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Identification of 332G>A Polymorphism in Exon 3 of the Leptin Gene and Partially Effects on Body Size and Tail Dimension in Sanjabi Sheep

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***Abstract*—**The objective of the present study was to determine the polymorphism in the leptin (332G>A) and its association with biometric traits in Sanjabi sheep. For this purpose, blood samples from 96 rams were taken, and tail length, width tail, circumference tail, body length, body width, and height were simultaneously recorded. PCR was performed using specific primer to amplify 463 bp fragment including exon 3 of leptin gene, and PCR products were digested by Cail restriction enzymes. The 332G>A (at 332th nucleotide of exon 3 leptin gene) that caused an amino acid change from Arg to Gln was detected by Cail (CAGNNNCTG) endonuclease, as the endonuclease cannot cut this region if G nucleotide is located in this position. Three genotypes including GG (463), GA (463, 360and 103 bp) and GG (360 bp and 103 bp) were identified after digestion by enzyme. The estimated frequencies of three genotypes including GG, GA, and AA for 332G>A locus were 0.68, 0.29 and 0.03 and those were 0.18 and 0.82 for A and G alleles, respectively. In the current study, chi-square test indicated that 332G>A positions did not deviate from the Hardy–Weinberg (HW) equilibrium. The most important reason to show HW equation was that samples used in this study belong to three large local herds with a traditional breeding system having random mating and without selection. Shannon index amount was calculated which represent an average genetic variation in Sanjabi rams. Also, heterozygosity estimated by Nei index indicated that genetic diversity of mutation in the leptin gene is moderate. Leptin gene polymorphism in the 332G>A had significant effect on body length ($P<0.05$) trait, and individuals with GA genotype had significantly the higher body length compared to other individuals. Although animals with GA genotype had higher body width, this difference was not statistically significant ($P>0.05$). This non-synonymous SNP resulted in different amino acid changes at codon positions111(R/Q). As leptin activity is localized, at least in part, in domains between amino acid residues 106-1406, it is speculated that the detected SNP at position 332 may affect the activity of leptin and may lead to different biological functions. Based to our results, due to significant effect of leptin gene polymorphism on body size traits, this gene may be used a candidate gene for improving these traits.

***Keywords*—**Body size, Leptin gene, PCR-RFLP, Sanjabi sheep.

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I. Introduction

SHEEP is an important source of meat in Iran. In industry of sheep meat, it is favorable to have animals that produce massy carcasses, great amount of muscles, and enough amounts of fat with good composition [1]. In meat-producing species such as sheep, body conformation and growth rate are important selection criteria [2]. Measures of size and body form are desired in many experiments with sheep, including studies of growth, inheritance and nutrition [2]. These measurements, in addition to the weight measurements, explain more completely an individual or population than do the conventional methods of weighing and grading [3]. These measurements have value in predicting live body weight [4] and also in judging the quantitative characteristics of meat [5]. Meat quality and carcass traits are classified as quantitative traits, being influenced by environmental factors and genetic [6]. The genotype influence study is important to improve productivity since the environment can be controlled to some extent.

As a result of the revolution of molecular biology, it was feasible to determine the genetic markers with great correlation with many aspects of DNA structure specially for the genes with major effect on the economical features [7]. Leptin gene as a biological key is associated with the important traits in animal breeding [8]. In the other words, the genetic character of leptin gene was evaluated as a "marker" that is capable to refer on the relative differences between individuals [8].

The leptin gene was detected by positional cloning techniques in 1994. This gene consists of three exons and two introns [9]. Leptin is the product of the obese (OB) gene, is a 16-kDa protein expressed in adipose tissue, and is involved in the regulation of feed intake, energy balance, fertility and immune functions. Leptin binds to its receptor localized mainly on neuropeptide Y-neurons, which results in a reduction of feed intake and an increase of energy expenditure. Many researches indicated that leptin plays an important role in the regulation of growth, development, and feed conversion efficiency [10].

The present study was designed to identify LEP (Exon 3) gene variation using PCR-RFLP and then to test whether those variants are associated with body size and dimensions of the tail traits in Iranian Sanjabi sheep because these indigenous sheep form a very valuable genetics resource. Despite the importance of indigenous sheep in Iran, breed information is scarce on their genetic make-up.

