JAPANESE QUAIL MICROSATELLITE LOCI AMPLIFIED BY CHICKEN-SPECIFIC PRIMER PAIRS

by

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ABSTRACT

Japanese quail (*Coturnix japonica*) is an important food species as well as a popular animal model for research. Yet, despite their importance, very little is known about their genome. Only allozyme markers have been used to determine the genetic variation of Japanese quail populations and the extent of the protein polymorphism seemed to be relatively low. The use of more variable DNA markers would provide a better understanding of the population structure of these birds. Recently, some scholars suggested that microsatellite markers could detect much higher variation in populations where the detectable allozyme variability is low.

Microsatellite loci are often conserved among related species and successful cross-species amplification using heterologous microsatellite primers have been shown in many closely related species of mammals and birds. Microsatellite markers have not been developed for the Japanese quail. The purpose of this study was to examine whether polymorphic microsatellite primers from chicken could amplify corresponding microsatellite loci in Japanese quail DNA templates. Forty-eight chicken microsatellite primer-pairs were tested on Japanese quail genomic DNA using polymerase chain reaction (PCR). Post-PCR DNA fragments were first analyzed on 2% agarose gels and then on Spreadex™ gels. Amplification products were obtained from 28 chicken microsatellite primer-pairs (58.3%) after optimizing the PCR condition for each primer set. Of the 28 primer-pairs that gave PCR products, 12 (25.0%) generated specific products and 16 yielded non-specific amplification products. Specific amplification products from each of the 12 PCR primers were sequenced. Nine of these markers were polymorphic and 3 were monomorphic from 4

Japanese quail populations. Seven of the 9 polymorphic and one of the 3 monomorphic markers contained microsatellite repeats. Three microsatellite loci were confirmed to be homologous to their corresponding loci in chicken.

The results indicate the possible use of chicken-specific microsatellite primers in the analysis of Japanese quail genome. Furthermore, results from cross-species characterizations are also presented in the hope of stimulating interest in the use of these markers in various genetic studies.

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LIST OF ABBREVIATIONS

ABI	Applied Biosystems
ADL	USDA ARS Avian Disease and Oncology Lab
BLAST	Basic Local Alignment Search Toll
EtOH	ethanol
HUJ	Hebrew University of Jerusalem
IAM	infinite alleles model
JAP	Japan
MCW	Microsatellite Chicken Wageningen
Mg^{++}	magnesium ion (divalent)
mtDNA	mitochondrial DNA
NAGRP	National Animal Genome Research Program
NAPs Unit	Nucleic Acid-Protein Service Unit Laboratory
NCW	North Carolina wild type
PCR	polymerase chain reaction
QGRC	Quail Genetic Resource Center
RAPDs	randomly amplified polymorphic DNAs
RFLP	restriction fragment length polymorphism
SMM	stepwise mutation model
SSRs	short simple sequence repeats
STRs	short tandem repeats
VNTRs	variable number tandem repeats

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SECTION 1

INTRODUCTION AND OVERVIEW

1.1 The Japanese Quail

1.1.1 Classification

The Japanese quail (*Coturnix coturnix japonica*) was previously classified as a subspecies of the common quail (also known as the European or migratory quail) (*C. coturnix*) based mostly on examination of museum specimens (Taka-Tsukasa, 1935). However, due to non-hybridization of the 2 taxa both in the wild and in captivity (Lepori, 1964; Pala and Lissia-Frau, 1966), the Japanese quail has now been classified as a distinct species, *C. japonica* (American Ornithologists' Union, 1983; Howard and Moore, 1984). However, 'coturnix' is still often used as the common name for both the European and the Japanese quail. It has been reported that the coturnix quail have the widest natural distribution of any galliform birds owing to their ability to adapt to a wide range of ecological conditions (Cheng *et al.*, 1992). However, due to the loss of breeding habitat, both species have suffered extensive population reduction in the last 3 decades (Kimura, 1991).

1.1.2 Domestication and early history

Many scholars believe that Japanese quail were domesticated in Japan, China, or Korea by the 11th century (Crawford, 1990). Very little information is available concerning early history of domestication because of the lack of written records. While *C. coturnix* was

domesticated by the Romans for game purposes, there was no record of any domestic birds after the fall of the Roman Empire (Crawford, 1990). It is generally believed that *C. japonica* is the sole ancestor of all commercial Japanese quail stocks (Crawford, 1990). However, the monophyletic origin of the domestic strains cannot be confirmed and it remains possible that some laboratory strains and strains kept by aviculturists may have an origin involving *C. coturnix* (Cheng *et al.*, 1992). There is a need to seek further into its history to ascertain if in fact all commercial and most laboratory strains today are descended from *C. japonica* (Cheng *et al.*, 1992).

According to Howes (1964), the first written records of domestication in Japan date from the 12th century. Japanese quail were first kept and bred as caged song birds for centuries, especially by the Samurai (warrior) caste in Japan. However, it is believed that during World War II, most of these song quail in Japan were destroyed. Because only a few domestic quail survived after World War II, it has been speculated that some quail may have been reintroduced into Japan from Korea, China, and Taiwan to rebuild the quail industry (Howes, 1964). Recovery of the quail stocks was very rapid and by the 1950s, Japanese quail became an important food species mainly for egg production in Japan (Wakasugi, 1984; Cheng *et al.*, 1992). In the 1960s, heavy body weight strains were first developed for meat production in Germany and France, then, later in the United States using domestic strains imported from Japan (Cheng *et al.*, 1992; Wakasugi, 1984).

1.1.3 Japanese quail as a laboratory animal

Nowadays, Japanese quail are often used as animal models for biomedical research and for teaching because of their adaptability to captive rearing, small size (90-160 gm), high productivity, relative resistance to disease, accelerated growth and short generation interval. The use of the Japanese quail as a research animal has been increasing and various specialized strains have been developed for research purposes (Cheng and Nichols, 1992; Hammad *et al.*, 1995). A large volume of literature in physiological, biological, medical, pathological, virological, toxicological and agricultural studies, involving the Japanese quail as an animal model, have been published. For example, in atherosclerosis studies, the Japanese quail are often the preferred animal over rabbit and pigeon because quail are naturally deficient in apolipoprotein E and hence develop atherosclerosis rapidly when exposed to a high cholesterol diet. Furthermore, they develop lesions exhibiting structural features (in their aorta) that closely resemble those in the human disorder (Day *et al.*, 1977; Siegel *et al.*, 1995; Stocker *et al.*, 1995). In toxicological studies, Japanese quail are used as an indicator species for all avian species and the effects of toxins/chemicals, acute gamma radiation and neurotoxins on these birds have been determined (Poonacha, 1972; Hitchcock, 1980; Kaltner *et al.*, 1993; Varghese, 1995; Winsor, 1973; Chilgren, 1968).

Due to ease of manipulation, Japanese quail eggs are frequently used in studies of pathology and virology in which the effects of tested materials on embryonic development were investigated (Miyagawa-Tomita *et al.*, 1996; Kocisova, 1994; Soliman *et al.*, 1994; Kubota *et al.*, 1995; Wilson *et al.*, 1997; Ratnamohan, 1976). Japanese quail have also been used as replacement for chickens in virological research owing to their susceptibility to many

oncogenic viruses such as retroviruses and Herpes virus. According to Ratnamohan (1985), Japanese quail could be a potential important model for arbovirus research.

Since some of the laboratory strains of Japanese quail may have an origin involving *C. coturnix*, it is important to identify some molecular markers that are species-specific for *C. japonica*. This will enable researchers to clarify genetic relationships among different laboratory strains of coturnix quail and thus, to compare results from different studies.

1.1.4 Japanese quail as a food species

While the Japanese quail has been used extensively as animal models in biological and medical research, the domestic quail is also an important food species. Although Japanese quail is used widely as poultry in Asia and some parts of Europe, it has never been as popular as chicken in North America because of its small body size (Cheng *et al.*, 1990). Nevertheless, changing social trends in North America has contributed to an increase in consumption of Japanese quail in Southeastern United States and in Canada (Paulson *et al.*, 1989). This increase in consumption is probably due to a stronger preference for white meat versus red meat by the general population. Furthermore, the population of ethnic groups here in British Columbia has been increasing and a large and varied ethnic group from Middle East, Asia and Europe traditionally consume game bird dishes. All these changes would provide a strong niche market potential for quail production (Paulson *et al.*, 1989). In fact, there is an expanding market for specialty products such as fresh or pickled eggs and fresh or frozen quail carcass. However, commercial success requires thorough market and genetic research. More genetic research efforts are necessary in order to develop appropriate

management strategies and selection for domestic Japanese quail populations.

Therefore, with the increasing popularity of Japanese quail as a research animal and as a meat and egg producer, the paucity of information about the quail genome becomes obvious.

1.1.5 Genetics of Japanese quail

Many researchers have conducted genetics research not only on mutant and laboratory strains (e.g. Cheng and Kimura, 1992), but also on commercial strains of Japanese quail (e.g. Kimura and Fujii, 1989). For instance, in Henry Marks' study (Marks, 1995), both heterosis and overdominance following long-term selection for heavy body weight were estimated. In addition, heritability for different traits (e.g. Lauber *et al.*, 1989; Cheng *et al.*, 1997) have been reported. On the other hand, very little is known about the genome of these birds.

Genetic variation in Japanese quail has been studied only using allozyme electrophoresis. Enzyme and protein polymorphic loci have been used as markers to determine the genetic variability among different coturnix quail populations. Baker and Manwell (1967) reported that the coturnix quail had higher polymorphic loci and average individual heterozygozity than any other related species surveyed (see also Baker and Manwell, 1975). Lucotte and Kaminski (1975) and Kimura and Fujii (1989) examined domestic Japanese quail by allozyme electrophoresis and reported the values of 0.285 and 0.109, respectively, for the expected average heterozygozity. Genetic variability between commercial strains of Japanese quail from different origins were also compared by Cheng *et al.* (1992). According to Kimura and Fujii (1989) and Cheng *et al.* (1992), the levels of

genetic variability in the domestic quail populations seemed to be much less than that of coturnix quail (Baker and Manwell, 1967; Baker and Manwell, 1975). It should be pointed out that in both Kimura and Fujii's (1989) and Cheng *et al.*'s (1992) studies a fairly small sample size was used. In addition, domestic populations may have relatively low levels of genetic variation due to small effective population size. Clarification of the genetic variability within and among selected lines will be important to evaluate genetic changes associated with selection. While it is possible to gain some understanding of population structure using data obtained from allozyme studies, the use of more variable markers such as DNA markers could clearly facilitate such analysis. The use of DNA markers may be a more appropriate tool to determine the degree of genetic variation among individuals and populations where the use of enzyme electrophoresis could only detect low levels of variation (Lucotte and Kaminski, 1975; Kimura and Fujii, 1989; Cheng *et al.*, 1992).

1.2 DNA markers

There are many different tools/markers available for genetics studies. For example, minisatellites (multilocus and single-locus), allozyme electrophoresis, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNAs (RAPDs), microsatellites and mitochondrial DNA-RFLP (mtDNA-RFLP) (Hoelzel, 1992). In allozyme electrophoresis, a protein solution is electrophoresed through a gel and an enzyme specific reaction highlights one locus; the protein products from different alleles may migrate different distances due to charge differences. As with microsatellite loci, minisatellites also consist of tandem repeats of sequence unit, however, the variable tandem repeats within

minisatellites contain longer repeat units, sometimes, may be as large as 200 bp each. In DNA multilocus fingerprinting, minisatellite variation are revealed at multiple loci simultaneously using conventional Southern blotting techniques. When specific probes are designed to hybridize to a minisatellite sequence and identify a single locus, the technique is called single-locus minisatellites or variable number tandem repeats, VNTRs. The RFLPs are similar to DNA fingerprinting but the probe hybridizes to a single locus in which alleles differ in the presence or absence of nearby restriction sites. For RAPDs, like microsatellites, no prior knowledge of nucleotide sequences of the genome of the target species is required. Short arbitrary oligoprimers (about 10 bases long) are used randomly in PCR reactions to generate different DNA profiles. These fragments are usually non-allelic and are scored simply as present or absence of bands. Finally, mtDNA is used to trace the maternal background of a particular species and thus, is particularly useful for studies inferring phylogenetic relationship among components of a species (Estoup *et al.*, 1995).

1.3 Microsatellites

1.3.1 Characteristics of microsatellites

Microsatellites, also called simple sequence repeats (SSRs) or short tandem repeats (STRs), are tandem stretches of very short nucleotide sequence motifs (of 1-6 bases long) that frequently occur as randomly and widely dispersed repetitive elements in all eukaryotic genomes (Tautz and Rentz, 1984; Kashi *et al.*, 1990; Weber, 1990). These loci are often highly polymorphic due to variation between multiple alleles in the number of repeat units (Weber and May, 1989; Litt and Lutty 1989; Weber, 1990). It has been proven that the

number of iterations may also vary between microsatellite loci (Ellegren *et al.*, 1997). Most polymorphic microsatellite loci harbour alleles in the range of 10-30 repeat units and rarely, some loci may display alleles with their motifs repeated up to ~100 times (Tautz, 1993). It is generally believed that microsatellite loci with more than 10 repetitions of the basic motif are highly variable in taxa ranging from plants to vertebrates (Lagercrantz *et al.*, 1993). Heterozygozity of 0.8 and as many as 20 or more alleles at a locus are often observed in these studies (Bowcock *et al.*, 1994; MacHugh *et al.*, 1994; Deka *et al.*, 1995; Goldstein and Clark, 1995). In addition, most microsatellite mutations that are embedded in non-coding repetitive regions of the genome, are neutral. However, there are microsatellite mutations, such as the disease-causing trinucleotide repeats in disease loci in human that are not neutral and are being selected against (Chakraborty *et al.*, 1997).

1.3.2 History of microsatellites

Simple sequence polymers (the building blocks of microsatellite loci) were the first synthetic DNAs to be produced *in vitro* using short primers with the respective complementary motifs and a DNA polymerase (Kornberg *et al.*, 1964; Byrol *et al.*, 1965). Due to poor sensitivity of the earlier methods such as ultra-centrifugation and reassociation kinetics for genome analysis, these repetitive sequences were undetected and thus, not recognized. The status of microsatellites as repetitive elements in eukaryotic genomes were first documentated in 1982 by Hamada *et al.* when hundreds of copies of the poly (dT-dG)n

motif in evolutionary diverse eukaryotic genomes were discovered. These repetitive sequences were found by the sequencing of genomic clones and Southern blot hybridization experiments. In 1984, Tautz and Renz hybridized different microsatellite sequences to genomic DNA from a variety of organisms and confirmed that many different types of simple sequences were ubiquitous repetitive components of eukaryotic genomes. Subsequently, the broad distribution of microsatellites and their high number of simple "cryptic" repeats (repeats that did not have regular spacing) were demonstrated by Tautz (1986) when many of the simple sequences occurring in eukaryotic genome were shown to be 5- to 10-fold more frequent than equivalent-sized random motifs.

Since those early publications, microsatellites have been proven extremely useful as genetic markers in studies of gene mapping and linkage analysis (Todd *et al.*, 1991; Weissenbach *et al.*, 1992; Cheng *et al.*, 1992; Barendse *et al.*, 1994; Dietrich *et al.*, 1994), in studies of gene expression and function (Nelson and Warren, 1993) and human genetic diseases (Thibodeau *et al.*, 1993), and in evolutionary and population studies (e.g. Crouas-Roy *et al.*, 1993; MacHugh *et al.*, 1994; Estoup *et al.*, 1995; Goldstein *et al.*, 1995; Slatkin, 1995; Bowcock *et al.*, 1994). Microsatellites were also valuable in the identification of quantitative trait loci (QTL) (Berrettini *et al.*, 1994), in forensic identification (Edwards *et al.*, 1992) and in analysis of ancient samples from museum specimens (Jeffreys *et al.*, 1992; Gill *et al.*, 1994). Their high level of polymorphism, broad genomic distribution, nature of codominant inheritance and amenability to PCR technology make them potentially a powerful genetic marker available for various genetics studies.

1.3.3 Hypothesized mechanisms of microsatellite variability

Microsatellites frequently show high level of multiallelic polymorphism probably due to their high rate of mutations. For a long time, it has been assumed that variation in allele length in microsatellite loci is caused by changes in the numbers of tandem repeats at these loci (Sirugo *et al.*, 1992; Kwiatowski *et al.*, 1992; Oudet *et al.*, 1995; Weber and Wong; 1993). However, current studies suggest that differences between alleles of homologous loci in different species are not only due to change in the number of tandem repeats, but also variation in the non-repeated 5' and 3' flanking regions that flank the microsatellite repeats (Grimaldi and Crouau-Roy, 1997; Stallings, 1995; Blanquer-Maumont and Crouau-Roy, 1995; Callen *et al.*, 1993; Koorey *et al.*, 1993; Garza *et al.*, 1995). Although, the precise nature of mutations of microsatellite sequences is still unclear, there are 2 competing thoughts about the mechanisms of microsatellite variability.

