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Original scientific paper

ASSOCIATIONS OF POLYMORPHISMS IN THE PROLACTIN RECEPTOR GENE WITH GROWTH TRAIT IN JAPANESE QUAIL (*Coturnix coturnix japonica*)

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Prolactin receptor (*PRLR*), as an important regulatory gene about growth and differentiation, might be a condition gene for reproductive traits. The prolactin receptor is a specific receptor for prolactin (*PRL*), which is an anterior pituitary peptide hormone involved in various physiological activities and is essential for reproductive improvement. In chickens, the PRLR gene is on the Z chromosome. In the present study, the polymorphism of exon 2 of PRLR gene in Japanese Quail (*Coturnix coturnixjaponica*) was investigated. Blood samples of 180 Japanese Quail were collected randomly. DNA was extracted from blood samples and amplified. Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis of the exon 2 of *PRLR* gene. PCR-SSCP analysis of the exon 2 region revealed two banding patterns. Two different SSCP patterns, representing two different genotypes, were identified. The frequencies of the observed genotypes were

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0.939 and 0.061 for AA and BB. Allele frequencies were 0.939 and 0.061 for A and B. Observed heterozygosity (Hobs) value was 0.0. The chi-square test showed significant (P<0.05) deviation from Hardy-Weinberg equilibrium for this locus in studied population. The effect of these genotypes on weight gain was investigated, and the BB genotype was found to be associated with body weight at 46 days. This was the first study on polymorphism of PRLR gene in Japanese quail.

Key words: Genetic Polymorphism, Japanese Quail, *PRL*, SSCP

INTRODUCTION

Japanese quail is widely used in laboratory studies because this bird has small body size (*80-300 gr*), short intergeneration interval, production of more eggs and high resistance to diseases. *PRL* is the peptide hormone made and secreted in the anterior pituitary by special cells (ALAMER, 2011). PRL has a role in more than 300 biological activities such as reproduction, growth, metabolism and immunity reactions in the immunity system (MALAGUARNERA *et al*., 2004; NICOLL *et al*., 1986; PEIRCE *et al*., 2004). In bird species; *PRL* is an important hormone in inducing and preserving the act of incubation and adjusting follicle development (KISHIMOTO *et al*., 2000; VLAHOS *et al*., 2001). The increase of *PRL* in blood circulation can influence a hen's life, although its amount isn't definitely clear (HIYAMA *et al*., 2009). High density of *PRL* hormone has a relation with the appearance of broodiness (BACON *et al*., 1983; BAILES *et al*., 2007; BOLE-FEYSOT *et al*., 1998; BURKE *et al*., 1980). Broodiness in birds is usually accompanied by the increase of body temperature, the decrease of water and food consumption, frequent nest occupancy, turning and adjusting eggs, aggressive defensive behaviors and special noises (ROMANOV *et al*., 2002). Therefore, the increase of the level of *PRL* in plasma stimulates the act of incubation and then the end of laying (RASHIDI *et al*., 2012) that is the result will be the decrease of egg production (REDDY *et al*., 2002).

PRLR gene has an important role in the process of sending the signals of *PRL* hormone making broodiness start, and it seems that *PRL* applies its biological roles with its receptor action (BOLE-FEYSOT *et al*., 1998). *PRLR* and growth hormone receptor are approximately similar in structural similarity point of view, and both are membrane signal chain conveyors and have the same structural and functional features (KELLY *et al*., 1991). *PRLR* gene is located in the Z chromosome (DUNN *et al*., 1998) and in the hen, it has been mapped on Zp23-222 chromosome (CHENG *et al*., 1995). The size of this gene is more than 34 kb and includes 15 exons and 14 introns (LEUNG *et al*., 2005). The purpose of the present study was to determine the polymorphism of exon 2 of *PRLR* gene and to assess its association with body weight in Japanese quail.

MATERIAL AND METHODS

Blood sample collection and genomic DNA extraction

This experiment was carried out at the poultry research station of Bonab of agricultural science and natural resources, East Azarbaijan, Iran. Blood samples (approximately 2-3 mL) were obtained from 180 unrelated Japanese quail and stored in *ethylenediaminetetraacetic acid* (EDTA)-coated tubes. Genomic DNA was extracted from 10 µl blood using Pronase method (MALAGUARNERA *et al*., 2004) according to manufacturer instructions. The quality and quantity of extracted DNA were measured on 0.8% Agarose gel prepared in 0.5X *TBE* buffer (45 mM

Tris-base, 45 mM boric acid, 1 mM EDTA, pH 8.0), visualized with ethidium bromide (1.0 *μg/mL*) under ultraviolet light, and photographed.

