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Full Length Research Paper

Study of polymorphism of leptin gene receptor in Mazandaran fowls

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In chickens, leptin is expressed mainly in the liver and adipose tissue. In Iran, Mazandaran native fowls are under recording and breeding programs, but according to the action modes and importance of the leptin receptor, its polymorphisms can be related to economical traits such as body weight. In this study, in order to identify allelic polymorphism in leptin gene receptor, a restriction fragment length polymorphism (RFLP) method was used. Blood samples were collected randomly from 100 individuals. The DNA extraction was based on a salting-out method, while an amplified polymerase chain reaction technique was used. The quantity and quality of extracted DNA were examined using spectrophotometric and agarose gel electrophoresis. A strategy, employing polymerase chain reaction, was used to amplify a 374 bp fragment of 9 to 11 exon leptin gene receptor. Digestion of amplicons with HaeIII revealed leptin gene receptor. The obtained results from restriction digestion showed none of the polymorphism in leptin receptor gene, so all samples were monomorph due to the fact that there was no mutation that was related to polymorphism.

Key words: Leptin gene receptor, PCR- RFLP, polymorphism, fowl, HaeIII.

INTRODUCTION

In farm animals, the control and prediction of fatness is of a high economic interest. The exaggerated adipose tissue development negatively affects the whole body metabolism, production efficiency, reproduction and meat quality. The word leptin comes from the Greek leptos, meaning thin, referring to the anti-obesity effect which was believed to be the primary physiological function of the hormone. Leptin is the product of the ob gene, discovered by Zhang et al. (1994) using the positional cloning technique.

Leptin was first identified as the gene product found deficient in the obese ob/ob mouse. The 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). The full coding sequence contains 167 amino acids (a.a.) and is composed of a 21-amino acid signal peptide and a 146-amino acid circulating, bioactive hormone. However, leptin is a 16-kDa hormone that has been shown to play an important role in the regulation of food intake, energy expenditure and hypothalamus endocrine function in response to nutritional changes (Friedman and Halaas, 1998; Elmquist et al., 1999). 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). Thus, it represents an excellent candidate gene for polymorphism investigation and association with economic traits in livestock species. Organization of this gene is conserved among mouse, human and bovine, presenting three exons and two introns (Taniguchi et al., 2002). In bovine, polymorphisms on LEP gene has been associated with body fat, feed intake and milk yield (Buchanan et al., 2002; Liefers et al., 2002; Nkrumah et al., 2005). Unlike

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Abbreviations: PCR, Polymerase chain reaction; **RFLP,** restriction fragment length polymorphism; **LEP,** leptin; **LEPR,** leptin receptor; **NPY,** neuropeptide Y.

mammals, little is known about the avian LEP gene function. In chicken, only its coding sequence was identified and sequenced by Taouis et al. (1998). The leptin signal is mediated through a species receptor localized in the target tissues, and the leptin receptor (LEPR) belongs to the class I cytokine receptor superfamily that shares common structural features and signal transduction pathways (Tartaglia et al., 1995). Leptin mediated its central effect through specific receptors located in the hypothalamus. 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 hypohypothalamic NPY (orexigenic effector) to inhibit ingestive behavior (Schwartz et al., 1997).

Mammalian adipocytes produce and secrete more leptin in bloodstream as fat storage increases (Maffei et al., 1995), signalling the brain via leptin receptor (Hakansson et al., 1996; Banks et al., 1996) and modulating the hypothalamic neuropeptide system to suppress appetite and increase energy expenditure (Rohner et al., 1996; Kristensen et al., 1998). More interestingly, it has been shown that chicken leptin gene expression is sensitive to hormonal treatment in liver, but not in adipose tissue (Ashwell et al., 1999). These observations are thought to be due to the role of the avian liver as the primary source of lipogenesis (Goodridge and Ball, 1967; Leveille et al., 1968). The peripheral action of leptin in the chicken is poorly documented except in the pancreas, where it has recently been demonstrated that leptin has a profound inhibitory influence upon insulin secretion in the perfused chicken pancreas (Benomar et al., 2003). Several studies have showed that exogenous administration of leptin decreased feed intake in chicks, which was similar to the one described in mammals, but the anorexigenic effect within chicken hypothalamus was mediated via selective neuropeptides, such as NPY and orexin (Dridi et al., 2005).

