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Genetic variation and bottleneck in Japanese quail (*Coturnix japonica*) strains using twelve microsatellite markers

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The genetic structure of four strains of Japanese quail (Pharach, Panda, Tuxedo and Golden) was investigated by 12 microsatellite markers in Iran. Whole blood samples were collected from 200 individuals belonging to four strains and genomic DNA was extracted by salting out procedure. The 12 microsatellite markers were amplified through polymerase chain reaction (PCR). The results indicated that the average heterozygosity between strains ranged from 0.4343 to 0.7902. The Chi-square and likelihood ratio test performed to examine strains for Hardy-Weinberg equilibrium showed some highly significant deviations from deficiency. F_{IS} value, which indicates the degree of departure from random mating, was particularly high in the four strains when compared to that of other breeds, indicating heterozygosity deficiency. Maximum Nei's genetic distance was observed between Tuxedo and Golden strains; whereas the minimum Nei's genetic distance was observed between Pharach and Tuxedo strains. Tuxedo strain revealed bottleneck event under three models of microsatellite evolution for sign, standardized differences and Wilcoxon sign rank tests. The power of the microsatellite marker as a useful tool for evaluating genetic variation within and between Japanese quail strains was also noted.

Key word: Bottleneck, genetic distance, genetic variation, microsatellite, Japanese quail.

INTRODUCTION

The Japanese quail, originally domesticated around the 11th century as a pet song bird (Howes, 1964; Crawford, 1990), is valued for its eggs and meat. It is also a valuable laboratory species because of its small body size, rapid generation interval and high prolificacy (Mills et al., 1997). Japanese quail is phylogenetically closely related to the chicken (Stock and Bunch, 1982). Both species have a karyotype of 2n = 78 chromosomes and a similar genome length of 1.2×10^9 bp, consisting of morphologically distinct macrochromosomes (1–8 and the ZW sex chromosomes) and cytologically indistinguishable microchromosomes (Shibusawa et al., 2001).

Thus, the Japanese quail has been recommended as a model species for poultry (Baumgartner, 1994; Mills et al., 1997; Kayang et al., 2004). Recently, 100 microsatellite markers were developed for Japanese quail (Kayang et al., 2000, 2002) and used to build the first microsatellite linkage map, which spans 576 cM and contains 58 loci assigned to 12 linkage groups (Kayang et al., 2004). Microsatellites are valuable genetic markers due to their dense distribution in the genome, great variation, co-dominant inheritance and easy genotyping. They were extensively used in parentage testing, linkage analyses, population genetics and bottleneck studies (Goldstein and Pollock, 1997).

Genetic diversity in populations and the evolutionary forces that affect it are central to both evolutionary (Wright, 1931) and conservation biology (Frankham, 1995b). When populations undergo temporary large reductions in size of so-called population bottlenecks (Nei et al., 1975), they lose genetic diversity through random drift. Identifying populations that have experienced a

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severe reduction in size (a bottleneck) is important because bottlenecks can increase demographic stochasticity, rates of inbreeding, loss of genetic variation and fixation of mildly deleterious alleles, thereby reducing evolutionary potential and increasing the probability of population extinction (Frankel and Soule, 1981; Frankham, 1995a, b; Rall et al., 1988; Bryant et al., 1986; Goodnight, 1987; Luikart et al., 1998). In this study, the bottleneck and genetic diversity within and among four strains of Japanese quail was analyzed (Panda, Pharach, Golden and Tuxedo) by 12 microsatellite markers as recom-mended by Kayang et al. (2002).

MATERIALS AND METHODS

Sampling and DNA extraction

Japanese quail strains, reared at Bonab Research Center, Northwest of Iran, were used as the experimental animals. These strains were reared for 17 generations and were phenotypically selected for high weight gain. Any new individual has not been introduced to the strains.

Whole blood samples were randomly collected from 200 individual belonging to four strains: 70 individuals from Pharach strain, 40 individuals from Panda strain, 50 individuals from Tuxedo strain and 40 individuals from Golden strain. About 200 μl of blood per individual was collected in 0.5 mM EDTA (pH 8). Genomic DNA was extracted by the salting out procedure (Miller et al., 1988).

