95% ethanol. Crude DNA was washed with 1.5 ml of room temperature 70% ethanol and the ethanol was allowed to evaporate briefly. The DNA was stored in 1 ml of TE buffer (1M Tris pH 8.0, 0.5 M EDTA pH 8.0, $dH_2O$). Approximately 20 ng/μl of genomic DNA was used for each PCR reaction.

### 2.2.5 Microsatellite markers

24 highly polymorphic markers (Appendix 1) were selected from the Japanese quail microsatellite markers developed by Kayang *et al.* (2002). These markers were screened based on polymorphism, wide distribution over the genome, and clear gel bands. Finally, 14 markers, GUJ0001, GUJ0024, GUJ0030, GUJ0034, GUJ0040, GUJ0044, GUJ0057, GUJ0059, GUJ0060, GUJ0065, GUJ0068, GUJ0070, GUJ0071, and GUJ0085, were selected. The characteristics of the markers are shown in Table 2.1.

### 2.2.6 Microsatellite PCR product analyses

0.5-2 μl of 20 ng/(l of genomic DNA was added to a mixture of 1.5mM of MgCl2, 4.3-5.8 μl of dH2O, 1 μl of 2.0 mM dNTP's (Roche LTD., ON, Canada), 0.5 μl of 1.0 pmol/(l forward and reverse primers (Operon Biotechnologies, Inc., Alabama, USA), 0.5 μl of 1 pmol/(l M 13f-29 primer (LiCor), and Taq DNA Polymerase (Roche LTD., ON, Canada). After the PCR reactions, the products were analyzed on a LiCor 4200 (LiCor Inc., Lincoln, NEB). The bands were scored using RFLPscan (LiCor Inc., Lincoln, NEB). If the gel did not show any band, or showed faint, or non-specific bands, the annealing temperatures for the PCR reactions were adjusted ±2-3℃ or the amount of genomic DNA was either increased or decreased to get a better resolution.

### 2.2.7 Microsatellite data analysis

The expected heterozygosity (He), the observed heterozygosity (Ho) and inbreeding coefficient ($F_{IS}$) from the microsatellite information were calculated by Genetic Data Analysis, v1.0 (GDA; Lewis and Zaykin, 2001). $F_{IT}$ of the inbred line based on the randombred line was calculated by the calculations of 2.2.1, and $F_{IT}$ per each locus was also calculated to estimate sampled standard deviation by following:

$$s = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \overline{x})^2}$$, where N is the number of microsatellite loci, which is 14 in this study, $x_i$ is the $F_{IT}$ of each locus, and $\overline{x}$ is the mean of the $F_{IT}$ values of the loci.

## 2.3 Results

The inbreeding coefficient, $F_{IT}$, of the inbred line was 0.687 with the sampled standard deviation of 0.069 (Table 2.5). From microsatellite analysis, $F_{ST}$ was estimated as 0.497 and $F_{IT}$ was calculated as 0.567 with the sampled standard deviation of 0.329. The number of alleles for each locus was counted for the randombred line and the inbred line, and the means were 3.29 and 1.93, respectively (Tables 2.2, 2.3 and 2.4). In the randombred line, all of the markers were polymorphic or highly polymorphic. Four loci (GUJ0001, GUJ0024, GUJ0040, and GUJ0085) had two alleles, four loci (GUJ0044, GUJ0060, GUJ0065, and GUJ0068) had three alleles, and five loci (GUJ0034, GUJ0057, GUJ0059, GUJ0070, and GUJ0071) had four alleles. The locus, GUJ0030 had six alleles. For the inbred line, the loci were found to be either monomorphic or less polymorphic than the randombred line. Five loci (GUJ0001, GUJ0024, GUJ0034, GUJ0059, and GUJ0085) were monomorphic and six loci (GUJ0040, GUJ0057, GUJ0065, GUJ0068, GUJ0070, and GUJ0071) had two alleles, while three alleles were observed for GUJ0044 and GUJ0060, and GUJ0030 had four alleles.

The observed and expected heterozygosity for the randombred line and for the inbred line is shown in Table 2.2, 2.3, and 2.4. For most of the markers, the expected and observed heterozygosity for the inbred line was lower than for the randombred line. Both the mean observed and expected heterozygosity for the random bred line were approximately twice as high as the inbred line, with the mean observed heterozygosity for the random bred line being 0.430, and that for the inbred line being 0.207. The mean expected heterozygosity for the randombred line was 0.475 and that for the inbred line was 0.239. The estimated mean inbreeding coefficients ($F_{IS}$) for the random bred line and for the inbred line were 0.095 and 0.140, respectively. Annealing temperatures for the markers are mentioned in Appendix 2.

## 2.4 Discussion

The purpose of this research was to investigate the level of inbreeding in the inbred line and to compare the two methods (microsatellite markers and pedigree analysis) for estimating the level of inbreeding in Japanese quail.

From the microsatellite marker analyses, the mean number of observed alleles in the randombred line was 3.29 and the mean observed heterozygosity was 0.430. The mean number of alleles per locus for the randombred line is higher than that reported by Pang et al.

(1999) (2.45 per locus) and Inoue-Murayama et al. (2001) (2.3 per locus) for their Japanese quail populations. Our result is more in line with that reported by Kayang et al. (2002) (3.7 per locus). Chang *et al.* (2005) reported 4.67 alleles per locus for the wild Japanese quail in China.

Mean observed heterozygosity for the randombred line was higher than that (0.31) of the populations studied by Inoue-Murayama et al. (2001). Our randombred line had similar heterozygosity to those of Kayang et al. (2002) (0.423). In all three studies, the heterozygosity observed for domestic quail populations is lower than that reported for wild Japanese quail (0.66) (Chang *et al.,* 2005). Cheng et al (1992) and Kimura and Fuiji (1989) used isozyme polymorphism to assess genetic variability in domestic and wild Japanese quail also reported higher proportion of polymorphic loci in wild populations than in domestic populations.

The mean number of observed alleles in the inbred line was 1.93 and the mean observed heterozygosity was 0.207. These values were less than half of those for the randombred line and indicated that the inbreeding scheme applied was effective in reducing heterozygosity (Maeda and Hashiguchi, 1981; Curik *et al.,* 2003; Markert *et al.,* 2004; Slate *et al.,* 2004).