Amplification of exon 2 of the PRLR gene

Two polymerase chain reaction (PCR) primers-PRLR-up (5' TTTTGCTCCTTGTGTTTTAGGA-3') and PRLR-down (5' TGGTTTCCTACCGAAAGGATT3')-targeting a fragment of 162 bp were used for DNA amplification as described by RASHIDI *et al*. (2012). The reaction PCR was performed at 25 µl final volume, including 1 U Taq DNA polymerase, 0.2 mM dNTP, 1.5 mMMgCl2, 10 µM of each primer, 30 *ng* of genomic DNA and 1X buffer. The following cycles were used for the development of PRLR: 95oC for the first denaturation for 5 min, 35 cycles with 95oC for 30 sec, 53oC for 35 sec for initiator's connection (it was determined according to temperature gradient technique), 72oC for 40 sec for extension of multiplication section and finally 72oC for 10 min the extension of all the products.

SSCP

We used the SSCP method only to identify genotypes at the exon 2 locus. PCR products were mixed with 8 µL denaturing loading dye [95% (*w/v*) deionized formamide, 0.05% (*w/v*) xylene cyanol, 0.05% (*w/v*) bromophenol blue and 0.02 *M* EDTA] in a total volume of 15 *μL*. The mixture was denatured at 95°C for 5 min and then snap-chilled on ice (PIPALIA *et al*., 2004). The total volume was electrophoresed on 8% polyacrylamide gel, as described by HERRING *et al*. (1982). The electrophoresis was performed in 0.5X TBE buffer at room temperature (18°C) and a constant 100 V. Polyacrylamide gels were stained using silver nitrate according to the protocol described by HERRING *et al*. (1982) Genotypes were recorded according to the band patterns. The 100 bp ladder was used as molecular size marker.

Statistical analysis

Alleles and genotype's frequency and their accordance to Hardy-Weinberg equilibrium were calculated from POPGENE software (YEH *et al*., 1997). The observed and expected heterozygosity were calculated using Pop Gene software. The allelic and genotypic frequencies and observed and expected Nei's heterozygosities ($HE= 1 - \Sigma P_{2i}$, where P_{iis} the frequency of allele *i*) were estimated using PopGene32 version 1.31 (YEH *et al*., 1997). PopGene32 was also used to perform the Hardy-Weinberg equilibrium test. The association of genotypes with body weight was investigated using the MIXED procedure of SAS 9.0 software. The following linear equation was applied to analysis the genetic effects the PRLR:

$$Y_{ijk}= \mu + G_i + D_j + A_k + (G\times D)_{ij} + e_{ijk}$$

Where Y_{ijk} is the trait measured in the Japanese Quail, μ is the population mean, G_i is the fixed effect the genotypes, D_j is the fixed effect the times of recording, A_k is the random effect the animals, $(G\times D)_{ij}$ is the effect of interaction between genotype and time of recording, and e_{ijk} is the random error.

RESULTS

We successfully amplified the exon 2 region within the PRLR gene (a fragment of 162 bp in length) on our first attempt. All extracted DNA from Japanese quail blood samples yielded a specific, single-band PCR product without nonspecific bands. Therefore, the PCR products were directly used for SSCP analysis. The allelic variation within the PRLR gene was examined using a PCR-SSCP method. The non-denaturing gel electrophoresis allowed visualization of single-stranded DNA and SSCP band patterns. Two SSCP patterns were observed in the Japanese quail (Figure 1).

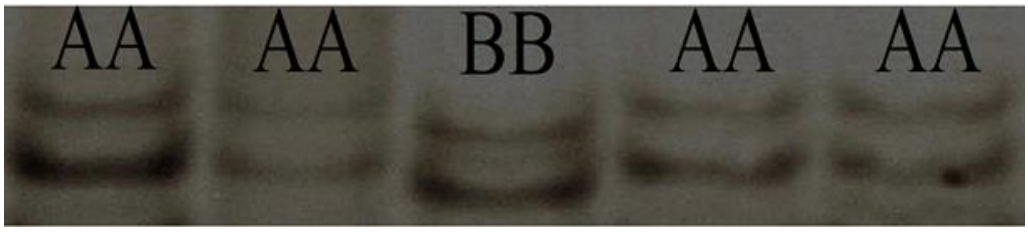

Figure 1. Genotypes of PRL gene on 8% polyacrylamide gel.

