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Original scientific paper

ASSOCIATION OF POLYMORPHISMS IN THE PROMOTER REGION OF TURKEY PROLACTIN WITH EGG PERFORMANCE

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The induction and regulation of broodiness is of the most important role of prolactin in avian species. In this study, the association between prolactin promoter region alleles and reproductive traits in Fars native turkey was investigated . These traits consisted of mean egg weight (MEW), number of egg (EN) and egg mass, during the first laying period .In total, 115 laying turkeys, randomly selected from the flock of the Breeding Center for Fars Native turkey, and DNA was purificated from blood samples, 231 bp of prolactin promoter region was amplified and Genotype of Samples was determinate by PCR-SSCP technique were genotyped . Two alleles D and I were identified. Based on the results obtained, the frequency of D and I alleles were 0.67 and 0.33, respectively. Frequencies of DD, II and ID genotypes were 0.385, 0.044 and 0.571, respectively. The association analysis between the polymorphism PRL gene promoter region and egg performance was carried out. Significant relationship was found between genotypes with egg production ($P<0.01$). Individuals with II genotype produced higher egg production than DD and ID genotype. The results of current study showed that using information of genes related to egg production could be used to improve the performance of native turkey of East Azerbaijan province.

Key words: egg performance, polymorphism, prolactin promoter, turkey

INTRODUCTION

The broody instinct, actually, consists of two phases, incubation of egg (nesting) and raising the chicks (SAEKI and TANABE.1955). Incubation behavior is associated with the cessation

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of egg laying, and selection for persistency of egg production has resulted in a reduction in the incidence of the behavior (HUTT, 1949) The neurobiology of incubation behavior has been extensively studied in birds (SHARP,P.J.1989), the principal hormones involved being estrogen ,progesterone and plasma prolactin (PRL),and brain center that control incubation behavior being rich in progesterone and prolactin receptors (LUCASE *et al.,*1998). As has been long considered, broodiness apparently results from the PRL secretion by the anterior lobe of the pituitary (BURROWS and BYERLY.1936;PAYAN,1943 ;HUTT, 1949; SAEKI and TANABE.,1955). The onset of incubation in chickens and other birds was thought to be caused by an increase in pituitary PRL (BURROWS and BYERLY.1936). BURROWS and YERLY. (1938) stated that the pituitaries of broody hens, as compared with those of laying hens, showed no indication of an increase in PRL-like substance and suggested that PRL is not essential to the broody instinct. WONG *et al* .(1991) Isolated cDNA-encoding turkey PRL from a turkey pituitary library and established the increased levels of PRL mRNA and the corresponding increases in plasma PRL levels in photo stimulated , laying, and incubating hens relative to that found in nonphoto stimulated hens. Active immunization against PRL in bantam hens decreases broodiness (CRISOSTOMO *et al.* ,1997), and passive immunization of the vasoactive intestinal peptide, a PRL-releasing stimulator, can be effective in preventing or interrupting broodiness (CRISOSTOMO *et al.* ,1997). Therefore, it is obvious that PRL or the PRL gene, an autosomal gene, has a role in the onset and maintenance of broodiness. The expression of PRL depends on the 5′-flanking region sequence. Studies with mammals and birds have shown that Pit-1/GHF-1 (KURIMA *et al*.,1995), estrogen receptors (MAURER and NOTIDES 1987), and the CCAAT-enhancer binding protein-α (DAY et al .,2003),and other proteins are essential in regulating the expression of PRL via specific promoter binding sites. Physiologically, it has been well established that prolactin in poultry play an important role in the onset of incubation of hens. Increased plasma prolactin concentration is associated with the occurrence of broodiness. During incubation, prolactin mRNA reaches its highest level which infers that prolactin is important in the maintenance of broodiness. Egg laying pattern in domestic hen is characteristic to the breed of birds. Genetically superior birds' take fewer pauses compared to native breed of birds developed for dual purpose, resistant to diseases and adverse climatic variables as backyard poultry in rural areas (REDDY and RAJU.,2006). Broodiness is observed in most breeds of domestic fowl with the exception of the White Leghorn which has undergone long-term artificial selection to minimize phenotypic expression of this behavior (ROMANOVE *et al* .,2002). All authors agree that incubation behavior is a polygenic trait but although some authors have presented evidence of contributory sex-linked genes (SAEKI and INOUE. 1979), others have concluded that incubation behavior is controlled by a small number of dominant autosomal genes with no sex-linkage (HAYSE,. 1940). The purpose of this study was to estimate the allelic frequencies at the promoter prolactin gene and its association with egg traits of native Turkeys of East Azerbaijan breeding station.