Microsatellite primers

12 microsatellites with high polymorphism information content (PIC) value, indicating the informative of the markers, recommended by Kayang et al. (2002) were used in this study. The primers were encoded by GUJ0001, GUJ0021, GUJ0023, GUJ0034, GUJ0041, GUJ0049, GUJ0052, GUJ0055, GUJ0059, GUJ0070, GUJ0097 and GUJ0099 characters. Primers were synthesized by TIBMO-LBIOL Company, Germany.

PCR amplification

Genomic DNA was amplified by PCR containing 50 ng of template DNA, 2.5 μl of 10 x buffer (10 Mm Tris, 50 mM Kcl, 0.1% gelatin, pH 8.4), 1.5 to 2.0 mM $MgCl_2$ (as optimized for each marker), 200 μM of each dNTPs , 1 μl of 5 pmol/μl each for forward and reverse primers and 1 u of Taq DNA polymerase; ddH_2O was added to the volume of 25 μl. Reactions were carried out on a thermal cycler (Biometra) using an initial 2.5 min denaturation at 95°C, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 50 to 62°C for 30 s, extension at 72°C for 30 s, and a final extension step at 72°C for 5 min. The PCR products were electrophoresed on 8% denatured urea-polyacrylamide gel and bands visualized by rapid silver staining method (Sanguinetti et al., 1994). The gel was photographed using Gel-Doc XR (BioRad). Patterns of the different genotypes for each microsatellite locus were analyzed using Gel-Pro analyzer, version 3.1 for windows TM, which determines the allele's sizes in each animal.

Statistical analysis

Allelic frequencies were estimated using genotype counting. Hardy-Weinberg equilibrium (HWE) based on likelihood ratio were tested for different locus-population combinations by POPGENE software Version 1.31 (Yeh et al., 1999). Nei's (1972) standard genetic distance and Reynolds genetic distance (Reynolds et al., 1983) matrices were calculated by tools for population genetic analysis (TFPGA) software version 1.3 (Miller, 1997) and dendrograms were constructed using unweighted pair-group method with arithmetic mean (UPGMA) by TFPGA software (Miller, 1997) with 1000 bootstrap replications. Polymorphism criteria such as PIC (Botestein et al., 1980) and the number of observed and effective alleles (Hedrick, 1999) were also estimated by HET software version 1.8 (Ott, 2001) and POPGENE software packages, respectively with assumption of Hardy-Weinberg equilibrium. Average expected heterozygosity was calculated by POPGENE software (Yeh et al., 1999).

To accomplish the goal of finding evidence of fluctuations in population sizes, bottleneck software package was used (Cornuet and Luikart, 1996) to detect deviations from mutation drift equilibrium. This method consisted three excess heterozygosity tests developed by Cornuet and Luikart (1996): (i) sign test, (ii) standardized differences test and (iii) a Wilcoxon sign-rank test. The probability distribution was established using 1000 simulations under three models: infinite allele model (IAM), stepwise mutation model (SMM) and two-phase model of mutation (TPM) by using Bottleneck v1.2.02 Software (http://www.ensam.inra.fr/URLB).

RESULTS

All loci were polymorphic in the four Japanese quail strains, except GUJ0001 and GUJ0041, which were monomorphic in the Panda and Tuxedo, respectively. The number of alleles per locus varied from 9 (GUJ0059) to 3 (GUJ0001, GUJ0021 and GUJ0041). New alleles were found in several loci that have not been previously reported in the same loci for other Japanese quail by Kayang et al. (2002). Significant deviation from HWE in all population-locus combinations were observed except for GUJ0041 in Pharach strain, GUJ0023, GUJ0099, GUJ0021, GUJ0034, GUJ0041 and GUJ0097 in Panda strain and GUJ0034, GUJ0055, GUJ0049 and GUJ0070 in Golden strain (P < 0.05). Effective number of alleles, PIC, observed heterozygosity and expected heterozygosity are given in Table 1. All the expected heterozygosities were larger than the observed heterozygosities, except for GUJ0097 in Tuxedo.