II. Material and Methods

A. Sampling

This study was performed on Sanjabi sheep breed in Kermanshah province, Iran. Samples were collected from 96 rams belonging to three herds using artificial vagina over two years during autumn and spring seasons. At the same time, the dimensions of tail length, width tail, circumference tail, body length, body width, and height were measured. Blood samples were collected from jugular vein.

B. DNA Extraction, PCR and Genotyping

Genomic DNA was purified from 100 µL of blood sample using the Diatom DNA Prep100. Primers were designed using the Oligo software, based on published sequence information (GenBank No.: HE605296.1) to amplify a 463 bp fragment including exon 3 of leptin gene. The forward and reverse primers were F: 5′ - TGTTGTCCCCTTCCTCCTG - 3′ and R: 5′ - CCCACATAGGCTCTCTTCTGC - 3, respectively. PCR was performed in a final volume of 45 µl containing 50-100 ng DNA template, 0.25 µM of each primer, 18 µl Master Mix (2X PCR Master Mix Red-MgCl2:1.5mM), and 22.2 µl distilled water. The PCR was programmed as follows: an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 63 °C for 1 min, and 72 °C for 1 min. A final extension step was performed at 72 °C for 5 min. Electrophoresis of the amplicons was carried out in 1% agarose gels containing Green Viewer in 0.5x TBE buffer, and the gels were visualized under ultraviolet light. Suitable restriction enzyme for RFLP method was found by web cutter program. The PCR products were digested by for Cail restriction enzymes in order to detect 332G> A polymorphism. The digestion of amplicon was performed at 37 °C for 10 hours using 5 U enzyme and 8 µl PCR product. Digestion products were separated in 4% agarose gel for 70 min at 70 V and stained with ethidium bromide.

C. Statistical Analysis

The genotypic and allelic frequencies were calculated using the PopGene software (version32). The Hardy-Weinberg equilibrium in the populations was also tested. The data were analyzed by Generalized Linear Model (GLM) on SAS program (version 9. 2). The effect of various factors was investigated, and significant factors were kept in the model. So that different models were fitted for different traits. The statistical fixed model is shown as follows:

$$y_{ijkl} = \mu + HYS_i + G_l + Age_k + W_j + e_{ijkl}$$

where y_{ijkl} is the considered dependent variable (body size and dimensions); µ is the overall mean, HYS_i is the effect of Herd-Year-Season, G_l is the effect of the l^{th} genotype, Age_k is fixed effect of ram age, W_j is the effect of ram weight (as covariate), and e_{ijkl} is the residual effect. Least square means of different genotypes for body size and tail dimension trait were compared at level 0.05 to show significant statistical differences.

III. Results and Discussion

A. PCR- Genotyping

Quality of extracted DNA was assessed by 1% agarose gel electrophoresis (Fig. 1). A 463bp fragment of the leptin gene exon 3 containing mutation at position 332G> A was successfully amplified (Fig. 2).

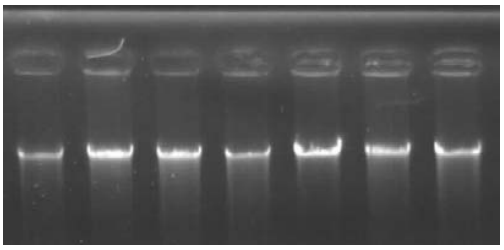

Fig. 1 Extracted DNA from Sanjabi ram bloods

The 332 G>A (at 332th nucleotide of exon 3 leptin gene) that caused an amino acid change from Arg to Gln was detected by by C*ai*l (CAGNNNCTG) restriction enzyme. Three genotypes including GG (463), GA (463, 360, and 103 bp) and GG (360 bp and 103 bp) were identified after digestion (Fig. 3). The frequency of G allele was higher than that of A allele (Table I). The genotype frequency showed a tendency of GG > GA>AA.

