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GENETIC DIVERSITY OF LEPTIN GENE INTRON 2 IN WILD AND NATIVE AND EXOTIC GOAT BREEDS IN IRAN

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ABSTRACT

The aim of this study was to investigate the intron 2 of l*eptin* gene polymorphism in wild, native and exotic goat breeds in Iran using PCR-RFLP. Blood samples were collected from 14 wild and domestic goat breeds including (Cashmere Abadeh, Torki-Ghashghaei, Naeini, Robati, Nadoushani, Adani, Shahrbabaki, Birjandi, China goat, Sannen, Pakistani, Raeini cashmere, Najdi and Wild goat) and then the genomic DNA was extracted. A 422bp fragment from intron 2 of the *leptin* gene was amplified. PCR products were digested by *Sau3AI* restriction enzyme and were separated and visualized on the agarose gel. Three genotypes including MM, Mm and mm with genotype frequency of 94%, 1% and 5%, respectively, were observed in the studied populations. The number of observed alleles, number of the effective alleles, Nei's Index and Shanon's Index were 2, 1.10, 0.09 and 0.19, respectively. The studied populations were not found to be in a Hardy-Weinberg equilibrium. Our investigation demonstrated that MM genotype and M allele had a very high frequency (0.94 and 0.95, respectively) in Iranian goats. Hence, it can be concluded that this finding can provide basis for selection when considering evolution and differentiation among breeds, however further studies should be carried out on a larger population of different domestic and wild breeds to verify the final conclusions.

Keywords: Diversity, domestic goat, leptin gene, PCR-RFLP, wild goat

INTRODUCTION

Goat breeding is a growing industry in the world and its products have a desirable landscape (**Moghadaszadeh *et al.*, 2015**). Despite significant achievements related to the agricultural technology and knowledge of developed countries, but the number of goats has increased in these countries as well (**Shamsalddini *et al.*, 2016**). Meat production is the first reason for breeding goats in the world and dairy production is in the next priority. Asia and Africa are the most important goat breeding continents (**Mohammadabadi and Tohidinejad, 2017**).
According to FAO (2008), about 96% of the total world goat population are in the developing countries and only 4% are found in the developed countries (FAO, 2008). There are 30 million heads of cashmere goats around the world and 4.5-5 million heads of them are in Iran that is 16% of all in the world (Baghizadeh et al., 2009). Goat breeding plays an important and economic role for farmers in the arid and semi-arid regions. Increasing meat production using scientific, and precise selective programs is one of the most important goals of genetic improvement of goats. To identify the genotype of animals and their relationship with productive and reproductive traits, determining the polymorphism and the phylogenetic relationships of domestic animals is also very important (**Soufy *et al.*, 2009; Ruzina *et al.*, 2010; Moghbeli *et al.*, 2013; Mohammadabadi & Tohidinejad, 2017**).
Leptin (a product of the *ob* gene with weight of 16 KD) was derived from the Greek word *leptos* (Greek) and encodes for a 167 amino acid. This hormone is mainly produced by the white adipose tissue and to a lesser extent, by muscle cells, stomach epithelium, placenta, fetal tissues, and mammary glands (**Gregorio *et al.* 2014**). The most important role of the leptin is to regulate feed intake, energy balance, fertility, and immune functions (**Javanmard *et al.*, 2008**). This gene has 3 exons and 2 introns, however only 2 exons are translated to protein (**Shojaei *et al.*, 2010**). The leptin gene is located on the chromosome 4 of the cattle, sheep and goats (**Gregorio *et al.* 2014**), and on the chromosome 8 of water buffalo (**Gregorio *et al.* 2014**). This gene has both endocrine performance, in the brain and in the various peripheral tissues and also autocrine/paracrine signal within tissues where it is expressed (**Zieba *et al.*, 2003**). It is demonstrated that leptin is expressed in adipocytes (**Chilliard *et al.*, 2001**), fetus (**Yuen *et al.*, 2002**), breast (**Bartha *et al.*, 2005**), rumen (**Yonekura *et al.*, 2002**), small intestine (**Yonekura *et al.*, 2002**) and hypophysis (**Yonekura *et al.*, 2003**) of the ruminants. Some effects of this gene are feed intake reduction, body weight loose, lower fat deposits and energy metabolism elevation (**Javanmard *et al.*, 2008**). Leptin seems to be important in controlling reproduction through adequite body fat deposits for assisting prosperous conception and pregnancy (**Liefers & Veerkamp, 2002**). It is demonstrated that there is direct correlation between plasma level of leptin and body fat mass and energy balance in cattle and sheep (Shojaei *et al*., 2010). Leptin gene affects the milk performance in cattle and reproduction in beef cattle. According to ***Bartha et al.*** (2005), expression of this gene is changed in various physiological and growth stages of animals, hence leptin can be used as a proper marker for evaluating the growth, feed efficiency and health of animals. **Celi *et al.* (2008)** showed that plasma and milk leptin concentrations will not be affected by maternal food variation in dairy goats and milk leptin concentration has a negative correlation with kids' liveweights and average daily growth rate. A relatively limited number of studies have been conducted on leptin in goats compared with cattle and in particularIranian wild goats. Therfore, the aim of this study was to study the intron 2 of leptin gene in some Iranian native goat breeds, Iranian imported goat breeds and wild goats.