Nei's (1972) standard genetic distance (D_a), corrected for bias, due to sampling of individual, and Reynolds genetic distance ($D_{Reynolds}$) matrices (Reynolds et al., 1983) are reported in Table 2.

The expected heterozygosity (H_{Exp}) ranged from 0.841 (GUJ0070) in the Pharach strain to 0.486 (GUJ0052) in the Golden strain. Polymorphism criteria such as PIC values and number of alleles indicate high polymorphism at some of the loci. The PIC is a good standard for evaluating genetic markers. The highest and the lowest PIC values belonged to GUJ0059 in Golden (0.815) and GUJ0041 in Panda strain (0.427), respectively. Effective number of alleles is a reciprocal of gene homozygosity (Hartl and Clark, 1989). The highest and the least effective numbers of alleles were in GUJ0059 locus (6.04) in Pharach strain and in GUJ0041 locus (1.91) in

Table 1. Statistical results of 12 loci of quail strains.

Microsatellite primer	Genetic parameter of microsatellite loci	Sample size (n)			
		Pharach 70	Panda 40	Tuxedo 50	Golden 40
GUJ0001	N_O	5	1	3	4
	Ne	3.37	1.00	2.95	3.07
	PIC	0.641	0.000	0.586	0.613
	H_{Obs}	0.230	0.000	0.000	0.000
	H_{Exp}	0.717	0.000	0.676	0.687
	F_{IS}	0.672	0	1	1
GUJ0021	N_O	6	3	5	5
	Ne	4.48	2.57	3.66	3.58
	PIC	0.751	0.535	0.680	0.677
	H_{Obs}	0.526	0.666	0.428	0.055
	H_{Exp}	0.798	0.666	0.727	0.730
	F_{IS}	0.322	-0.091	0.410	0.922
GUJ0034	N_O	5	5	5	7
	Ne	3.10	4.57	3.20	4.78
	PIC	0.627	0.745	0.636	0.761
	H_{Obs}	0.200	0.750	0.250	0.571
	H_{Exp}	0.690	0.883	0.709	0.805
	F_{IS}	0.705	0.040	0.636	0.277
GUJ0041	N_O	6	3	1	6
	Ne	2.65	1.91	1.00	2.75
	PIC	0.590	0.427	0.000	0.568
	H_{Obs}	0.321	0.250	0.000	0.250
	H_{Exp}	0.629	0.491	0.000	0.646
	F_{IS}	0.484	0.475	0	0.601
GUJ0049	N_O	6	6	5	7
	Ne	4.00	4.26	3.50	4.00
	PIC	0.715	0.735	0.668	0.715
	H_{Obs}	0.600	0.666	0.400	0.583
	H_{Exp}	0.769	0.787	0.733	0.766
	F_{IS}	0.200	0.129	0.441	0.222
GUJ0059	N_O	5	4	6	8
	Ne	3.27	3.57	4.96	6.03
	PIC	0.650	0.762	0.767	0.815
	H_{Obs}	0.333	0.000	0.250	0.071
	H_{Exp}	0.709	0.757	0.815	0.849
	F_{IS}	0.520	1	0.687	0.914
GUJ0070	N_O	7	5	6	7
	Ne	6.04	4.00	4.88	4.57
	PIC	0.813	0.707	0.765	0.750
	H_{Obs}	0.300	0.333	0.600	0.500
	H_{Exp}	0.841	0.782	0.815	0.797
	F_{IS}	0.640	0.555	0.245	0.360
GUJ0097	N_O	6	6	6	6
	Ne	4.84	3.66	4.76	3.52
	PIC	0.762	0.690	0.758	0.685
	H_{Obs}	0.500	0.625	0.846	0.647
	H_{Exp}	0.807	0.7500	0.805	0.727
	F_{IS}	0.369	0.139	- 0.071	0.096

Table 1. Contd.