The frequencies of the observed genotypes were 0.939 and 0.061 for AA and BB respectively. Allele frequencies were 0.939 and 0.061 for A and B respectively. The observed heterozygosity value was 0.0. The chi-square test showed significant ($P \leq 0.05$) deviation from Hardy-Weinberg equilibrium for the locus under study in the Japanese quail population (table 1).

Table1. Study on genetic diversity of PRLR locus in Japanese quail population

Genetic diversity statistics	Value	Allele frequencies	value	Genotype frequencies	Value
NA	2	A	0.939	AA	0.939
NE	1.129	B	0.61	BB	0.61
I	0.23				
Observed hemozygosity	1				
Observed hetorozygosity	0				
Expected hemozygosity	0.884				
Expected heterozygosity	0.115				
PIC	0.112				

NA= observed number of alleles,

NE= effective number of alleles

I = Shannon's information index

PIC=Polymorphic information content

Effective (ne) and observed (NA) number of alleles, PIC, Shannon's information index (I) of exon 2 locus and heterozygosity values are presented in (Table 1). The number of observed alleles were 2. The effective number of alleles were 1.129, Shannon's

information index estimations 0.23, The expected heterozygosity 0.115, while the observed heterozygosity was 0.0, the expected homozygosity and the observed homozygosity were 0.884 and 1.0, respectively. The average polymorphism information content (*PIC*) for exon 2 of PRLR gene calculate 0.112, and the number of polymorphism loci is 1.0 (Table 1).

The results of the *MIXED* model analysis of body weight traits were analyzed and the least means ± SE is shown in Table 2.

Table 2. Estimated least mean body weight and their standard errors (±SE) depending on number of genotype and time of recording.

Effect	G	Dat	Estimate	Standard Error
G	1		126.28	0.8033
G	2		134.3	3.1487
Dat		1	33.5334	0.8031
Dat		2	102.15	2.3589
Dat		3	170.68	3.6077
Dat		4	214.79	4.7973
G*dat	1	1	33.2485	0.3971
G*dat	1	2	100.02	1.1663
G*dat	1	3	162.64	1.7837
G*dat	1	4	209.21	2.3718
G*dat	2	1	33.8182	1.5564
G*dat	2	2	104.27	4.5714
G*dat	2	3	178.73	6.9914
G*dat	2	4	220.36	9.2967

G= Genotypes (1=AA and 2= BB)

Based on the results in Table 2, animals with genotype 2 (BB) in the total recording time (from birth day to 64 day) had more body weight than animals with genotype 1 (AA).

The results of the MIXED model analysis of associations between PRLR gene polymorphisms and body weight traits were analyzed and the least means ± SE is shown in Table 3.

According to the result of Table 3, the genotypes (overall) had the significant effect on body weight ($P<0.05$) in whole recording time. Interaction effects between two genotype and four-time recording are shown in Table3. The interaction between genotype and the different recording time in all cases showed significant differences with the exception of 3 cases that genotype 1 with genotype 2 at the time of birth, genotype 1 with genotype 2 at the age of 32 days, and genotype 1 with genotype 2 at the age of 64 days. But the interaction of genotype 1 and 2 with a time of recording at the age of 45 days, which is the same as the age of majority has indicated significant differences of quail. Due to the least mean squares of the animal with genotype 2 (BB) at the age of 45 days (178.73 ± 6.9914) most of the animals with the genotype 1 (AA) (162.64 1.7837.) at the same age. The BB genotype in exon 2 was signnniffficant association with body weight at all times recording.

Table 3. Comparisons of least square means and their standard error (±SE) for Weight gain depending on genotype, data recording and statistical significance.