The first hypothesis suggests that variation in tandem repeats are probably due to the unequal homologous recombination between misaligned arrays of repeats (Tautz and Renz, 1984; Jefferys *et al.*, 1985; Sia *et al.*, 1997). Simple repeats located on different DNA molecules pair in a misaligned configuration, resulting in arrays with reciprocal additions and deletions (Sia *et al.*, 1997; Haymer, 1994; Levinson and Gutman, 1987). Another recombination hypothesis involving a double strand break occurring within the repeated tract is also possible. This break could be followed by exonuclease degradation, hybridization of complementary single-stranded regions, and DNA synthesis and religation, leading to a reduction in repeat units. Furthermore, if the broken ends could invade an array on another

DNA molecular, it might result in an expansion in the repeated tract (Sia *et al.*, 1997).

The competing hypothesis involves intra-allelic polymerase slippage mutations during DNA replication (Levinson and Gutman, 1987; Schlotterer and Tautz, 1993; Tautz and Schlotterer *et al.*, 1994; Sia *et al.*, 1997). A transient dissociation occurs as the DNA polymerase progresses across the repeated sequence. When the 2 strands reassociate, they may form a misaligned configuration with an unpaired repeat present on either the nascent or template strand. If the mismatch is not corrected due to defective DNA repair, it will lead to either gain or loss of a small number of repeats (Levinson and Gutman, 1987; Schlotterer and Tautz, 1992; Sia *et al.*, 1997; Messier *et al.*, 1996).

Strand slippage during DNA replication has been suggested by Sia *et al.* (1997) as the most likely mechanism involved in loss or gain of a small number of repeats. It is likely that unequal recombination event or other unknown mechanism influence microsatellite instability resulting in larger reductions and/or expansions of repeats. Although random point mutations within a simple sequence could lead to changes in nucleotide composition (Tautz *et al.*, 1986), Nave *et al.* (1997) emphasized that the variability in microsatellites is most likely due to the extreme mutability of microsatellite repeat number structure and not to the relatively stable structure of a single point mutation.

Changes in composition of sequence flanking the repeats could also cause multiallelic length polymorphism observed in microsatellite loci. Several studies have suggested that sequences flanking microsatellites could be quite unstable and their variability could lead to microsatellite allelic homoplasy (Grimaldi and Crouau-Roy, 1997; Stallings, 1995; Blanquer-Maumont and Crouau -Roy, 1995; Callen *et al.*, 1993; Koorey *et al.*, 1993). The instability

of flanking sequences could be caused by association with the repeat region (Stalling, 1995). Koorey *et al.* (1993) suggested that sequence adjacent to a microsatellite would be likely to accumulate mutations due to slippage events in the microsatellites. It was found that at PCR-based microsatellite loci, a mutation which had occurred in one of the priming sites could cause one primer not to recognize and bind onto that priming region. This would lead to the formation of "null" or non-amplifying allele and generation of polymorphism within the priming site (Koorey *et al.*, 1993; Callen *et al.*, 1993). The presence of null alleles can complicate linkage studies employing polymorphism detected by the PCR by producing apparent non-Mendelian inheritance, unless one could type both parents for specific allelic length. In addition, null alleles may also have important implications for paternity testing since their apparent non-Mendelian inheritance in a pedigree may be incorrectly attributed to non-paternity or non-maternity.

1.3.4 Evolution of microsatellites

Microsatellite evolution is also poorly understood. Due to the inconsistency of the pattern of allele frequency distributions that have been observed, it is difficult to determine the mechanism of microsatellite evolution. It is unclear whether the allelic distributions are consistent with the infinite alleles model (IAM) of mutation (Kimura and Crow, 1964), or with the stepwise mutation model (SMM) (Kimura and Ohta, 1978). Both the IAM and SMM are 2 extreme models of mutations that are often considered in both mini- and micro-satellites. The IAM implies that new mutations occurring are always different from the existing alleles in the population because a mutation can involve any number of tandem

repeats (MacHugh *et al.*, 1994). In contrast, under the simple SMM, an allele mutates only by gaining or losing single repeat units. If this is true, a new mutation may have similar number of repeat arrays and may not be differentiable from an allele already present in the population (MacHugh *et al.*, 1994; Estoup *et al.*, 1995; Valdes *et al.*, 1993; Di Rienzo *et al.*, 1994; Shriver *et al.*, 1993; Tautz and Schlotteres, 1994). More recent works favored an extended SMM, in which most mutations are single-step changes, but rare large jumps in the number of repeat units (multistep SMM) also occur (Di Rienzo *et al.*, 1994; Farber *et al.*, 1994; Chung *et al.*, 1993; Zhong *et al.*, 1993; Shibata *et al.*, 1994; Wooster *et al.*, 1994). In accordance with a replication slippage mechanism of mutational change, current studies supported that microsatellite evolution favors the extended SMM (Levinson and Gutman, 1987; Ellegren *et al.*, 1997; Valdes *et al.*, 1993; Shriver *et al.*, 1993; Di Rienzo *et al.*, 1994).

1.3.5 Mutations in microsatellites

Microsatellite represent inherently unstable regions of the eukaryotic genome with mutation rates that are much greater than that reported for non-repetitive DNA sequences (Sia *et al.*, 1997). Microsatellite loci typically exhibit high variability also due to their dispersed distribution in the genome and are largely unlinked to one another (Boyce *et al.*, 1997). The rates of mutation in microsatellites are estimated to vary between 10^{-3} and 5×10^{-6} per locus per gamete in mammals (Dallas, 1992; Dietrich *et al.*, 1992; Haymer, 1994; Edwards *et al.*, 1992; Queller *et al.*, 1993), and between 10^{-2} and 10^{-5} per generation in human (Edwards *et al.*, 1992; Mahtani and Willard, 1993). Jin *et al.* (1996) demonstrated that the mutation rate among alleles within a single microsatellite locus could vary drastically, and he also

suggested that microsatellite alleles with a larger number of repeats tended to have higher mutation rates. Since most mutations in microsatellites are neutral (occur in non-coding regions), theoretically, any nucleotide changes that occur in these repetitive sequences will persist. Because of their high mutation rate, microsatellite markers may have a large number of alleles (varying in the number of repeats) which increases the likelihood of being polymorphic in any resource population (Litt and Luty, 1989; Weber and May, 1989; Tautz, 1989: Weber, 1990; Cheng and Crittenden, 1994). This high level of polymorphism and broad genomic distribution of microsatellites make microsatellites extremely useful as markers in studies for genome mapping or paternity analysis.

1.3.6 Constraints acting on microsatellite loci

Very little is known about the constraints affecting allele size at microsatellite loci. In most evolutionary studies, the dynamics under random drift and mutation have been often investigated assuming no constraints on allele size. However, the absence of alleles of very large size suggests that the number of repeats at microsatellite loci is under range constraint (Goldstein and Pollock, 1997; Saino *et al.*, 1996; Amos and Rubinsztein, 1996). Furthermore, microsatellites may be unstable above a certain threshold and quickly degrade through large deletions or the induction of imperfections (Goldstein and Pollock, 1997; Levinson and Gutman, 1987). It has been suggested that mutation bias and range constraint may limit the variation at microsatellite loci and may have a significant effect on the estimation of microsatellite genetic distances (Feldman *et al.*, 1997; Nauta and Weissing, 1996; Zhivotovsky *et al.*, 1997). Under mutation bias (Zhivotovsky *et al.*, 1997), alleles of

large size mutate preferentially to alleles of smaller size, and *vice versa* for alleles of small size. On the other hand, range constraint (Bowcock *et al.*, 1994; Goldstein *et al.*, 1995; Nauta and Weissing *et al.*, 1996; Feldman *et al.*, 1997) selects against multiple repeats beyond some size threshold and hence, restricts microsatellite variation to bounded intervals. Both mutation bias and range constraint could be rather small, possibly less than the mutation rate. Nevertheless, a cautious approach should be taken when using microsatellite markers for evolutionary studies as these constraints might have a significant effect on evolutionary statistics. For instance, Zhivotovsky *et al.* (1997) has demonstrated that mutation bias could significantly increase the estimated divergence time compared to estimates obtained assuming no bias.

1.3.7 Comparing microsatellites and allozymes as molecular markers

Many studies have shown that much more variation could be detected at the DNA level than what had been seen in allozyme studies (Bancroft *et al.*, 1995; Begun and Aquadro, 1993; Queller *et al.*, 1993; Estoup *et al.*, 1995). In terms of nucleotide sequence changes, considerably more variation can be found in non-coding than in coding DNA (Haymer, 1994; Cheng *et al.*, 1992). It is generally believed that microsatellite markers, especially those embedded in non-coding regions, are more neutral to selection than allozymes (Queller *et al.*, 1993). Codon degeneracy and selection pressure against lethal or harmful mutations contribute to little variation at particular allozyme loci. Codon degeneracy allows the same amino acid to be encoded by more than one set of nucleotide triplets, as a results, only point mutations that could change the overall charge of a protein could be

detected using allozyme electrophoresis (i.e., not all genetic differences at an allozyme locus can be detected) (Engel *et al.*, 1996). Point mutations may not alter the mobility of the protein and thus, remain undetected at allozyme level. In fact, the point mutations that could change the overall charge of a protein represent only a third of the possible point mutations (Engel *et al.*, 1996). Codon degeneracy, therefore, greatly reduces variation at the allozyme level. Furthermore, mutations in single-copy genes occur in coding regions and some mutations create lethal effects while others can be deleterious, and both subject to intense selection pressure, reducing the probability that they will persist in a population over time. In addition, loci under either directional or balancing selection may have patterns of variation that primarily reflect past selective events and are not necessarily consistent with evolutionary history or population structure of the taxa (Boyce *et al.*, 1997). Since microsatellites are generally embedded within non-coding sequences (between genes and within introns), i.e., they are neutral, the amount of differential selection acted upon these markers should be very low, and thus, selection pressure and codon degeneracy would not affect the variability of number of repeats (Haymer, 1994; Boyce *et al.*, 1997). The results from an allozyme study conducted by Cheng *et al.* (1992) had showed that little variation (heterozygozity of 0.27) from 2 polymorphic allozyme loci in the domestic Canadian strains of Japanese quail. A more powerful molecular tool is required to detect polymorphic loci within and among strains of Japanese quail, and microsatellite marker is a good candidate owing to its high level of polymorphism and broad genomic distribution. Currently, microsatellites have been widely used in many population studies involving estimation of demographic and evolutionary parameters of natural populations (MacHugh *et al.*, 1994;

Estoup *et al.*, 1995; Goldstein *et al.* 1995; Slatkin, 1995) and of phylogenetic relationship among populations and closely related species (Bowcock *et al.*, 1994; MacHugh *et al.*, 1994; Deka *et al.*, 1995; Estoup *et al.*, 1995; Goldstein *et al.*, 1995; Pepin *et al.*, 1995).

1.3.8 PCR-based assay to score polymorphism in microsatellite loci

Genetic polymorphism is defined as the existence of more than one allele in a locus and that the least frequent allele is present at frequency of more than 1% in the population (Lewin, 1997). In 1989, the isolation of microsatellites and the characterization of allelic variability at microsatellite loci using polymerase chain reaction (PCR) was first reported by Tautz, (1989) and Litt and Luty (1989). In these early studies, microsatellites were either cloned and sequenced, or were identified from sequence databases for a repeat region (e.g., the Genbank and EMBL DNA sequence data-banks). Length polymorphism in the repeat was detected by first using a pair of microsatellite primers designed to recognize the unique sequences flanking the tandem repeat in a PCR, followed by amplifying a specific microsatellite repeat of a particular locus, and by resolving the PCR amplification products using gel electrophoresis on polyacrylamide gels (Saghai Maroof *et al.*, 1994; Tautz, 1989; Litt and Luty, 1989; Weber and May, 1989; Gupta *et al.*, 1994; Love *et al.*, 1990). The unique flanking regions allow each microsatellite to be analyzed as a single locus. Microsatellite loci tend to be relatively short (generally less than 400 bp) and are readily amplified by using the PCR technology (Weber and May, 1969; Houlden *et al.*, 1996). In contrast, the repeat units in minisatellite may be as large as 200 bp each, and their allele sizes can range up to 50 kb (Bruford *et al.*, 1996). Due to the large sizes of many minisatellite

alleles, only a limited subset of variation can be analyzed by PCR. Since PCR is most efficiently performed with sequences shorter than 1 kb, microsatellites are more adaptable to PCR than minisatellites due to their smaller fragment size. Variability in microsatellite loci having allele sizes smaller than 1 kb could be assayed by PCR and resolved by gel electrophoresis, avoiding the need the construction of locus-specific-probe to visualize the allelic difference on Southern blots. This PCR-based assay to score microsatellite polymorphism is fast and reliable (Cheng and Crittenden, 1994). In addition, PCR-based assay requires only minute amount of DNA, unlike restriction fragment length polymorphism (RFLP), and hence, avoiding the need for direct assaying of high quality and quantity DNA. Microsatellite loci are also very widely and evenly distributed throughout the eukaryotic genome (Tautz and Rentz, 1984; Kashi *et al.*, 1990; Weber, 1990; McCouch *et al.*, 1997) and therefore, the entire genome can be examined. In contrast, minisatellite loci are sometimes clustered in particular regions such as proterminal regions that may reduce their usefulness (Queller *et al.*, 1993). Furthermore, due to the high frequency with which microsatellite sequence are found in eukaryotic genomes, microsatellite loci are relatively easy to be isolated.

Microsatellites can be very helpful for population and conservation genetics, especially in species where allozyme variability is low (e.g. Hughes and Queller, 1993). Since microsatellites are single-locus, codominant DNA markers, they can be used to identify paternity and estimate relatedness in natural population (Queller *et al.*, 1993; Ishibashi *et al.*, 1995). In contrast, random amplified polymorphic DNAs (RAPDs) are usually interpreted as dominant markers and can detect only 2 alleles of a locus by the presence or absence of the

marker (Queller *et al.*, 1993). A failed RAPDs reaction is not readily recognizable because it also shows the absence of a band (Engel *et al.*, 1996). Moreover, the dominant mode of inheritance of RAPDs marker requires the assumption of random mating for the calculation of genotype frequencies and subsequent population genetic statistics and this assumption is invalid for many mammal groups (Engel *et al.*, 1996). Besides, the finite number of restriction enzymes available generally which limits the number of loci that can be examined in RFLP; probes for specific loci are also not always readily available (Engel *et al.*, 1996). Furthermore, because multiple loci are analyzed simultaneously in multilocus DNA fingerprinting and multilocus minisatellites, the alleles of a particular locus usually cannot be identified and only average heterozygozity overall loci can be estimated. On the other hand, the simple PCR-based assay allows the heterozygozity and number of alleles to be estimated for each locus.

In summary, microsatellites have many advantages over alternative marker systems, including broad distribution and abundance of microsatellite loci, nature of codominance inheritance, the quantity of DNA required, fast and reliable screening, and adaptability to PCR. Microsatellite markers provide more genetic information than RFLP, RAPDs and DNA multilocus fingerprinting in studies which require information on allele frequency distributions (e.g. in studies of genetic relatedness of individuals and populations). Many studies have also shown that analysis of microsatellite locus variation could be extremely informative in studies of kinship (Queller *et al.*, 1993) and population genetics (Houlden *et al.*, 1996; Bruford and Wayne, 1993).

1.4 Cross-species Amplification

1.4.1 Limiting factor of using microsatellite markers

A perceived limitation to widespread use of microsatellite markers has been the need and effort required to synthesis unique oligonucleotide DNA sequences that are used as primers to produce PCR products containing microsatellites for each new species of interest. Identifying and sequencing species-specific sequences that flank microsatellite loci can be a time-consuming process that involves the construction and screening of libraries of genomic DNA, DNA sequencing of clones, and design of primers (Bruford, 1993; Queller *et al.*, 1993; Engel *et al.*, 1996). A different set of primer-pairs is generally developed for each species. Species-specific primers are often necessary because PCR primers require a high degree of homology to the target sequence in order to anneal onto the target sequence and initiate amplification of microsatellite locus in that species during PCR reaction. Since microsatellites have a comparably high mutation rate (Dallas, 1992; Dietrich *et al.*, 1992) and are generally embedded within non-coding regions, accumulated mutations in microsatellite flanking sequences will eventually inhibit amplification in one species with primers developed for a distantly related species (Primmer *et al.*, 1996). Unfortunately, primers for microsatellite loci are presently available for only a limited number of taxa and the development of microsatellite primers for each new species of interest can be labor-intensive, time-consuming and expensive.

Moderate levels of microsatellite conservation have been found across closely related species of mammals (Fredholm and Winterø, 1995; Moore *et al.*, 1991), and studies have

shown that microsatellite primers designed for various species can often amplify the corresponding and sometimes polymorphic loci in closely related species (Calvas *et al.*, 1994; Coote *et al.*, 1996; Schlotterer *et al.*, 1991; Stallings *et al.*, 1991), or even in widely divergent taxa (Fitz-Simmons *et al.*, 1995). The ability to use heterologous microsatellite primer-pairs to amplify microsatellite loci in a variety of species within the same genus, family, or order, would reduce the number of microsatellite primers needed to specifically develop for every species of interest (Engel *et al.*, 1996).

