



ORIGINAL ARTICLE

Prolactin and NPY Gene Polymorphism and its Associations with Production and Reproductive traits in West-Azarbaijan Native chicken

Mohammadbager Abdi *¹, Hamidreza Seyedabadi ¹, Abolfazl Gorbani ¹

1. Department of Animal Science, Shabestar Branch, Islamic Azad University, Shabestar, Iran

* Correspondence email: m.abdi@yahoo.com

ABSTRACT

The objective of this study was to determine the PRL and NPY gene polymorphisms and their associations with some reproductive traits, including body weight at 12 weeks of age, age at sexual maturity, egg numbers and average egg weight in West Azerbaijan native Fowls. For this purpose, a total of 200 blood samples were collected with equal numbers of male and female and DNA samples extracted by modified salting out method. After extraction, genotype was determined directly by PCR-RFLP method and agarose gel electrophoresis. Direct sequencing of some samples was used to results confirmation. To determine the genotypes of the animals, the 439 bp fragments from the 5' region of prolactin gene and 240 bp fragments from transcription starting site of the NPY gene were amplified and these fragments digested with Alul and Dral restriction enzymes. Data obtained from enzymatic digestions showed transition mutation (C→T) in the promoter region of prolactin gene and 4 bp deletion in the transcription starting site (TSS) of the NPY gene. Two genotypes (CC and CT) were obtained for prolactin gene and TT homozygous genotype was not seen in this study. For NPY gene three genotypes including AA, Aa and aa were detected. Allele frequencies showed that C (0.78) and a (0.73) alleles are most for prolactin and NPY genes, respectively. Results in this study showed that there are no significant association ($P>0.05$) between these sites and studied traits. Based on the results, we can conclude that these sites from the PRL and NPY genes cannot be appropriate marker for these traits and we must to study another candidate genes related with these traits.

Keywords: PCR-RFLP, PRL, NPY, reproductive traits, West-Azarbaijan Native chicken

Received 10.03.2014

Revised 19.04.2014

Accepted 05.05. 2014

INTRODUCTION

Prolactin (PRL) is a polypeptide hormone secreted by the anterior pituitary gland and has been shown to have a diverse spectrum of biological activities and functions in all vertebrates. The chicken prolactin hormone plays a crucial effect in egg production, because the onset of poultry incubation behavior (broodiness) is induced by an increase in prolactin secretion. Extensive studies on the mechanism of broodiness have been carried out to inhibit incubation behavior and improve the egg production of domestic fowl [1].

The expression of prolactin (PRL) depends on the 5'- flanking region sequence. Studies with mammals and birds have shown that Pit-1, CCAAT-enhancer binding protein- α estrogen receptors and other proteins are essential in regulating the expression of PRL via specific promoter binding sites [2]. 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 [1]. At around the time of hatch, the secretion of PRL significantly increases in chickens and turkeys in concert with increases in PRLR. Presumably, the increase in circulating PRL is associated with adaptation of the embryo to ex ovo life although in what capacity is not clear [3].

Neuropeptide Y (NPY) is known to be involved in the regulation of reproductive function at the hypothalamic level through the control of GnRH secretion [4, 5]. a commercial broiler breeder hen population, Dunn et al. [6] detected polymorphisms of the GnRHR and NPY genes and discovered an SNP in the intron 1 of the GnRHR gene and a 4-bp indel located 700 bp upstream of the NPY transcription start site. More than 77% of the world poultry population is in village production systems, contributing up to

88% of poultry products in some developing countries. In some developing countries, native stocks may reach up to 90 percent of the poultry population [7, 8]. A national project for increasing the number of native poultry breeds was started in Iran in 1983. In this project, various native breeds were reared in 14 poultry centers in different parts of the country and the vaccinated pullets distributed to the villagers. The project increased the number of native poultry, which was about 52 million pieces in the year 2006 (in contrast to 16 million pieces in 1982) [9]. Among the native chicken breeding stations in Iran, the West Azarbaijan breeding station, located in the West Azarbaijan province, Iran is one of the most important poultry strains in Iran.

Based on the literatures, the chicken PRL and NPY are considered to be the most important candidate genes that can influence chicken performance traits including growth, body measurement, carcass and reproduction. So, the hypothesis of the current study was to investigate PRL and NPY gene polymorphisms and their associations with some growth traits in West-Azarbaijan native chicken.

