

*Full Length Research Paper*

# Genetic polymorphism of exon 9-11 of the leptin gene receptor in breeder hens of Mazandaran native fowls

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Accepted 31 August, 2011

Leptin is a 16 kDa protein synthesized by white adipose tissue and involved in regulation of feed intake, energy balance, fertility and immune function. In order to evaluate the leptin gene receptor polymorphism, we used a restriction fragment length polymorphism (RFLP) method. Blood samples were collected from 100 randomly chosen Mazandaran native fowls. Genomic DNA was extracted using modified salting-out method and amplified polymerase chain reaction technique. Exon and intron 9-11 of the fowl leptin gene receptor was amplified to produce a 382 bp fragment. The PCR products were electrophoresed on 1% agarose gel and stained by etidium bromide. Then, amplicons with Tsp509I were digested and revealed two alleles, A and B. Data were analysed using PopGene 32 package. In this population, AA, AB, BB genotype have been identified with the 69.14, 30.16 and 0.7% frequencies. A and B alleles frequencies were 0.84 and 0.16, respectively.  $\chi^2$  test did not show Hardy-Weinberg equilibrium in this population ( $p < 0.05$ ). Further association analysis is required to clarify the effects of these marker genotypes on production traits in this breeder flock.

**Key words:** Leptin gene receptor, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), polymorphism, breeder hen.

## INTRODUCTION

Leptin is a 16 kDa hormone that has been shown to play an important role in the regulation of feed intake, energy expenditure and hypothalamus endocrine function in response to nutritional changes (Friedman and Halaas, 1998; Elmquist et al., 1999). The leptin gene is located on chromosome 6 in the mouse and chromosome 7 in humans, and encodes a protein that shows a high degree of homology between species. Mutations in this ob gene revealed the pivotal role of leptin in energy balance (Zhang et al., 1994).

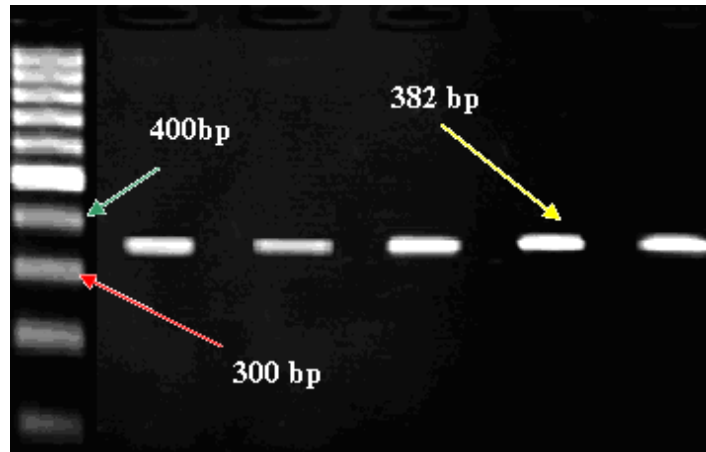
Recent studies have demonstrated that leptin is produced by other tissues, such as brain, pituitary gland, skeletal muscles and stomach. In mammals, leptin is expressed primarily in adipose tissue (Zhang et al., 1994) and at a lower level in the placenta and stomach (Masuzaki et al., 1997; Bado et al., 1998). Organization of this gene is conserved among mouse, human and bovine, presenting three exons and two introns (Taniguchi et al., 2002). Leptin receptors have been located on

neurons producing neuropeptide Y (NPY), and when activated by leptin binding, it is hypothesized to function in part by down regulating the production of hypothalamic NPY (orexigenic effector) to inhibit ingestive behavior (Schwartz et al., 1997). Several studies have shown that exogenous administration of leptin decreased feed intake in chicks, which is similar to that described in mammals, but the anorexigenic effect within chicken hypothalamus was mediated via selective neuropeptides, such as NPY and orexin (Denbow et al., 1997; Dridi et al., 2005). The objective of the present study was to evaluate genetic polymorphisms of exon 9-11 of the leptin gene receptor in breeder hens of Mazandaran native fowls using PCR-RFLP methodology. Association between different allelic and genotypic forms of gene and economical important traits can be found if the polymorphisms are seen. Finding association can improve accuracy and genetic gain in fowls.