1.4.2 Heterologous amplification of microsatellite loci

It has been suggested that the chance of a successful cross-species (heterologous) amplification of microsatellite loci would be inversely related to the evolutionary distance between the 2 species (Primmer, 1996). Several studies have shown the successful heterologous amplification of the corresponding microsatellite loci in closely related species (Moore *et al.*, 1991; Schlolterer *et al.*, 1991; Stallings *et al.*, 1991). For instance, in Moore's (1991) study, around 50% of cattle microsatellites could be amplified by primers from sheep, and *vice versa*. In some whales, cognate DNA sites in species thought to have been diverged about 20 million years ago were detected by PCR assays based on microsatellite-flanking primer pairs from unique sequences (Schlolterer *et al.*, 1991). The degree of conservation of microsatellite loci across the avian taxa has also been extensively studied in passerines (Hanotte *et al.*, 1994; Primmer *et al.*, 1997). Hanotte *et al.* (1994) found that microsatellite primers developed for the reed bunting (*Emberiza schoeniclus*) amplified loci in 13 other passerine species. Primmer *et al.* (1997) used primers developed for the swallow (*Hirundo*

rustica) and the pied flycatcher (*Ficedula hypoleuca*) and tested them on 48 different bird species and in 162 cases (species/marker combinations) showed heterologous amplification. Together, these studies suggest that microsatellite primers that were developed from various species could be used to amplify true homologues of microsatellite loci in closely related species.

Chicken (*Gallus gallus*) microsatellite primers have been tested in another galliform, turkey (*Meteagridis galopario*), by 2 different laboratories (Levin *et al.*, 1995; Liu *et al.*, 1996). The majority of chicken primers generated amplification products in turkey genomic DNA in both studies. However, 2 very different conclusions were drawn by these groups. Levin *et al.* (1995) took all amplifiable products into consideration and concluded that more than 33% of the microsatellite primer-pairs developed for the chicken might be useful for analyzing turkey DNA. On the other hand, Liu *et al.* (1995) considered only the polymorphic products (generated by 9% of all the chicken markers tested) and concluded that most chicken microsatellites are not very useful for the mapping and analysis of the turkey genome. Therefore, a more general investigation of cross-species microsatellite amplification to determine the extent to which heterologous microsatellite primers can be applied in other galliform birds is necessary. The ability to amplify microsatellites using heterologous primer-pairs in *Galliformes* within the same order or family could greatly reduce the number of microsatellite primers needed to be designed for every avian species of interest (Engel *et al.*, 1996).

1.4.3 Using chicken-specific microsatellite primers in Japanese quail

Many studies have shown that microsatellite loci are often conserved among closely related species (Fredholm and Winterø, 1995; Moore *et al.*, 1991; Calvas *et al.*, 1994; Coote *et al.*, 1996; Primmer *et al.*, 1997). Since Japanese quail (*Coturnix japonica*) and chicken (*Gallus gallus*) are very closely related (Crawford, 1990; Mindell *et al.*, 1997: Sibley and Ahlquist, 1990), the locations and sequences of their microsatellite loci could be very similar. In addition, according to Primmer *et al.* (1997), there is a significant and negative relationship between microsatellite performance and evolutionary distance between the original species and the tested species. The evolutionary distance between Japanese quail and chicken is believed to be much shorter than that between turkey and chicken (Crawford, 1990; Wetmore A, 1960; Delacour, J; 1964). The chance of successful heterologous amplification is therefore expected to be high in this study.

To my knowledge, microsatellite markers have not been specifically developed for the Japanese quail and no genetic mapping study has been conducted for this species. In contrast, 575 pairs of chicken-specific microsatellite primers are available and more than 200 polymorphic chicken microsatellite markers have been mapped on the chicken genome (Khatib *et al.*, 1993; Cheng and Crittenden, 1994; Crooijmans *et al.*, 1994, 1995, 1996; NAGRP, 1998). Since the development of microsatellite primers is a laborious and expensive process which involves cloning and sequencing, the possible use of chicken-specific primers in the analysis of Japanese quail genome could significantly reduce the number of microsatellite primers needed to be developed for such an analysis. Likewise, primers flanking microsatellite loci in the genome of Japanese quail might be useful in the

analysis of the chicken genome (Levin *et al.*, 1995).

If chicken-specific primers could be used to detect corresponding microsatellite loci in Japanese quail, genetic variability between and within Japanese quail strains could be determined using the identified microsatellite markers. If a Japanese quail microsatellite map is going to be generated, one can exploit the chicken interval mapping information for generating selection markers in the Japanese quail. Since the Japanese quail has a much shorter generation interval (6 weeks) and requires less space than the chicken, the Japanese quail will be more suitable model for marker-assisted selection projects.

1.5 Objectives of thesis

The objectives of this research were (1) to examine whether microsatellite primer-pairs, flanking chicken microsatellites and giving polymorphic PCR products in that species, can amplify microsatellite loci in Japanese quail DNA templates, (2) to characterize any polymorphic PCR products amplified, and (3) to use these markers to determine the expected and observed heterozygozity and the inbreeding coefficient for 4 different Japanese quail populations.

SECTION 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Experimental birds

Ten individuals (5 males and 5 females per strain) from each of 4 different strains of the Japanese quail (UBC, JAP, NCW and ALT) that are being maintained at the University of British Columbia's Quail Genetic Resource Center (QGRC) were sampled.

The researcher was blind to the genetic background of the strains prior to and during the study. The UBC strain was a cross between a strain acquired from the University of California at Davis and another obtained from Korea in 1976. The population has been closed for more than 75 generations and was maintained randombred with 48 males and 96 females per generation. The NCW strain was acquired from the North Carolina State University in 1990. The strain originated from wild Japanese quail imported by the University of California in the 1920's. The strain was maintained as a randombred strain with 24 males and 48 females per generation. The JAP strain was acquired from the University of Nagoya, Japan, in 1989 and has gone through a bottleneck (6 males and 8 females) after arriving at the QGRC. The strain has been maintained as a randombred strain with 24 males and 48 females. The ALT strain was acquired from the University of Alberta in 1977 and has been maintained with 48 males and 96 females until 1995, when the population size was reduced to 10Males/10Females.

2.1.2 Extraction of Japanese quail genomic DNA

Approximately 75 µl of blood was collected from each of the 40 individuals by venipuncture of the wing vein. Samples were stored in 1 ml of Queen's Lysis buffer (0.01M THAM, 0.01M NaCl, 0.01M Disodium-EDTA, 1% Lauroyl sarcosine, pH8.0) (Seutin *et al.*, 1991). Approximately 250 µl of cell lysate was diluted with distilled water (v/v, 1:2) and then digested with 50-90 µl of 18 mg/ml proteinase K solution at 55°C for at least 3 hours. The volume of the content was brought up to 2.0 ml with distilled water. Extraction was conducted using standard protocol described in Sambrook *et al.* (1989) with minor modifications. DNA was extracted with equal volume of phenol-chloroform-isoamyl alcohol (v/v, 25:24:1) twice, then with equal volume of chloroform-isoamyl alcohol (v/v, 24:1) once. The volume of the extract was brought up to 2.0 ml with distilled water. 200 µl of 2 M NaCl was then added into each tube. Crude DNA was washed and precipitated first with 3 volume of -20°C 95% ethanol (EtOH) and then washed with one volume of -20°C 70% EtOH, air-dried briefly, and resuspended in 250 µl of 1 x TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.8). The yields of DNA were calculated from the absorbance at 260 nm using a spectrophotometer (Ultrospec-3000, Pharmacia Biotech., Canada) and by comparison with a molecular weight DNA standard on a 2% agarose gel stained with ethidium bromide. Approximately 25-50 ng of Japanese quail genomic DNA were used as template for each PCR reaction.

2.1.3 Source of chicken microsatellite primers

Chicken microsatellite population mapping kits #1 and #2 were obtained from NAGRP, USA. Forty-eight chicken microsatellite primers were randomly selected and tested for their usefulness in cross-species amplification with individual Japanese quail template using PCR (see section 2.2.1). These primers were 19-21 bases in length (mostly 20 nucleotides long) and represented microsatellites of di-, and tri-nucleotide repeats (Appendix 1). Primers for the 12 microsatellite markers that generated specific PCR amplification products in Japanese quail template were synthesized by GIBCO BRL, Canadian Life Technologies, Ontario (Appendix 1). Dried desalted primer-pairs were dissolved in TE buffer for sequencing on ABI Automated Sequencer Model 373.

2.2 Methods

2.2.1 Polymerase Chain Reaction (PCR)

The PCR conditions were optimized for each primer set. Both the preferential amplification temperature and final Mg^{++} ion concentration were optimized for each primer set (Tables 1, 4 and Appendix 2) in order to minimize the likelihood of obtaining false bands. The optimum PCR conditions (which yielded a significant quantity of desired product) were determined by attempting to amplify genomic DNA samples using the following strategies. The annealing temperature in the PCR reaction was first tested at the suggested annealing temperature used on chicken DNA. If gel analysis of the PCR products did not give a single band on 2% agarose gel, then the PCR was repeated either at a different temperature or with

an adjusted final Mg^{++} ion concentration. The annealing temperature was altered by using a five-degree incremental series of temperature (±5°C) until a specific band was observed or until the range of ±10-15°C of the suggested annealing temperature was reached. The final Mg^{++} ion concentration was also adjusted according to the specificity of the PCR reaction. For example, if many non-specific bands were observed, a decrease in the final Mg^{++} ion concentration would increase the specificity, and hence, would increase the intensity of the major band. If gel analysis of the PCR products gave a single but not clear and well-defined band, the annealing temperature would be altered by using a 2-degree incremental series of temperature (±2°C) until a reasonably clear band was obtained. In general, decrease in the annealing temperature and increase in the final Mg^{++} ion concentration would decrease the specificity of the PCR reaction, and hence, allowed a certain mismatch between the primers and the heterogeneous sequences. On the contrary, an increase in the annealing temperature and a decrease in the final Mg^{++} ion concentration would increase the specificity and increase the number of copies of the target DNA fragments. The final Mg^{++} ion concentrations and annealing temperatures were also dependent on several factors that influenced PCR including the type of PCR machine, the enzyme supplier, and other components of the reaction buffer (Love *et al.*, 1990).

PCR amplifications were performed in a 25-µl reaction volume containing 2.0-3.0 mM $MgCl_2$ (final concentration), 2 µl of 2 mM deoxyribonucleoside triphosphate mix (GIBCO BRL), 25-50 ng total Japanese quail genomic DNA, 15 pmol of each cold primer (forward and reverse primers), and 1.25 U *Taq* DNA Polymerase (GIBCO BRL) in the supplied reaction buffer (200 mM Tris-HCl pH 8.4; 500 mM KCl). The PCR amplification

was started with the first denaturation at 94°C for 3 minutes, and then 35 cycles of PCR, each consisting of thermal denaturation at 94°C for 1 minute, at optimized annealing temperature for 45 seconds, and at 72°C for 45 seconds, and finally followed by a prolonged extension step at 72°C for 15 minutes. Each primer-pair was first tested using 2 individuals from each strain under different PCR conditions until optimized condition was determined for each primer set. When a locus specific amplification was observed in at least one of those samples, all the samples (a total of 40) were analyzed as well. Chicken DNA samples of known genotype were amplified and run on every gel to serve as positive controls and to aid in size determination of alleles in Japanese quail. In addition, a negative control involved PCR master mix without added DNA (blank PCR control) was also always included to enable detection of handling errors and contamination. The PCR reactions were performed on a PTC-100 Programmable Thermal Controller (MJ Research, Inc.).

2.2.2 Amplification product analysis

In order to examine the products of the amplification procedure, a 10-µl aliquot of each reaction was analyzed by electrophoresis through 2.0% agarose gel in 1x 30 mM TAE buffer (Tris(hydroxymethyl) aminomethane, $Na_2EDTA2H_20$, glacial acetic acid). A 20/100 bp DNA ladder was used to ascertain the size of a particular DNA band. The gel was subjected to 80 Volts (V) (100 milliamps (mA)) for 2 hours to give optimal migration and resolution. Amplification products were stained with 0.3-0.4 µg/ml of ethidium bromide for 30 minutes, destained with distilled water for 30 minutes, and visualized by ultraviolet (UV) exposition on Polaroid film.

2.2.3 Screening for polymorphism

2.2.3.1 Screening for length variation

In order to examine the reaction products for length polymorphism, a 5-10 μl aliquot of reaction was analyzed on a recently available precast Spreadex™ gels with appropriate exclusion limit for fragment size (a polyacrylamide gel manufactured by Elchrom and distributed by Helixx Technologies Inc., Toronto) in 1x TAE buffer on an Elchrom Scientific system. A M3 marker (Helixx) was run adjacent to the samples to provide a size marker for the microsatellite alleles. The gel was subjected to 100-120 V (400-650 mA) for 3-7 hours to give optimal migration and resolution. Post PCR fragments were stained with 0.3-0.4 μg/ml of ethidium bromide for 30 minutes and destained with distilled water for 30 minutes. DNA bands were observed with UV light and photographed using Polaroid 667 system.

2.2.3.2 Gel interpretation

Screening for polymorphism was performed using Spreadex gels. Spreadex gels with different exclusion limit (EL) (of 400, 500 and 800 bp) and with different optimal separation range (of 65-250, 100-300, 200-500 bp, respectively) were run in this experiment. In general, the distances were larger between adjacent ladder bands on the Spreadex gel with lower exclusion limit numbers, in agreement with their ability to resolve smaller DNA size differences. For instance, for the EL500 Spreadex gel, the exclusion limit was 500 bp and the optimal range of separation was 100-300 bp. The expected allele length or PCR amplification product size should fall into this optimal separation range. High molecular

weight DNA fragments, that were generated in PCR reaction due to non-specific amplification and whose size was above 500 bp, could not migrate on the gel and remained in the sample well. This method of separation of the amplification products could detect differences in size of as little as 2 bp in an environmentally safe and cost-effective manner. This technique has many advantages in terms of speed and ease of analysis. For instance, bands can be visualized by ethidium bromide staining since a sufficient amount of DNA is examined in a non-denaturing form. Therefore, more complex detection methods, such as the use of radioactive- or digoxigen-labeled probes for Southern blotting or silver staining, are not needed.

Electrophoresis of 4-10 μl of 25 μl PCR was sufficient to detect DNA fragment differing by 4 bp on all Spreadex gels using this system (Helixx). Screening for polymorphism using Spreadex gels was possible because this technique took advantage of the formation of heteroduplexes in the PCR between different alleles with several mismatches from heterozygous individuals. Heating and renaturation of PCR products from different alleles generated heteroduplexes. After denaturation, the PCR products became single stranded. At renaturation temperature, these strands folded back on themselves in a sequence-dependent manner. As a result, both homoduplexes and heteroduplexes were formed. Only one band would be detected in homozygotes on the gels. Because heteroduplexes had more "loops" in the double-stranded configuration surrounding the mismatched bases, they would migrate slower than their corresponding homoduplexes on Spreadex gels (White *et al.*, 1992). Therefore, 2 bands of the same intensity would be detected in heterozygous individuals on the gels. Meanwhile, some individuals might exhibit

triple bands of the same intensities consistent with a locus-specific amplification. These individuals would also be heterozygotes because the 2 alleles would be so different (differed by more than 4 bp) in length of repeats that 01/01 and 02/02 homoduplexes could be detected as 2 distinctive bands on the gel. As a result, 3 bands would be observed on the gel: 01/01, 02/02, 01/02 (2 homoduplexes and one heteroduplex). In some cases, even 4 bands would be detected on the gel. The presence of 4 bands was thought to correspond to an asymmetry of 2 possible strand combinations in each cross-hybridization (i.e., 01/01, 02/02, and 2 different forms of 01/02). Sorrentino *et al.* (1991) suggested that the quality of the mismatches, i.e., in their being either transition- or transversion- type substitutions, might affect electrophoretic migration of these heteroduplexes.

To determine whether or not the true homologues of corresponding microsatellite loci were detected in cross-species amplification, a selection of specific amplification products were analyzed using DNA sequencing. Determination by sequencing is very important especially when the repeat units are dinucleotides (e.g. (TG)n), where the alleles could be very similar in size, and thus, heterozygotes could not be easily differentiated from homozygotes using this heteroduplex polymorphism technique.

2.2.4 Sequencing on ABI Automated Sequencer Model 373

2.2.4.1 Preparation of DNA fragments (PCR products) for direct sequencing

Specific amplification products, which showed as a single reasonably well defined band were sequenced by the Nucleic Acid-Protein Service Unit Laboratory (NAPs Unit) at the University of British Columbia using an ABI Model 377 automated sequencer (Perkin-

Elmer-Cetus, CA, USA). Prior to sequencing, the PCR sample was separated on 0.8% agarose gel (using 1x TA). The band of interest was cut out into as small a slice as possible. Then, the slice was placed on top of a plugged tip (200µl, Fisher cat#0270740) inside a microcentrifuge tube, and was spun at 13,000 rpm for 4-5 min. The gel was discarded and the liquid (containing DNA fragment) was saved. The quantity of DNA was estimated using a spectrophotometer. The sample was dried down using a SpeedVac and the DNA was then resuspended with appropriate volume of autoclaved distilled water to achieve the final concentration of approximately 90 ng/µl.

2.2.4.2 Sequencing reaction using ABI AmpliTaqFS OldDeoxy Terminator Cycle Chemistry

The sequencing reaction using this chemistry was carried out on the Perkin Elmer DNA Thermal Cycler Model 480. ABI AmpliTaqFS OldDeoxy Terminator Cycle Sequencing chemistry was applied: 8µl of Taq Terminator Reaction Premix (ABI's) was used, approximately 90 ng of the PCR amplification product was used as the sequence template and 3.2 pmol of one of the original cold PCR primers was used as the sequencing primer. PCR products were sequenced in both directions. Before the sample was run on a sequencing gel, purification of DNA fragment was required to remove excess and unincorporated dye terminators, nucleotides and primers in the sequencing reaction. To accomplish this, each sample was purified by using the Centri-Sep column (NAPs Unit).

