# Polymorphism in prolactin and PEPCK-C genes and its association with economic traits in native fowl of Yazd province

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***Abstract***
The objective of the present study was to investigate the polymorphism of prolactin promoter and cytosolic phosphoenol pyruvate carboxykinase (PEPCK-C) intron 3 to exon 3 regions, and its association with economic traits in native fowl of Yazd province. These traits consisted of body weight at 8 (BW8) and 12 (BW12) weeks of age, age at sexual maturity (ASM), weight at sexual maturity (WSM), mean egg weight at 28, 30, and 32 weeks (MEW), and the number of eggs during the first 12 weeks of laying period (EN). Blood samples were collected from 159 pedigreed fowl at native fowl breeding center of Yazd province, and DNA was extracted from the samples according to salting-out protocol. PCR amplification together with restriction fragment length polymorphism was used to identify different genotypes of prolactin and PEPCK-C genes. The effect of prolactin genotypes on economic traits was analyzed using general linear model. A 24-bp indel (insertion or deletion) at nucleotide position (np) 358 was identified, but no polymorphism was found for PEPCK-C. Based on our 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 incresased EN ($P<0.01$). Meanwhile, the genotypes of the 24-bp indel site were not significantly associated with BW8, BW12, ASM, WSM and MEW ($P> 0.05$). The results of current study showed that using information of genes related to egg production could be used to improve the performance of native fowl of Yazd province.

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## INTRODUCTION

Native fowl of Yazd province is a breed with moderate egg production and broodiness. The breed produces 150 eggs per year in average. Prolactin (PRL) is a peptide hormone synthesized and secreted by specialized cells in anterior pituitary of vertebrates. It has been well established that PRL plays an important role in the onset of incubation and brooding behavior of hen (Sharp *et al*., 1988; Shimada *et al*., 1991; Jiang *et al*., 2005). Elevated levels of PRL decrease the egg sequence lengths (clutch length) by increasing the intersequence pauses between the sequences of egg lay. This is particularly pronounced in native birds (Reddy *et al*., 2006; 2002). PRL is also involved in crop-sac development of columbiforms, induction and maintenance of broody behavior, regulation of gonadal function and immune responsiveness in a variety of species (Kansaku *et al*., 2008). Earlier studies revealed that PRL inhibits gonadotrophin stimulated ovulation and estrogen production at ovarian level in chicken. Furthermore, a decrease in PRL is found before and during the pre-ovulatory LH surge (Reddy *et al*., 2001). In avian species, dopamine has a dual role and can either stimulate or inhibit releasing of PRL. Dopamine has two types of receptors including D1 and D2. D1 and D2 receptors are presented in hypothalamus and anterior pituitary, respectively. When dopamine binds to D1 receptor, vasoactive intestinal

polypeptide (VIP) is released and consequently PRL is secreted. However, when dopamine binds to D2 receptors in the anterior pituitary, the release of PRL is inhibited (Youngren *et al*., 1996). 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). Liang *et al*. (2006) suggested that these loci might serve as potential genetic markers for chicken breeds, but also provide valuable insights into the regulation of chicken PRL gene expression. Compared to the extensive studies on the promoter region of mammalian PRL gene, the information on the 5′ promoter region of chicken PRL gene is rather limited. The flanking regions in chicken and turkey show high similarity (Liang *et al*., 2006). Unlike mammals where the synthesis and release of PRL is under inhibitory control, avian PRL is under tonic regulation and the major releasing factor is VIP (Kansaku *et al*., 2008).

Phosphoenolpyruvate carboxykinase (PEPCK; E.C. 4.1.1.32) catalyses the conversion of oxaloacetate to phosphoenolpyruvate and is an important rate-limiting enzyme involved in hepatic gluconeogenesis (Matte *et al*., 1997). There are two isozyme forms of the enzyme in vertebrates: cytosolic (PEPCK-C), and mitochondrial (PEPCK-M). However, both enzymes are encoded by distinct nuclear genes (Granner *et al*., 1983). Cytosolic PEPCK gene transcription is positively regulated by glucagon (through cyclic AMP) and glucocorticoids, whereas it is inhibited by insulin and glucose (Cournarie *et al*., 1999; Scott *et al*., 1998; Hanson and Reshelf, 1997). The sizes of PEPCK-C and PEPCK-M genes are 8 kb and 3.4 kb, respectively (yaccov *et al*., 1984). Birds are unique among animals studied to date in that their intracellular distribution of PEPCK varies among tissues, with the liver containing only PEPCK-M, but the kidney having both isozymic forms present. This tissue-specific pattern of distribution of PEPCK may contribute to the differing abilities of chicken liver and kidney to support gluconeogenesis from a variety of precursors (savon *et al*., 1997). PEPCK enzyme regulates a key step in de novo synthesis of glucose. Given the importance role of PEPCK in energy metabolism, it could be considered a potential candidate gene for production traits in poultry, where the development and evolution of such characters are highly dependent on energy metabolism (Torkamanzehi and Kuhnlein, 2007). Parsanejad *et al*. (2002) showed that for PEPCK gene, the C to T transition at locus R41 corresponds with a loss of an AciI restriction site (AACGTT to AATGTT) and the G to A transition at locus F22 with an acquisition of a BstEII restriction site (GGTGGCC to GGTGACC). These RFLPs can be used for rapid screening by PCR and to group haplotypes.