The inbreeding coefficient ($F_{IT}$) of the inbred strain estimated from pedigree information was 0.687 (± 0.069). This would be comparable to inbreeding achieved by 5 generations of consecutive full-sib matings ($F_{IT}$ = 0.672). The less intensive inbreeding scheme would allow purging by natural selection to play a bigger role in shaping the population (Kulenkamp *et al.*, 1973), and would allow the population to go into a higher degree of inbreeding without suffering the expected inbreeding depression (Ballou, 1997; Kirkpatrick and Jarne, 2000; Boakes and Wang, 2005).

The inbreeding coefficient ($F_{IT}$) of the inbred line estimated from microsatellite marker analyses was 0.567 (± 0.329), which shows that the high standard deviation makes microsatellite markers less accurate for estimating inbreeding in an inbred line of Japanese quail. Baumung and Sőlkner (2003) concluded from their simulated study that good pedigree information, traceable to the base population, would be a good measure of autozygosity. On the other hand, microsatellite information, without multi-generation genotyping, tends to over-estimate heterozygosity and provides a lower than true estimate of inbreeding coefficient. Length variation of sequences flanking the microsatellites (Angers and Bernatchez, 1997; Grimaldi and Crouau-Roy, 1997; Schug *et al.,* 1998) would cause individuals with identical microsatellite repeats to show different length alleles because of the

difference of the flanking region. Such flanking region can also generate "homoplasy", where individuals with different microsatellite repeats can show the same alleles because of the flanking region. Therefore, heterozygosity of microsatellite markers can underestimate inbreeding. Our empirical data seem to support the simulation carried out by Baumung and Sölkner (2003).

In many situations, especially when working with wild populations, good pedigree information is not available. Using poor pedigree that includes gaps and false parentages appears to have many weaknesses and may not be a good measure of autozygosity (Roughsedge *et al.,* 2001; Baumung and Sölkner, 2003). However, Baumung and Sölkner (2003) argued that low quality pedigrees were still better indicator of autozygosity than microsatellite analysis. Never-the-less, in situations where no pedigree information is available, microsatellite analysis would still provide a comparable measure of autozygosity.

In conclusion, we have (1) demonstrated that our less intensive inbreeding scheme is effective in developing inbred Japanese quail inbred line, and (2) provided empirical data to support the notion that whenever pedigree information is available, it would provide a better estimate of inbreeding coefficient than a single generation microsatellite information.

Table 2.1. List of 14 Japanese quail microsatellite loci used for final analysis and their size range and annealing temperature described by Kayang *et al.* (2002).

| Locus name[a] | GenBank accession number[b] | Repeat array[c] | Forward primer (5'-3') | Reverse primer (5'-3') | Size range (bp)[d] | $T_A$ |
|---|---|---|---|---|---|---|
| GUJ0001 | AB035652 | (CA)7TG(CA)13 | GAAGCGAAAGCCGAGCCA | CAGCACTTCGGAGCACAGGA | 231-239 | 56 |
| GUJ0024 | AB035834 | (CA)13AA(CA)3 | TCACACCTTCGGGCTGATCT | ATGCGACGGGGTGCCTTAAA | 162-174 | 55 |
| GUJ0030 | AB035840 | (CA)31 | TGCACCAATCCCAGCTGTTT | AACGCACAATGGAAAGTGGG | 167-179 | 64 |
| GUJ0034 | AB035844 | (CA)9CG(CA)2 | CGTAACGGTCCAATATGGAT | TCCACGATGCAGAGGTATTT | 219-241 | 55 |
| GUJ0040 | AB035850 | (CA)12 | GTTGAAGCTCCCATCCCTCC | ACACCCCCACGGTCTTTGCA | 176-192 | 55 |
| GUJ0044 | AB035854 | (CA)16 | GCCTTGAAACCTGAGTGATC | TGCATTTCAGCAGCTCTCAG | 180-220 | 55 |
| GUJ0057 | AB063125 | (CA)12 | GGAATGGAAAATATGAGAGC | CAGGTGTTAAAGTCCAATGT | 132-154 | 62 |
| GUJ0059 | AB063127 | (CA)10 | GACAAAGTTACAGCTAGGAG | TAGGTGCGAAAATCTCTGAC | 207-219 | 50 |
| GUJ0060 | AB063128 | (CA)9 | ATGCTATGGGAACCTCACTC | TATAAAGCAGGGGGACATGG | 132-168 | 60 |
| GUJ0065 | AB063133 | (CA)13 | GCGTGCCATTTACTTCCCGG | AGCCAGGATGACCAGGAAGG | 109-131 | 55 |
| GUJ0068 | AB063136 | (CA)13 | TAGGAGAGGTCACGATTTGC | ATCTTAACTCGCCCAGCCTT | 204-216 | 54 |
| GUJ0070 | AB063138 | (CA)9 | AAACCCCAAAGAAGCTGTCC | ACGTTGTCACCATCAGCTTG | 196-206 | 54 |
| GUJ0071 | AB063139 | (CA)8 | AGATCCTGCTCCTGGAATTG | CAGCTGCACTTAATACAGGC | 160-178 | 54 |
| GUJ0085 | AB063153 | (GT)14 | ACAACCACTTCTCCAGCTAC | GCTTGTGCTGCTGTTGCTAA | 245-265 | 55 |

These 14 microsatellite markers are from 100 microsatellite markers Kayang et al. (2002) published.

[a] GUJ stands for Gifu University Japanese quail.

[b] Genbank accession number

[c] Repeat array at their 14 microsatellitemarkers as reported by Kayang et al. (2002).

[b & c] Size range (bp) and $T_A$: annealing temperature of 14 microsatellite markers reported by Kayang et al. (2002).