In Iran, Mazandaran native fowls are under recording and breeding programs, but according to the action modes and importance of leptin gene receptor, its polymorphisms can be related to economical traits such as body weight. The aim of the present study is to investigate the polymorphisms of leptin receptor gene in 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 an association between these caused an improved accuracy and genetic gain in fowls.

MATERIALS AND METHODS

Experimental population

The study, which was conducted at the Native fowls breeding station of Mazandaran located in the North of Iran, have been established in 1988 with the objective of conserving the endangered population of native fowls in rural areas. The location is typically hot and semi-arid with yearly minimum and maximum temperature ranges between 4 and 34°C, respectively. The station has two main activities, namely extension and genetic improvement. Genetic improvement is done by selecting the best 100 cocks and 800 hens as parents of the next generations. Parents of each generation are selected among 7000 pedigreed and performance recorded birds produced by each generation. The extension part is continuously producing and distributing 8 weeks old chicks among rural communities with the aim of increasing the population of native fowls in Northern provinces of Iran. Rearing chicks in a 30 to 60 days period and distributing them in rural areas to enhance meat and egg production are quantitative goals of Mazandaran native fowl breeding station.

Sample collection and DNA isolation

A total of 100 blood samples (5 ml) were collected in EDTA (1 mg ml^{-1}) treated tubes as an anticoagulant from randomly chosen individuals. The chicks were sampled randomly from four rearing salons, and each contains chicks with same hatching. Samples were transferred to the laboratory with ice flask and stored at -20°C for further analysis. DNA was isolated by standard salt procedure described by Miller et al. (1988). The quality and quantity of the extracted DNA was checked by spectrophotometer and agarose gel electrophoreses.

DNA samples were adjusted to a concentration of 25 µl reaction mixture, containing 200 ng/µl, and exactly 1/5 µl of the DNA samples were used as template for polymerase chain reaction.

Primers design

One set of primers was designed using Primer3 primer design software [(Rozen and Skaletzky, 2000) - 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 and 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 dimmer. Specific primer pairs were prepared from the Sina gene (Iran) company in a lyophilized form and were solved in double steriled water and stored at -20°C. Blood purified DNA showed better quantity and quality.

Leptin gene receptor amplification

The design primer, primer premier 3(2000) software, was used for the DNA sequence in exons 9 to 11 leptin gene receptor, while the Net Primer was used to compare the produced sequence and the saved sequence. The sequences of the forward and reverse primers for the amplification of the leptin gene receptor were: F 5´-GTGTGATAGCTTTGAATGTTGGTG -3´ and R 5´- CTCTTCTG TTGCCAGCTGTGAT -3´. The polymerase chain reaction for the leptin gene receptor was performed in a 25 µl reaction mixture, containing 3 mM $MgCl^2$, 200 µM of each dNTPs, 0.4 µM of each primers, 1X PCR buffer, 1U Taq polymerase (Cinagen, Iran) and 100 ng of genomic DNA template. The reaction mixture was placed in a DNA thermal cycler (Perkin Elmer 9700). Thermal cycling conditions included an initial denaturation step at 93°C for 3 min followed by 33 cycles of 93°C for 45 sec, 57°C for 45 s, 72°C for 45 s and a final extension at 72°C for 4 min.