Effect	G	Dat	G	dat	Estimate	SE	Pr > |t|
G	1		2		-8.0159	3.2496	0.0139
G*dat	1	1	1	2	-66.7692	1.232	<.0001
G*dat	1	1	1	3	-129.39	1.8274	<.0001
G*dat	1	1	1	4	-175.96	2.4048	<.0001
G*dat	1	1	2	1	-0.5697	1.6063	0.723
G*dat	1	1	2	2	-71.0242	4.5886	<.0001
G*dat	1	1	2	3	-145.48	7.0027	<.0001
G*dat	1	1	2	4	-187.12	9.3052	<.0001
G*dat	1	2	1	3	-62.6213	2.1311	<.0001
G*dat	1	2	1	4	-109.2	2.6431	<.0001
G*dat	1	2	2	1	66.1996	1.9449	<.0001
G*dat	1	2	2	2	-4.255	4.7178	0.3674
G*dat	1	2	2	3	-78.7095	7.088	<.0001
G*dat	1	2	2	4	-120.35	9.3696	<.0001
G*dat	1	3	1	4	-46.574	2.9677	<.0001
G*dat	1	3	2	1	128.82	2.3673	<.0001
G*dat	1	3	2	2	58.3663	4.907	<.0001
G*dat	1	3	2	3	-16.0882	7.2154	0.0261
G*dat	1	3	2	4	-57.7246	9.4663	<.0001
G*dat	1	4	2	1	175.39	2.8369	<.0001
G*dat	1	4	2	2	104.94	5.15	<.0001
G*dat	1	4	2	3	30.4857	7.3828	<.0001
G*dat	1	4	2	4	-11.1506	9.5945	0.2455
G*dat	2	1	2	2	-70.4545	4.8291	<.0001
G*dat	2	1	2	3	-144.91	7.1626	<.0001
G*dat	2	1	2	4	-186.55	9.4261	<.0001
G*dat	2	2	2	3	-74.4545	8.3533	<.0001
G*dat	2	2	2	4	-116.09	10.3599	<.0001
G*dat	2	3	2	4	-41.6364	11.6323	0.0004

G= Genotypes (1=AA, 2= BB)

Dat= data recording (1=16 day, 2= 32 day, 3=46 day, 4= 64 day)

G*dat= interaction between genotype and time of recording

DISCUSSION

Animal reproduction involves the activity of many hormones, chemokine's and cytokines, and their receptors play crucial roles in the regulation of reproduction (OU *et al.*, 2009). According to the results of this study, the PRLR gene was effectively selected and used to study genetic association with body weights.

Compared to other birds, PRLR locus is poorly studied in Japanese Quail. Different methods have been used to study genetic variation in the PRLR gene in various birds and animals. Among different methods, PCR-SSCP analysis has been found a valuable technique in identifying genetic variation of the PRLR gene in farm animals (KONNAI *et al.*, 2003; GRUSZYNSKA *et al.*, 2005, ASHRAFI *et al.*, 2014).

To detect the potential association between the PRLR gene and body weight traits, we sought polymorphisms in the PRLR gene Japanese Quail.

Two alleles and two genotypes were identified in the exon 2 region of the PRLR gene in Japanese Quail population. The most frequent allele and genotype were A allele and AA genotype in the same frequencies of 0.939. Also our results showed that, the Japanese Quails with BB genotype had more body weight increase than those with AA genotype and the body weight at maturity age was also better with BB genotype.

Our results partly were in agreement with the results of RASHIDI *et al*. (2012) and LIU *et al*. (2012).

RASHIDI *et al.* (2012) have observed 2 alleles and 2 genotypes in exon 2 and exon 5 using PCR-SSCP and PCR-RFLP method respectively in breeder hens of indigenous chicken of Mazandaran province. Their results showed that significant association between SNP in exon 2 with body weight at hatch, age at sexual maturity, and between SNP in exon 5 and egg number. Individuals with AA genotype produced higher eggs than BB genotype ($p<0.05$).

LIU *et al*. (2012) have used polymerase chain reaction single strand conformation polymorphism (PCR-SSCP) and DNA sequencing method to detect polymorphism of PRLR gene in Erlang Mountainous chicken. Their results have shown that one SNP, two allele (G, C) and two genotypes (GG, CC) with frequencies 56.22% and 43.78% respectively. In a study from FATHI *et al*, (2014) in the promoter region of turkey prolactin and their association with egg performance, two alleles (D and I) and three genotypes (DD, II, and ID) were found. The frequencies of genotype ID and allele D were the highest. The II turkeys had a significant difference with in egg number and egg mass than those of DD and ID turkeys ($P < 0.01$). There was no significant association between PRLR polymorphism and the mean egg weight ($P >0.05$) in their study.

In exon 3 of PRLR, a single nucleotide polymorphism (SNP), was detected, which led to a nucleotide transition in the 5′-untranslated region (5′-UTR) of PRLR cDNA. Two SNP, were detected in exon 6 of the PRLR. Their results showed that there is no association between genotypes with broodiness at exon 3 and exon 6 ($P>0.05$) (JIANG *et al*., 2005). The results of association analysis showed the CC genotype had extremely significant effect on age at first egg ($P<0.01$). There was no association between two genotypes (GG, CC) with body weight at first egg (BWFE), weight at first egg (WFE), egg number at 300 days of age after hatch (EN), body weight at 300 days of age (BWTA), egg weight at 300 days of age (WTE).