Table 2.2. Profiles of 14 Japanese quail microsatellite markers amplified in the random bred line (QO).

| Locus name[a] | Allele size (bp)[b] | $T_A$[c] | N[d] | $A_O$[e] | $H_O$[f] | $H_E$[g] | $F_{IS}$[h] |
|---|---|---|---|---|---|---|---|
| GUJ0001 | 230, 234 | 58 | 39 | 2 | 0.026 | 0.026 | 0.000 |
| GUJ0024 | 162, 164 | 58 | 39 | 2 | 0.000 | 0.144 | 1.000 |
| GUJ0030 | 171, 173, 175, 177, 179, 181 | 64 | 40 | 6 | 0.425 | 0.692 | 0.389 |
| GUJ0034 | 217, 233, 237, 239 | 55 | 40 | 4 | 0.450 | 0.683 | 0.344 |
| GUJ0040 | 178, 182 | 55 | 31 | 2 | 0.419 | 0.455 | 0.080 |
| GUJ0044 | 188, 198, 216 | 57 | 28 | 3 | 0.536 | 0.649 | 0.177 |
| GUJ0057 | 138, 144, 146, 152 | 64 | 24 | 4 | 0.667 | 0.696 | 0.043 |
| GUJ0059 | 211, 215, 217, 235 | 50 | 39 | 4 | 0.462 | 0.548 | 0.159 |
| GUJ0060 | 246, 252, 258 | 60 | 38 | 3 | 0.737 | 0.590 | -0.253 |
| GUJ0065 | 118, 122, 134 | 53 | 32 | 3 | 0.688 | 0.589 | -0.170 |
| GUJ0068 | 208, 212, 218 | 58 | 25 | 3 | 0.440 | 0.489 | 0.102 |
| GUJ0070 | 196, 202, 204, 206 | 56 | 36 | 4 | 0.639 | 0.543 | -0.179 |
| GUJ0071 | 165, 167, 175, 181 | 54 | 37 | 4 | 0.486 | 0.490 | 0.007 |
| GUJ0085 | 257, 263 | 58 | 40 | 2 | 0.050 | 0.049 | -0.013 |

$T_A$: annealing temperature; N: the number of individuals amplified; $A_O$: the number of observed alleles; $H_O$: observed heterozygosity; $H_E$: expected heterozygosity; $F_{IS}$: inbreeding coefficient.

[a] GUJ stands for Gifu University Japanese quail.

[b] product size according to LiCor 4200 (LiCor Inc., Lincoln, NEB) and RFLPscan (LiCor Inc., Lincoln, NEB).

[c] annealing temperature that showed amplification for each locus in the both lines.

[d] the number of individuals that showed amplification for each locus in QO line.

[e] the number of observed alleles for each locus in QO line.

[f] observed heterozygosity for each locus in QO line.

[g] expected heterozygosity for each locus in QO line.

[h] inbreeding coefficient for each locus in QO line.

[d, e, f, g, & h] calculated by GDA (v1.0: Lewis and Zaykin, 2001)

Table 2.3. Profiles of 14 Japanese quail microsatellite markers amplified in the inbred line.

| Locus Name[a] | Allele size (bp)[b] | N[c] | $A_O$[d] | $H_O$[e] | $H_E$[f] | $F_{IS}$[g] |
|---|---|---|---|---|---|---|
| GUJ0001 | 230 | 13 | 1 | 0.000 | 0.000 | 0.000 |
| GUJ0024 | 162 | 31 | 1 | 0.000 | 0.000 | 0.000 |
| GUJ0030 | 171, 173, 175, 177 | 31 | 4 | 0.484 | 0.515 | 0.061 |
| GUJ0034 | 239 | 31 | 1 | 0.000 | 0.000 | 0.000 |
| GUJ0040 | 178, 182 | 28 | 2 | 0.250 | 0.363 | 0.315 |
| GUJ0044 | 188, 198, 216 | 25 | 3 | 0.320 | 0.523 | 0.393 |
| GUJ0057 | 146, 152 | 14 | 2 | 0.357 | 0.516 | 0.316 |
| GUJ0059 | 211 | 31 | 1 | 0.000 | 0.000 | 0.000 |
| GUJ0060 | 246, 252, 258 | 31 | 3 | 0.581 | 0.544 | -0.068 |
| GUJ0065 | 118, 122 | 27 | 2 | 0.370 | 0.352 | -0.053 |
| GUJ0068 | 212, 242 | 9 | 2 | 0.111 | 0.111 | 0.000 |
| GUJ0070 | 202, 204 | 9 | 2 | 0.111 | 0.111 | 0.000 |
| GUJ0071 | 165, 181 | 26 | 2 | 0.308 | 0.317 | 0.029 |
| GUJ0085 | 257 | 31 | 1 | 0.000 | 0.000 | 0.000 |

N: the number of individuals amplified; $A_O$: the number of observed alleles; $H_O$: observed heterozygosity; $H_E$: expected heterozygosity; $F_{IS}$: inbreeding coefficient.

[a] GUJ stands for Gifu University Japanese quail.

[b] product size according to LiCor 4200 (LiCor Inc., Lincoln, NEB) and RFLPscan (LiCor Inc., Lincoln, NEB).

[c] the number of individuals that showed amplification for each locus in the inbred line.

[d] the number of observed alleles for each locus in the inbred line.

[e] observed heterozygosity for each locus in the inbred line.

[f] expected heterozygosity for each locus in the inbred line.

[g] inbreeding coefficient for each locus in the inbred line.

[c, d, e, f, & g] calculated by GDA (v1.0: Lewis and Zaykin, 2001).

Table 2.4. The summary of the analysis done by GDA (v1.0: Lewis and Zaykin, 2001) of the 14 Japanese quail microsatellite markers amplified in the random bred line and in the inbred line.

| Population[a] | N[b] | $A_O$[c] | $H_O$[d] | $H_E$[e] | $F_{IS}$[f] |
|---|---|---|---|---|---|
| Randombred | 34.86 | 3.29 | 0.430 | 0.475 | 0.095 |
| Inbred | 23.93 | 1.93 | 0.207 | 0.239 | 0.140 |

[a] The random bred line is QO and the inbred line was derived from QO and maintained inbred for 17 generations.

[b] N: average number of individuals amplified for each line.

[c] $A_O$: average number of observed alleles for each line.

[d] $H_O$: average observed heterozygosity calculated by GDA for each line.

[e] $H_E$: average expected heterosygosity calculated by GDA for each line.

[f] $F_{IS}$: inbreeding coefficient calculated by GDA for each line.

Table 2.5. $F_{IT}$ of 2 individuals in the last generation in the pedigree of the inbred line calculated by ENDOG (v3.0: Gutiérrez and Goyache, 2005).

| Individual[a] | $F_{IT}$[b] |
|---|---|
| 44614-1 | 0.746 |
| 44614-2 | 0.746 |
| 44228-1 | 0.644 |
| 44228-2 | 0.613 |
| Mean[c] | 0.687 (± 0.069) |

[a] 2 individuals chosen in the last generation in the pedigree of the inbred line and two different pedigrees per each individual chosen to calculate $F_{IT}$ by ENDOG.