The objective of the present study was to estimate the frequency of PRL and PEPCK-C gene variants and to find possible association between genotypes and reproductive and growth traits in native fowl of Yazd province.

## MATERIALS AND METHODS

**Population:** Native fowl of Yazd province were from a small population selected for individual phenotypic value of body weight at 8 and 12 weeks of age (BW8, BW12) and the number of eggs (EN) during the first 12 weeks of laying period. In the first generation, eggs were randomly collected from rural areas without any history of introduction of exotic birds, and were hatched to constitute the base population. Day-old chicks were wing-banded. All birds were reared on floor, intermingled during the rearing period. Parents of the next generation were selected in two steps. In the first step, females and males were selected based on their BW8 and BW12. After 20 weeks of age, hens were transferred into individual cages and their egg production was recorded for 12 weeks. In the second step, hens were selected based on age at sexual maturity (ASM), EN and mean egg weight at 28$^{th}$, 30$^{th}$ and 32nd weeks (MEW); and cocks were selected based on the performance of their sisters. Average selection proportion of about 40% for hens and 5% for cocks were applied in each generation, in which 800 hens and 100 cocks were selected to produce next generation. Birds were fed with a diet containing 17.5% CP and 2,900 kcal of ME/kg during the growing period and a diet containing 16% CP and 2,900 kcal ME/kg during the laying period. Drinking water was provided ad libitum throughout the experiment. After 6 generations of selection, blood samples were collected from 159 pedigreed fowl at 15 weeks of age. Blood samples were kept into 3 ml tubes containing EDTA as coagulant agent. Genomic DNA was isolated from 200 µl blood samples using modified salting out method (Miller *et al*., 1988). The experimental birds were hatched in three different hatches. Economic traits including BW8, BW12, ASM, weight at sexual maturity (WSM), MEW and EN were recorded on mentioned birds.

**PCR and DNA sequencing:** Gel monitoring and spectrophotometry were used to determine quality and

quantity of the DNA. The primers used for the amplification of a fragment of PRL gene (130 or 154 bp, containing the 24 bp indel at np 358) and PEPCK-C gene (R4F4 region), were those described by Cui *et al*. (2006) and Parsanejad *et al*. (2002), respectively. The primer pair used for the amplification of the 24-bp indel site was 5′-TTT AAT ATT GGT GGG TGA AGA GAC A-3′ (forward); and 5′-ATG CCA CTG ATC CTC GAA AAC TC-3′ (reverse). The primer pair for the R4F4 region was 5′-GTC TCT CCC AAC GAA CCC AAC ATG-3′ (forward), and 5′-CCT CTT CTG ACA TCC AGC GAC C-3′ (reverse). The PCR was performed in 25 µl mixture containing 100 ng of genomic DNA, 1X PCR buffer, 0.5 µM of each primer (Takapoo Zist, Iran) and 12.5 µl of Sina Gene™ master mix (Iran). The following cycles were applied for the PRL gene amplification: 94°C for 5 min; followed by 35 cycles of 30 sec at 94°C, 30 sec at 54°C, and 30 sec at 72°C; and a final extension of 5 min at 72°C. The following cycles were applied for PEPCK-C gene: 95°C for 5 min; followed by 35 cycles of 60 sec at 94ºC, 80 sec at 62°C, and 90 sec at 72°C; and a final extension of 5 min at 72°C. The fragment amplified by the primer pair for R4F4 was digested with AciI endonuclease. The PCR-products of the 24 bp and R4F4 regions were run on 3 and 1 percent agarose gels, respectivly. Ethidium bromide was used for staining the gels. Allele and genotype frequencies were estimated by direct counting.