2.2.5 Observed and expected heterozygozity levels

The degree of observed and expected heterozygozity was estimated at 3 loci that were polymorphic from the 4 Japanese quail populations. The observed heterozygozity (H_o) was calculated as the number of heterozygotes over the total number of individuals being examined (Hedrick, 1985). The expected heterozygozity (H_e) was estimated using equation 2 in Appendix 3. These estimates were unbiased since the values were adjusted for small sample size (Hedrick, 1985). The mean observed and expected heterozygozity were calculated using equation 3 in Appendix 3. Inbreeding coefficients ($F_{IS} = 1 - H_o/H_e$) were also calculated (Wright, 1951).

SECTION 3

RESULTS

3.1 Heterologous amplification

A total of 48 chicken-specific microsatellite primers were applied to Japanese quail to analyze the possibility of amplifying homologous microsatellite loci. Amplification products were obtained in 28 cases (58.3%) after optimizing the PCR condition for each of these microsatellite primer sets. From these 28 markers, 16 generated non-specific products even after many attempts to try to eliminate/reduce the number of these non-specific bands by optimizing different parameters of the PCR assay. The PCR conditions tested in Japanese quail template were showed in Table 4. In some cases, increasing the annealing temperature reduced the number of non-specific bands, but did not completely eliminate them. The high amount of non-specific signals or non-specific false bands seen in the amplification made these bands unscorable and therefore, these 16 markers were not selected for further screening.

Twelve out of 48 microsatellite heterologous primers tested (25%) showed a successful specific cross-species amplification and 9 of which revealed length polymorphism (18.8%) (Table 1). The optimized PCR conditions for each primer set used in Japanese quail were shown in Table 1. Four primer pairs (ADL024, ADL143, ADL315 and ADL366) could amplify specific product in Japanese quail template using the suggested annealing temperature reported in chicken. The optimized annealing temperatures for the remaining 8 primer sets were differed from those recommended for chicken by 2°C to 11°C (Table 1).

These 12 markers generated specific PCR products in Japanese quail template and their product sizes were also shown in Table 1. The difference between the observed PCR product size in Japanese quail template and the reported average allele length in chicken using the same primer-pair varied greatly from locus to locus (Table 1). The size difference between the average allele size in these two species was small (≤5 bp) for markers ADL037, ADL038, ADL315 and ADL366, moderate (between 12-15 bp) for markers ADL111 and HUJ006, and great (between 22-69 bp) for markers ADL023, ADL024, ADL142, ADL143, ADL206 and ADL257 (Table 1). All 3 monomorphic markers (ADL023, ADL024 and ADL257) detected in Japanese quail generated PCR products that were very different in sizes from the corresponding PCR products reported in chicken (Table 1).

3.2 Gel interpretation

The specific amplification products from 7 markers of the 12 examined (ADL023, ADL037, ADL206, ADL257, ADL315, ADL366 and HUJ006) showed clear bands which lacked any non-specific signals (Figs. 3A, 1A, 1D, 1E, 1F and 1G, respectively). At loci ADL024, ADL038, ADL111, ADL142 and ADL143 (Figs. 3B, 2B, 2A, 1B, and 1C, respectively), individuals exhibited one or a few fainter bands (appearing immediately below the main/expected product) consistent with a locus-specific amplification. Several criteria were set in order to distinguish the true band from the extra band detected at loci ADL024, ADL038, ADL111, ADL142 and ADL143 (Figs. 3B, 2B, 2A, 1B, and 1C, respectively). First, the location of the major/expected band was critical. Bands of the expected size (based on sequence of observed allele length in chicken) were considered as the true bands. Bands

that were reasonably smaller or larger than the expected product (within the range of 90-300 bp in allele length) would also be considered and examined since we could not rule out the possibility that these bands could be true microsatellite products in Japanese quail genome. Finally, the intensity of any bands with other bands of similar size were only compared with samples in the same lane and not with samples of different lanes.

3.3 Number of alleles detected at each locus

The number of alleles detected at each of the 12 loci in this test panel is shown in Table 2. Loci ADL023, ADL024 and ADL257 were monomorphic in the 4 populations of Japanese quail. Loci ADL111, ADL 143, ADL206 and ADL366 might contain null alleles (see sections 4.4 and 4.5). The number of alleles detected in these 4 loci did not include the possible null alleles since further investigation is required to proof that these loci do indeed contain true null alleles. ADL315 and HUJ006 seemed to be highly polymorphic because relatively high number of alleles (5 and 4, respectively) were detected at these loci. The number of alleles at loci ADL038 and ADL142 could not be determined. The bands were too faint to be scored at locus ADL038 (Fig. 2B) and due to high amount of stutter bands present at locus ADL142, it was also difficult to estimate the number of allele present (Fig. 1B).

The sequencing results showed that microsatellite repeats were detected in 8 out of the 12 loci examined (ADL024, ADL037, ADL142, ADL143, ADL206, ADL315, ADL366 and HUJ006) (Table 2). No repeats were detected at loci ADL023, ADL111 and ADL257 and the signals were too weak at loci ADL038 for sequencing analysis (Table 2).

The amplification product generated by primer pair ADL037 contained microsatellite repeat and both forward and reverse primers could be sequenced. The repeat units detected in Japanese quail was $(CAG)_3N_9(CA)_3TA(CA)_5$ and was reasonably similar to that reported in chicken, $(CAG)_3N_9(CA)_8$ (Table 2). The amplification product from primer set ADL315 was also sequenced in both directions. The repeat $(CT)_6(CA)_5$ was detected was very similar to the one reported in chicken, $(CT)_8(CA)_9$ (Table 2).

The repeats detected at the other 6 loci were very different than respective ones reported for chicken (Table 2). Locus ADL143 in Japanese quail was $(AT)_9N_{18}(AG)_3$ whereas the one reported in chicken was $(TG)_{10}(TA)_5$. Similar to locus ADL143, the repeats detected at ADL142 and ADL206 were also very different from the repeat reported at the corresponding loci in chicken. For ADL206, the $(AG)_3(TG)_7$ repeat units detected in Japanese quail showed no similarity to the repeat reported at the corresponding locus in chicken ($(CA)_6CG(CA)_{10}$). At HUJ006, an $(AC)_9$ repeat was detected in Japanese quail template while the repeat reported at the corresponding locus in chicken was also an (AC) repeat but the exact number of repeat unit was unknown (Table 2). It is possible that this locus in chicken was highly polymorphic and the range of allele size (due to the variation in the number of repeats) was large. In contrast, an (AG) repeat, instead of the expected (AC) repeat, was detected at locus ADL366 in Japanese quail. At locus ADL024, groups of very short repeats were detected, $(CT)_3N_{77}(CT)_3TT(AT)_3N_{43}(CT)_4$. This detected repeat in Japanese quail had no similarity to the repeat reported in the corresponding locus in chicken, $A_{10}N_{66}(CA)_6$.

3.4 Primer and flanking sequences

Primer sequences examined in Japanese quail were shown in Table 3. No base substitution at the primer sequences was detected at locus ADL111, ADL143, ADL257 and ADL366. Both forward and reverse primer sequences were identified from the sequenced strands of allele at locus ADL037 and there was only an A→C base substitution at the 15th position of the forward primer sequence (Table 3). There was also a base substitution, T→C, at the 9th position of the forward primer of locus ADL206 and an A→C substitution at the 12th position of the reverse primer of locus ADL142 (Table 3). It is interesting to note that there were 3 base substitutions in the forward primer sequence and none in the reverse primer sequence at locus HUJ006 (Table 3). At loci ADL023 and ADL024, 2 base substitutions were detected in each reverse primer sequence (Table 3).

The BLAST algorithm (Basic Local Alignment Search Toll, developed at National Center for Biotechnology Information) was used to search for homology in the non-priming flanking sequences between the quail and the chicken. A considerable amount of homology (see section 4.3) with the chicken DNA in the flanking sequences immediate to the repeats at 3 loci (ADL023, ADL037 and ADL366).

3.5 Observed and expected heterozygozity

ADL023, ADL024 and ADL257 were monomorphic from the 4 Japanese quail populations (Table 1, Figs. 3A, 3B and 3C). ADL111, ADL143, ADL206 and ADL366 might have null alleles (Table 2, also see sections 4.4 and 4.5). It was impossible to estimate the number of alleles at ADL142 due to the high amount of stutter bands present at this locus.

ADL038 was too weak to be analyzed and required further optimization of PCR conditions (Table 2, Fig. 2B). The degree of observed and expected heterozygozity was therefore only determined at 3 loci (ADL037, ADL315 and HUJ006) (Table 5).

The observed heterozygozity (H_o) for the 4 populations ranged from 0.11 to 0.47 at locus ADL037, 0.32 to 0.63 at locus ADL315, and 0.36 to 0.69 at locus HUJ006 (Table 5). The expected heterozygozity (H_e) ranged from 0.27 to 0.52 at locus ADL037, 0.44 to 0.65 at locus ADL315, and 0.48 to 0.65 at locus HUJ006. At locus ADL037, the observed heterozygozity (0.21 and 0.11) were much lower than the expected heterozygozity (0.51 and 0.27) in JAP and ALT strains, respectively. The inbreeding coefficient (F_{IS}) for these 2 strains at locus ADL037 were therefore high, 0.58 and 0.61 respectively. Similar to locus ADL037, the values for the observed heterozygozity (0.53 and 0.32) were lower than the estimates for the expected heterozygozity (0.65 and 0.44) in JAP and ALT strains at locus ADL315. Meanwhile, the estimates for the observed and the expected heterozygozity for the other 2 strains, UBC and NCW, were exactly the same. The inbreeding coefficient (F_{IS}) for these 2 strains at locus ADL315 were therefore low (Table 5). At locus HUJ006, the estimate for the observed heterozygozity in the JAP strain (0.36) was found to be lower than the expected one (0.48). The mean observed heterozygozity for loci ADL037, ADL315 and HUJ006 were 0.33, 0.49 and 0.58, respectively and the mean expected heterozygozity were 0.45, 0.55 and 0.56, respectively. The average observed heterozygozity over these 3 microsatellite loci was 0.46 and the average expected heterozygozity was 0.52.

Table 1. List of 12 chicken-specific primer sets that generated specific amplification products in Japanese quail template.

Locus name[a]	Observed PCR product in Japanese quail template (bp)[b]	Optimized PCR condition in Japanese quail DNA template: T (°C), final Mg^{++} (mM)[d]	Suggested PCR annealing temp. in chicken DNA template (°C)[c]	Average allele length reported in chicken template (bp)[c]	Polymorphic vs. Monomorphic[d]
ADL023	132	47; 2.0	52	164	monomorphic
ADL024	206	46; 2.5	46	145	monomorphic
ADL037	176-180	47; 3.0	49	178	polymorphic
ADL038	130-140	34; 3.0	44	131	polymorphic
ADL111	112-114	42; 2.5	49	128	polymorphic
ADL142	204-214	47; 2.0	52	231	polymorphic
ADL143	138	47; 2.0	47	168	polymorphic
ADL206	95-101	38; 2.5	49	125	polymorphic
ADL257	118	40; 2.5	47	187	monomorphic
ADL315	236-250	51; 2.5	51	248	polymorphic
ADL366	226-232	54; 2.5	54	227	polymorphic
HUJ006	99-107	45; 2.5	55	115	polymorphic

[a]the first 3 letters designate the laboratory name: ADL=USDA ARS Avian Disease and Oncolgy Lab; HUJ=Hebrew University of Jerusalem

[b]product size according to results test panel.

[c]PCR conditions and average allele length in chicken reported by NAGRP.

[d]opimized PCR condition and PCR product length variation detected in a panel of 40 Japanese quail derived from 4 different populations: University of British Columbia (UBC), Japan (JAP), North Carolina wild type (NCW) and Alberta (ALT).

Table 2. The number of alleles detected at each of the 12 loci in this test panel and their repeats.

Locus name[a]	Number of alleles[b]	Observed PCR product size range[b] (bp)	Repeat detected in Japanese quail[b]	Repeat reported in chicken[c]
ADL023	1	132	X	$(CA)_5(CG)_4(CA)_9$
ADL024	1	206	$(CT)_3N_{77}(CT)_3TT(AT)_3N_{43}(CT)_4$	$A_{10}N_{66}(CA)_6$
ADL037	2	176-180	$(CAG)_3N_9(CA)_3TA(CA)_8$	$(CAG)_3N_9(CA)_8$
ADL038	NA[d]	130-140	NA[d]	$(GT)_7(AT)_9$
ADL111	2[e]	112-114	X	$(TG)_{15}(T_4G)_5T_7$
ADL142	y[f]	204-214	$(AT)_3T(CT)_{11}A(AC)_7$	$(AC)_{11}AG(AC)_7$
ADL143	1[e]	138	$(AT)_9N_{18}(AG)_3$	$(TG)_{10}(TA)_5$
ADL206	4[e]	95-101	$(AG)_3(TG)_7$	$(GT)_{23}$
ADL257	1	118	X	$(CA)_{14}(CAA)_{10}$
ADL315	5	236-250	$(CT)_6(CA)_5$	$(CT)_8(CA)_9$
ADL366	3[e]	226-232	$(AG)_3$	$(AC)_{14}$
HUJ006	4	99-107	$(AC)_9$	$(AC)_n$

[a]the first 3 letters designate the laboratory name: ADL=USDA ARS Avian Disease and Oncolgy Lab; HUJ=Hebrew University of Jerusalem

[b]number of allele, alelle size range and repeat detected at each of these 12 loci in a panel of 40 Japanese quail derived from 4 different populations: University of British Columbia (UBC), Japan (JAP), North Carolina wild type (NCW) and Alberta (ALT).

[c]repeat at these 12 microsatellite loci in chicken reported by NAGRP.

[d]these bands were too faint to be scored. Further modification of PCR conditions for this locus is required in order to determine the number of alleles in the test panel and to sequence strand of alleles.

[e]this locus may contain a null allele. However, the presence of this possible null allele was not counted for the number of alleles detected in the test panel since further investigation is required to proof that this locus indeed contains a true null alelle.

[f]difficult to estimate the number of allele present at this locus due to high amount of non-specific signals present at this locus.

NA = no data

X = no repeats

Table 3. Primer sequences detected in Japanese quail and primer sequences of the 12 chicken-specific primer sets.

Primer ID[d]	Primer sequence in Japanese quail[a]		Primer sequence in chicken[b]	
	Forward[c]	Reverse[c]	Forward[c]	Reverse[c]
ADL023	NA	CCT G**T**C **C**NT GTA TGT GTT GC	CTT CTA TCC TGG GCT TCT GA	CCT GGC TGT GTA TGT GTT GC
ADL024	NA	**A**TT CCA TTA CAG AG**A** GAG GT	TGA AGC AAA AAC CCA GCA AG	GTT CCA TTA CAG AGT GAG GT
ADL037	ATG CCC CAA ATC TC**C** ACN TT	TNT CTA AAA TCC AGC CCT NA	ATG CCC CAA ATC TCA ACT CT	TCT CTA AAA TCC AGC CCT AA
ADL038	NA	NA	TCG CCT GTG AAC TCT TAC CC	AGA CAT TCT CTA ATC ATT CC
ADL111	CCT TCC TGA CCT TCC ACT TC	CCA CAA AAA TAC CCA CCA TC	CCT TCC TGA CCT TCC ACT TC	CCA CAA AAA TAC CCA CCA TC
ADL142	NA	CTN TAG ATN CC**C** NGG AGT GC	CAG CCA ATA GGG ATA AAA GC	CTG TAG ATG CCA AGG AGT GC
ADL143	CCN GTN TCT GNT CTT TAT CC	NGT TTA CTT CCT TTT CTT GC	CCT GTC TCT GGT CTT TAT CC	AGT TTA CTT CCT TTT CTT GC
ADL206	TTT CTA TCC **C**TC ATC TCC AG	NA	TTT CTA TCC TTC ATC TCC AG	AGA CAT CCT GCT TTC TCG TG
ADL257	ATC TTG AAA CCT CAC AAA GC	TCT TCC AAC CTA TTT TTA GT	ATC TTG AAA CCT CAC AAA GC	TCT TCC AAC CTA TTT TTA GT
ADL315	NA	NA	TCC TTG GGC AGT AGT TTC AA	CTC CCA TGT TGC TTC TTT AG
ADL366	NA	CNC CAT TTG CCT CAC CAA CT	AGC TCC TTG TAC CCC TTT GC	CAC CAT TTG CCT CAC CAA CT
HUJ006	GGN ACN TGT ANA C**GC** A**G**G C	AGT AGT CCA TTT CCA CAG CCA	GGA ACA TGT AGA CAA AAG C	AGT AGT CCA TTT CCA CAG CCA

[a]according to sequenced strand of allele.

[b]primer sequences of the 12 chicken-specific primer sets reported by NAGRP.

[c]primer sequences are written in the 5' to 3' direction.