[b] $F_{IT}$ per each pedigree of each individual calculated by ENDOG.

[c] The mean $F_{IT}$ of the 4 $F_{IT}$ values.

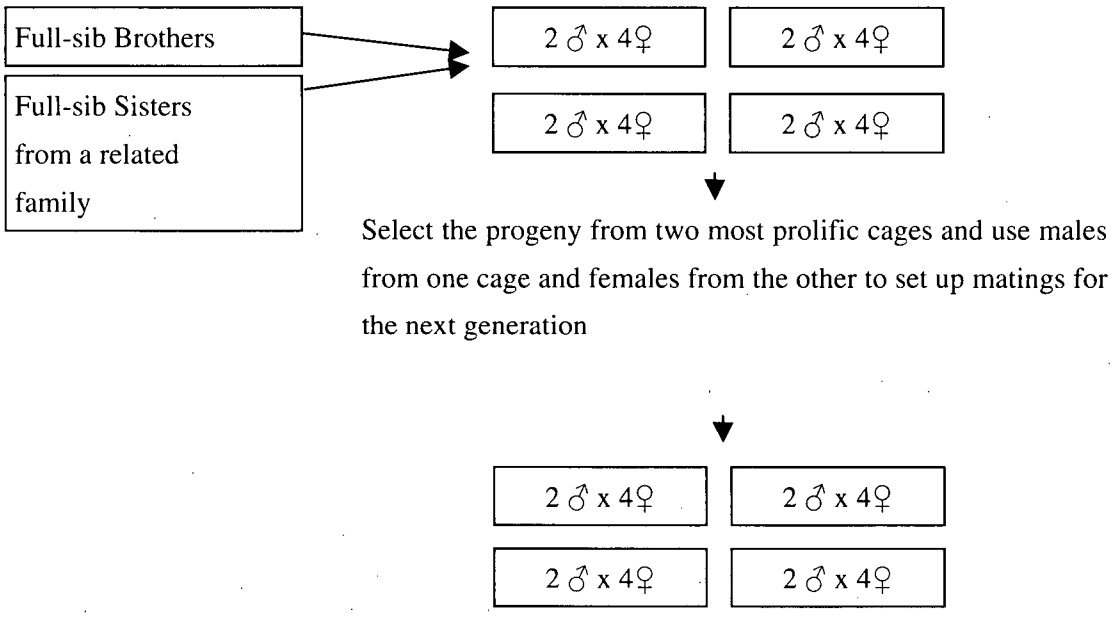



Figure 2.1. The less intensive inbreeding scheme used at the Quail Genetic Resource Centre (QSRC) of the University of British Columbia.

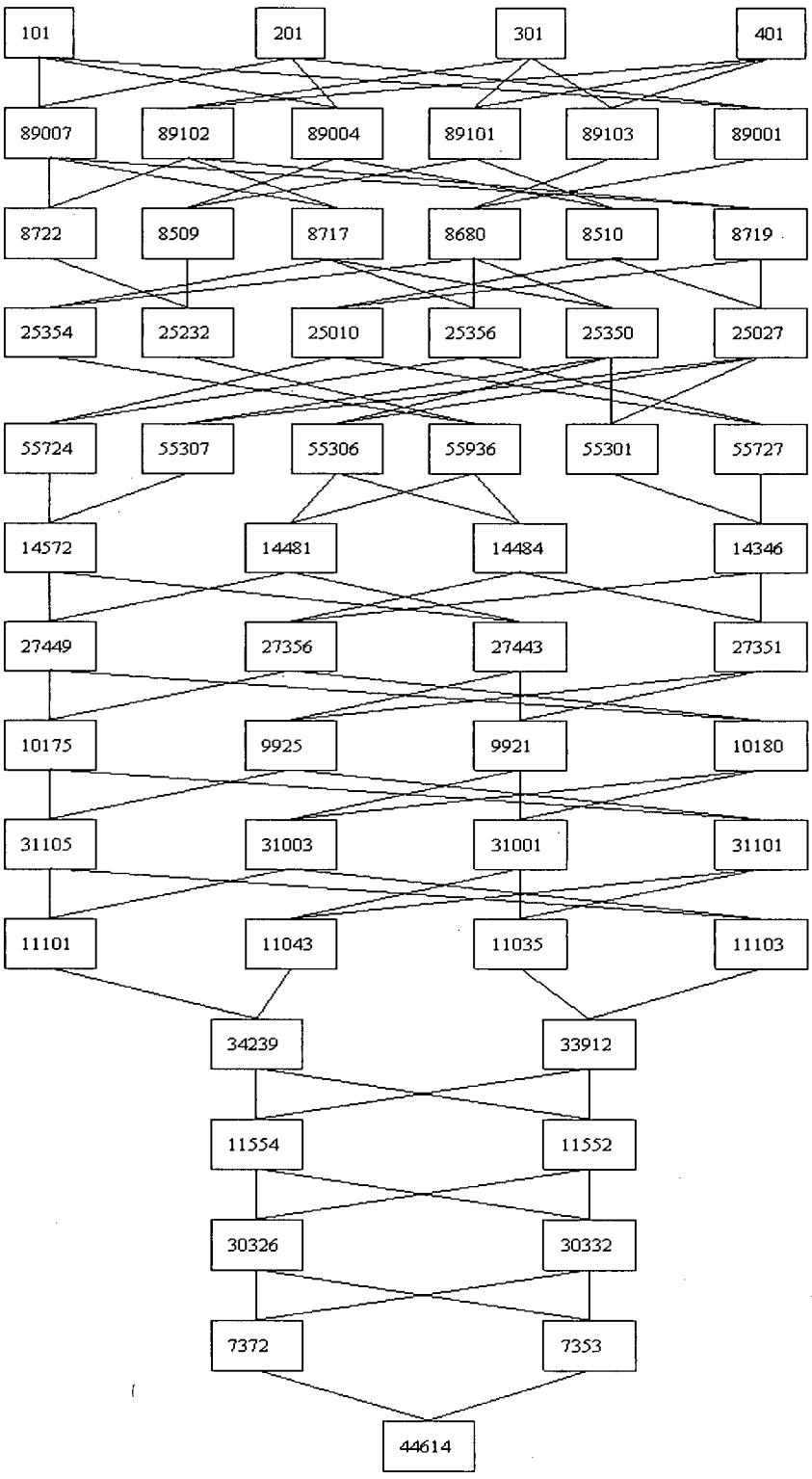
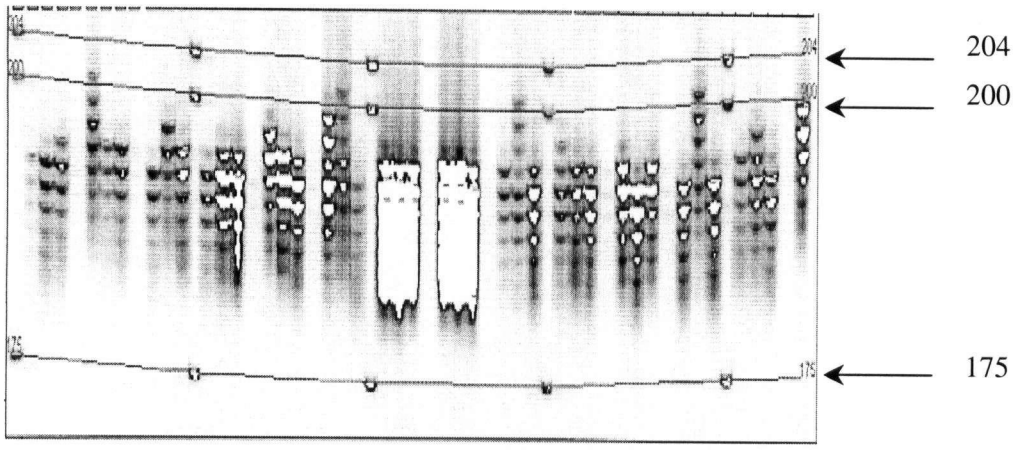