MATERIAL AND METHODS

Animal and Blood Samples

West Azerbaijan native poultry lines which their reproductive characteristics studied for 12 generation were used in this study (Tala Tabeh, west Azerbaijan province, Iran). A 200 West-Azarbaijan native birds (100 hens and 100 roosters) were randomly selected, blood samples (2ml in EDTA containing tubes) collected from via wing vein using disposable syringes in all birds and stored at -20 °C until used at hematology laboratory.

Establishment of a PCR-RFLP assay

The PCR primers in the 439 bases were used to propagation intron 2 of prolactin chicken using fragment as Forward: 5'-CTAAAggACCTggAAgAAggg-3' and reverse: 5'-AACTTGTCGTAGGTGggTCTg-3' (Fermentas, Germany) and in the 240 bases for NPY was forward: 5'-TCTCAgAgCTCAACCGTATgA-3'; reverse: 5'-ATATTTCTgTgTgAACACA-3' and accuracy of primers checked using blast procedure from NCBI database. The PCR was performed in a total volume of 15 µL, containing 5 µL of genomic DNA, 10 pmol of each oligonucleotide primer, 2 µL 25 mM MgCl₂, 2 µL of 1 mM deoxynucleotide triphosphate mixture, and 1 U of Taq DNA polymerase; cycle parameters were 94 °C for 5 min then 35 cycles of 94 °C for 45 sec, 60 °C for 45 sec, and 72 °C for 60 sec, with a final extension step for 10 min at 72 °C; the PCR products with length 439 bp were digested at 37 °C overnight with 10 U of AluI.

Carcass characteristics

To investigate polymorphism, phenotypic characteristics of the birds at 12 weeks of age include maturity age, mean of number and weight of eggs were used.

Number of alleles and effective alleles

Number of alleles and effective alleles were determined using PCR-RFLP. Genotype information processed using excel and determined using Pop Gene Version 1.31 software as described by Yeh *et al.*, [10].

Also, the hardy-Weinberg equilibrium test was performed using χ square test through Pop Gene Version 1.31 software [10].

$$\chi^2 \text{ square test} = \sum_u \sum_{v \neq u} \frac{2nD_{uv}^2}{p_u p_v} \quad X_T^2 = \sum_u \frac{nD_{uu}^2}{p_u^2}$$

STATISTICAL ANALYSIS

Obtained results was processed and analyzed using GLM procedure in SAS ver. 9.1 using Least Squares Means (LSM).

$$y_{ijk} = \mu + \text{Genotype}_i + \text{Sex}_j + e_{ijk}$$

RESULTS AND DISCUSSION

PRL5 allele in C2402T site (*ALU I*) in West Azarbaijan native birds

To our knowledge this is the first study to investigate correlation between prolactin and NPY gene polymorphism with production and reproductive traits in west-Azarbaijan native chicken. The number of confirmed causative genes associated with traits of biomedical, economic and evolutionary importance is still small and the list of identified candidate genes is limited. However, the candidate gene approach is useful for quickly determining the association of a specific genetic variant with phenotype [11]. The transition of C→T in 2402 bases in 5' (promoter) in prolactin gene was done by *ALU I* enzyme. Digestion of 439 bases using *ALU I* resulted to 4 segments in 54, 81, 144 and 160 bases for homozygote CC genotype

and 5 segments in 54, 81, 144, 160 and 304 bases for heterozygote CT genotype as well as 3 segment for homozygote TT genotype were in 54, 81 and 304, respectively (figure 1).

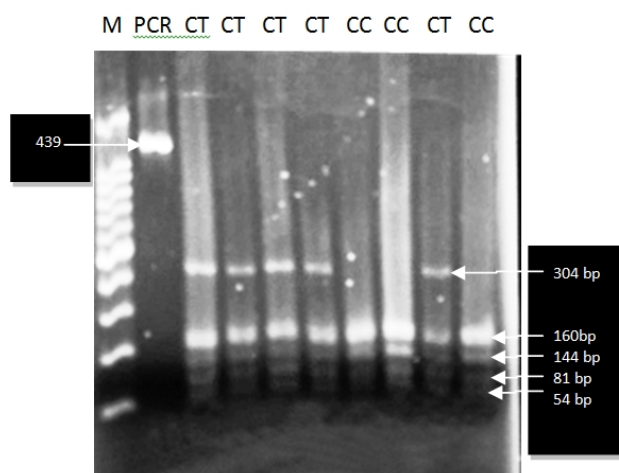


Fig 1. The *AluI* enzyme products on 439 fragments on intron 3 % agarose gel of prolactin gen. CC and CT genes, K: PCR product.