Restriction digest

For the PCR- RFLP assays, 15 U of the PCR products were digested

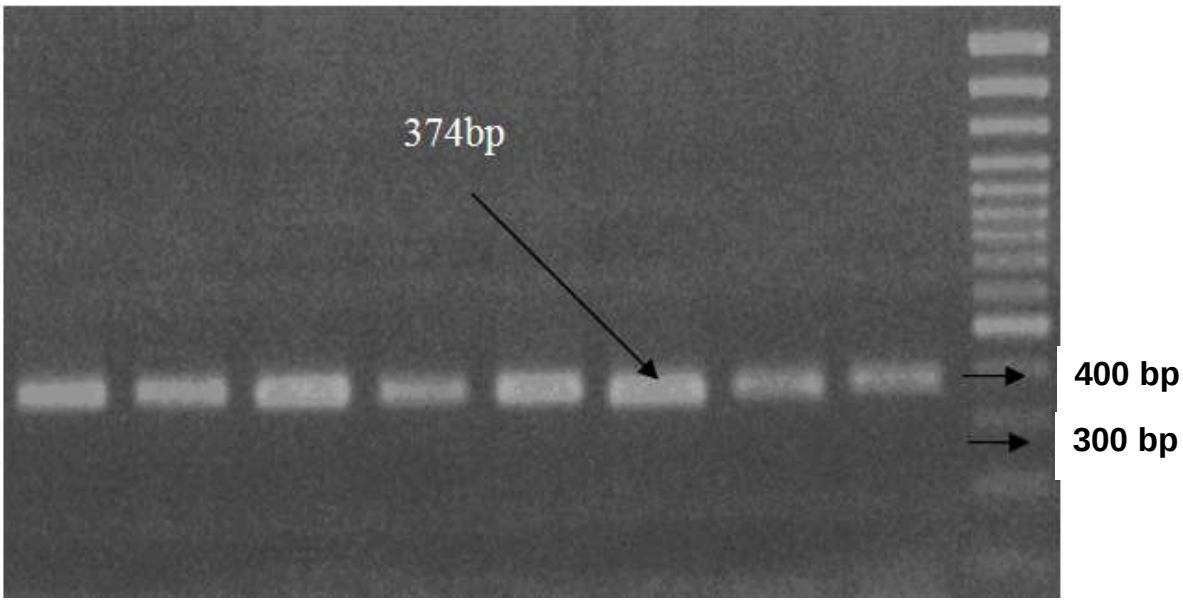

Figure 1. Various samples obtained from PCR.

55 GTGTGAT AGCTTTGAAT GTTGGTGTTC TGTTTTCATT GGCTCCTTAT
TACGTCTTCC TCTACAGTCA GTGCTGCCAA CTGTAACAGCACAGAACTG
ATCATCCTGGA AAAACAGAAC GCTAAGCTAA ACCCTCTAAGCAGCAGAC
AACTGTTAATCA GTATTAGCTG TGTTGACAGGAAGAGGAAGGATTCAGC
TAAGGCTAACTTT TGTGGTCCAG CCGAATAATG AATACCCTATCTGGCC
ACTCTTACTGCAAG TCTGAGCTGT GCAGAAGCTT AGGAGGAATT
TGGAGCCTGTCCTTCTCACC TCCCATAAGA CATTACTGCT ATTTTGTCCA
429 GAGCCCAGGTTTCCTCTCTT CTGTTGCCAG CTGTGAT

Figure 2. Schematic of the 374 bp sequence of leptin gene receptor.

with 5 units of HaeIII (Gibco BRL, life Technologies, USA) at 37°C for at least 24 h, respectively. Digested products were separated by electrophoresis on 1% agarose gel in 1×TBE (Tris-Boric acid-EDTA) buffer at 85 V for 1 h. The 100 bp DNA ladder (Fermentas SM0331) was used in each gel as a molecular size standard. The gels were stained with ethidium bromide and the fragments were visualized using UV transilluminator.

RESULTS AND DISCUSSION

One of the challenges of the poultry industry is to improve chicken's carcass quality and reduce fat content, without prejudicial effects on the genetic gains already obtained. In poultry, the animal's rapid growth rate has led to excessive body fat associated with impairment of the total body metabolism, disorders in the reproductive functions and muscular development resulting in low performance with high mortality.