Figure 2.2. The pedigree of 44614-1 in Table 2.5.

Figure 2.3-2.5. Examples of polyacrylamide gel showing PCR products. Each lane represents a sample and the empty lanes either are non-emplified products or were unused for the analyses.

**A**




**B**

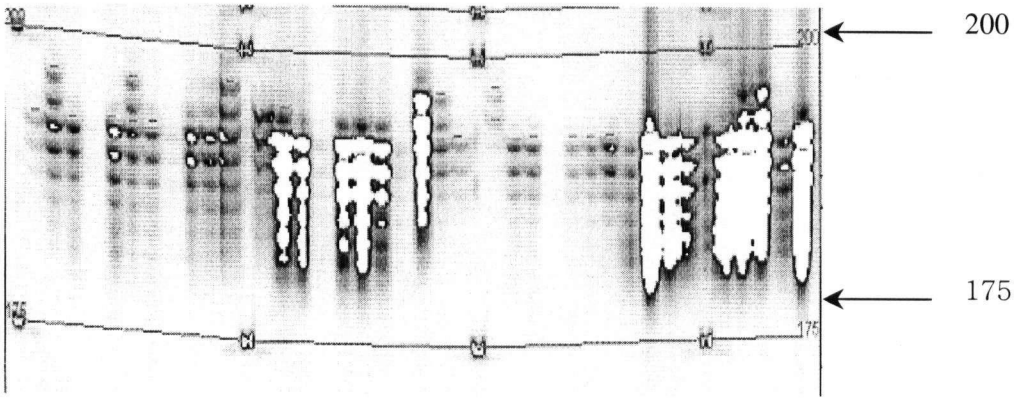
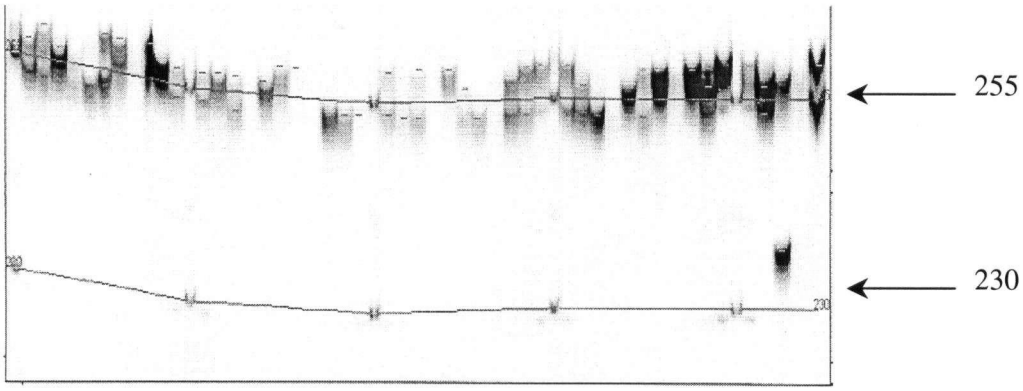



Figure 2.3. Polyacrylamide gels, analyzed by RFLPscan (LiCor Inc., Lincoln, NEB), showing PCR products for the most polymorphic locus, GUJ0030, in both lines of Japanese quail.