Previously, Cui et al., [1] using PCR-RFLP technique was reported that the promoter on prolactin gene in several poultry strain was in C→T location on 2402 bases and a 24 bases segment was detected in 358 fragments with 3 genotypes for each. In this regard Rashidi et al [2] using PCR-SSCP and PCR-SSCP-RFLP revealed that the prolactin promoter was in 2402 in C→T transition in native Mazandarani poultry.

Genotype and allele frequencies and hardy-Weinberg test PRL5 gene in West Azarbaijan native birds

Genotype and allele frequencies and hardy-Weinberg test PRL5 gene is presented in table 1. According to the data, frequency for C allele was higher than T allele but there was no difference between 2 alleles which might relate to selection strategies. In a similar study on Zaboli birds the same results reported which C allele frequency was higher than T allele (0.67 and 0.33, respectively) [12].

In this study the number of observed and prospect population revealed that this population was out of hardy-Weinberg equilibrium (table 1). In this regard Rashidi et al [2] reported the prolactin promoter standard deviation was out of hardy-Weinberg equilibrium and our data was similar to their findings.

Table 1. Genotype and allele frequencies and hardy-Weinberg test PRL5 gene in C2402T site (*ALU I*)

Genotype		Allele frequencies		hardy-Weinberg	
	Number	Frequencies		χ^2	P value
CC	56	0.56	C	0.88	
CT	44	0.44		7.896	0.005
TT	0	0.00	T	0.22	

Correlation of polymorphism on PRL5 gene with treats in West Azarbaijan native birds

According to the data, there was no polymorphism between prolactin gene and reproductive traits (body weight in 12 weeks of age, puberty age, number of eggs and mean egg weight) ($P>0.05$). The obtained results revealed that the birds with CC genotype were better than the other for reproductive traits (table 2).

Table 2. Correlation of polymorphism on PRL5 gene with treats

	CC	CT	P-value
Body weight on 12 weeks of age	179.777±4.987	175.605±5.578	0.361
Puberty age	12.902±0.513	11.827±0.574	0.167
Mean egg production	1.524±0.114	1.658±0.128	0.464
Mean egg weight	0.180±0.058	0.024±0.065	0.073

In comparison, Cui *et al.*, [1] reported that there was no correlation between reproductive traits and polymorphism on prolactin gene in native and commercial strains. Also, same investigations were observed on Mazandarani native poultry [2].

NPY genotype in West Azarbaijan native birds

Promoter for transcription (TSS)

As seen, a 4 bases I 495 location ~ 700 bases above the promoter of NPY transcription location results to polymorphism. The PCR reaction using 240 bases via *DraI* results showed that 79 and 161 segments were for homozygote (aa) whereas 79, 161 and 240 were for heterozygote (Aa) and the 240 segment related for (AA) homozygote genotype (figure 2). The same results previously reported in other poultry [6, 13-15].

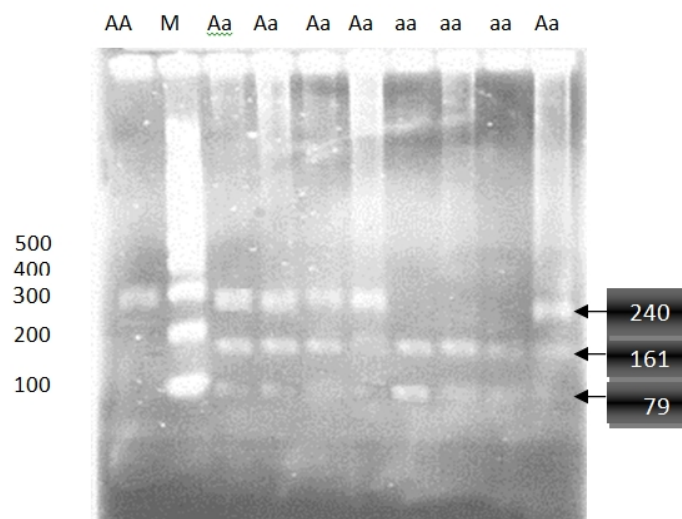


Fig 2. The *DraI* enzyme products on 240 fragments on intron 3 % agarose gel of prolactin gen. AA, Aa and aa gene PCR product.

Results of NPY gene sequence in promoter for transcription (TSS) in West Azarbaijan native birds

Results of PCR-RFLP on NPY gene sequence is presented in fig. 3-5.