**Statistical analysis:** Frequencies of alleles and genotypes and their accordance to Hardy-Weinberg equilibrium were calculated using POPGENE (Ver. 1.31) software (Yeh *et al*., 1997). The frequencies of alleles and observed and expected genotypes were compared by the chi-square test. Association of PRL genotypes with BW8, BW12, ASM, WSM, MEW, and EN were analysed using the GLM procedure of SAS software (SAS Institute, 2001). 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

The electrophoretic profile of PCR products of PRL gene is shown in Figure 1. The frequencies of alleles and genotypes for PRL gene are shown in Table 1. Based on present results, the frequencies of I and D alleles were 0.761 and 0.239, respectively. Frequencies of II, ID and DD genotypes were 0.566, 0.390 and 0.044, respectively. The observed and expected genotypes of PRL were calculated. The analysis of chi-square test showed that the genotypes were in Hardy-Weinberg equilibrium (Table 2). The effect of polymorphism of PRL gene on economic traits was estimated. Insertion-insertion (II) and insertion-deletion (ID) genotypes were significantly associated with increase in EN ($P<0.01$) (Table 3). Nevertheless, genotypes of the 24 bp indel were not significantly associated with BW8, BW12, ASM, WSM and MEW ($P> 0.05$). Genotypes II and ID showed significantly higher EN.

The electrophoretic profile of PCR-RFLP products of PEPCK-C gene is shown in Figure 2. Based on

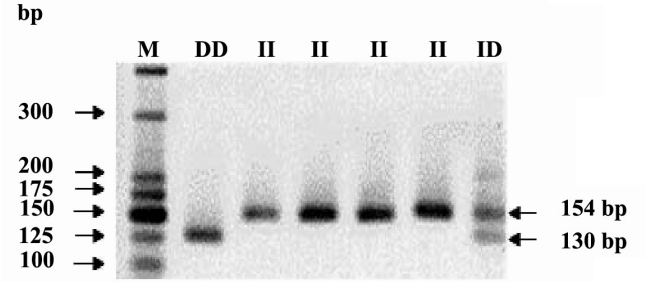


**Figure 1.** Genotypes of the 24-bp indel at np 358 by PCR with agarose gel electrophoresis. II: insertion-insertion; DD: deletion-deletion; ID: insertion-deletion; and M: size marker (300, 200, 175, 150, 125, and 100bp respectively).

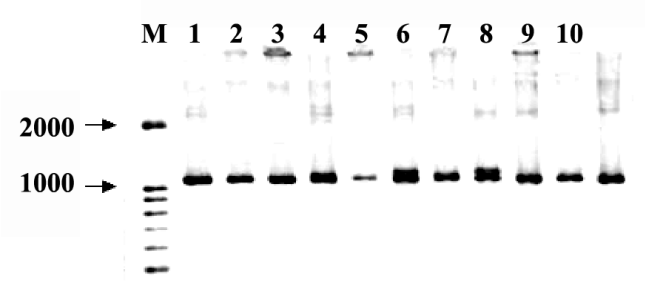


**Figure 2.** Genotypes of the R4F4 at np 1150 by PCR-RFLP, showing enzyme digestion products (1,2,…,11). M: size marker.

**Table 1.** Allelic and genotypic frequencies of the 24-bp indel site.

| | Allele | | Genotype | | |
|---|---|---|---|---|---|
| | I | D | II | ID | DD |
| Frequency | 0.761 | 0.239 | 0.566 | 0.390 | 0.044 |

[1]I = insertion allele; D = deletion allele; II = insertion-insertion; ID = insertion-deletion; DD = deletion- deletion.

**Table2.** Chi-Square test for Hardy-Weinberg equelibrum in prolactin genotypes.

| Genotypes | Observed (O) | Expected (E) | $(O-E)^2/E$ | $X^2$ |
|---|---|---|---|---|
| II[1] | 90 | 92.08 | 0.047 | 0.822 [ns 2] |
| ID | 62 | 57.84 | 0.299 | |
| DD | 7 | 9.08 | 0.476 | |

[1]II = insertion-insertion; ID = insertion-deletion; DD = deletion-deletion. [2]ns: non significant (p>0.05).