MATERIALS AND METHODS

Studied animals

In this study, 516 blood samples were collected from fourteen different goat breeds (Figure 1) in Iran (Cashmere Abadeh (CAB), n=40; Torki-Ghahghaei (TOG), n=34; Naeini (NAE), n=30; Robati (ROB), n=28; Nadoushani (NAD), n=38; Adani (ADA), n=14; Shahrbabaki (SHB), n=28; Birjandi (BIR), n=34; China goat (CHI), n=34; Sannen (SAN), n=60; Pakistani (PAK), n=60; Raeini cashmere (RAC), n=60; Najdi (NAJ), n=28; and Wild goat (capra aegagrus) (WIL), n=28). Wild goat (capra aegagrus) were collected from 3 protected regions and Zoos of Iran (Sistan and Baluchistan, Kerman and Fars provinces). The wild goat is distributed from Europe to Asia (particularly, central Asia and the Middle East). It has been listed as vulnerable on the IUCN Red List since

1996 (Figure 2). Wild goat (capraaegagrus) is a species of mountain goat. Males are commonly called Kal and have long and sword horns. Blood samples were collected through the jugular vein in K3, EDTA containing tubes to get plasma.

DNA extracting, PCR and gel electrophoresis

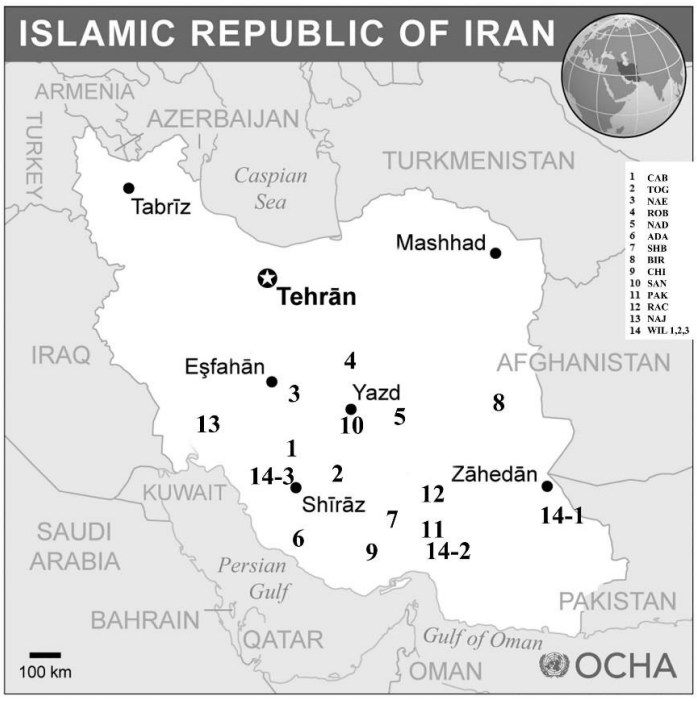
The modified salting-out method (**Abadi *et al*., 2009**) was employed to extract genomic DNA. Both spectrophotometry and agarose gel (1%) were applied to determine the quality of extracted DNA. A 422-bp fragment within the intron 2 of leptin gene was amplified using PCR primers 5'-TGGAGTGGCTTGTTATTTTCTTCT-3' and 5'-GTCCCCGCTTTGGCTACCTAACT-3 '(Singh *et al*., 2009). CinnaGen PCR Master Kit was applied to perform PCR amplification in a 25 µl reaction volume, containing negative controls according to the instructions by the manufacturer (CinnaGen Co., Iran). PCR protocol was done at 3 steps: step 1; 5 min at 95°C, step 2 had 30 cycles consisted of 3 stages; 60 s at 95°C, 60 s at 62°C, 60 s at 72°C and step 3 for final extension 5 min at 72°C. Electrophores on 1% agarose gel at constant voltage and 1X TBE for approximately 2 h was used for visualization of PCR products. The gels were visualized by ethidium bromide staining and photographed under ultra-violet light. All PCR products were digested with 5 U of *Sau3A*I restriction enzyme (Fermentas) at 37°C overnight, and the resulting products were separated by the 3% agarose gel and visualized by ethidium bromide staining.

Data analysis