## MATERIALS AND METHODS

In this study, blood samples were collected from 100 randomly chosen chicken in Native fowls breeding station of Mazandaran,

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**Figure 1.** The PCR product of leptin gene receptor.

located in the north of Iran. Approximately 5 ml blood sample was gathered in EDTA tube and was transferred to  $-20^{\circ}\text{C}$  freezer. Genomic DNA was isolated by using DNA Extraction Kit and was based on Miller et al. (1988) method. Exon and intron region from a portion of the 9-11 repetitive domain of the leptin gene receptor amplified to a product of 382 bp using primers based on the sequence of the fowl. Spectrophotometer was used for the investigation of quality and quantity of DNA.

#### Primers design

One set of primer was designed using Primer3 primer design software (Rozen and Skaletsky, 2000-[www.frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)). These primers were designed based on the chicken sequence leptin gene receptor (GenBank – NC006095.2) *Gallus gallus* chromosome 8, reference assembly (based on *Gallus gallus*-2.1). The primers were analyzed on NetPrimer (<http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html>), in order to avoid secondary structures, such as hairpins and loops, and primer dimer. Specific primer pairs were prepared from sina gene (Iran) company in lyophilized form and were dissolved in double sterile water and stored at  $-20^{\circ}\text{C}$ . Blood purified DNA showed better quantity and quality.

#### Method of detection

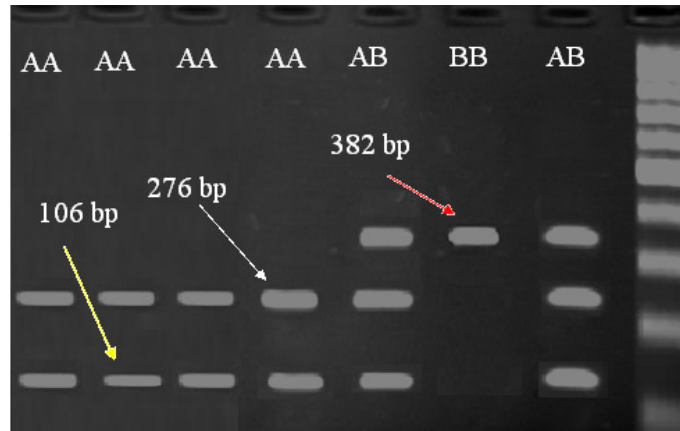
The sequences of the forward and reverse primers for the amplification of the leptin gene receptor were: F 5'-ATAGCTTTGAATGTTGGTGTCTG-3' and R 5'-AGCTGTGATACTGACTGCCTGT-3'. The polymerase chain reaction for the leptin gene receptor was performed using a buffer PCR1X, 200  $\mu\text{M}$  dNTPs, 3  $\mu\text{M}$   $\text{MgCl}_2$ , 8 pmol each primer, 0.15 U taq DNA polymerase, 200 ng genomic DNA and  $\text{H}_2\text{O}$  up to a total volume of 25  $\mu\text{l}$ . 35 cycles of preliminary denaturation at  $93^{\circ}\text{C}$  (4 min), denaturation at  $94^{\circ}\text{C}$  (1 min), annealing at  $58^{\circ}\text{C}$  (1 min), extension at  $72^{\circ}\text{C}$  (1 min) and final extension at  $72^{\circ}\text{C}$  (4 min) was used. The PCR products were separated by 1% (w/v) agarose gel electrophoresis. The amplified fragment of leptin gene was digested with Tsp509I, 11  $\mu\text{l}$  of PCR production with 2  $\mu\text{l}$  buffer, 5 U (0.5) of Tsp509I and 11.5  $\mu\text{l}$   $\text{H}_2\text{O}$  up to a total volume of 25  $\mu\text{l}$ , following the manufacturers instruction for 12 to 16 h at  $37^{\circ}\text{C}$ . The digestion products were electrophoresis on 1% agarose gel in 1X TBE and visualized by ethidium bromide staining for 1 h at  $85\text{ V}$ .

#### Statistical analysis

Pop Gene 32 package (Yeh et al., 1999) was used to calculate genotypic and allelic frequencies and to detect the state of population with Hardy-Weinberg equilibrium.