MATERIALS AND METHODS

Population

Native turkey of East Azerbaijan were from a small population selected for individual phenotypic value of number of eggs (EN), mean egg weight (MEW) and egg mass during the first week of laying period.

DNA extraction and amplification of PRL promoter gene

A total of 115 blood samples were collected randomly in EDTA treated tubes as an anticoagulant. The collected blood samples were transferred to the laboratory using cooling chain and stored at -20°C for further analysis. Genomic DNA was isolated by using DNA extraction Kit based on BOOM et al. (1998). Quality and quantity of extracted DNA was measured on 0.8% Agarose gel prepared in 0.5 X TBE buffer (45 mM Tris base, 45 mM boric acid, 1mM EDTA, pH 8.0) and visualized with Ethidum Bromide (1.0 mg ml^{-1}) and photographed under UV light using a Gel-Doc image analysis system (Gel Logic 212 PRO, USA). The primer used for the amplification of a fragment of PRL gene (231 bp).The primer sequences were as follows:

PRL-Pro F:5´-TTGTATTATTTCCTTTCCAGAAATAGC-3´

PRL-Pro R: 5´- AAAATTTCAGTTGTGGGATGC- 3´.

The PCR were performed in a final volume of 25 µl containing 100 ng template DNA , 0.5 µl of each primers, 2.5 µl of 10 × PCR buffer, 4 µl of 1.25 mM dNTP (BioFluxbiotech, http://biofluxbiotech.com), 1 µl of 50 mM $MgCl_2$ (Cinna Gen, Tehran, Iran), 0.5 µl of Taq DNA polymerase (CinnaGen, Tehran, Iran) by using a 96-well Eppendorf Mastercycler Gradient. The following cycles were applied for the PRL gene amplification: initially denatured at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for30 sec, annealing 45 sec at 59°C, and extension 45 sec at 72°C; and a final extension of 10 min at 72°C.

Polymorphism detection of PRL promoter gene using single strand conformation polymorphism (SSCP)

The single strand conformation polymorphism (SSCP) analysis was used for detection of polymorphism of prolactin promoter gene(5′-flanking region). For SSCP analysis, 5 µl of PCR product were mixed with 10 µl of denaturing/loading buffer (formamide , NaOH , xylene cyanol and bromophenol blue). The samples were heat-denatured at 95°C for5 min and immediately chilled on ice, and loaded onto 6% polyacrylamide gel .Gels were run at 75V. After the run the gel was removed from the apparatus and the DNA bands were visualized through silver staining. The alleles were scored manually from the silver staining gel. Genotypes of individual birds at the different polymorphic loci were recorded by direct counting of the bands. The 100 bp ladder was used as molecular size marker.

Statistical Analysis

The allelic and genotypic frequencies and observed and expected Nei's heterozygosities (HE = 1- P^2i, where Pi is the frequency of allele i) were estimated by using PopGene32 version 1.31 (YEHET *et al* .,1997). Hardy-Weinberg equilibrium test was performed in the PopGene32. The association of genotypes with egg performance was investigated using the GLM procedure of SPSS software .The following model was used :$Yij = \mu + Gi + Hj + eij$, Where Yij is the average performance of ith genotype in jth hatch, µ is mean of the population, Gi is fixed effect of ith genotype (i=1,2,3), hj is fixed effect of jth hatch (j=1,2,3), and eij is random residual error .Number of recording days was included as a covariate for EN.

RESULTS

Genotype and Allele Frequencies

The electrophoretic profiles of SSCP analysis of the fragment obtained from primer pair are shown in Figure1. Two alleles, D and I and three genotypes, namely, DD, II and ID were observed in the population (As shown in Figure). The observed frequencies of alleles and genotypes for the PRL gene are shown in Table 1. The frequencies of D and I alleles were 0.67 and 0.33, respectively. The observed frequencies of DD, II and ID genotypes were 0.385, 0.044 and 0.571 , respectively. Observed heterozygosity value was 0.5702. Expected heterozygosity value was 0.4434. Effective number of alleles (Ne) was 1.7905 and Shannon's Information index (I) was 0.6334. The chi - square test revealed that the turkey population is not in Hardy-Weinberg equilibrium for this region of the PRL gene.

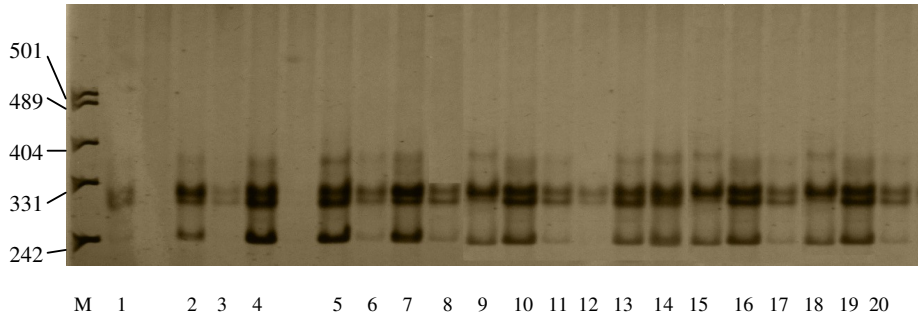

Figure 1. Genotypes of PRL promotor gene on 6% polyacrylamide gel.