[d]the first 3 letters designate the laboratory name: ADL=USDA ARS Avian Disease and Oncolgy Lab; HUJ=Hebrew University of Jerusalum

NA = data not available; primer sequence was not detected in the test panel due to technical problem during direct sequencing.

Bold and Underlined letter = nucleotide that is different from the reported primer sequence in chicken.

Table 4. List of 16 chicken-specific microsatellite primer-pairs generated non-specific PCR amplification products in Japanese quail template.

Locus name[a]	Repeat reported in chicken[c]	PCR condition tested in Japanese quail DNA template: T (°C), final Mg^{++} (mM)[b]	PCR annealing temp. in chicken DNA template (°C)[c]	Average allele length (bp)[c]
ADL036	$(GT)_8(AT)_5(AGAT)_4$	35-47; 2.0-3.0	40	288
ADL101	$(GT)_3G_2(GT)_7$	45-57; 2.0-3.0	51	171
ADL114	$A_8(CAA)_7$	35-55; 2.5-3.0	45	185
ADL115	$(TA)_8(GTT)_9$	39-50; 2.0-3.0	45	109
ADL117	$(TTG)_{10}$	37-50; 2.0-3.0	45	184
ADL121	$(AAC)_7$	43-55; 2.5-3.0	50	135
ADL124	$T_6(GTT)_6$	35-55; 2.5-3.0	45	252
ADL158	$(CA)_{12}$	48-57; 2.0-3.0	52	216
ADL176	$(GT)_{12}$	48-57; 2.0-3.0	52	192
ADL190	$(TA)_5(TG)_{14}$	42-57; 2.0-3.0	47	220
ADL219	$(CA)_8A_{10}$	35-55; 2.5-3.0	45	107
ADL267	$(CA)_{12}$	40-55; 2.0-3.0	50	117
ADL288	$(GT)_6GC(GT)_4$	38-53; 2.5-3.0	48	152
ADL299	$(TG)_{24}$	49-59; 2.0-2.5	54	159
ADL312	$(CA)_6CG(CA)_{10}$	40-55; 2.0-3.0	50	153
HUJ005	$(AC)_n$	40-60; 2.0-2.5	55	150-170

[a]the first 3 letters designate the laboratory name: ADL=USDA ARS Avian Disease and Oncolgy Lab; HUJ=Hebrew University of Jerusalum

[b]PCR conditions tested at each of these 16 loci in a panel of 40 Japanese quail derived from 4 different populations: University of British Columbia (UBC), Japan (JAP), North Carolina wild type (NCW) and Alberta (ALT).

[c]repeat, average alelle length in sequenced clone and suggested annealing temperature for each microsatellite primer set reported by NAGRP. All primer-pairs were highly polymorhic on the East Lansing and/or Compton reference mapping populations.

Table 5. Estimates of the observed and the expected heterozygozity for 3 microsatellite loci in Japanese quail.

Locus name[a]	Unbiased estimates of heterozygozity[b] and F_{IS}[e]	Strain				Average[d]
		UBC[c]	JAP[c]	NCW[c]	ALT[c]	
ADL037	Observed[b]	0.47	0.21	0.53	0.11	0.33
	Expected[b]	0.52	0.51	0.52	0.27	0.45
	F_{IS}[e]	0.10	0.58	-0.01	0.61	0.32
ADL315	Observed[b]	0.63	0.53	0.47	0.32	0.49
	Expected[b]	0.63	0.65	0.47	0.44	0.55
	F_{IS}[e]	-0.01	0.19	0.00	0.28	0.11
HUJ006	Observed[b]	0.67	0.36	0.69	0.59	0.58
	Expected[b]	0.56	0.48	0.65	0.57	0.56
	F_{IS}[e]	-0.20	0.25	-0.05	-0.04	-0.01

[a]the first 3 letters designate the laboratory name: ADL=USDA ARS Avian Disease and Oncolgy Lab; HUJ=Hebrew University of Jerusalum

[b]estimates of heterozygozity calculated using equation (2) on Appendix 3.

[c]4 different populations of Japanese quail: University of British Columbia (UBC), Japan (JAP), North Carolina wild type (NCW) and Alberta (ALT).

[d]mean values for this locus.

[e]inbreeding coefficient of Wright (1951): $F_{IS} = 1 - H_o/H_e$

Fig.1. Representative modified polyacrylamide gels showing length polymorphism for 7 microsatellite loci in Japanese quail.

A. ADL037

B. ADL142

C. ADL143

D. ADL206

E. ADL315

F. ADL366

G. HUJ006

1A. Lanes 1 and 13 are M3 markers. Lanes 2 to 6 are individuals from North Carolina wild type population (NCW) and lanes 7 to 12 are individuals from Alberta population (ALT).

1B. Lanes 1, 2 and 13 are M3 markers. Lanes 3 to 8 are individuals from North Carolina wild type population (NCW) and lanes 9 to 12 are individuals from Alberta population (ALT).

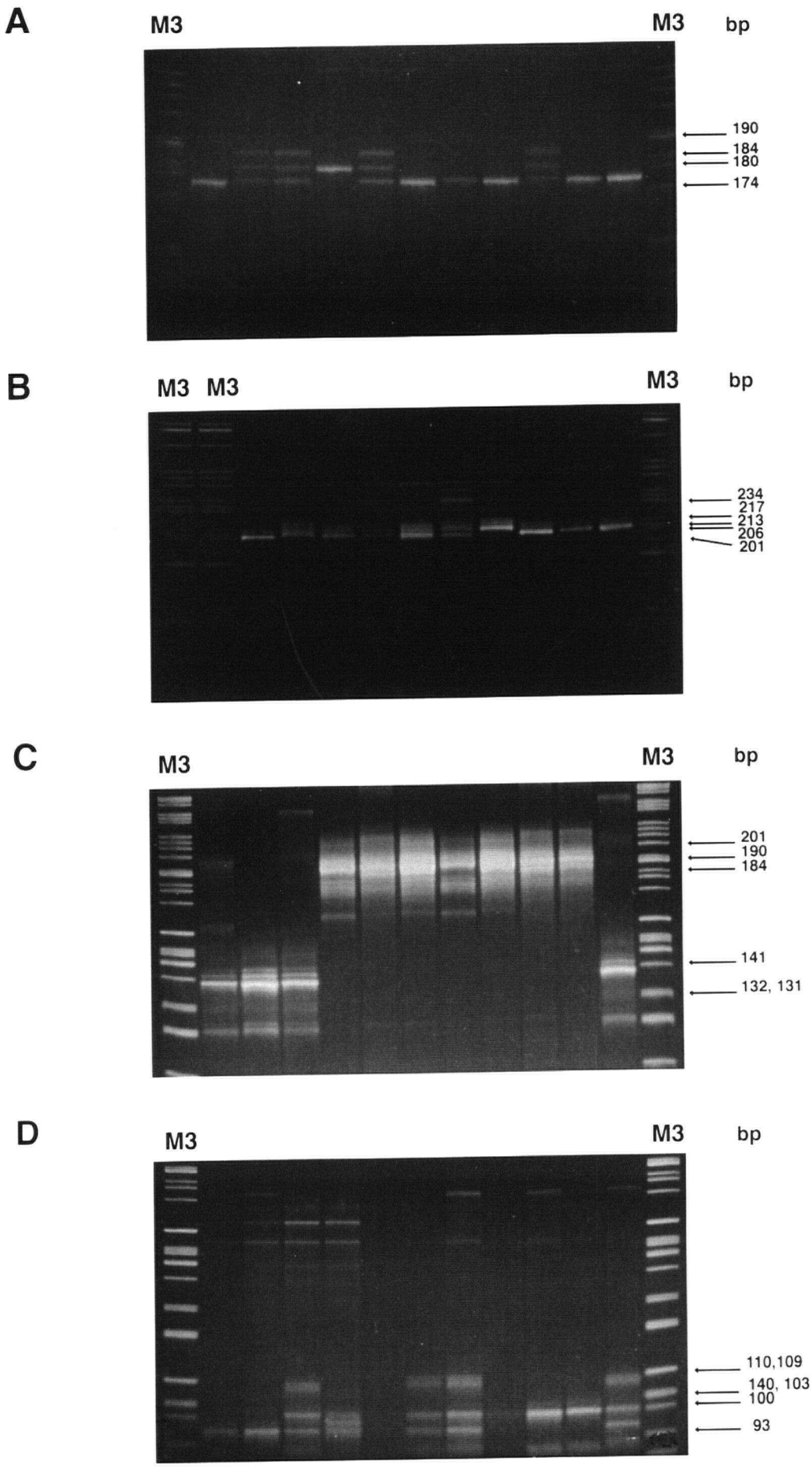
1C. Lanes 1 and 13 are M3 markers. Lanes 2 to 3 are individuals from University of British Columbia population (UBC). Lanes 4 and 12 are individuals from North Carolina wild type population (NCW) and lanes 5 to 11 are individuals from Alberta population (ALT).

1D. Lanes 1 and 13 are M3 markers. Lanes 2 to 5 are individuals from Japan population (JAP). Lanes 6 to 7 are individuals from North Carolina wild type population (NCW) and lanes 8 to 12 are individuals from Alberta population (ALT).

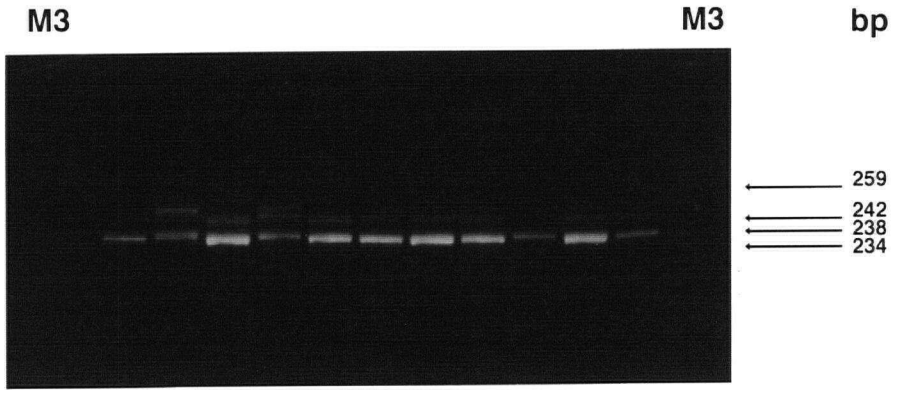
1E. Lanes 1 and 13 are M3 markers. Lanes 2 to 6 are individuals from University of British Columbia population (UBC). Lanes 7 to 11 are individuals from Japan population (JAP). Lanes 12 is an individual from North Carolina wild type population (NCW).

1F. Lanes 1 and 13 are M3 markers. Lanes 2 to 5 are individuals from North Carolina wild type population (NCW) and lane 6 is blank. Lanes 7 to 12 are individuals from Alberta population (ALT).

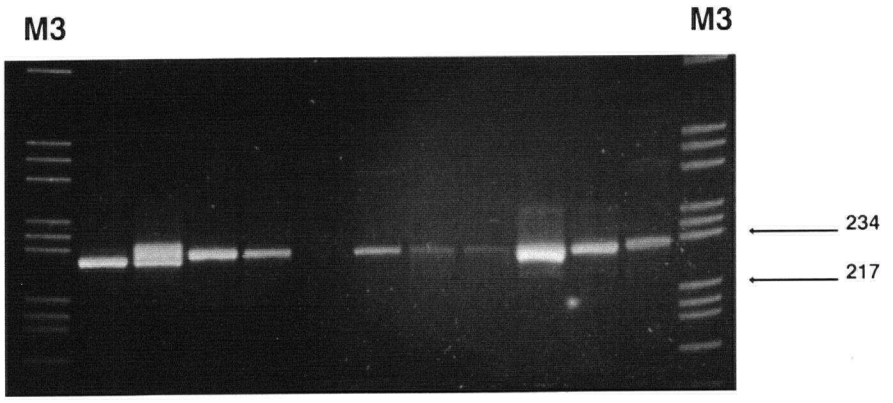
1G. Lanes 1 and 13 are M3 markers. Lanes 2 to 10 are individuals from North Carolina wild type population (NCW) and lanes 11 and 12 are individuals from Alberta population (ALT).


A
M3
M3
bp
190
184
180
174
B
M3 M3
M3
bp
234
217
213
206
201
C
M3
M3
bp
201
190
184
141
132, 131
D
M3
M3
bp
110,109
140, 103
100
93

E



F



G

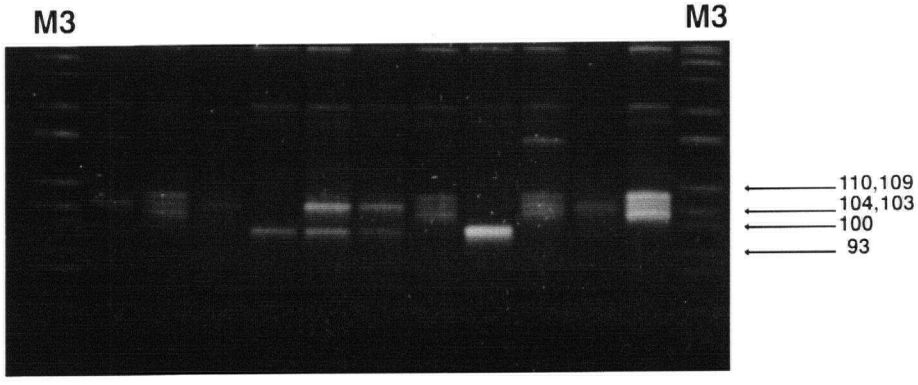


Fig.2. Representative modified polyacrylamide gels showing length polymorphism for 2 loci in Japanese quail.

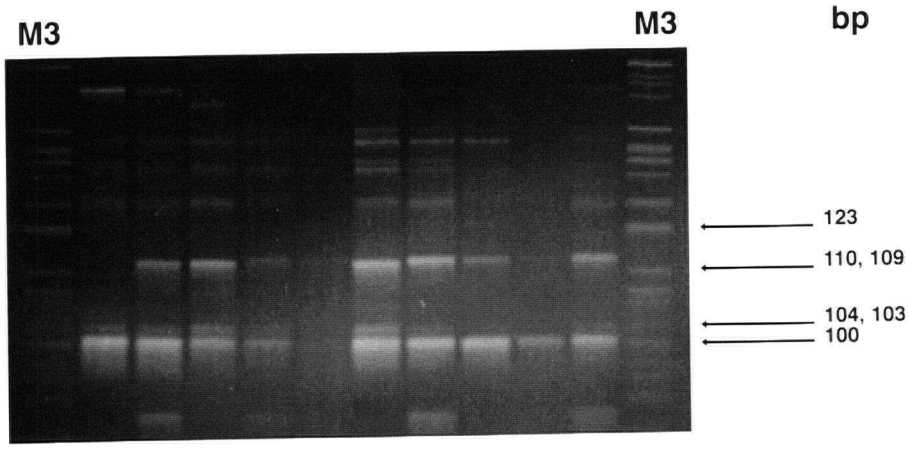
A. ADL111

B. ADL038

2A. Lanes 1 and 12 are M3 markers. Lanes 2 to 3 are individuals from Japan population (JAP). Lanes 4 and 9 are individuals from North Carolina wild type population (NCW) and lanes 10 to 11 are individuals from Alberta population (ALT).

2B. Lanes 1 and 12 are 20/100 bp markers. Lanes 2 to 7 are individuals from University of British Columbia population (UBC) and lanes 8 to 11 are individuals from Japan population (JAP).

A



B

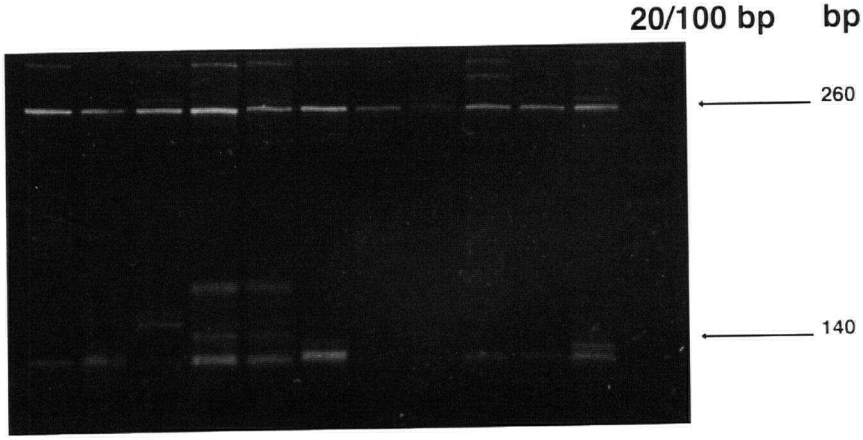


Fig.3. Representative modified polyacrylamide gels showing monomorphic PCR products for 3 loci in Japanese quail.