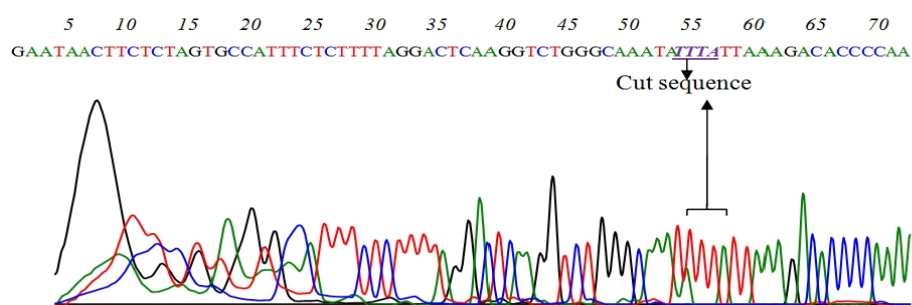


Fig 3. AA genotype frequency

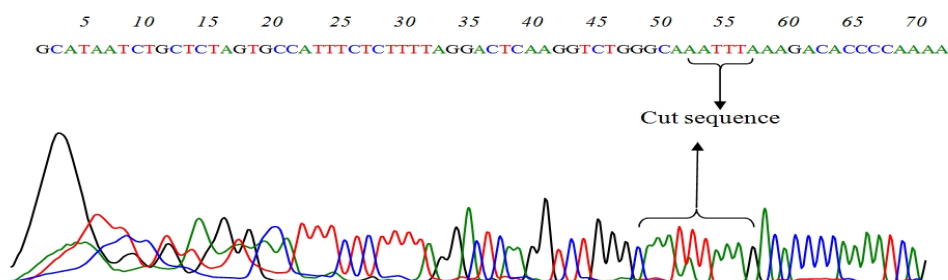


Fig 4. Aa genotype frequency

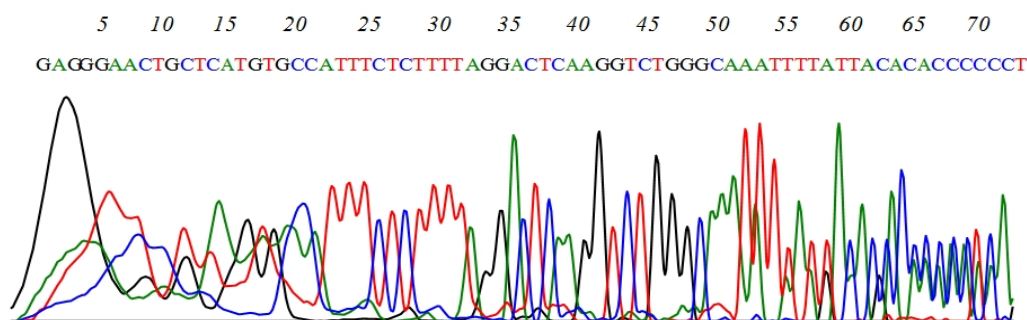


Fig 5. aa genotype frequency

Genotype and allele frequencies and hardy-Weinberg test NPY gene in West Azarbaijan native birds

As seen in table 3, genotype and allele frequencies for a allele was higher than A allele which was similar to previous observation on Chinese native poultry ($a=0.54$ and $A=0.46$) [14]. In comparison, in native Mazandarani poultry they were 0.780 and 0.221 for A and a alleles, respectively which our data was different from their observations [8]. To our knowledge the observed differences might related to difference in strains, sample selections and/or study population. Also, the study population was out of hardy-Weinberg equilibrium which there was differences between observed and prospects (table 3). It seems, the differences may related to correlation of NPY gene with other selected genes in West Azarbaijan native birds.

Table 3. Genotype and allele frequencies and hardy-Weinberg test NPY gene

Genotype	Allele frequencies		hardy-Weinberg	
	Number	Frequencies	χ^2	P value
AA	6	0.057	7.008	0.008
Aa	34	0.326		
aa	56	0.538		

Correlation of polymorphism on NPY gene with treats in West Azarbaijan native birds

Results of correlation of polymorphism on NPY gene is presented in table 4. As seen, there was no significant difference between NPY gene and reproductive traits ($P>0.05$). It is reported that there is a significant difference in NPY gene between 494-499 nucleotides with egg production age [6]. In study population, the heterozygote (Aa) genotype had lower age for egg production age than AA and aa genotypes. Also, they revealed that there was a correlation between total egg production and number of double yolk eggs with NPY which was similar to our observations.