## RESULTS AND DISCUSSION

RFLP is a rapid, simple and exact technique for single nucleotide polymorphism genotyping. After a forced restriction site was introduced into one of the primers, the PCR produced contained a certain restriction enzyme site. The specific primer pairs were designed from sequence of leptin receptor gene in genbank NCBI site. The amplified leptin gene receptor resulted in a DNA fragment with 382 bp including the sequences of exon and intron regions from a portion with PCR technique (Figure 1). The Tsp509I restriction enzyme has restriction site of (AATT) in the amplified segment and cut this site after second (A) base. If the enzyme cut the segment, two alleles (A and B) were observed, resulting in three genotypes. The Tsp509I digests the allele A amplicon, but not allele B. The animals with both alleles were designated as AB genotype, whereas those possessing only A or B alleles were designated as AA or BB genotypes, respectively. Genotype AA showed two band patterns (bands of approximately 276 and 106 bp). Genotype BB had one band pattern (approximately 382), while AB animals displayed a pattern with all three bands (382, 276 and 106) (Figure 2). The digestion of all samples showed that breeder hens Mazandaran native fowls were polymorph for leptin gene receptor. This result shows that there were polymorphisms in leptin gene receptor segment, as previously observed by Mokhtarzadeh et al. (2009). A and B allele frequencies were 0.84 and 0.16, respectively. The genotype frequencies within 100 breeder hens examined were 69.14 for AA, 0.7 for BB and 30.16 for AB.  $\chi^2$  test did not show Hardy-Weinberg equilibrium in this population ( $p < 0.05$ ).



**Figure 2.** Genotyping for native fowl leptin gene receptor by restriction enzyme Tsp509I, genotype AA (276 and 106 bp size), genotype AB (382,276 and 106 bp size) and genotype BB (382 bp size).

The observed and expected heterozygosity were 0.19 and 0.27, respectively. To confirm accuracy of digestion, this process was performed twice. The low diversity and the difference between effective and true allele number is due to more frequency of allele A as compared to allele B that reduced frequency in this locus. This number is more, if there are more loci with same combination of alleles.

Investigation of mRNA of leptin gene receptor showed eighteen exons in it (Almeida et al., 2003). Cloning study revealed expression of leptin gene in chicken's liver (Taose et al., 1998). The polymorphisms of leptin gene receptor were surveyed in Khoozestan native fowl population using RFLP-PCR (Mokhtarzadeh et al., 2009). They showed that change in restriction site of HaeIII generated different restricted segments. Three genotypes and two alleles were seen in Khoozestan fowls. The allelic frequencies of leptin gene receptor in Khoozestan fowls were 31.19 and 61.81 for alleles A and B, and genotypic frequencies were 18.81, 24.75 and 54.44 for genotypes AA, AB and BB, respectively. Results show deviation from Hardy-Weinberg equilibrium in population of Khoozestan fowls. Association study revealed that allele A had positive effect than allele B on economical traits. The observed and expected heterozygosities were 0.243 and 0.568. Obtained results from Mazandaran native fowls are antonymous with Khoozestan fowls. The frequency of mutant allele is higher than wild type allele in Mazandaran native fowls. It could be due to physiological role of allele A in Iranian fowls. Inbreeding and family selection can be one of the major factors enhancing AA genotype in Mazandaran native fowls. Reared chickens in Mazandaran native fowls breeding station are prepared from state center and in addition, it is a closed population and therefore open for disequilibrium factors. Inbreeding coefficient is high in the closed population that in turn, caused decreases of

diversity in population. Increasing effective population size, controlling mating and preparing independent populations with large number of primitive individuals are necessary for preventing decrease of diversity in Mazandaran native fowls.

## Conclusion

The main objectives of the current strategy in commercial broiler breeding programs were to increase growth rate, increase breast muscle yield, decrease abdominal fat pad content, increase feed efficiency and increase overall fitness. The obtained results in the present study indicated that the A allele frequency in leptin gene receptor loci was the dominant allele. It can be concluded that the incidence of higher A allele frequency for leptin gene receptor loci may be as a result of long term selection strategy used in this population. Further association analysis will be required to clarify the effects of these marker genotypes on production traits in this breeder flock.

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