Diversity indice including gene diversity (H), observed number of alleles (Ne), Shannon's information index and Nei's index were calculated using POPGEN 3.2 software (**Yeh et al., 1999**). The animals were cared for in accordance with the local Ethical Committee laws and regulations.

RESULTS AND DISCUSSION

The extracted DNA had a good quality (Figure 3) and its concentration was approximately 100 nanograms per microlitters. The tested DNA of the goats in our study was amplified by the specific primers and yielded PCR products at the expected size of 422 bp (Figure 4). Amplification of the leptin gene produced 422 bp fragments. After digesting these fragments with the restriction enzyme *Sau3AI*, the MM genotype produced two bands: 390 and 32 bp (one restriction site in the M allele), the mm genotype produced three bands: 303, 88 and 32 bp (two restriction sites in the m allele), and the Mm produced four bands: 390, 303, 88 and 32 bp (heterozygote genotype).



Map Sources: ESRI, UNCS.
The boundaries and names shown and the designations used on this map do not imply official endorsement or acceptance by the United Nations. Map created in Sep 2013.

Figure 1 Location of the fourteen study sites in Iran. The putative subspecies are indicated as Cashmere Abadeh (CAB), Torki-Ghahghaei (TOG), Naeini (NAE), Robati (ROB), Nadoushani (NAD), Adani (ADA), Shahrbabaki (SHB), Birjandi (BIR), China goat (CHI), Sannen (SAN), Pakistani (PAK), Raeini cashmere (RAC), Najdi (NAJ), and Wild goat (WIL-1, 2 and 3).

The different alleles from the digestion of the PCR products with the *Sau3AI* restriction enzyme after running on the agarose gel electrophoresis are presented in Figure 5. Different genotypic and allelic frequencies of leptin gene were observed in the various breeds (Table 1). In this study, the Hardy Weinberg equilibrium was estimated with Chi-square and G-square tests. The studied populations were not found to be in a Hardy-Weinberg equilibrium ($P<0.05$).

Figure 2 Iranian wild goat.

The values of the population genetics parameters in the studied polymorph breeds have been shown in Table 2. Cashmere Abadeh, Naeini, Robati, Adani, Shahrbabaki, China, Raeini cashmere, Torki-Ghahghaei, Nadoushani, Birjandi, Najdi and Wild breeds were monomorph based on intron 2 of leptin gene. A UPGMA dendrogram based on the Nei's standard genetic distance among studied animals has been shown in figure 6.