A. ADL023

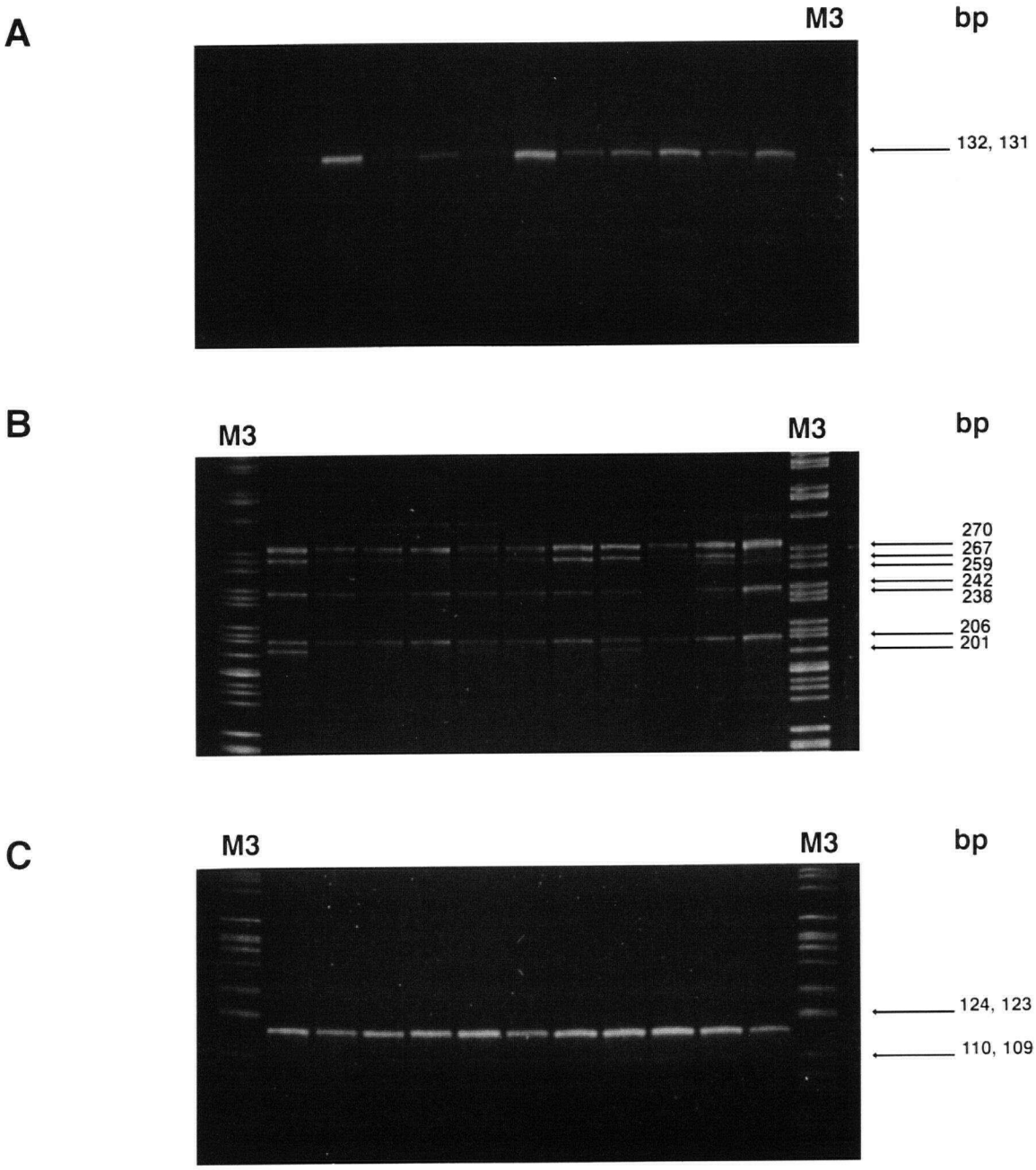
B. ADL024

C. ADL257

3A. Lanes 1 and 13 are M3 markers. Lanes 2 to 6 are individuals from University of British Columbia population (UBC) and lanes 7 and 12 are individuals from Japan population (JAP).

3B. Lanes 1 and 13 are M3 markers. Lane 2 is an individual from University of British Columbia population (UBC) and lanes 3 to 6 are individuals from Japan population (JAP). Lanes 7 to 9 are individuals from North Carolina wild type population (NCW) and lanes 10 to 12 are individuals from Alberta population (ALT).

3C. Lanes 1 and 13 are M3 markers. Lane 2 to 3 are individuals from University of British Columbia population (UBC) and lanes 4 to 5 are individuals from Japan population (JAP). Lanes 6 to 8 are individuals from North Carolina wild type population (NCW) and lanes 9 to 12 are individuals from Alberta population (ALT).


A
M3
bp
132, 131
B
M3
M3
bp
270
267
259
242
238
206
201
C
M3
M3
bp
124, 123
110, 109

SECTION 4

DISCUSSION

The conservation of flanking DNA allows microsatellite primer sets designed for chicken to amplify the corresponding loci in Japanese quail. Out of 48 chicken-specific primer sets tested in this study, 16 generated non-specific amplification products and 12 yielded specific products. Sequencing the specific amplification products confirmed that 8 of the 12 were indeed microsatellite loci in Japanese quail. This study is the first to detect microsatellite loci in Japanese quail. The results estimate that 25% of the primer sets designed for chicken could be useful in analysis of Japanese quail genome. The ability to use these heterologous microsatellite primer-pairs to amplify loci in closely related avian species alleviates some of the problems associated with developing species-specific primers for each new species of interest.

Much of the chicken genome has been mapped (NAGRP, 1998). Mapping efforts will shift increasingly towards other poultry species. A reference genome map will be developed for turkeys and will be used to compare with the chicken map. Further efforts will also be made to develop and use reference maps for ducks and quail which are next on priority list of the NAGRP. The results from this study may provide some insights on using heterologous microsatellite primer-pairs in other avian species.

4.1 Heterologous Amplification

The observed non-specific signals at loci ADL024, ADL038, ADL111, ADL142 and ADL143 (Figs. 3B, 2B, 2A, 1B, and 1C, respectively) is most likely due to introduction of primer mismatch positions from the sequence of Japanese quail. This could partially or completely prohibit the amplification of a targeted locus which were already associated with some amplification difficulties (Primmer *et al.*, 1997). These "stutter" or "ghost" bands seen in these 5 loci were inevitable artifacts in PCR amplification resulting from either slipped strand mismatch events as the *Taq* polymerase synthesizes the complementary strand of the repeat region during the amplification reactions, or prematurely termination of DNA extension. Cloning experiments carried by Luty *et al.* (1990) and Riess *et al.* (1990) have demonstrated that extra bands lack one or a few repeat units. The presence of these stutter bands did not prevent scoring at these 4 loci since the occurrence of these additional bands was recognized as less intensive bands which were usually smaller in size than the main band (Love *et al.*, 1990). Distinct high-molecular-weight bands were observed in some of the amplification products with primers ADL288, ADL124, ADL121 and ADL107 which were much greater than any of the expected sizes of bands (Table 4). These non-specific large bands could be due to incomplete extension by the *Taq* polymerase and/or terminal transferase activity of the enzyme (Love *et al.*, 1990), or, more likely due to amplification of non-target regions of the genome.

The size difference between the observed PCR products in Japanese quail template and the reported average allele length in chicken using the same primer sets varied greatly from locus to locus. Since only the average allele length of these polymorphic markers were

reported in chicken, the average products size (not the allele size range) could be compared between these 2 species. Markers ADL037, ADL038, ADL315 and ADL366 generated PCR products of about the same size in Japanese quail and in chicken (Table 1). The remaining 8 markers yielded PCR products that were quite different in size (ranging from 12 bp to 69 bp) than the ones reported in chicken. However, 2 of these 8 markers (ADL111 and HUJ006) generated PCR products with average sizes only 12 bp and 15 bp, respectively, apart from the reported ones in chicken. If these 2 loci were highly polymorphic in chicken, it was possible that there might be some overlapping in the product size range at these 2 markers in chicken and in Japanese quail. In summary, most of these 12 markers tested generated PCR products that were different in size than their corresponding PCR products in chicken (Table 1). There are two possible explanations for these results. First, these loci were probably subjected to different rates and directions of mutation in Japanese quail and chicken during evolution and thus, one could not expected their nucleotide structure to be the same. Second, it is possible that some of these amplified loci in Japanese quail were not homologous to the chicken loci.

In general, the optimized annealing temperatures used in Japanese quail were lower than the ones used in chicken (Table 1). This is expected because decreased in the annealing temperature would allow a certain degree of mismatches between the chicken primers and the heterogeneous template sequences, and hence, would allow amplification of DNA fragments in Japanese quail.

4.2 Detection of the repeat units

An alternative approach for confirming that a microsatellite locus has been amplified would be to hybridize a microsatellite probe, e.g. (CA)n and/or (CT)n, to the amplification products using Southern blot analysis. However, homology confirmation by sequence analysis is a much quicker and easier method since Southern blots may require substantially more DNA and the use of labeled probes and is more time-consuming to perform than any other PCR-based methods. Besides, Primmer *et al.* (1997) have suggested that Southern blots might incorrectly indicate a false amplification in cases where the repeat region has decreased in size or acquired interruptions to an extent (during microsatellite evolution) which no longer allows detection through hybridization. For instance, the short repeats seen at loci ADL024 and ADL366 in Japanese quail (Table 2) would probably not be detected through Southern blot hybridization.

4.3 Sequence characteristics and analyses

The BLAST program was used to search for similarity in nucleotide sequences between the Japanese quail and the chicken. Since the comparison between the primer sequences detected in Japanese quail and the reported ones in chicken has been examined earlier, only comparison between the non-priming flanking sequences in these 2 species would be discussed here. The results from the BLAST search showed that there was considerable homology between the non-priming flanking sequences in these 2 species at ADL037, ADL023 and ADL366. At locus ADL037, there were 82% (92 nucleotides) and 85% (20 nucleotides) identity to the non-priming flanking sequence reported in chicken.

There was 76% (47 nucleotides) identity and 93% (181 nucleotides) identity to the non-priming flanking sequence reported in chicken at loci ADL023 and ADL366, respectively. At the first glance, the results from the BLAST search showed no homology between nucleotide sequences in Japanese quail and in chicken at loci ADL023, ADL038, ADL111, ADL142, ADL143, ADL257, ADL206, ADL315 and HUJ006. Sequences of these 9 chicken primer sets were entered into the BLAST program and no matching sequences could be found. These results might indicate that the nucleotide sequences of these loci were not submitted to the database. Therefore, it is impossible to conclude whether these 9 loci detected in Japanese quail were homologous to the ones in chicken.

ADL037

The observed polymorphic variation of this locus in Japanese quail could be represented by a repeat number variation within the microsatellite repetitive regions (Fig. 1A). The repeat units detected in Japanese quail was $(CAG)_3N_9(CA)_3TA(CA)_5$; whereas, in chicken, the repeat array was $(CAG)_3N_9(CA)_8$. The differences in the composition of the microsatellite repeats between the 2 species seemed to be an insertion of a TA dimer within the (CA) repeat motif. Another explanation is that the alteration was caused by first an expansion of CA repeat by 1 unit, followed by a single C→T substitution in the 25th base pair of the repeat, resulting in a CATA motif interrupting the CA repeat array. This hypothesized pattern of mutation was supported by Tautz's finding (1986) that random point mutations within a simple sequence could lead to changes in nucleotide composition and would fit with a simple SMM through a strand-slippage mechanisms (Strand *et al.*, 1993). Other alternative

mechanisms for the variation observed cannot be ruled out. Nave *et al.* (1997) claimed that a single point mutation would be very unlikely to be the cause for the variability in microsatellites. Therefore, the underlying molecular mechanism remains unclear and further investigation of the mechanisms of microsatellite evolution at this locus is required.

An A→C base substitution within the forward primer sequence was detected from the sequenced strands of allele at locus ADL037. Even though it is possible that the A→C transition could be due to sequencing error, the chance is very small (0.2%-0.9%) (Tracy Evans, NAPs Unit, UBC, personal communication). The change in the composition of the primer sequence was probably due to point mutation (base substitution) since these primers were designed for chicken and were used to examine the genome of Japanese quail.

ADL315

The repeat $(CT)_6(CA)_5$ was detected in Japanese quail and was very similar to the one reported in chicken, $(CT)_8(CA)_9$ (Table 2). The observed length polymorphism at this locus in Japanese quail was probably caused by variation in the number of repeat units within the microsatellite repetitive regions (Fig. 1E). The shorter repeats found in Japanese quail support the general observations in diverse taxa that longer microsatellite repeats are usually found in the focal species (species which microsatellite markers have originally been developed) than in related species (e.g. Deka *et al.*, 1995; Ellegren *et al.*, 1995). Rubinsztein *et al.* (1995) have interpreted the results from these unidirectional analyses as evidence for a

difference in evolutionary rate between species, and have suggested that microsatellite evolution may be directional and proceed with different paces in different lineages. In support of Rubinsztein's view, Amos *et al.* (1996) also showed that mutation rate was correlated with heterozygozity and the difference in length between alleles in a heterozygote could increase instability relative to a homozygote. In this case, microsatellite loci would evolve faster in larger populations than those in smaller ones. On the other hand, Ellegren *et al.* (1997) have cautioned that in order to properly compare the rate and direction of microsatellite evolution in 2 species, a reciprocal analysis is necessary. They have demonstrated that microsatellites developed from the bovine genome are longer in cattle than in sheep and, conversely, microsatellites developed from the ovine genome are longer in sheep than in cattle. This bias could be explained by the usual selection of loci from the upper end of the repeat length distribution in the genome (which is known to harbor the most polymorphic markers) (Weber, 1990) when isolating informative molecular markers. This simple selection bias related to the cloning procedure would allow a non-random choice of loci with particularly longer-than-average repeat units in the focal species but would give shorter repeat units at homologous loci in related species (Ellegren *et al.;* 1997). The reciprocal analysis of markers developed from species will be necessary to provide a good mean for comparison of the rate and direction of microsatellite evolution in any 2 species.

ADL142, ADL143 and ADL206

Results from direct sequencing (in both directions) of the PCR product obtained from amplification with primer-pairs ADL142, ADL143 and ADL206 indicated that microsatellite loci were amplified in Japanese quail. The observed polymorphic variation at these loci is likely due to varying number of repeat units within the microsatellite regions (Fig. 1B, 1C, 1D, respectively, and Table 2).

ADL111

Two major products of different sizes were detected at locus ADL111: 112-114 bp and 100 bp, respectively (Fig. 2A). To clarify whether these bands were alleles that were very different in fragment size, or they were 2 different PCR products, strands of alleles from each band (114 bp and 100 bp) were sequenced. The results showed that they were 2 different PCR products because there was no homology in the flanking sequences between the 2 products. In fact, both PCR products did not contain any repeats. Even though there was 100% homology in the primer sequences in Japanese quail and chicken, but no repeat was detected and the percentage of nucleotide sequence identical to the corresponding chicken locus is unknown, it is difficult to conclude if this locus ADL111 was indeed the homologous locus in chicken. The length variation of PCR products (112-114 bp) observed at locus ADL111 in Japanese quail was probably due to mutations occurred within the flanking sequences since no repeats were detected at this locus (Fig. 2A).

HUJ006

Length polymorphism was observed at the detected locus with chicken-specific primer-pairs HUJ006 in Japanese quail (Fig. 1G, Table 2). The observed length variation at this locus seemed to be due to variation in the number of AC repeat. An $(AC)_9$ repeat and both primer sequences were detected at this locus by direct sequencing. It is unlikely that a non-homologous locus was amplified in Japanese quail since the priming sequences in both species were very similar and the frequency of $(AC)_{\geq 10}$ was low (7,000-9,000) in avian genome (Raudsepp *et al.*, 1997).

ADL023 and ADL366

Amplification products from loci ADL023 (Fig. 3A) and ADL366 (Fig. 1F) in Japanese quail were sequenced and only the reverse primer could be identified in both forward sequencing reactions. No microsatellite repeats were detected in locus ADL023 and a very short microsatellite repeat, $(AG)_3$, were detected in locus ADL366 in Japanese quail.

Results from the BLAST search showed 76% identity (47 nucleotides) between the microsatellite DNA in chicken and the flanking sequences in Japanese quail at locus ADL023. This mild similarity between the DNA sequences in these 2 species may imply that the homologous microsatellite locus amplified in the Japanese quail has been subjected to different rate and direction of evolution than that in the chicken. Another explanation is that non-target amplification products were detected in Japanese quail and the observed similarity at this locus between the 2 species is purely due to coincidence.

Results from BLAST search for the microsatellite product from locus ADL366 showed that this sequence has 93% (181 nucleotides) identical to the corresponding chicken ADL366 locus. This sequence similarity strongly suggests that the homologous locus of the chicken ADL366 was amplified in Japanese quail. Surprisingly, an (AG) repeat, instead of the expected (AC) repeat, was detected in Japanese quail (Table 2). There was high conservation of sequences flanking the repeat but no conservation of the repeat itself. On one hand, constraints to restrict the expansion and loss of repeat units to a bounded intervals should have prevented complete loss of the (AC) repeat at this locus (Bowcock *et al.*, 1994; Goldstein *et al.*, 1995; Nauta and Weissing *et al.*, 1996; Feldman *et al.*, 1997). On the other hand, the large differences between the composition and the length of the repeat units observed in ADL366 of these 2 species may be due to an exceptionally high mutation rate of microsatellite repeat at this locus. Microsatellites have been reported as the inherently unstable regions of the eukaryotic genomes that undergoing mutational changes at rates much greater than that reported for non-repetitive DNA sequences (Sia *et al.*, 1997). If the latter is true, the results might indicate that there was little constraint acting on this ADL366 microsatellite locus. The 3 sequences from amplification products at loci ADL023, ADL037 and ADL366 were found to have considerable similarity between the quail and the chicken in this study. This dissimilarity between the quail and chicken ADL366 implied that constraints acting on microsatellite loci might not be as stringent as suggested by researchers in recent studies (Bowcock *et al.*, 1994; Goldstein *et al.*, 1995; Nauta and Weissing *et al.*, 1996; Feldman *et al.*, 1997). Further studies such as reciprocal analysis will be necessary to

determine the nature of microsatellite evolution at this locus in both chicken and Japanese quail (Ellegren *et al.*, 1997).