GUJ0055	N_O	5	4	4	4
	Ne	4.458	3.446	3.188	3.173
	PIC	0.740	0.658	0.637	0.489
	H_{Obs}	0.45	0.555	0.307	0.411
	H_{Exp}	0.783	0.730	0.699	0.547
	F_{IS}	0.399	- 0.217	0.552	0.237
GUJ0052	N_O	5	4	5	4
	Ne	3.913	3.677	4.083	1.933
	PIC	0.701	0.685	0.712	0.563
	H_{Obs}	0.166	0.666	0.642	0.166
	H_{Exp}	0.750	0.755	0.768	0.486
	F_{IS}	0.864	- 0.077	0.148	0.589
GUJ0023	N_O	5	4	4	4
	Ne	3.161	2.976	3.881	2.919
	PIC	0.647	0.616	0.694	0.597
	H_{Obs}	0.414	0.375	0.500	0.235
	H_{Exp}	0.689	0.685	0.755	0.667
	F_{IS}	0.357	0.435	0.326	0.687
GUJ0099	N_O	5	4	3	4
	Ne	4.304	3.521	2.945	3.085
	PIC	0.729	0.662	0.586	0.625
	H_{Obs}	0.607	0.666	0.143	0.388
	H_{Exp}	0.774	0.736	0.672	0.685
	F_{IS}	0.209	- 0.014	0.785	0.234

N_O, observed number of alleles; Ne, effective number of alleles; PIC, polymorphism information content; H_{Obs}, observed heterozygosity; H_{Exp}, expected heterozygosity; F_{IS}, is the inbreeding coefficient.

Table 2. Distance matrices estimated by D_a (above diagonal) and $D_{Reynolds}$ (below diagonal) distance using 1000 bootstrap replications.

Strain	Pharach	Panda	Tuxedo	Golden
Pharach	***	0.307	0.247	0.571
Panda	0.050	***	0.402	0.743
Tuxedo	0.044	0.071	***	0.794
Golden	0.112	0.142	0.128	***

Panda strain, respectively (Table 1). F_{IS} of the inbreeding coefficient, measure the relative heterozygote deficit and non-random mating in samples. Its value ranges between -1 (all individuals heterozygote), 0 (random association of alleles) and 1 (all individuals homozygote). If inbreeding is avoided, F = 0; negative F indices are usually from selection in favor of the heterozygotes, whereas positive values indicate that the considered population has an inbreeding system of mating (Liu et al., 2008). The mean values of F_{IS} estimates obtained 0.45, 0.3, 0.47 and 0.46 for Pharach, Panda, Tuxedo and Golden strains, respectively indicating the high level of inbreeding in the four strains. This could be as a result of a bottleneck effect. Bottlenecks influence the distribution of genetic variation within and among populations, thus the genetic effects of reductions in population size requires evaluation. To characterize it, sign, standardized differences and Wilcoxon sign rank tests were utilized. The values of average heterozygosity (He) and their probabilities (H > He) in the sign test, under three models of microsatellite evolution (IAM, SMM and TPM) were calculated and used to measure the expected number of loci with heterozygosity excess (Table 3). If the probability values for each model were less than 0.05, then null hypothesis was rejected, indicating that the bottleneck event occurred in this model. For example, the expected number of loci with heterozygosity excess in Pharach strain was 7.10 and 7.15 for TPM and SMM, under null hypothesis in the sign test, respectively (Table 3). The probability values were, 0.0749 and 0.0792, respectively, meaning that the null

Table 3. Test for null hypothesis testing under three microsatellite evaluation models, the IAM, SMM and TPM.

Parameter	IAM	TPM	SMM
Sign test: number of loci with heterozygosity excess (probability)			
Pharach	Expected = 7.01(0.0151) *	7.10 (0.0749)	7.15(0.0792)
Panda	Expected = 6.37(0.0024) *	6.46(0.0028) *	6.59 (0.1179)
Tuxedo	Expected = 6.32(0.0023) *	6.49(0.0030) *	6.54 (0.0032) *
Golden	Expected = 7.03(0.0690)	7.11 (0.2095)	7.07 (0.3649)
Standardized differences test (Ti values and probability)			
Pharach	3.272 (0.00053) *	2.077 (0.0189) *	0.131 (0.4480)
Panda	4.204 (0.00001) *	3.435 (0.0003) *	2.497 (0.0062) *
Tuxedo	4.998(0.00001) *	4.490(0.0000) *	3.729 (0.0001*)
Golden	2.666 (0.0038) *	1.461 (0.0720)	-0.441 (0.3295)
Wilcox sign-rank test (probability of heterozygosity excess)			
Pharach	0.03418*	0.04248*	0.2334
Panda	0.00049*	0.00049*	0.00342*
Tuxedo	0.00049*	0.00049*	0.00049*
Golden	0.00122*	0.05225	0.67725