Table 4. Correlation of polymorphism on NPY gene with treats

	AA	Aa	aa	P-value
Body weight on 12 weeks of age	164.110±15.432	174.820±6.499	183.920±5.069	0.436
Puberty age	11.322±1.534	13.124±0.646	12.382±0.504	0.485
Mean egg production	1.957±0.337	1.372±0.142	1.666±0.111	0.157
Mean egg weight	0.294±0.181	0.074±0.076	0.126±0.592	0.493

Formerly, Li et al., [14] reported that polymorphism of NPY in 494-499 nucleotides of NPY transcription had positive correlation with number of egg produced at 300 days of age. In our study, animals with AA genotype had much more egg produced until 300 days of age ($P<0.05$) but there was no significant correlation among NPY polymorphism and egg produced until 400 days of age, double yolk egg and mean egg days ($P>0.05$). Furthermore, no significant report was on NPY gene and I31391359D and I31394761T haplotypes using *DraI* and *KpnI* enzymes, respectively at 300 days egg production [16]. For instance, there was no significant on 494-499 nucleotides for NPY gene and reproductive traits in Mazandarani native birds [8]. In a commercial broiler breeder hen population [6] detected polymorphisms of the GnRHR and NPY genes and discovered an SNP in the intron 1 of the GnRHR gene and a 4-bp *indel* located 700 bp upstream of the NPY transcription start site. Subsequently, marker-trait association analyses were carried out, but no association of these two variations with total egg production was found. Similarly, in this study, the same sites including the site I31391359D of the NPY

gene and the A19960831G of the GnRHR gene were selected to analyze their effects on egg number at day 300, and the results were compatible with findings of Dunn *et al.* [6]. The PRLR gene seems to be a promising candidate, because it may regulate important physiological functions ranging from well-known effects in mammalian reproduction to broody traits in birds [17].

In birds the role of PRL are not yet understood precisely, but it is believed that the major function of PRL is manifested during incubation and feeding of nestlings (Hui-Fang *et al.*, 2009). The allelic frequencies obtained for 5' flanking region of prolactin gene by primer pairs of PRL5 in the present study differ from those reported by Cui *et al.* [1]. The associations detected by the analysis within the single generation of hens from the heterozygous sires suggest that the GnRHR gene and the NPY genes play a role in controlling the traits of double-yolked eggs and age at first egg, respectively [6].

The PRLR mutations might play a role in selecting for individuals with advantageous phenotypes. After domestication the reproduction performance of the pig has increased several folds due to an increase in litters and piglets. The PRLR knockout mice was accompanied by a 5–14% decrease in body weight, and a 50–60% decrease in abdominal fat mass, suggesting potential novel roles for PRLR in animal development and growth. absence of PRLR led to the decreases in the rate of bone formation and bone mineral density in mice, suggesting PRLR mutations may be required for the maintenance of normal bone formation and bone mass [17].

In Conclusion, Egg production is an important economic trait in the poultry industry. The search for molecular markers that influence reproductive traits of chickens has been well reported [18]. Number of eggs at 300 days of age (EN) and age at first egg (AFE) are the most economically important reproductive traits and much effort has been put into improving those [19]. Dunn *et al.* [6] identified polymorphisms in the GnRHR (gonadotropin-releasing hormone receptor) and NPY genes but discovered no association of the two polymorphisms with total egg production. In recent years, DNA polymorphisms have been widely studied in the GH gene of various animals. This suggested that the prolactin and NPY genes are, therefore, a potential marker for use in marker-assisted selection programmers. This needs to be further confirmed by evaluating polymorphism and traits of growth in other native breed and lines of chickens.