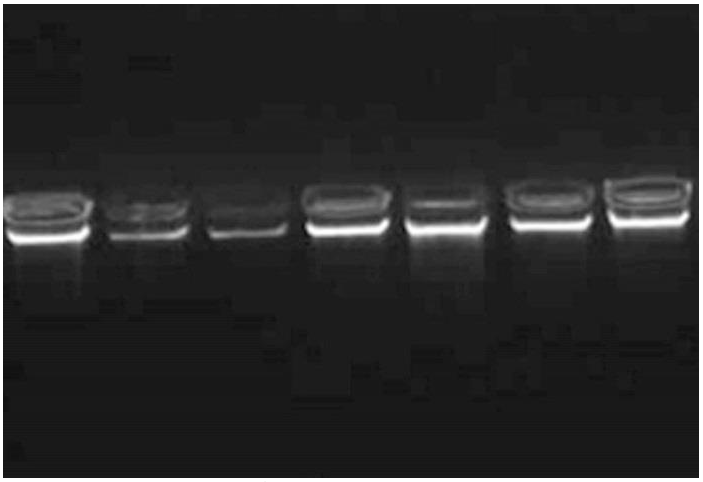


Figure 3 The extracted DNA from the studied animals on the 1% agarose gel.

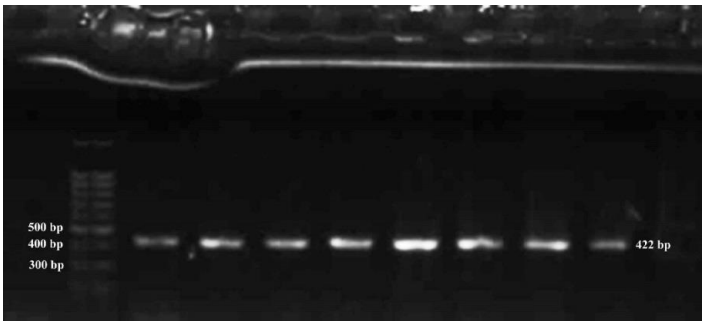


Figure 4 Ethidiumbromide-stained agarose gel of amplified PCR products representing amplification of intron 2 of leptin gene in some studied goats.