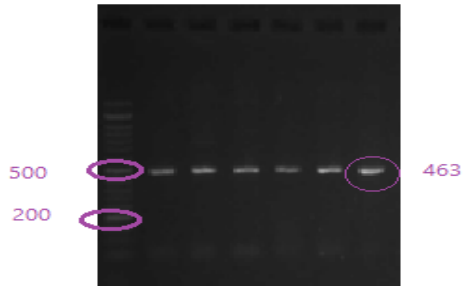

Fig. 2 The 463 bp fragments (PCR) of exon 3

The allelic and genotypic frequencies and chi-square ($\chi 2$) and diversity indexes values are summarized in Table I.

At 332 G>A locus, the estimated frequencies of GG, GA, and AA genotypes were 0.68, 0.29, and 0.03, and those were 0.82 and 0.18 for G and A alleles, respectively.

In Herri sheep, the A(mutant allele) alleles frequencies for 332 G>A positions were 0.886 [11]. This difference is due to different sample size and studied breeds in the previous studies compared to our study.

In this study, Sanjabi sheep indicated did not deviation from Hardy-Weinberg equilibrium. This non-deviation might represent that samples used in this study belong to large local herds with a traditional breeding system having random mating and no selection.

Population diversity indexes were also estimated and are shown in Table II. Shannon index was calculated for 332G>A and was equal to 0.46, which represent an average genetic variation in Sanjabi rams.

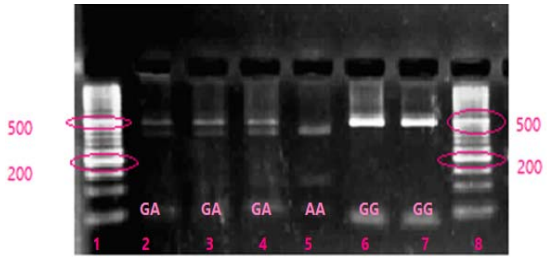

Fig. 3 The PCR–RFLP results of the 463 bp fragments by Cail Restriction enzyme

Also, heterozygosity estimated by Nei index (0.29) for 332G>A position indicated that genetic diversity of mutations in the leptin gene is moderate showing a high potential for selection inbreeding programs.

TABLE I
THE GENOTYPES AND ALLELE FREQUENCIES AND CHI-SQUARE OF LEPTIN GENE POLYMORPHISM IN IRANIAN SANJABI RAM

Locus	Allele frequency	Genotype frequency	(Chi-square P-Value)
G332A	G= 0.82 A=0.18	GG(65)= 0.68 GA(28)=0.29 AA(3)=0.03	0.001 P=0.96

TABLE II
THE ESTIMATION OF POPULATION DIVERSITY INDEXES IN SANJABI RAM

Locus	Nei	I	Ne
G332A	0.291	0.466	1.411

B. Association with Testicular Dimension

Iran has 27 sheep populations which vary in their genetic potential for production of milk, meat and wool, disease resistance, fecundity, etc. [12]. The Sanjabi sheep is one of the local breeds in Iran. The population size of the Sanjabi breed is more than the other breeds of sheep (except Baluchi breed) in Iran and 3 ecotypes were detected for this breed [12]. This breed has an average adult body weight of 42.3 kg and has better adaptability to extensive management in Western area of Iran, better immunity against diseases, semi-high reproduction rate, and better meat-wool quality [13].

Many polymorphisms have been identified or verified in ovine LEP despite the limited number of published studies: in the 3 untranslated region, intron 2 [9], and exon 3 [14]-[16] in sheep populations in Iran (Shal, Zandi, Zel, Makooei,Baluchi, Kermani). So far, no study has been done on leptin gene polymorphism in Sanjabi sheep. This article is the first study on leptin gene polymorphism in Sanjabi sheep.

As shown in Table III, leptin gene polymorphism had no significant effect on chest, circumference tail, tail length, width tail, height, and body width. However, this polymorphism showed significant effect on body length ($P<0.05$) trait, as animals with GA genotype had significantly the higher body length ($P<0.05$) compared to other genotypes. Although this genotype had higher body width, this difference was not statistically significant ($P>0.05$).