*Rejection of null hypothesis/bottleneck.

hypothesis was accepted when using sign test. These results indicate that, due to mutation-drift equilibrium, the Pharach strain has not undergone a recent genetic bottleneck. However, the expected number of loci with heterozygosity excess was 7.01 for IAM with probability of 0.0151 and thus rejected the null hypothesis, indicating bottleneck under this model.

The standardized difference test for Pharach strain provided the T_i (probability) statistics equal to 3.272 (0.00053), 2.077 (0.0189) and 0.131 (0.4480) for the IAM, TPM and SMM models, respectively. The probability values were less than 0.05 for IAM and TPM, thus hypothesis of mutation-drift equilibrium was accepted under SMM only. Using the Wilcoxon rank test, the probability values were 0.03418 (IAM), 0.04248 (TPM) and 0.2334 (SMM) under the three models, indicating that the null hypothesis was accepted under SMM model. Among the used tests, Wilcoxon rank test and standard difference test showed bottleneck event in golden strain under IAM. However other P values tests were not significant, demonstrating that the null hypothesis of mutation–drift equilibrium was fulfilled in this strain. The results of all the three tests together showed that the bottleneck event did not occur in this strain. Golden strain is a wild strain that was collected from its natural niche and suffered few selective breeding programs. Results revealed bottleneck event in Tuxedo strain under three models for sign, standardized differences and Wilcoxon sign rank tests.

DISCUSSION

The Hardy-Weinberg equilibrium test showed several deviant loci in the four strains. There were many causes of disequilibrium such as small population, selection at or near the genomic locus, non-random mating, genetic drift and inbreeding. In addition, the possible occurrence of null alleles could have led to false observation of homozygotes which could have accounted for more deviations from HWE. The mean observed heterozygosity for these four strains were 0.387, 0.462, 0.364 and 0.325 for Pharach, Panda, Tuxedo and Golden, respectively. In all the four strains, the heterozygosity observed was lower than that reported for wild Japanese quail (0.66) (Chang et al., 2005). The average PIC of the 12 microsatellite loci in the four strains was close to the results of Kayang et al. (2002). Based on the classification of Botstein et al. (1980), PIC > 0.5 is highly informative, 0.25 < PIC < 0.5 is middle informative and PIC < 0.25 is slightly informative. In this study, all loci were highly informative (PIC > 0.5), except GUJOO41 (PIC = 0.427) in Panda strain. Reynolds distance were used to estimate pairwise genetic distance between the breed. This measure is recommended by Eding and laval (1999) for population with short divergence time. Maximum Nei's genetic distance was observed between Tuxedo and Golden strains; whereas the minimum Nei's genetic distance was observed between Pharach and Tuxedo strains. Both cluster resulted from Nei's standard genetic distance and
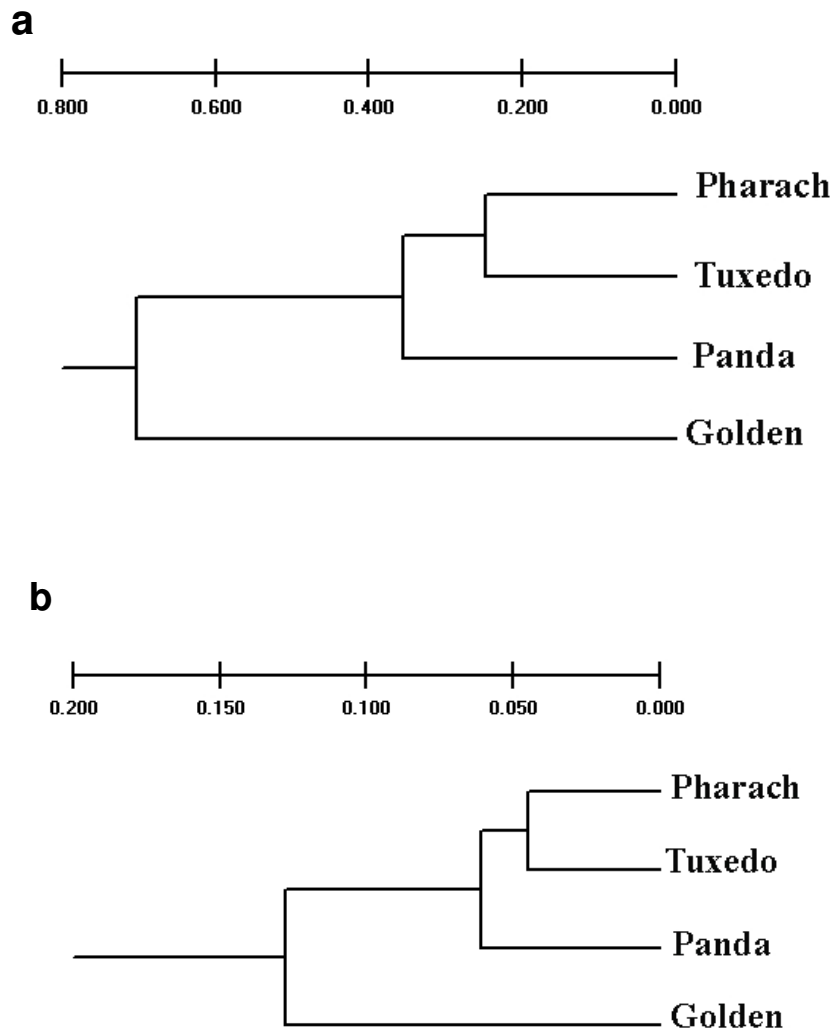
a