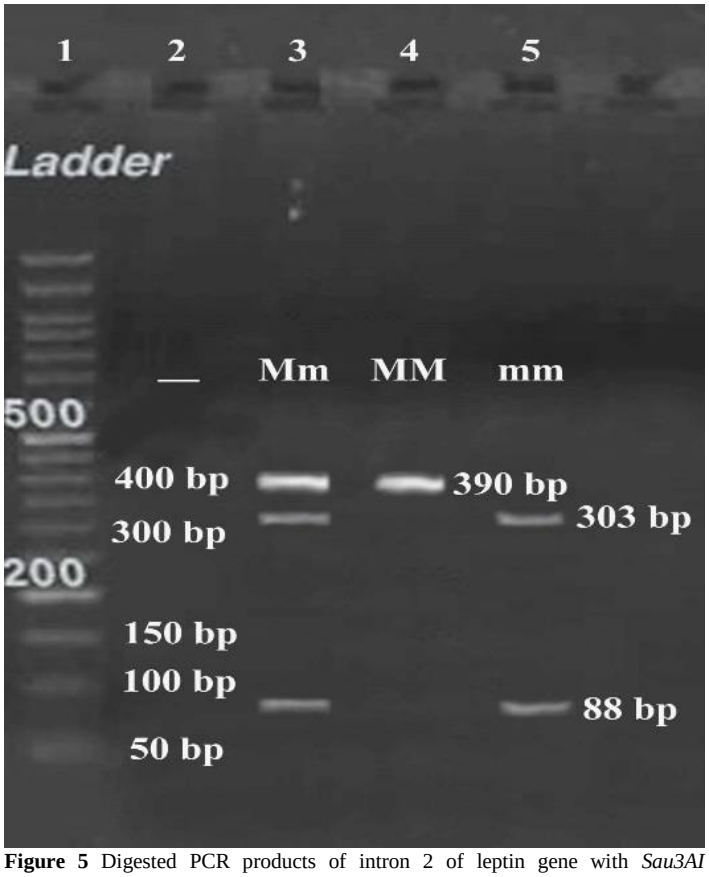


Figure 5 Digested PCR products of intron 2 of leptin gene with *Sau3AI* restriction enzyme in some studied goats. Lane 1 is ladder M50, lane 2 is negative control, lane 3 is Mm genotype, lane 4 is MM genotype and lane 5 is mm genotype.

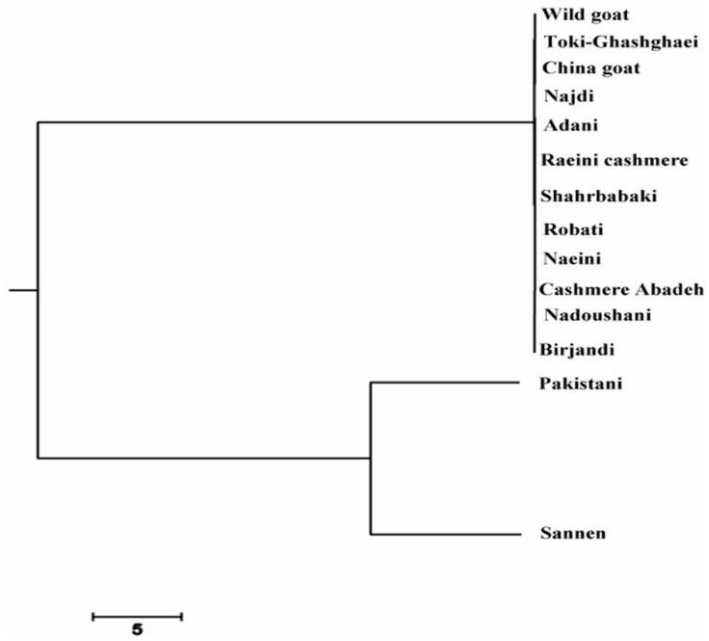


Figure 6 UPGMA phylogenetic tree based on Nei genetic distance.

Based on UPGMA phylogenetic tree, breeds including Cashmere Abadeh, Naeini, Robati, Adani, Shahrbabaki, China, Raeini cashmere,Torki-Ghahghaei, Nadoushani, Birjandi, Najdi and Wild breeds were clustered in the same branch and Sannen and Pakistani were clustered in a separate branch from the other breeds. Results of UPGMA phylogenetic tree showed that the two breeds; Sannen and Pakistani breeds that clustered separately from the other ten breeds are genetically different from the rest breeds. The reason for this could be the origin of these two breeds, mutation or selection. The frequencies of leptin alleles in 14 studied breeds showed that frequency of M allele is higher than frequency of m allele. The low frequency of allele m as observed in the 516 different domestic and wild goats of our study is consistent with the results of other researchers (**Kulig *et al*. 2001; Korwin-Kossakowska *et al*. 2002; Kmieć *et al*. 2003; Kołodziej *et al*., 2009**).

Three genotypes (MM, Mm and mm) were observed in 14 studied domestic and wild populations. These results, in terms of genotype number were similar to results of other researchers on leptin gene (**Křenková *et al*. 1999; Kulig *et al*. 2001; Korwin-Kossakowska *et al*. 2002; Kmieć *et al*. 2003; Kołodziej *et al*., 2009**), although some of these researchers reported only two genotypes from three possible genotypes.