It is very interesting that polymorphism was observed in this locus (ADL366) in Japanese quail. There were 2 detected alleles (232 bp and 228 bp). The longer PCR product was sequenced and found to contain an $(AG)_3$ repeat. According to Messier *et al.* (1996), a minimum number of repeat units will be necessary before initial expansion occurs. Therefore, while possible, it is very unlikely that the length variation between the 2 PCR products was due to variation in the number of repeat units. Point mutations may have created 3 repeat units of AG but not enough repeat units for subsequent microsatellite expansion. However, it is also possible that the $(AG)_3$ repeat could be caused by contraction/reduction in repeat units assuming that there was no constraint acting upon this locus. In either cases, mutations within the flanking sequences immediately follow the primers would have resulted in the observed allelic length differences between the 2 PCR products at this locus.

ADL024 and ADL257

Of the 12 loci that were examined, 3 were monomorphic: ADL023, ADL024 and ADL257 (Fig. 3A, 3B and 3C respectively). The nature of the ADL023 locus detected in Japanese quail has already been discussed earlier in this section. The nature of the remaining 2 monomorphic loci were very different from each other.

Although the $(CT)_3N_{77}(CT)_3TT(AT)_3N_{43}(CT)_4$ repeat was detected in the ADL024 locus, it is unlikely that there would be any variation in the number of repeat units within the

microsatellite sequence since they were small in numbers and dispersed. It is possible that some of these short dinucleotide repeats seen at this locus may be the result of point mutations similar to the case in old world monkeys (Messier *et al.*, 1996).

Another monomorphic locus detected in Japanese quail is ADL257 (Fig. 3C). No repeat was detected in the sequenced strand and no sequence similarities were observed between this detected locus in Japanese quail and the one reported in chicken (Table 2). Therefore, the PCR products generated in Japanese quail was probably amplified DNA fragments from a locus that was non-homologous to the one in chicken.

ADL038

A major monomorphic band of 260 bp in size and a polymorphic fainter band of 130-140 bp in size were detected with primer-pairs ADL038 in Japanese quail (Fig. 2B). Sequencing these alleles was unsuccessful due to weak signals. The fainter bands (range from 130-140 bp in length) seem to be polymorphic and closer to the allele size reported in chicken (NAGRP). Further optimization of PCR condition is necessary for proper PCR product scoring and sequencing analysis.

Successful amplification and sequencing of PCR products for each of 12 loci suggests that the degree of sequence conservation around microsatellites and the level of microsatellite repeat conservation was variable among different loci. Due to the small number of microsatellite markers involved in this study, it is impossible to determine the preferential conservation of a particular repeat type. It is interesting to note that out of those 8 markers

that generated specific amplification products with microsatellite repeats, 6 were compounds (combination of 2 or more different kinds of repeat units) and 2 were perfects (uninterrupted runs of repeats). Further investigation of the 3 monomorphic loci detected in this study is warranted. Polymorphism may be revealed when larger sample size is applied or when different populations of Japanese quail are examined.

4.4 Possible null alleles at loci ADL111, ADL143, ADL206 and ADL366

The absence of strain-specific amplification product was observed in loci ADL111, ADL143, ADL206 and ADL366. Microsatellite products were not detected in 5 individuals within the UBC strain in locus ADL206, in all the individuals in both JAP and ALT strains in locus ADFL143 and in 1 individual from the UBC strain in locus ADL366. In addition, PCR products (112-114 bp in size) were not detected in 3 individuals within the JAP strain at locus ADL111. There are a few possible explanations for this observation. First, the primer sets were designed for chicken, not for Japanese quail. Even though the PCR conditions were optimized for each primer pair, the conditions were not optimized for each strain tested. Perhaps with certain quail strain/individual, further optimization on the PCR conditions is required in order to detect the targeted microsatellite product. Therefore, a positive control from each strain should be included in each optimization of PCR condition for each primer set. Second, such observation could be explained by the presence of null alleles. The occurrence of null alleles have long been known in allozyme loci (Foltz, 1986) and, more recently, in variable number tandem repeats (VNTR) loci (Chakraborty *et al.*, 1992 Chakraborty and Jin, 1992). Callen *et al.* (1993) showed that the incidence of null alleles

was 30% (7 out of 23 loci) in human microsatellites. The term "null" alleles may be somewhat misleading because it implies that there are absence of alleles. In fact, the alleles are not missing but are not visualized on the gel due to insufficient or loss of PCR amplification product. There are a few hypotheses involving mechanisms for the generation of "null" or non-amplifying alleles in microsatellite loci. The most popular and generally accepted hypothesis involved modification or mutation at one of the non-repeated primer-binding sites used for the PCR amplification (Grimaldi and Crouan-Roy, 1997; Stallings, 1995; Blanquer-Maumont and Crouau-Roy, 1996; Callen *et al.*, 1993; Koorey *et al.*, 1993). Mutations such as duplication, substitution, deletion and insertion within the primer-binding sequence would cause changes in the composition of the primer-binding sequence may inhibit or completely prevent their binding, resulting in either reduced or complete loss of amplification product (Callen *et al.*, 1993). Stalling (1995) suggested that instability/variability of these primer-binding regions could be caused by the repeat region itself or association with the repeat region.

In order to determine whether the absence of amplifying alleles in loci ADL111, ADL143, ADL206 and ADL366 is, in fact, due to the occurrence of null alleles, (1) large sample size of F_1 progeny has to be examined for the apparent non-inheritance of a parental allele in some offspring, (2) additional Japanese quail oligoprimers flanking the original chicken oligoprimers have to be constructed to avoid possible mutation sites and to generate PCR products, and (3) stringency for PCR amplification has to be lowered to permit certain levels of mismatch between the primers and the binding sites. First, F_1 population should be tested for deficiency of apparent heterozygozity (or excess homozygozity) observed at these

loci (i.e., significant deviations from Hardy-Weinberg equilibrium). The reasoning behind this proposal is that many scholars have suggested that the occurrence of null alleles in microsatellites could be recognized by the apparent non-inheritance of a parental allele in some offspring (Foltz, 1986), and the absence of apparent heterozygozity observed at some loci in some populations (Bruford and Wayne, 1993; Callen *et al.*, 1993; Pemberton *et al.*, 1995). The frequency of null alleles are usually underestimated especially when only the parents are being examined. When there is a PCR product from the other allele, the presence of segregating null allele would be undetected and individuals who are heterozygotes for a null allele may in fact be mistaken and scored as homozygotes. As a result, the inability to recognize the presence of null alleles would cause deficiency in apparent heterozygozity and marker heterozygozity would be underestimated and genotypes within a family would have apparent incompatibility. On the other hand, null alleles generally indicate the presence of polymorphism in the sequence flanking the repeat region. Second, PCR walking can be used to develop appropriate primers for rescoring all homozygotes and individuals that had produced no PCR products with the original primers (Lin *et al.*, 1995). These new Japanese quail oligoprimer flanking the original chicken oligoprimer would enable the amplification of the null alleles and hence, would allow the differentiation between the homozygotes and heterozygotes for these loci. By using an alternative sets of redesigned oligoprimers, Lehmann *et al.* (1996) discovered that 2/3 of the homozygotes examined at one mosquito (*A. gambiae*) locus (AG2H46) were indeed heterozygotes for a null allele. Third, Pemberton *et al.* (1995) has suggested that by allowing a certain level of mismatch involving different apparent homozygotes (i.e., by lowering primer stringency), the number of null alleles that

could be revealed on the gel would increase, and hence, revealing potential parents that carry a null allele.

4.5 Locus heterozygozity

The inability to detect null alleles can lead to apparent heterozygote deficiencies in comparison with Hardy-Weinberg expectations. The presence of null alleles can produce pseudohomozygozity if null heterozygotes are mistakenly scored as active homozygotes (Chakraborty *et al.*, 1992; Chakraborty and Jin, 1992; Foltz, 1986). Thus, it is inappropriate to calculate levels of heterozygozity at those 4 loci (ADL111, ADL143, ADL206 and ADL366) in which the presence of null alleles were suspected.

Since each heterozygote carries different alleles and represents the existence of variation, the amount of heterozygozity (i.e., the frequency of heterozygotes) observed in a population is a simple measure of genetic variation in that population. In general, individuals from random-mating populations may be considered to represent the species. However, in this context, it should be emphasized that even though individuals were randomly selected from random-mating populations, it is possible that the allelic frequency estimates from the samples may not accurately reflect the allelic frequency in the population because of small sample size. Thus, the estimates of the observed heterozygozity calculated were adjusted for small sample size (Appendix 3).

Detailed pedigree information of these populations was made known to the researcher only after the data were analyzed. The results of the analysis agreed with the history of these Japanese quail populations. The observed and the expected heterozygozity for 3

microsatellite loci (ADL037, ADL315 and HUJ006) were calculated (Table 5). The observed heterozygozity was close to or equal to the expected heterozygozity in the UBC and the NCW strains in all 3 loci (Table 5). This observation suggested that the UBC and NCW populations were close to Hardy-Weinberg equilibrium. Both the UBC and the NCW strains have been closed for many generations and were maintained as random-mating populations (Cheng and Nichols, 1992). Table 5 also showed that in the JAP and the ALT strains, the observed heterozygozity was lower than the expected heterozygozity, and indicated that they have less genetic variability than the other 2 populations. The JAP strain was initiated with a small number of individuals at the QGRC in 1989 and was maintained with a fairly small effective population size. Although the ALT strain was acquired from the University of Alberta in 1977 and had been maintained with 48 males and 96 females, the effective population size had just been reduced to 10 breeding pairs per generation 2 years ago. It is generally believed that when a population was exposed to founder/bottleneck effects, the subsequent genetic variation in the population would be determined by the founder size, the allelic frequency distribution and the intrinsic rate of increase after the initiation of the population (Hedrick, 1985). Inbreeding would further reduce the genetic variability in these 2 strains. As expected, the inbreeding coefficients were high in the JAP and the ALT strains at loci ADL037 and ADL315 when compared to the UBC and the NCW strains (Table 5). It should be pointed out that only 5 individuals from the UBC strain and 6 individuals from the JAP strain were successfully amplified at locus HUJ006. The extremely small sample size would make the heterozygozity estimates inaccurate.

The average observed heterozygozity over 3 microsatellite loci (ADL037, ADL315 and HUJ006) was 0.46 and the average expected heterozygozity was 0.52. This estimate of 0.46 is higher than the average heterozygozity of 0.11 and 0.08 (domestic and wild quail populations, respectively) estimated by Kimura and Fujii (1989) from 34 allozymes. In Cheng *et al.*'s study (1992), the expected heterozygozity for 2 polymorphic allozyme loci and the average was only 0.27. Data from this study clearly showed that microsatellite analysis detected greater variation in Japanese quail populations than allozyme.

4.6 Comparison between results from cross-species amplification among chicken, turkey (*Meteagridis galopario*), and Japanese quail

The first heterologous amplification study using chicken-specific primers was published by Levin *et al.* (1995) where 48 chicken microsatellite markers, previously developed in ADOL (USDA), were tested for their ability to amplify polymorphic fragments using turkey genomic DNA. Their results indicated that 92% (41 out of 48) of chicken-specific microsatellite markers generated some amplification products in turkey. Only 14 of 41 primer-pairs that they had tested for the presence of microsatellite repeat hybridized to a detectable $(TG)_n$ oligonucleotide probe. Among 18 primer-pairs that they had tested for polymorphism, only 5 were found to exhibit length polymorphism and 3 of which did not contain a detectable TG repeat. Based upon these results, Levin *et al.* (1995) claimed that a significant portion of chicken microsatellite markers were useful for genomic mapping and linkage analysis in the turkey.

In 1996, Liu *et al.* tested 88 chicken microsatellite marker (developed in Wageningen Agricultural University) in turkey genomic DNA. Their results showed that 61 out of 88 chicken-specific microsatellite markers (69%) gave rise to amplification products in the turkey. 16 of these 61 markers yielded non-specific products, 8 generated length polymorphic and the remaining 37 were monomorphic in 3 divergent turkey lines. Of the 8 chicken markers (9%) exhibited length polymorphism in turkey, only 5 of them contained a detectable (TG) repeat. The average number of alleles over these 8 loci, was found to be 2.1, lower than the average value of 3.0 that they had detected over 26 turkey microsatellite loci using microsatellite markers designed for turkey. Based upon these results, Liu *et al.* (1996) concluded that most chicken microsatellite markers were not very useful in turkey and the development of specific turkey microsatellite markers would be more efficient for the mapping and analysis of the turkey genome.

PCR products were separated and polymorphism were revealed on a 6% denaturing polyacrylamide gels in both turkey studies and the presence of a (TG) repeat was confirmed by Southern blotting (Levin *et al.*, 1995; Liu *et al.*, 1996). Among 18 primer-pairs tested for polymorphism by Levin *et al.* (1995), 5 revealed length polymorphism. Levin *et al.* (1995) claimed that more than 33% of the microsatellite primer-pairs developed for the chicken might be useful for analyzing turkey DNA. In contrast, Liu *et al.* (1996) reported 8 out of 88 primer-pairs (9%) tested were polymorphic from 3 turkey lines. In Japanese quail, 19% (9 markers) of the 48 markers exhibited length polymorphism (Table 1). This value was higher than the proportion of polymorphic markers reported in Levin *et al.*'s (1995) (10%; 5 out of 48 markers) and Liu *et al.*'s (1996) (9%; 8 out of 88 markers) studies.

Three out of 5 primer-pairs (60%) tested for the presence of a microsatellite repeat did contain a detectable repeat in Levin *et al.*'s study (1995). Similar results were obtained in Liu *et al*'s experiment where 5 out of 8 polymorphic markers (63%) contained a detectable repeat. A higher portion of polymorphic markers harbouring detectable microsatellite repeat was found in Japanese quail in this study. Among 9 polymorphic markers tested for the presence of a microsatellite repeat, 7 (78%) harboured a repeat (Tables 1 and 2). The proportion of DNA specific products harbouring microsatellite repeats was higher in quail than in turkey (Levin *et al.*, 1995; Liu *et al.*, 1996). The presence of a microsatellite repeat in amplified fragments were detected by hybridizing them to a end-labeled $(TG)_n$ probe in these studies. According to Levin *et al.* (1995), their hybridization conditions could detect a microsatellite sequence $\leq(TG)_5$ because little or no signal could be obtained with $\leq(TG)_4$. Therefore, it was possible that the turkey microsatellites that had significantly contracted were undetected via hybridization. Direct sequencing was used to detect microsatellite repeats in amplified fragments in Japanese quail. Among the 7 microsatellite markers, 2 harboured microsatellite repeats that were shorter than 5 repeat units (ADL024 and ADL366). If Southern blotting were used instead, the repeats present in amplified fragments of these 2 loci would not be detected. Therefore, the estimation for the proportion of polymorphic markers harbouring a microsatellite repeat (≥5 repeat units) would be reduced to 56% which was closer to the values reported in turkey by Levin *et al.* (1995) and Liu *et al.* (1996). Since ~60% of all the identified polymorphic markers generated amplification products containing microsatellite repeats, Liu *et al.* (1996) suggested that the chance of

finding polymorphism increased considerably when markers were first screened for the presence of a microsatellite repeat on the amplified fragments.

Even though the number of chicken-specific microsatellite markers exhibiting length polymorphism in turkey in Levin *et al.*'s study (1995) (9%) was similar to that of Liu *et al.* (1996) (10%), 2 very different conclusions were drawn by these 2 groups. Levin *et al.* (1995) claimed that a significant portion of the chicken microsatellite markers were useful in turkey; whereas, Liu *et al.* (1996) concluded that most chicken microsatellite markers were not useful in such analysis. Levin *et al.* (1995) reported that 92% of chicken-specific microsatellites generated amplification products. Their high proportion of microsatellite markers amplifying DNA fragments could be explained by the unusually high final concentration of $MgCl_2$ (4 mM) and relatively low annealing temperature (40°C) that they had used for all their PCR reactions. Such low PCR stringency would allow high degree of mismatches between primer and template sequence and hence, would also permit amplification of DNA sequences at the corresponding locus in chicken and/or at other non-target loci in the turkey genome. Liu *et al.* (1996) used only 1.5 mM as the final concentration of $MgCl_2$ and different annealing temperatures of 40-50°C for their PCR reactions. This relatively low final concentration of $MgCl_2$ would only allow template sequences that were very similar to the primers to be amplified. Therefore, only 69% of the 88 chicken microsatellite markers generated amplification products. In this study, even a lower percentage, 58%, of the 48 chicken-specific primer sets tested in Japanese quail yielded amplification products. The low proportion of chicken microsatellite markers amplifying DNA fragments in this study when compared to those reported in turkeys could be explained

by the higher PCR stringency that were employed in amplification reactions were used in Japanese quail templates (Table 1).