Using an appropriate thermal program in PCR, the 374 bp segment was amplified without unspecific bands (Figure 1). The specific primer pairs were designed from sequence of leptin receptor gene in genbank NCBI site. Using these primers, a 374 bp segment from exons 9 to 11, which started from nucleotide 55 to 429, was amplified (Figure 2). The HaeIII restriction enzyme has a restriction site of ((GGCC)) in the amplified segment and this site was cut after the second ((G)) base. If the enzyme cuts the segment, then two bands with lengths of 241 and 133 are seen after electrophoresis on the gel (Figure 3). However, if the mutation did not occur in the amplified segment, the HaeIII can not cut the segment and as such, only one allele (allele B) would be seen, but if the mutation occurs, two alleles (A and B) are seen after restriction of digestion. It is shown in the samples with two bands that the DNA was cut at both strands after digestion. The digestion products showed that Mazandaran native fowls were monomorph at the leptin receptor gene, but the entire samples showed one band

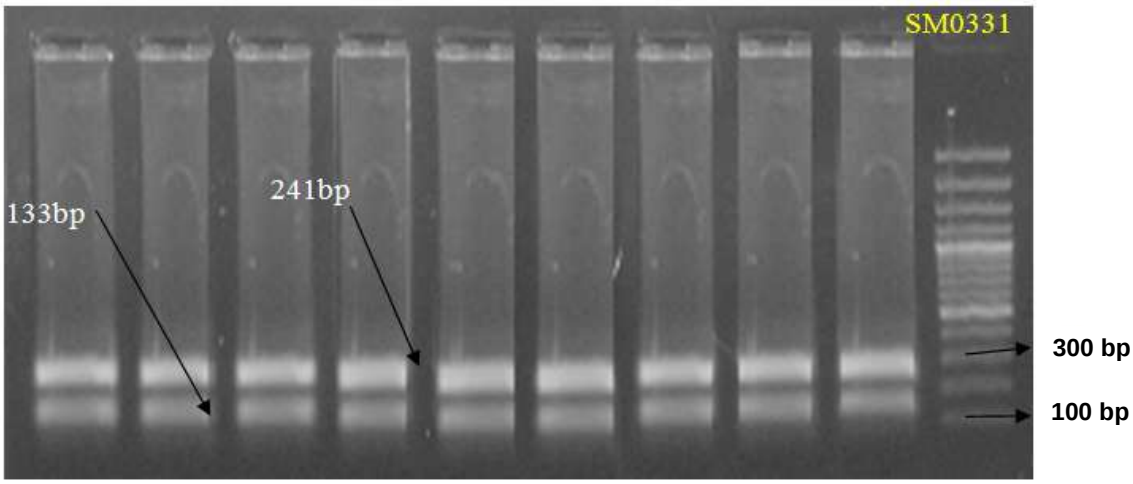

Figure 3. Results of PCR-RFLP analysis for leptin gene receptor by restriction enzyme HaeIII on 1% agarose gel and on ladder 100 bp (fermentas).

on agarose gel; therefore, all had BB genotype in this locus. To confirm the accuracy of digestion, this process was performed twice, and the cloning study revealed that the leptin receptor gene has six isoforms such as the long isoform. Other cloning studies showed three exons and two introns in the leptin gene. Investigation of the mRNA of the leptin receptor gene showed eighteen exons in it (Almeida et al., 2003).

Nevertheless, the cloning study revealed an expression of leptin gene in the chicken's liver (Taose et al., 1998). The polymorphisms of leptin receptor gene were surveyed in Khoozestan native fowl population using RFLP-PCR at 2009. It showed that change in the restriction site of HaeIII generated different restricted segments. Consequently, 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 the genotypic frequencies were 18.81, 24.75 and 54.44 for genotypes AA, AB and BB, respectively. Results showed deviation from Hardy-Weinberg equilibrium in the population of Khoozestan fowls. Furthermore, the association study revealed that allele A had positive effect on economical traits than allele B. The observed and expected heterozygosities were 0.243 and 0.568, respectively. However, the obtained results from Mazandaran native fowls were contrary to those of Khoozestan fowls. The frequency of the wild type allele is higher than the mutant allele in Mazandaran native fowls. This can be due to the physiological role of allele B in Iranian fowls. Inbreeding and family selection can be one of the major factors that enhance the BB genotype in Mazandaran native fowls. Reared chickens in the breeding station of Mazandaran native fowls are prepared from the state native fowls' breeding center, and in addition, it is a closed population and therefore is open for disequilibrium factors. Inbreeding coefficient is high in the closed population which, in turn, causes a decrease of diversity in the 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. Nonetheless, designed primers in this study were first used in Iranian chickens.

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