Table 1 Genotypic and allelic frequencies of intron 2 of leptin gene in studied goats

Breed	Genotype	N	Genotypic frequency	Allele	Allelic frequency
Cashmere Abadeh (CAB)	MM	40	1.00	M	1.00
Torki-Ghahghaei (TOG)	MM	34	1.00	M	1.00
Naeini (NAE)	MM	30	1.00	M	1.00
Robati (ROB)	MM	28	1.00	M	1.00
Nadoushani (NAD)	MM	38	1.00	M	1.00
Adani (ADA)	MM	14	1.00	M	1.00
Shahrbabaki (SHB)	MM	28	1.00	M	1.00
Birjandi (BIR)	MM	34	1.00	M	1.00
China goat (CHI)	MM	34	1.00	M	1.00
Sannen (SAN)	MM	34	0.57		
	Mm	4	0.07	M	0.60
	mm	22	0.36	m	0.40
Pakistani (PAK)	MM	58	0.97	M	0.97
	mm	2	0.03	m	0.03
Raeini cashmere (RAC)	MM	60	1.00	M	1.00
Najdi (NAJ)	MM	28	1.00	M	1.00
Wild goat (WIL-1, 2 and 3)	MM	28	1.00	M	1.00
All	MM	488	0.94		
	Mm	4	0.01	M	0.95
	mm	24	0.05	m	0.05

Table 2 The v*alue*s of the population genetics parameters in studied goats

Breed	Number of observed alleles	Number of effective alleles	Nei's Index	Shanon's Index
Sannen (SAN)	2	1.92	0.48	0.67
Pakistani (PAK)	2	1.06	0.07	0.14
All	2	1.10	0.09	0.19

In the current study, we observed only one genotype (MM) in the 12 of 14 studied breeds, similar to the wild breed. This might be explained by the originatation of Iranian domestic breeds from the wild breed, which have conserved their genotype for intron 2 of leptin. Furthermore, the low number of the studied samples might also influence the distribution of MM genotype as observed in our study. So it is suggested to investigate more samples from any intact breeds which were kept seperatedly. Our results implied that the studied breeds have a very suitable gene pool for the selection and breeding programs in regards to improving the desired traits. According to **Askeri *et al*. (2011)**, high genetic diversity is the most important factor for the animal genetic improvement. **Vitali *et al*. (2005)** showed that body weight, body fat deposits and diet quality affect the onset of puberty in goats, but not the plasma leptin levels. They also concluded that higher plasma leptin during the onset of the puberty maybe operates as permissive sympom for developing the sexual maturity, which can be applied as a detecting instrument to predict the imminent approaching of the event. In another study, **Maitra *et al*. (2014)** identified and characterised leptin gene polymorphism and whole sequence in Indian goats. They analysed the sequences using bioinformatics procedures and reported 22 variations in comparison to the exotic goats. In this study, seven SNPs were detected among the 22 variations in exon 2 (g.1029T_C), intron 2 (g.1621G_A) and 3'UTR (g.3968T_C, g.3971C_T, g.4026G_A, g.4105G_A and g.4225T_C). These researchers demonstrated that part of the meat quality and tenderness polymorphism in the Indian goat populations is under the effect of the seven detected novel SNPs in the leptin gene. They also concluded that the relationship between theses novel SNPs and the meat quality trait in the animal breeding can be used as a basis for the subsequent precise investigation of leptin genotype correlation with performance. **Gregorio *et al*. (2014)** compared leptin gene in goats, sheep, cattle and water buffalos and also evaluated the effects of the Intron

1 microsatellite polymorphism in goats and showed that leptin gene can be used as a proper marker for studying the metabolism and mammary gland health in dairy goats.

CONCLUSION

The results of our investigation demonstrated that MM genotype and M allele have a very high frequency (0.94 and 0.95, respectively) in goats bred in Iran, hence this finding could provide basis information for animal selection when considering evolution and differentiation among breeds. Taking into account all aspects and results, goat *leptin* gene can be useful for achieving genetic relationship of goat breeds and the selection of economic traits. Considering the observed differences between the results of our study and others, it can be concluded that more investigation is needed to perform on leptin gene polymorphism and its effects on the important traits to verify the final conclusions.

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