M: PUC19, PCR Marker .2,4,5,6,7,10,11,13,14,16,17,19,20.ID; 1,3,8,12.II; 9,15,18.DD.

Table 1. Genetic diversity of PRL promoter locus in native turkey.

Genetic diversity statistics	Value	Allele frequencies	Value	Genotypic frequencies	Value
N_A	2.0000	D	0.6711	DD	0.385
N_E	1.7905	I	0.3289	II	0.044
I	0.6334			ID	0.571
Observed homozygosity	0.4298				
Observed heterozygosity	0.5702				
Expected homozygosity	0.5566				
Expected heterozygosity	0.4434				
Average heterozygosity	0.4415				

N_A = observed number of alleles,N_E = effective number of alleles.I =Shannon's information index

The association between PRL promoter region gene polymorphism and egg performance:

Association of gene polymorphism with egg performance was analyzed using the GLM procedure of SPSS11.0 . The results are presented in the Table2.In the turkey population, association analysis demonstrated that the Prolactin Promoter polymorphism was significantly associated with egg production, with the II genotype associated with higher egg production than the DD and ID genotype. Meanwhile, there was not significant difference in MEW, among the three genotypes.

Table2. The interaction of prolatin promoter region on egg performance of Turkeys .

PRL genotypes	Trait		
	Egg Number	Egg Mass	Mean egg Weight
DD	$50.84^{b} \pm 17.99$	$3937.18^{b} \pm 1411.96$	77.34 ± 3.5
ID	$48.48^{b} \pm 19.17$	$3686.06^{b} \pm 1456.6$	76.065 ± 3.6
II	$71.6^{a} \pm 14.34$	$5501.76^{a} \pm 1175.72$	76.79 ± 3.34
Total	50.40 ± 18.99	3862.6193 ± 1465.67	76.592 ± 3.56

Note: Within rows different superscripts (a – b) indicate significant differences (P <0.05).

DISCUSSION

More recent studies have demonstrated that incubation behavior is not controlled by major genes on the Z chromosome and proposed that at least 2 dominant autosomal genes are involved, one causing and the other inhibiting the behavior with equal influence (ROMANOV *et al* .,2002) Physiologically, it has been well established that prolactin (PRL) plays an important role in the onset of incubation of hens (SHARPE,.1989).Most of reported data are limited to native or commercial chickens in studies of CUI *et al* (2006), EMAMGHOLI-BEGLI *et al* .,(2010) , ALI PANAHI *et al*., (2011), RASHIDI *et al*,. (2012) and NIAZI *et al.,* (2013) . It has been reported that the avian PRL gene is highly conserved most of sequence polymorphisms in the chicken PRL gene occur in 5′ flanking region, 3′ flanking region, and the coding region of the signal peptide (WONG *et al* .,1991; CUI *et al*,. 2006). CUI *et al* .,(2006) reported that 3 SNPs had been detected in cPRL exon 2 and exon 5, and 1 SNP had been detected in cPRL intron 2. In study of EMAMGHOLI-BEGLI *et al* (2010), based on their results, the frequency of I and D alleles were 0.761 and 0.239 , respectively. Frequencies of II, ID and DD genotypes were 0.566, 0.389 and 0.044, respectively. Genotypes II and ID were significantly associated with increased EN (P<0.01). In study of HUI-FANG *et al*., (2009) in duck, three genotypes were found AA, AB, and BB. The frequencies of genotype BB and allele B were the highest. The BB ducks had