A. GUJ0030 in the randombred line

B. GUJ0030 in the inbred line

**C**




**D**

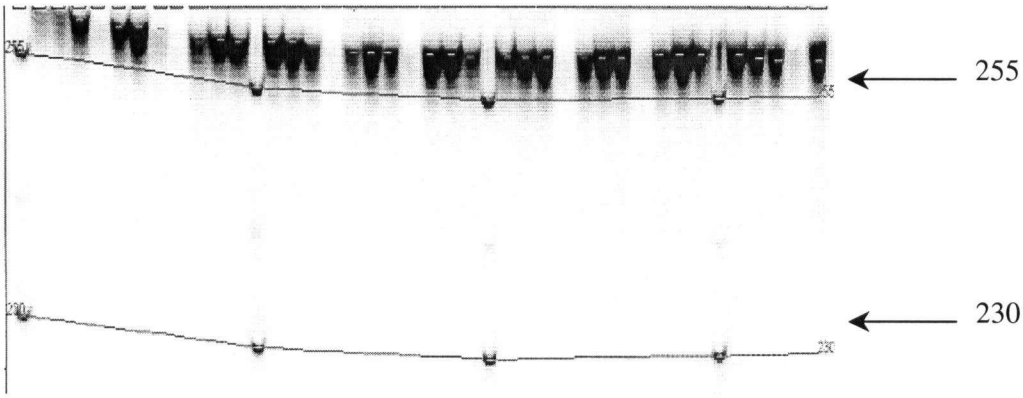
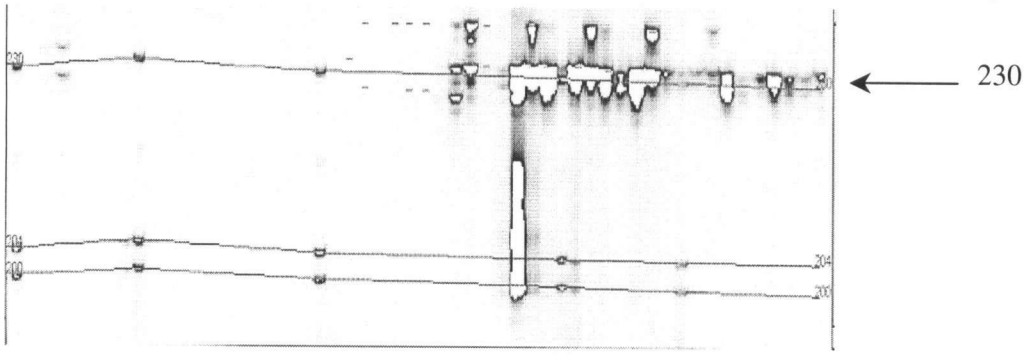



Figure 2.4. Polyacrylamide gels showing PCR products for GUJ0034, which was polymorphic in the randombred line and monomorphic in the inbred line.

C. GUJ0034 in the randombred line

D. GUJ0034 in the inbred line

**E**




**F**

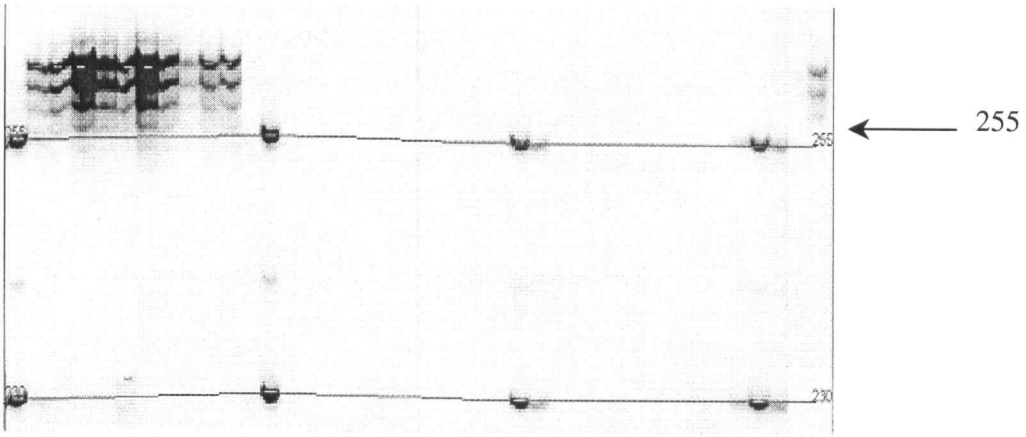



Figure 2.5. Polyacrylamide gels showing PCR products for GUJ0068 having many non-emplified products.

E. GUJ0068 in the randombred line

F. GUJ0068 in the inbred line

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CHAPTER 3

# Conclusion And General Discussion

## 3.1 Amplification of Markers

In this research, 24 Japanese quail specific primer sets were chosen from 100 markers Kayang *et al.* (2002) reported, but 3 of GUJ0079, GUJ0080, and GUJ0082 did not show any PCR product. Besides, GUJ0029, GUJ0067, GUJ0074, GUJ0076, GUJ0089, and GUJ0097 were excluded because they showed non-specific or unscorable bands for so many individuals, or they had too much contamination between individuals during LiCor 4200 (LiCor Inc., Lincoln, NEB) analysis. Moreover, GUJ0047 was monomorphic for both lines, and was not considered further. As a result, for calculation of $F_{IT}$, 14 of them, GUJ0001, GUJ0024, GUJ0030, GUJ0034, GUJ0040, GUJ0044, GUJ0057, GUJ0059, GUJ0060, GUJ0065, GUJ0068, GUJ0070, GUJ0071, and GUJ0085, were finally analyzed. Even though a total of 71 quail including 40 from the random bred line and 31 from the inbred line were sampled, not all the samples were used for the final analysis for many reasons: drop-outs or amplification failure and LiCor 4200 (LiCor Inc., Lincoln, NEB) gel conditions or contaminations between individuals, and the number of individuals for each locus is shown in Table 2.2 and 2.3. Therefore, the data from this research does not represent all 71 individuals,

and it must be important to take care of LiCor 4200 (LiCor Inc., Lincoln, NEB) gel condition and minimize contamination during LiCor 4200 (LiCor Inc., Lincoln, NEB) analyses to be cost effective.

## 3.2 The Genetic Diversity and The Level of Inbreeding in The Lines

From the calculations of the number of alleles, the mean observed and expected heterozygosity, and $F_{IS}$ for both lines, it was found that the inbred line was more inbred than the randombred line, and the randombred line seemed to have a lower level of diversity than wild populations of Japanese quail. $F_{IT}$ calculated from the pedigree of the inbred line, 0.687 (± 0.069) on average and it was estimated 0.567 (± 0.329) from the 14 microsatellite loci analysis, which shows that the high standard deviation of microsatellite markers makes themselves less reliable for calculating inbreeding in the inbred line of Japanese quail, compared to pedigree analysis. Therefore, unless multi-genotyping is available with microsatellite markers, pedigree analysis will be more accurate for estimating inbreeding in an inbred line of Japanese quail. However, the result shows that microsatellite marker analyses can be another option for investigating the level of inbreeding in an inbred line of Japanese quail, when pedigree is not available. Moreover, the results show that the less

intensive inbreeding scheme at the Quail Genetic Resource Centre (QSRC) at the University of British Columbia seems efficient for generating an inbred line of Japanese quail because $F_{IT}$ of $5^{th}$ generation through full-sib is calculated as 0.672.