The average observed number of alleles over 7 polymorphic loci (ADL037, ADL111, ADL143 ADL206, ADL315, ADL366 and HUJ006) was 3.0 in Japanese quail. Whereas, only an average of 2.1 alleles was detected in turkey over 8 chicken markers that Liu *et al.* (1996) had tested. The low number of alleles detected in turkey could be due to higher inbreeding (caused by artificial insemination) in 3 commercial turkey lines that Liu *et al.* (1996) had used for their study. The mean observed heterozygozity estimated in Japanese quail were 0.33, 0.49 and 0.58 for loci ADL037, ADL315 and HUJ006 (Table 5). These values were also higher than the observed heterozygozity of 0.15 to 0.50 reported in turkey by Liu *et al.* (1996). Both the observed heterozygozity (0.33-0.58) and the average number of alleles (3.0) observed in Japanese quail is high compared with turkey (Liu *et al.*, 1996). On the basis of these results, the chicken microsatellite markers should be more useful for mapping and analysis of the genome in Japanese quail than in turkey.

It is interesting to note that 38 of the 48 chicken-specific microsatellite markers used in Japanese quail have also been tested on turkey by Levin *et al.* in 1995. They did not, however, reported the proportion of markers that generated specific products in the cross amplification. On the other hand, they reported that only 10% of those microsatellite markers revealed polymorphism (Levin *et al.*, 1995). In my study, 19% of the markers tested revealed polymorphism in Japanese quail. Assuming that the frequency of polymorphic markers reflected the frequency of markers that generated specific products in cross amplification, chicken microsatellite primers could amplify more specific amplification

products in Japanese quail than in turkey. This is expected because chicken and Japanese quail are more closely related (Sibley and Ahlquist, 1990; Crawford, 1990; Wetmore A, 1960; Delacour, J: 1964) than turkey. Many recent studies suggested that the relative success of applying heterologous markers would increase with decreasing genetic distance (Primmer *et al.*, 1996; Moore *et al.*, 1991; Schlolterer *et al.*, 1991; Stallings *et al.*, 1991). Chicken and Japanese quail are in the same order, family and subfamily (*Galliformes Phasianidae Phasianinae*) (Lewis, 1991). Whereas chicken and turkey are only in the same family and was thought to diverge more than 12 million years ago (Lewis, 1991). Besides, ancestors of both the chicken (jungle fowl) and Japanese quail are native to Asia whereas the wild turkey is endemic to Central America.

4.7 Special primers

It is interesting to note that primers ADL037 and ADL038 have not only been able to amplify corresponding microsatellite loci in Japanese quail, but also in turkey (Liu *et al.*, 1996). Moreover, these 2 markers have shown conservation of sequences in these 2 fairly diverse avian species. The fact that these markers successfully amplified microsatellite loci in all 3 species analyzed suggests that these markers may be potential universal markers for avian population studies. They may be at least applicable to other *Galliformes*. One potential use for these markers will be to test if there are any species-specific alleles among the *C. coturnix* and *C. japonica*. If species-specific alleles can be found, they will facilitate the clarification of the genetic relationships among different laboratory strains of Japanese quail which may have origin involving *C. coturnix*. If some of the laboratory strains of

Japanese quail commonly used in comparative studies are shown to have an origin involving *C. coturnix*, then an establishment of a reference population is necessary for comparison of results from different studies. Microsatellite analyses have been proven very useful in discriminate the pure Asiatic lions from the hybrids based on polymorphism revealed at 2 loci (Shankaranarayanan *et al.*, 1997). If these marker have a more universal application, the high level of polymorphism and the availability in other avian species will make them useful genetic markers for future population studies of individual species and powerful tool in various applications when the genetic characteristics of 2 or more species are to be compared.

Microsatellites are favored over mtDNA in many studies because with microsatellite markers, both the paternal and the maternal background can be traced. Because of the exceptionally high mutation rates of microsatellites (which are higher than that of found in mitochondrial DNA), these markers may be used in phylogeny construction between very closely related species as well as among subpopulations of the same species (e.g. Weber and Wong, 1993; Primmer *et al.*, 1997; Buchanan *et al.*, 1994; Bowcock *et al.*, 1994). Two greatest concerns for extension of microsatellite loci to phylogenetic reconstruction of more distantly related species are the rate of degradation of microsatellites (their longevity) and range constraints on allele size (Goldstein and Pollock, 1997). First, it has been suggested that microsatellite may eventually lose their phylogenetic information in time (Goldstein *et al.*, 1995). Some microsatellite may degrade and their frequency fixed much quicker in some taxa than others causing their mutation rates to differ greatly from taxon to taxon (Ellegren *et al.*, 1997; Rubinsztein *et al.*, 1995). As a result, genetic distances at these loci may not appear well correlated with time for more divergent taxa. Second, microsatellite may fail to

reflect separation times among species due to constraints acting on these loci (see Introduction 1.3.6). This disparity may not reflect differences in evolutionary dynamics, but may result from the potentially large errors caused by concerns described above. For example, the microsatellite-derived divergence time between Zebu cattle and domesticated taurine ancestral populations is estimated to be 610,000-850,000 years ago which is approximately 3 times larger than the upper bound of the data derived from mtDNA sequence analysis (275,000). Until more reasonable adjustments for estimates of rate of degradation and range constraint are developed (Goldstein and Pollock, 1997; Feldman *et al.*, 1997; Nauta and Weissing, 1996), it will be wise to select microsatellite loci with lower allelic variation and higher mutation rate and use them only for making estimates on populations in closely related species (Buchanan *et al.*, 1994; Macaubas *et al.*, 1997).

SUMMARY AND CONCLUSION

In this work, 9 polymorphic markers (7 contained microsatellite repeats) were detected in Japanese quail using chicken-specific microsatellite primers. With larger sample size, these microsatellite markers will permit the assessment of genetic relatedness of individuals and populations of Japanese quail. Segregation and linkage analysis can be performed by examining a large F_1 population to test if these microsatellites are inherited in a Mendelian pattern and if these markers are randomly distributed in the genome. This genetic linkage map will provide the basis for quantitative trait loci (QTL) studies and/or marker-assisted selection (MAS) in Japanese quail. Based on the sequencing information on these markers, Japanese quail microsatellite primers can be designed to amplify and confirm the presence of the null alleles in a few loci where possible null alleles were suspected in this study. Since 40% of all detected primer sequences in Japanese quail contained base substitutions, Japanese quail primers with total homology to the priming sites can be designed for future use in quail population studies. The polymorphic markers can be also mapped using fluorescent *in-situ* hybridization (FISH) method and the sequences and location can be compared to the existing chicken maps. The 2 polymorphic markers that amplified in chicken, turkey and quail can be used to detect microsatellite loci in other closely related species such as pheasants and European quail, or in even more distant species. Analysis of these potential universal primers may identify species-specific markers which will be very useful in distinguish pure breeds from hybrids and in species identification. In summary, the use of heterologous microsatellite primers, coupled with comparative mapping

information, will make microsatellites very useful in gene mapping studies in other avian species.

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APPENDICES

Appendix 1. List of 48 chicken-specific microsatellite primer-pairs tested on Japanese quail and their sequences.

Locus name[a]	GenBank #[c]	Forward primer sequence[b]	Reverse primer sequence[b]
ADL019	L23887	TGC TGC CTA GAC CAG TTC AA	TCT GCT GGG ATT ATG TGT CA
ADL021	L23894	GCT GGT CGC TTT GCT CTG AA	GCT TAG CCT CAT CTC TTG TA
ADL022	L23899	GCA TCA GAG GAA GAA GGA AA	GGT CAA GGA AAT CAT AGA AA
ADL023	L23905	CTT CTA TCC TGG GCT TCT GA	CCT GGC TGT GTA TGT GTT GC
ADL024	L23906	TGA AGC AAA AAC CCA GCA AG	GTT CCA TTA CAG AGT GAG GT
ADL034	L23908	AAC CTA AAA ACT CCT GCT GC	GGG AAC CTG TGG GCT GAA AG
ADL036	L23911	GTT TGG CTT ACA TTT ATT AT	TTT TTA GGA GTT ATT TGA CA
ADL037	L23912	ATG CCC CAA ATC TCA ACT CT	TCT CTA AAA TCC AGC CCT AA
ADL038	L23916	TCG CCT GTG AAC TCT TAC CC	AGA CAT TCT CTA ATC ATT CC
ADL101	G01546	CCC CAA GGA GAA CTG ATT AC	GAA AAG TGA AAA CGC AAA CA
ADL102	G01547	TTC CAC CTT TCT TTT TTA TT	GCT CCA CTC CCT TCT AAC CC
ADL111	G01724	CCT TCC TGA CCT TCC ACT TC	CCA CAA AAA TAC CCA CCA TC
ADL114	G01726	GGC TCA TAA CTA CCT TTT TT	GCT CTA CAT TCC TTC AGT CA
ADL115	G01556	GGA TGA GAA GAA GAA AGG CA	CAA TGG TGG TTC AGG TAA TC
ADL117	G01728	TCT TGT TTT CCT TTT GTT GT	GCA TAC GGC TCC TTC AGT TG
ADL121	G01732	CTG GAA CAA GAG GGC TTT GC	GGA TGT GAA AAA TCT CCT GG
ADL124	G01734	ATG CGT TAC AAG GTN GGA GG	ATA TGA TGA CTG GAG GTT TT
ADL127	G01736	GAA CCA GCA ATT ATA TTA AAT A	TTA ACA CAA AAG AAC CAG GCA G
ADL136	G01561	TGT CAA GCC CAT CGT ATC AC	CCA CCT CCT TCT CCT GTT CA
ADL142	G01567	CAG CCA ATA GGG ATA AAA GC	CTG TAG ATG CCA AGG AGT GC
ADL143	G01568	CCT GTC TCT GGT CTT TAT CC	AGT TTA CTT CCT TTT CTT GC
ADL158	G01582	TGG CAT GGT TGA GGA ATA CA	TAG GTG CTG CAC TGG AAA TC
ADL171	G01593	ACA GGA TTC TTG AGA TTT TT	GGT CTT AGC AGT GTT TGT TT
ADL172	G01594	CCC TAC AAC AAA GAG CAG TG	CTA TGG AAT AAA ATG GAA AT

Locus name[a]	GenBank #[c]	Forward primer sequence[b]	Reverse primer sequence[b]
ADL176	G01598	TTG TGG ATT CTG GTG GTA GC	TTC TCC CGT AAC ACT CGT CA
ADL181	G01603	CCA GTG AAA TTC ATC CTT TT	CAA TCT TTT GTG GGG TAT GG
ADL188	G01609	CAC TTC CAG TAT TAA CGT GA	GTG GAC ACA ATG AGT TCC TC
ADL190	G01611	TCA GCT CTT CAG GCA AAA AG	AAC TTG GAC CAC AAT CTT AT
ADL206	G01626	TTT CTA TCC TTC ATC TCC AG	AGA CAT CCT GCT TTC TCG TG
ADL210	G01630	ACA GGA GGA TAG TCA CAC AT	GCC AAA AAG ATG AAT GAG TA
ADL219	G01639	AAT ATG TTA CAC TGC CAT TT	GGA CCA AGA ATC TGT TCC AG
ADL257	G01677	ATC TTG AAA CCT CAC AAA GC	TCT TCC AAC CTA TTT TTA GT
ADL267	G01687	AAA CCT CGA TCA GGA AGC AT	GTT ATT CAA AGC CCC ACC AC
ADL288	G01707	AAA ACT TGC CTC CCA ACA TT	ATC CCC CAC GTA ATA CTT AT
ADL299	G01751	GTC TAG GCC CCT TGC CAA AC	CCA CCC CCA TGT TCA GGT CA
ADL312	G16081	AAG CTG GAA CTT GAA GAA GA	TCA GGA GGG TTG GAG GTG TG
ADL315	G16117	TCC TTG GGC AGT AGT TTC AA	CTC CCA TGT TGC TTC TTT AG
ADL356	G29064	AAG CTT TTA AAC CAA TCT GA	TCC TGC ATG TGC CCT TTG TA
ADL366	G29072	AGC TCC TTG TAC CCC TTT GC	CAC CAT TTG CCT CAC CAA CT
HUJ001	STMSGGHUH72	CCC TTT GTT AAC ACC TAC TGC A	CAT CCG GCT TAT ACA GAG CAC A
HUJ003	STMSGGHU30.1	GAC AGC AAG GAT TAA CCT CAG	GTC TTC TTA TAC TCT TAC TTG G
HUJ004	STMSGGHU60-2A	TCC GAC TGA TAA CTT TTG TGG C	CTA TGA TTT GCG GTT CCT AG
HUJ005	STMSGGHU15-1	TCC CTT CCA ACC CTT ACA GT	AAA AGC AAC ACG AAT AAC AGA T
HUJ006	STMSGGHU105-1A	GGA ACA TGT AGA CAA AAG C	AGT AGT CCA TTT CCA CAG CCA
HUJ007	STMSGGHU4.1	CAT AAA CTA AAG TCT CAA CAC	TTC TTC CAC ACA TCC TTG CTA
MCW005	NA	ACC TCC TGC TGG CAA ATA AAT TGC	TCA CTT TAG CTC CAT CAG GAT TCA
MCW031	NA	ATACA CCATG TAGAC CCCTG T	TACAG CCATA CAGGA CTCAT TAAC
MCW069	NA	GCACT CGAGA AAACT TCCTG CG	ATTGC TTCAG CAAGC ATGGG AGGA

[a]locus code: ADL=USDA ARS Avian Disease and Oncolgy Lab; HUJ=Hebrew University of Jerusalum and MCW = Microsatellite Chicken Wageningen.

[b]primer sequences are written in the 5' to 3' direction.

[c]genbank accession number.

NA = not available

Appendix. 2. List of 20 chicken-specific microsatellite primer-pairs that did not generate any PCR amplification products in Japanese quail template.

Locus name[a]	Repeat[c]	PCR annealing temp. in chicken DNA template[c] (°C)	Average allele length in chicken template (bp)[d]	Range of PCR annealing temperatures (°C) tested with final Mg^{++} concentrations between 2.0 and 3.0mM[b]
ADL019	$(AC)_{10}AT(AC)_2$	50	104	45-55
ADL021	$(CA)_2TA(CA)_6$	48	165	45-53
ADL022	$T_{10}(GT)_{14}$	46	146	35-55
ADL034	$(AC)_{11}(AT)_2$	49	126	44-54
ADL102	$(GT)_{18}$	47	122	41-52
ADL127	$(AT)_{12}..(AC)_8$	43	148	37-53
ADL136	$(TG)_{10}TC(TG)_{10}$	52	145	47-54
ADL171	$(TG)_{18}$	46	104	41-53
ADL172	$(AC)_{18}$	49	154	44-54
ADL181	$(CA)_{12}$	48	178	42-52
ADL188	$(TG)_{12}T_2(GA)_7A_6$	47	129	41-51
ADL210	$(AC)_{15}$	46	130	41-51
ADL356	$T_{25}(TG)_{13}$	48	164	38-58
HUJ001	$(CA)_n$	55	192	40-60
HUJ003	$(TG)_n$	55	165	40-60
HUJ004	T_n, $(TG)_n$	55	132	40-60
HUJ007	A_n, $(AC)_n$	55	162	40-60
MCW005	$(TG)_{15}$	55	189-259	40-60
MCW031	$(GT)_{24}$	55	80-110	40-60
MCW069	$(TG)_{11}$	55	159-168	40-60

[a]locus code: ADL=USDA ARS Avian Disease and Oncolgy Lab; HUJ=Hebrew University of Jerusalum and MCW = Microsatellite Chicken Wageningen.

[b]range of PCR annealing temperatures (°C) tested with final Mg^{++} concentrations between 2.0 and 3.0mM in a panel of 40 Japanese quail derived from 4 different populations: University of British Columbia (UBC), Japan (JAP), North Carolina wild type (NCW) and Alberta (ALT).

[c]repeat and PCR annealing temperature at these 20 microsatellite loci in chicken reported by NAGRP.

[d]length in sequenced clone. All primer-pairs were highly polymorhic on the East Lansing and/or Compton reference mapping populations.

Appendix 3. Unbiased estimates of the Hardy-Weinberg heterozygozity

Assuming factors that change allelic frequency (selection, mutation, migration and genetic drift) were absent and random mating was present, the Hardy-Weinberg principle can be applied to the Japanese quail populations. Therefore, the frequency of the Hardy-Weinberg heterozygozity is

$$H = 1 - \sum_{i=1}^{n} p_i^2 \quad \text{----------(1)}$$

where p_i^2 is the expected genotypic frequencies for homozygotes and n is the number of alleles at the locus being examined (Hedrick, 1985).

However, the sample size in this study is small and a systematic bias would be introduced when heterozygozity is estimated using the above equation. In order to have an unbiased estimate, the expectation of heterozygozity should be lowered by a factor of 1/2N. Thus, an unbiased estimate can be calculated as

$$H = (1 - \sum_{i=1}^{n} p_i^2)[2N/(2N-1)] \quad \text{----------(2)}$$

(Hedrick, 1985)

When *m* loci are scored, the average (mean) heterozygozity is

$$\bar{H} = (1/m)(1 - \sum_{l=1}^{m} H_l) \quad \text{----------(3)}$$

(Hedrick, 1985)