b

Figure 1. UPGMA showing the genetic relationships among strains using Nei's standard genetic distance (a) and Reynolds genetic distance (b).

Reynolds genetic distance revealed two branches. One branch included Pharach, Tuxedo and Panda and another branch was consisted of Golden. Pharach and Tuxedo grouped closely (Figure 1). It was expected to find high genetic distance between Golden and other strains. Golden is the most prolific type among the Japanese quail strains. The mean values of F_{IS} among the four strains were higher than that of the population studied by Kim et al. (2007), indicating a certain level of heterozygote deficiency. Significant heterozygote deficiencies have been reported in chicken breeds (Qu et al., 2006; Liu et al., 2008). The heterozygote deficiency ($F_{IS} > 0$) might be attributed to a number of factors, namely, sample relatedness, linkage with loci under selection (genetic hitchhiking), population heterogeneity, null alleles (non-amplifying alleles) and inbreeding (Shahsavarani and Rahimi-mianji, 2010). Null alleles were most unlikely segregated at all the loci. The most plausible explanation for the heterozygote deficiency was inbreeding in the four strains. These four closed strains especially Tuxedo strain were under selective breeding program for 17 generations. When a breeder conducts a selective breeding program, his primary objective is to alter, not conserve gene and genotypic frequencies in order to improve the population. Inbreeding and genetic drift are inevitable during a selective breeding program, because each act of selection creates a bottleneck event that accelerate the accumulation of inbreeding and magnify genetic drift. There are some techniques that can be used to moderate inbreeding and finally bottleneck so that it does not counteract selection. Managing a population to minimize the effect of bottleneck event can

be accomplished only by managing the number of effective population (N_e). Bottleneck reduces the N_e and make it difficult, if not impossible, to achieve genetic goals. Unfortunately, it is difficult to maintain a constant N_e generation after generation because N_e can decline due to diseases, selective breeding program, etc (Tave, 1999). One way to maintain a constant N_e generation after generation can be pedigree mating. The weak performance of some economic traits (meat and number of eggs) in these strains, especially Tuxedo can be as a result of bottleneck event. Breeding strategies should therefore be designed to amplify the population size and simultaneously avoid inbreeding in these four strains. The information generated in this study will greatly aid in the establishment of effective breeding for these four strains and may further be utilized for studying differentiation and relationships among different quail strains.

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