## 3.3 Comments on Future Study

It was shown that the less intensive inbreeding scheme at the QSRC was efficient for generating an inbred line of Japanese quail. However, achieving higher level of inbreeding with this inbred line using the less intensive inbreeding scheme is remained to be seen. This is because up to $8^{th}$ generation of Japanese quail through full-sib mating has been generated and $F_{IT}$ for $6^{th}$, $7^{th}$, and $8^{th}$ generation through full-sib mating is calculated as 0.734, 0.785, and 0.826, respectively.

Next, even though we found that microsatellite marker and pedigree analyses were comparable methods for estimating the level of inbreeding in an inbred line of Japanese quail, because of the high standard deviation of the microsatellites, more inclusive studies will be required. It is suspected that purging by natural selection can account for the that thanks to the lack of neutrality of microsatellites that DNA segment are affecting to gene(s) expression directly or indirectly, or are linked to other functional gene(s) (DiFiglia *et al.*, 1997; Hagerman and Hagerman, 2004; Moody *et al.*, 2005; Mooers *et al.*, 2005; Bauer *et al.*,

2005; Vanita *et al.,* 2006). Huntinton disease is an example, where large CAG repeat expansion causes neurodegeneration disease (DiFiglia et al., 1997). Another interesting research published by Elizabeth *et al.* (2005) is a report that difference of microsatellite repeats to 5' region of the prairie vole vasopressin 1a receptor (avpr1a) gene contributes to the change of socio-behavioral traits. Besides, microsatellite mutation can contribute to the wrong interpretation of microsatellite analysis thanks to homoplasy caused by the variation of flanking site of the microsatellite, which can lead to underestimating inbreeding.

The high standard deviation can be caused by these factors or it is possible that there are other unknown reasons. Therefore, future studies should be more comprehensive to be able to reduce any bias, to understand more about microsatellite markers such as microsatellite mutation or neutrality, and to be able to generalize that microsatellite marker and pedigree analyses are comparable methods to estimate inbreeding in an inbred line of Japanese quail.

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## APPENDICES

Appendix 1. 24 Japanese quail microsatellite markers (Kayang *et al.*, 2002).

| Locus name | Repeat array | (5'-3') Reverse primer | (5'-3') Size | range (bp) | $T_A$ | $N_O$ | $N_E$ | $H_O$ | $H_E$ |
|---|---|---|---|---|---|---|---|---|---|
| GUJ0001 | (CA)7TG(CA)13 | GAAGCGAAAGCCGAGCCA | CAGCACTTCGGAGCACAGGA | 231-239 | 56 | 4 | 3.3 | 0.70 | 0.70 |
| GUJ0024 | (CA)13AA(CA)3 | TCACACCTTCGGGCTGATCT | ATGCGACGGGGTGCCTTAAA | 162-174 | 55 | 6 | 4.3 | 0.80 | 0.77 |
| GUJ0029 | (CA)11CT(CA)2 | GAGCATTTCTAGTCTGTCTC | ATACACAGGCTAAGGAAACC | 140-152 | 55 | 5 | 2.9 | 0.80 | 0.66 |
| GUJ0030 | (CA)31 | TGCACCAATCCCAGCTGTTT | AACGCACAATGGAAAGTGGG | 167-179 | 64 | 5 | 4.2 | 0.35 | 0.76 |
| GUJ0034 | (CA)9CG(CA)2 | CGTAACGGTCCAATATGGAT | TCCACGATGCAGAGGTATTT | 219-241 | 55 | 5 | 4.2 | 0.60 | 0.76 |
| GUJ0040 | (CA)12 | GTTGAAGCTCCCATCCCTCC | ACACCCCCACGGTCTTTGCA | 176-192 | 55 | 4 | 2.3 | 0.20 | 0.56 |
| GUJ0044 | (CA)16 | GCCTTGAAACCTGAGTGATC | TGCATTTCAGCAGCTCTCAG | 180-220 | 55 | 5 | 3.5 | 0.75 | 0.72 |
| GUJ0047 | (CA)23 | GAGATAAGACTGGCTGGGGC | TCACCGTGGCTGGCCAACTT | 262-292 | 55 | 5 | 2.4 | 0.55 | 0.59 |
| GUJ0057 | (CA)12 | GGAATGGAAAATATGAGAGC | CAGGTGTTAAAGTCCAATGT | 132-154 | 62 | 5 | 2.4 | 0.65 | 0.59 |
| GUJ0059 | (CA)10 | GACAAAGTTACAGCTAGGAG | TAGGTGCGAAAATCTCTGAC | 207-219 | 50 | 5 | 3.4 | 0.85 | 0.71 |
| GUJ0060 | (CA)9 | ATGCTATGGGAACCTCACTC | TATAAAGCAGGGGGACATGG | 132-168 | 60 | 5 | 1.6 | 0.40 | 0.38 |
| GUJ0065 | (CA)13 | GCGTGCCATTTACTTCCCGG | AGCCAGGATGACCAGGAAGG | 109-131 | 55 | 5 | 2.3 | 0.55 | 0.57 |
| GUJ0067 | (CA)14 | ACGTACGAGCTCAACATTTG | GCGTGCATAAAGGCAACTTA | 121-131 | 55 | 5 | 2.8 | 0.85 | 0.65 |
| GUJ0068 | (CA)13 | TAGGAGAGGTCACGATTTGC | ATCTTAACTCGCCCAGCCTT | 204-216 | 54 | 5 | 3.6 | 0.60 | 0.72 |
| GUJ0070 | (CA)9 | AAACCCCAAAGAAGCTGTCC | ACGTTGTCACCATCAGCTTG | 196-206 | 54 | 6 | 4.3 | 0.62 | 0.77 |
| GUJ0071 | (CA)8 | AGATCCTGCTCCTGGAATTG | CAGCTGCACTTAATACAGGC | 160-178 | 54 | 6 | 2.0 | 0.30 | 0.49 |
| GUJ0074 | (CA)10 | GTTGTCCTGGCTGAGATGGC | GGGTTTGAGGGCTTGGGGTT | 290-298 | 59 | 3 | 2.2 | 0.60 | 0.54 |
| GUJ0076 | (CA)4AA(CA)9 | GTATCAGTGCATGCTCGTCC | TCGAGGACTGGCTGGAAAAT | 208-230 | 57 | 5 | 2.3 | 0.80 | 0.57 |
| GUJ0079 | (CA)12 | GAAAGATAAGCATGAGTGAC | GTTTTGGCATTCACTTCAGA | 121-135 | 55 | 6 | 3.0 | 0.65 | 0.67 |
| GUJ0080 | (CA)9 | TTGAAGGGACATAGGGAAGC | GAAAACGGTGAAGTCTGGTG | 151-167 | 54 | 6 | 4.2 | 0.35 | 0.76 |
| GUJ0082 | (CA)9 | CTTGGAACACACGGGATGGC | TTACCCCTCTTTTCCCCCCG | 142-156 | 59 | 5 | 2.7 | 0.30 | 0.63 |
| GUJ0085 | (GT)14 | ACAACCACTTCTCCAGCTAC | GCTTGTGCTGCTGTTGCTAA | 245-265 | 55 | 5 | 2.4 | 0.65 | 0.59 |
| GUJ0089 | (CA)12 | CCAGTTTAAGCACCAGCATC | TGGCAAGTAGTCGTGGAAGA | 131-145 | 55 | 5 | 2.5 | 0.79 | 0.60 |
| GUJ0097 | (CA)14 | GGATGCTCAGTGTGGAAAAG | GAGCAAGAGGTGAGTGTTTC | 131-157 | 55 | 5 | 3.6 | 0.40 | 0.72 |