significant egg weight than that of AB ducks ($P < 0.01$). There were no significant association between PRL intron 1 polymorphism and the other traits ($P > 0.05$) in their study. In the present study, we found polymorphisms in Fars native turkey PRL gene ,which is in line with the results obtained by other researchers (CUI *et al*,. 2006; EMAMGHOLI-BEGLI *et al*,.2010;RASHIDI *et al* ,.2012; HUI-FANG LI *et al* ,.2009) . At the present study the frequency of D (0.6711) and I (0.3289) and frequencies of DD, ID and II genotypes 0.044, 0.571, 0.385, respectively was in agreement with the findings of EMAMGHOLI-BEGLI *et al.,(*2010). Amongst genotypes of PRL promoter region, egg performance (egg number , egg mass) of ID genotype was not significantly different from that of DD genotype, but egg performance of II genotype was significantly different from those of DD and ID genotypes (Table 2) which were approximately close to each other (EMAMGHOLI-BEGLI *et al*,.2010; CUI *et al*,. 2006, RASHIDI *et al* ,.2012; NIAZI et al ,.2013).Therefore, it may be assumed that the PRL gene affected egg production by regulating the activity of reproduction in turkeys .The frequency of allele D was 0.6711 , the frequency allele I was 0.3289,so the allele D was a preponderant gene in turkey population , but the frequency of heterozygous genotype (ID:0.571) was higher compared to homozygous (DD:0.044)and (II:0.385) genotypes . PRL gene as a candidate gene had been studied in laying hen (CUI *et al*,. 2006). In this study, the least square analysis showed that the II turkey had significant egg performance (egg number , egg mass) than DD and ID ($P < 0.01$). So, we presumed that the PRL gene influenced the egg weight by regulating the activity of reproduction. Prolactin, one of pituitary hormones, regulates important physiological functions, ranging from well known effects in mammalian reproduction to osmoregulation in fish and nesting behavior in birds . In turkeys, changes in plasma PRL levels are associated with the expression of PRL mRNA in the anterior pituitary (WONG et al ,.1991). The sequence variation in the 5′-flanking region of PRL may lead to changes in transcriptional factor binding sites and alter the expression of PRL. Polymorphisms in the promoter region, especially those that result in changes of promoter binding sites, most likely influence mRNA expression, thus influencing hen incubation behavior and egg production (CUI *et al*,. 2006). The 5′-flanking region (promoter region) of the PRL gene has been considered as an excellent experimental model for studying both tissue specific and hormonally regulated activation of gene transcription (SEYFRED and GORSKI .,1990). PRL is thought to be involved in modulating a great variety of physiological processes that are involved with development, metabolism, the immune and neural systems and reproduction. The variable effects of PRL may be due to receptor variations because alternative splicing gives rise to different isoforms of mammalian prolactin receptor (PRLR).The aim of the present study was to determine the polymorphism of Prolactin Promoter genes and to evaluate their association with some important economic traits in native turkey of East Azerbaijan. The effect of polymorphism of PRL gene on economic traits was estimated. In the present study, we showed that polymorphism occurring in the 5′-flanking regions of PRL gene in native turkey population were association with egg performance and that PRL polymorphism could be used as a marker for improving egg production in turkey. Improvement of reproductive traits in livestock has become of increasing interest where small increases in litter size can equal large gains in profit. Genetic improvement of reproductive traits has traditionally been restricted to the use of quantitative genetic methods but using these methods led to the limited gain only. Provided the major genes associated with reproduction are identified they can be utilized in breeding through marker-assisted selection (MAS). Reproductive traits are often suggested as prime targets for MAS for their low heritability and the fact that the trait can be measured only in one sex. In

conclusion, this study showed the presence of an association between prolactin gene polymorphisms and egg production in Fars native turkeys, but further studies are needed to confirm this association.

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ASOCIJACIJA POLIMORFIZMA PROMOTORSKOG REGIONA GENA PROLAKTINA I OSOBINA JAJA ĆURKE

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Izvod

Indukcija i regulacija zametka je najvažnija uloga prolaktina u vrstama ptica.Vršena su istraživanja povezanosti promotorskog regiona alela prolaktina i reproduktivnih osobina ćurke u pokrajini Istoćni Azerbejdžan u Iranu. Ove osobine su prosečna težina jajeta (MEW) broj jaja (EN) i masa u prvom period inkubacije u toku ležanja ćurke Odabrano je slučajno 115 Fars nativnih ćurki u leglu Centra za oplemenjivanje. Izvršeno je izolovanje i prečišćavanje DNA iz uzoraka krvi. Prolaktin promotor region veličine 231 bp je umnožen a identifikacija genotipa izvršena PCR-SSCP metodom. Identifikovana su dva alela, D i I. Dobijeni rezultati su pokazali da je frekvenca D alela 0,67 a alela I 0,33. Frekvenca DD, II I ID su utvrđene (0,385, 0,044 i 0,571). Izvršena je analiza asocijacije promotor regiona gena PRL polimorfizma i osobina jaja. Utvrđena je značajnost odnosa genotipa sa produkcijom jaja ((P<0.01). Jedinke II genotipa imaju veću produkciju jaja u poređenju sa DD I ID genotipovima. Dobijeni rezultati ukazuju da se informacija o asocijaciji gena i produkcije jaja može da koristi da se poboljšaju osobine nativnih ćurki u provinciji Istočni Azerbejdžan.

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