REFERENCES

1. Cui, J.X., Du H.L., Liang Y., Deng X.M., Li N., Zhang X.Q. (2006). Association of polymorphisms in the promoter region of chicken prolactin with egg production. *Poult. Sci.* 85:26-31.
2. Rashidi H., Rahimi-Mianji G., Farhadi A., Gholizadeh M. (2012). Association of prolactin and prolactin receptor gene polymorphisms with economic traits in breeder hens of indigenous chickens of Mazandaran province. *IRAN. J. Biotechnol.* Vol. 10, No. 2.
3. Hiyama G., Kansaku N., Kinoshita M., Sasanami T., Nakamura A., Noda K., Tsukada A., Shimada K., Zadwor D. (2009). Changes in post-translational modifications of prolactin during development and reproductive cycles in the chicken. *Gen. Comp. Endocrinol.* 161: 238-245.
4. Dhillon S.S., Gingerich S., Belsham D.D. (2009). Neuropeptide Y induces gonadotropin-releasing hormone gene expression directly and through conditioned medium from mHypoE-38 NPY neurons. *Regul. Pept.* 156: 96-103.
5. Klenke U., Constantin S., Wray S. (2010). Neuropeptide Y directly inhibits neuronal activity in a subpopulation of gonadotropin-releasing hormone-1 neurons via Y1 receptors. *Endocrinol.* 151: 2736-2746.
6. Dunn I.C., Miao Y.W., Morris A., Romanov M.N., Wilson P.W., Waddington D. (2004). A study of association between genetic markers in candidate genes and reproductive traits in one generation of a commercial broiler breeder hen population. *Heredity.* 92: 128-34.
7. Pym R.A.E., Guerne Bleich E., Hoffmann I. (2006). The Relative Contribution of Indigenous Chicken Breeds to Poultry Meat and Egg Production and Consumption in the Developing Countries of Africa and Asia. *Proceedings of the 7th European Poultry Conference, Verona.*
8. Fatemi S.A., Mehrabani-Yeganeh H., Nejati-Javaremi A., Niknafs S. (2012). Association of neuropeptide Y and gonadotrophin-releasing hormone receptor gene SNPs with breeding value for growth and egg production traits in Mazandaran native chickens. *Gen. Mol. Res.* 11 (3): 2539-2547.
9. Kamalzadeh A., Rajabbaigy M., Kiasat A. (2008). Livestock production systems and trends in livestock industry in Iran. *J. Agr. Soc. Sci.* 4: 183-188.
10. Yeh, F.C., Yang, R., Boyle, T. (1999). PopGene. Version 1.31. Microsoft window-based freeware for population genetic analysis. University of Alberta. Edmonton, AB, Canada.
11. Zhu M., Zhao S. (2007). Candidate gene identification approach: progress and challenges. *Int. J. Biol. Sci.* 3: 420-427.
12. Alipanah, M., K. Shojaian and H. Khani Bandani. 2011. The Polymorphism of Prolactin Gene in Native Chicken Zabol Region. *J. Anim. Vet. Adv.* 10(5): 619-621.
13. Li, G.H., zhang X.Y., Han W., Tu Y.J., Su Y.J. (2010). Effect of Single and Pyramiding Genotypes of PRL and NPY Genes on Baier Chicken Egg Production. *J. Hubei Agricultural. Sci.*
14. Li, H.F., Chen K.W., Wu X., Tang Q., Gao Y. (2008). Associations between GHR and IGF-1 gene polymorphisms, and reproductive traits in Wenchang chickens. *Turk. J. Vet. Anim. Sci.* 32(4): 281-285.

15. Li, H.F., Zhu W.Q., K.W. Chen, Zhang T.J., Song W.T. (2007). Association of polymorphisms in the intron 1 of duck prolactin with egg performance. *Turk. J. Vet. Anim. Sci.* 33(3): 193-197.
16. Xu H., Zeng H., Luo C., Zhang D., Wang Q., Sun L., Yang L., Zhou M., Nie Q., Zhang X. (2011). Genetic effects of polymorphisms in candidate genes and the QTL region on chicken age at first egg. *BMC Genet.* 12: 33, V. 12, PMC. 3096585, Published online 2011 April 15. doi: 10. 1186/1471-2156-12-33.
17. Lu A., Hu X., Chen H., Dong Y., Pang Y. (2011). Single nucleotide polymorphisms of the prolactin receptor (PRLR) gene and its association with growth traits in Chinese cattle. *Mol. Biol. Rep.* 38:261–266.
18. Zhou M., Du Y., Nie Q., Liang Y., Luo C., Zeng H., Zhang D.X. (2010). Associations between polymorphisms in the chicken VIP gene, egg production and broody traits. *British Poul. Sci.* 51: 195–203.
19. Li D.Y., Zhang L., Smith D.G., Xu H.L., Liu Y.P., Zhao X.L., Wang Y., Zhu Q. (2013). Genetic effects of melatonin receptor genes on chicken reproductive traits. *Czech J. Anim. Sci.* 58 (2): 58–64.

CITATION OF THIS ARTICLE

Mohammadbager A, Hamidreza S, Abolfazl G. Prolactin and NPY Gene Polymorphism and its Associations with Production and Reproductive traits in West-Azarbaijan Native chicken. *Bull. Env. Pharmacol. Life Sci.*, Vol 3 [6] May 2014: 39-45