# The locus code GUJ stands for Gifu University Japanese quail.

$T_A$: annealing temperature

$N_O$: observed number of alleles

$N_E$: effective number of alleles

$H_O$ & $H_E$: observed and expected heterozygosity

Appendix 2. PCR optimization

PCR reactions were performed to seek for the optimal conditions for each primer set using 2% agarose gel. 2 % agarose gel analysis was tested at 100V for 30-45 minutes in the mini electrophoresis boxes (COSMO BIO Co., LTD.). If the agarose gel did not show any product or many non-specific bands were observed, PCR reactions were repeated with the annealing temperature difference of ±2~3℃ until it showed any significant specific bands. The PCR reactions were performed using the following protocol. 1 μl of 20 ng/μl Japanese quail genomic DNA was added into the cocktail of 1.5mM of $MgCl_2$, 2 μl of 2mM dNTP's, 2μl of each of 10pmol/μl both forward and reverse primers, and 10.4 μl of dH2O, and 0.6 μl of 5 U/μl Taq DNA Polymerase. The PCR reactions was started at 94℃ for the first denaturation for 5 minutes, and then 30-40 cycles of each of denaturation at 94℃ for 1 minutes, annealing at optimized temperature for 45-60 seconds, and extention at 72℃ for 1 minutes, and followed by 72℃ for 5-10 minutes.

Appendix 3. Polyacrylamide gel production

7% of 25Cm Polyacrylamide gels for a LiCor 4200 were made with the following protocol. After mixing 12.6g of Urea (USB™, Ohio, USA), 4.2ml of Long Ranger® (FMC®, Maine USA), and 7.2ml of 5X TBE (Trisbase, Boric acid, and EDTA, with $dH_2O$), $dH_2O$ was added up to total volume of 30ml with 200 µl of 10% APS. And then, 10-20 µl of TEMED (Sigma Chemical Co., MO USA) was added after the solution was filtered.

Appendix 4. For GUJ0068, allele size of 242 observed only in the inbred line.

For GUJ0068, one allele, 242, was observed in the inbred line, which was not found in the random line (Figure 2.5). There are several possible reasons for this observation. First possibility is PCR error, where it is possible that the PCR products for the random bred line did not amplify the allele because of "false homozygosity" (Miller *et al.,* 2002). The quality of research using microsatellite markers with PCR depends on several factors, which are DNA contamination, amplification failure, and allele drop-out (Miller *et al.,* 2002; Piyamongkol *et al.,* 2003). Miller *et al.* (2002) mentioned that polymerase error causes amplification failure and stochastic sampling error might be the cause of allele drop-out, which means when DNA is pipetted into PCR mixture and the concentration of DNA is very low, by accident, one copy may be copied more than the other, and this situation even causes "false homozygote". It has been shown that using stochastic simulation, low DNA concentration accounts for more than 50 % of the rate of drop-out (Taberlet *et al.,* 1996). Yang *et al.* (2005) even argued that because of amplification process of PCR, tiny amounts of artifacts or contaminants can affect to the results. Many other studies have suspected about the accuracy of PCR (Wilhelm *et al.*, 2000; Borst *et al.*, 2004; Yang *et al.*, 2005) as well. Another possibility is that several individuals which were dropped out in the random bred

line could actually have included the allele, 242. The last and most probable possibility is microsatellite mutation, because this allele was not observed in the research (Kayang *et al.*, 2002) either, in which the range was 204-216.

Appendix 5. Annealing temperature

The range of annealing temperature for 14 markers was from 50℃ to 64℃. Only 6 primer sets, GUJ0030, GUJ0034, GUJ0040, GUJ0059, GUJ0060, and GUJ0071 were amplified at the suggested annealing temperatures in this experiment. The annealing temperatures for other 8 primer sets had to be adjusted.

One possible reason for the difference between the annealing temperatures for these 8 markers in this study and those of the study of Kayang *et al.* (2002) could be mutations in the flanking regions of the microsatellite markers (Eggleston-Stott *et al.,* 1997; Achmann *et al.,* 2001). The studies (Eggleston-Stott *et al.,* 1997; Achmann *et al.,* 2001) commented that the mutation to the flanking site of a particular locus can actually cause null allele, which will appear to be homozygote even if it is heterozygote and reducing the annealing temperature for the mutated allele in Lipizzan horses increased the amplification and found the locus thought to be homozygote to be actually heterozygote.

The concentration of $MgCl_2$ can affect the amplification as well (Pang *et al.,* 1999; Kayang *et al.,* 2000, Kayang *et al.,* 2002). However, the concentration of $MgCl_2$ used in the research of Kayang *et al.* (2002) was 1.5 mM, which is the same as in this research. Most of

the size ranges for the 14 primer sets from the LiCor 4200 analyses were within those published by Kayang *et al.* (2002) or were not very different from them (Table 2.2, Table 2.4 and Table 2.5).

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