Based on the ZHANG *et al*. (2012), three linked SNPs at the P1 locus, two linked SNPs at the P2 locus and one SNP at the P3locus have been reported. They used 396 female Erlang mountainous chicken using PCR-SSCP and PCR-sequencing methods. At the P1 locus there was a significant effect between GAG genotype and ACA genotype in body weight at first egg (BWFE) and total number of eggs with 300 days of age (EN). SNPs at P2 locus were associated with egg weight at 300 days of age (EWTA). There was not significantly association at the P3 locus.

Shannon's information index revealed genetic diversity within studied population. The chi-square test showed significant ($P < 0.05$) deviation from Hardy-Weinberg equilibrium at the PRLR gene in the studied population. This was the first report about studying the polymorphism in the PRLR locus in Japanese Quail and its association with body weight in different recording time in East Azerbaijan, IRAN. The previous breeding programs in the most research centers of Iran were based on only phenotypic characters. This study might be considered as an introductory to understanding the genetic variability on native species in the Azerbaijan regions by using molecular techniques that do not affect by environmental effects.

The size and composition of the egg depend on the chicken feed, body weight, age and genotype flock. Egg weight has high correlation with body weight. Therefore selection for egg weight caused increase egg size and conversely, choose to increase the size of hen's eggs leads to Enlargement. However, in applying this principle should be somewhat wary that large hens produce large eggs and small hens, produce smaller eggs. The importance of body weight in laying hens, mostly due to its relationship with food consumption, age at sexual maturity and egg weight. For females, low body weight requires less space to store less energy per chicken farmed and also seems appropriate. But due to the positive correlation between body weight with egg weight, decreases too much body weight can lead to the production of very small eggs that will have a negative role in producer income.

According to the above description, as well as high body weight of animals with genotype BB at puberty, it can be concluded that the choice of the genotype BB quail can be likely led to larger egg production. As a result, the marker assisted selection (MAS) could play an important role to choose the best birds for body weight and resulting in the production of eggs.

Results of this study agreement with the results of the most prior studies, it is confirmed that SSCP markers are powerful tools in genetic diversity studies. The results showed that the genotypes at the exon 2 locus were associated with body weight trait. Body weight in BB genotype was found to be significantly greater than in individuals with the AA genotype at the exon 2 locus. The present results showed that genotype BB in the population of Japanese quail had a significant effect on both the body weight in the whole period and their maturity age. Therefore, this genotype can be introduced as a desired genotype in exon of PRLR gene in the population of Japanese quail.

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POVEZANOST POLIMORFIZMA PROLAKTIN RECEPTOR GENA SA OSOBINAMA RASTA U JAPANSKIM PREPELICAMA (*Coturnix coturnix japonica*)

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Izvod

Prolaktin receptor (*PRLR*), kao važan regulatorni gen za rast i diferencijaciju, može biti uslovni gen za reproduktivna svojstva. Prolaktin receptor je specifičan receptor za prolaKtin (*PRL*), koji je prednji režanj hipofize peptid hormone uključen u različite fiziološke aktivnosti i neophodan za reprodukciju. Kod pilića, PRLR gen je na Z hromozomu. U ovom radu, polimorfizam exona 2 PRLR gena u Japanskim prepelica (*Coturnix coturnix japonica*) je ispitan. Uzroci krvi 180 japanskih prepelica su sakupljeni nasumično. DNK je izolovana iz uzoraka krvi i amplifikovana. Polimeraze lančana reakcija - jedno lančani polimorfizam (PCR-SSCP) analiza exon 2 regiona PRLR gena je detektovala dva uzorka traka. Dva različita SSCP uzorka predstavljaju dva različita genotipa. Frekvencije dobijenih genotipova su 0.939 i 0.061 za AA i BB. Frekvencije alela su 0.939 i 0.061 za A i B. Dobijena vrednost za heterozigotnost (Hobs) je 0.0. Chi-square test je pokazao značajnu devijaciju ($P<0.05$) od Hardy-Weinberg ekvilibrijuma za ovaj lokus u ispitanoj populaciji. Efekat ovih genotipova na težinu je ispitan , i BB genotip je povezan sa težinom tela sa 46 dana. Ovo je prvo ispitivanje polimorfizma PRLR gena u japanskoj prepelici.

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