**Table 3.** Effects of prolactin promoter region on economic traits.

| Traits | II[1] | ID | DD | F-statistic | p-value |
|---|---|---|---|---|---|
| BW8(gr) | 390.65±6.652 | 394.38±7.88 | 418.60±23.13 | 0.50 | 0.69 |
| BW12(gr) | 658.00±9.50 | 667.50±11.26 | 708.77±33.04 | 1.16 | 0.31 |
| ASM(day) | 194.18±2.73 | 193.49±2.97 | 198.51±9.93 | 0.12 | 0.88 |
| WSM(gr) | 1253.05±19.92 | 1255.12±23.15 | 1251.63±74.60 | 0.20 | 0.93 |
| MEW(gr) | 41.73±0.48 | 42.03±0.51 | 40.46±1.64 | 0.39 | 0.81 |
| EN(number) | 37.47±1.9a3 | 36.79±2.1[a] | 15.59±6.3[b] | 5.47 | 0.005 |

[1]II: insertion-insertion; ID: insertion-deletion; DD: deletion-deletion. [2]Least-square means ± standard errors. [3]diffrent letters in each row shows significant difference (p<0.01).

PCR-RFLP analysis, no mutation was found for PEPCK-C gene.

## DISCUSSION

**Prolactin gene:** The frequencies of alleles I and D were studied in native and commercial chickens in a study by Cui *et al*. (2006). In their study, the frequencies of alleles I and D were found to be 1 and 0 in White Leghorn, 0.05 and 0.95 in Yangshan, 0.20 and 0.80 in Taihe Silkies, 0.22 and 0.78 in White Rock and 0.17 and 0.83 in Nongdahe. The frequencies of I and D alleles in native fowl of Yazd province were closer to White Leghorn. The underlying mechanism regarding how the 24-bp indel in the 5′-flanking region of PRL gene affects the phenotypic differences in chickens egg production is not clear. Several studies have shown that pituitary transcription and growth hormon factors (Kurima *et al*., 1995; Frisch *et al*., 2000), estrogen receptors (Maurer and Notides, 1987) and CCAAT-enhancer binding protien-α (Day *et al*., 2003; Enwright *et al*., 2003) are essential in regulating the expression of PRL via specific promoter binding sites. However, the sequence variation in the 5′-flanking region of PRL may lead to changes in transcription factor binding sites and alter the expression of PRL. In the present study, there was a 24-bp nucleotide sequence insertion at np 358 of the 5′-flanking region of PRL. Insertion of this sequence in the promoter may

inhibit pituitary transcription factor 1 (Pit-1), VIP and other transcriptional factor binding sites for PRL (Jiang *et al*., 2005) or may reduce secretion of stimulatory factors like thyrotropin-releasing hormone, that affect PRL release (Culewise *et al*., 2002) and therefore, decrease the expression of PRL. A possible ecotropic viral integration site-1 (Evi-1) binding site was found in the 5′ flanking region of the chicken PRL gene due to the 24-bp insertion (Cui *et al*., 2005). The presence of Evi-1 binding site suggested possible transcriptional regulation of the chicken PRL gene by Evi-1. It is possible that Evi-1 represses the expression of PRL gene in chickens by binding the Evi-1 binding site, and further prevents broodiness (Jiang *et al*., 2005), which can improve egg production to some extent. The results obtained in current study showed that PRL polymorphism could be used as a marker of egg production in native fowl of Yazd province.

**PEPCK-C gene:** Based on PCR-RFLP analysis, no mutation was found for PEPCK-C gene and all obtained genotypes for this region showed same bands. Parsanejad *et al*. (2003) studied the polymorphism of PEPCK-C gene in White Leghorn Chickens. In contrast to the result of the present study, they identified three single nucleotide polymorphisms (SNPs) in intron 3 to exon 3 region**.** Omrani Bidi *et al*. (2009) observed only two genotypes (AA and AB) in promotor to exon 2 region of PEPCK-C gene in Sistani native fowl, although Parsanejad *et al*. (2002) identified four SNPs in the same region in White Leghorn chickens. Based on current results and Omrani Bidi *et al*. (2009), it could be concluded that the genetic diversity of PEPCK-C gene in Iranian native fowl is lower than commercial chickens. It must be considered that only one restriction enzyme was used in this study (AciI), as well as in Omrani Bidi *et al*. (BsteII), while Parsanejad *et al*. (2003) used both enzymes. Therefore, lack of polymorphism in R4F4 region in the present study may be due to may be due to using only one restriction enzyme. We suggest using more restriction enzymes in future studies. The results of current study showed that using information of genes related to egg production such as prolactin and PEPCK-C could be used to improve the performance of native fowl of Yazd province.

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