Linear body traits have been suggested as objective measures of body conformation in sheep. Body conformation highly influences market value of meat sheep in traditional markets [17].

The prosperity using of marker assisted selection in animal populations will depend on ability to test whether allelic variations at these loci are separating in the population, the ability to identify the genes or closely linked markers to the genes underlying the QTL, and realization of how these genes interact with another gene or with the environment factors that affecting economic traits. All these factors must be done in an efficient and cost effective manner in order for the technology to be adopted by the animal industries [18]. These genes can be closely associated to genetic markers, capable of being segregated jointly. A genetic marker should be polymorphic, must have an easy inherence mode, and should be simply detectable [19]. The study of genes underlying phenotypic variation can be applied in two different ways; one from phenotype to genome, which is applied by LD based association mapping or by candidate genes identified based on homology to known genes, and latter from genome to phenotype, which needs the statistical estimation of genomic data to recognize likely targets of late selection using selective sweep analysis [20], [21].

Considerable interest exists in distinguishing the relationship between SNP for different genes with productive and health traits of economic significance for the livestock industry [22].

Genetic polymorphism in the leptin gene was first reported in mice and humans [22]. In recent years, a lot of studies have been conducted on the association between production traits and leptin gene polymorphisms in farm animal [16], such as weaning weight, bodyweight, carcass weight, slaughter weight, fat-tail percentage, muscle thickness, loin eye area, pH, shear force and metabolic activity in the muscle, and subcutaneous fat in Iranian sheep [9], [14]-[16], [23].

Most association studies performed on leptin gene, focused on association between leptin gene polymorphism and carcass composition and beef quality traits [24].

Fitzsimmons et al. [25] found that the BM 1500 microsatellite, near the obese(leptin) gene, was associated with carcass trait in beef bulls. Lagonigro et al. [26] suggested an association between leptin and feed intake. Jiang and Gibson [27] analyzed genetic polymorphisms in the leptin gene and their association with fatness in four pig breeds, polymorphic locus of 3,649 bp in the leptin gene was possibly related with back fat thickness of pig. Xi et al. [28] showed that the RFLP band types were significantly different in fat-type and lean-type pigs. There was a 4.3 kb band in all lean-type pigs and a 3.5 kb band in all fat-type pigs.

Here is restricted evidence about connection between gene polymorphisms with fat-tail estimates in sheep. It has been reported that the association between band originals observed with tail length and tail down circumference in Kermanian sheep was close to the significant level in fourth exon of growth hormone gene. Also, statistical analysis displayed definitive association between the band patterns (exon 3 Ovine leptin gene) observed in the Zel breed with the tail, chest, abdomen and neck circumference ($P<0.05$), and body length

(P<0.01), and the models were related with character, gap tail length, middle and down tail Width (p <0.05) in the Bakhtiari breed. Also in Lori-Bakhtiari sheep and Zel sheep breeds, statistical analysis demonstrated definitive relation between band models 16–17 exon of DGAT1 gene. Sheep with CC genotypes at the DGAT1 locus, demonstrated the significantly lower fat-tail weight (P<0.05) and back fat thickness (P<0.01) [29].

IV. Conclusion

The effects of 332G>A position of leptin gene polymorphism on body size and tail dimension in Sanjabi sheep were determined. In this study, we found significant effect of 332G>A position of leptin gene polymorphism on body length (P<0.05), also of population diversity indexes indicated that genetic diversity of mutations in the leptin gene is moderate showing a high potential for selection inbreeding programs.

TABLE III
Least Square Means (± Standard Errors) of Leptin Genotypes for Semen Characteristics Sanjabi Ram

Leptin	Chest	circumference tail	tail length	width tail	height	body width	body length
GG	111.62±1.17	64.17±2.13	39.26±1.76	33.93±0.81 3	44.51±0.83	34.14±0.73	61.71±0.91[b]
GA	112.18±1.34	63.23±2.44	37.18±1.94	33.76±0.98	43.50±1.03	35.22±0.84	63.95±1.04[a]
AA	112.40± 3.69	62.34±6.72	39.53±4.77	32.37± 2.93	44.97±2.63	31.22±2.31	58.72±2.89[ab]

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