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Original Article

The Investigation of Mutations and Comparison of Leptin Gene Pro-Motor in Najdi Cattle with the Database NCBI Sequences

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ABSTRACT

Objective: Identity the genetic aspects and major gene influence on energy balance, milk production, fertility, food safety and consumer are the recent interests of genetic and breeding researchers. **Methods:** Najdi Cattle is the most prominent breeds in Khuzestan province. To do this plan in Shoushtar Najdi Cattle Station, blood samples were taken from 15 Najdi Cattles. DNA was extracted from whole blood using the method of Boom et al and polymerase chain reaction took for amplification of two fragments 544 bp and 566 bp. After ensuring the accuracy of the duplicated sequences on 1% agarose gel, sequencing was performed and the Leptin gene promoter components pointed out in Najdi Cattle. In this study, the six mutations identified in the first fragment 566 bp. In these mutations, 2 types of them were point mutations including Transition and Transversion, and 4 mutations were Frame shift which included Deletion (3kinds) and Insertion (1kind). In the second fragment 544 bp, 2 point mutations (transition and Transversion) and 1 Frame shift mutation (deletion) were appeared, in the Leptin gene pro-motor region. Also, in this study, the sequences related to each sample was compared in the two fragments in the aspect of similarity with other documented sequences, that BLAST results showed that high percentage of similarity. **Results:** According to the obtained results, it could be said that the sequence of leptin gene of Najdi cattle in Khuzestan province is similar to other documented sequences and there are a high-similarity percentage between current samples' sequences and those documented in global information bank.

INTRODUCTION

Biotechnology science with the remarkable progress had in recent years caused agree art evolution in the life sciences. By this technology we can no waccessto the changed products which are the natural products in originate. It is distinct of products synthesized chemically (Galehdari et al., 2006). DNA sequencing is the most important technology available in molecular biology, in which the exact order of nucleotides could be determined in a piece of DNA, probably (Terence Austen, 2011). Leptin and its receptor can be used as a genetic marker for enhancing the productivity in livestock and are also potential candidates for marker assisted selection (Agarwal et al., 2008). It is known that *obesity* is determined by Leptin gene (Barb et al., 2001). Ob gene was discovered in mutant mice in 1994 by Friedman et al at Rockefeller University (Zhang et al., 1997). This protein made of 167Amino Acids and released in to the blood, after separating 21 amino acids. Bovine Leptin

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gene includes three exons and two in trans located on chromosome 4 in Cattle breeds (Cunningham et al., 1999). This hormone is created as a result of mutations in the gene responsible for generated *obesity* (Zhou et al., 2009). Leptin is in various tissues such as adipose tissue, placenta, mammary glands, skeletal muscle, stomach, brain and pituitary (Nobari at al., 2010). And it is secreted mainly by white adipose tissue (lagonigro et al., 2003). The protein leptin, a satiety hormone, regulates appetite and energy balance of the body. These hormones have important roles in energy homeostasis, glucose and lipid metabolism, reproduction, cardiovascular function, and immunity (Meier and Gressner, 2004). Recently polymorphisms on gene are associated with it are reported strongly suggest that these gene are considered as candidates affecting increased production of milk and its compounds (Buchanan et al., 2002; Liefers et al., 2002; Madeja et al., 2004; Javanmard et al., 2010). Bovine Leptin pro-motor genomic structures were studies in 2002 for the first time. In this study, Leptin gene pro-motor transcription site, the sequence elements, regulatory factors Sp1 and CCAAT/enhancer (C/EBP) binding protein motifs identified, in the sidelong site in 5‘ upstream. One motif TATA was identified in the region -28 to -33 at transcription pro-motor site. The region sidelong5’ is an important region in Leptin pro-motor and identified C/EBPα could be an activator for bovine Leptin gene pro-motor, in this region, the obtained results showed (Taniguchi et al., 2002). Najdi Cattle it at is in the South and South-West of the country and is the most prominent breeds Khuzestan province. This breed plays a role as a good source to provide milk and meat and also in economical aspect. The purpose of this study was amplifying and determining the leptin gene sequences, regulatory region, also the related variants to, leading to the decrease and increase in gene expression.

2. MATERIALS AND METHODS

2.1. Sample collection

Blood sampling taken from 15 Najdi Cattles jugular vein, using vacuum tubes containing EDTA anticoagulant, this plan was performed in Najdi Cattle station located on the road Shoushtar-Ahvaz. The samples were transported with ice to the central laboratory of the Khouzestan Ramin Agricultural and Natural Resources University, and then stuck in fridge with -20 ° C till extracting DNA.

2.2. DNA extraction

By a DIA tom kit (Iso Gene Moscow) DNA extraction was performed from whole blood using (Boom et al., 1990) method. This method is based on the use of lubricating agent guanidinethiocyanate and silica adsorbents. At the first, 200 ml of blood picked, and then 400 ml of digestion buffer M5 guanidiniumthiocyanate (20mM EDTA, 10g/l acidic Tris, 40g/l Triton and 10g/l Dai Triton (DTT)) was added. The samples kept at 65 °C for 5 minute a water bath. The nucleolus absorber in the rate of 20 micro liters added to samples and then vortexed gradually for 10 min. In the next step, 400µl of buffered saline was added to the homogeneous environment, and finally DNA from other impurities isolated through the Extra Gene.1% agarose gel used to determine the quantity of extracted DNA.

2.3. Selecting primers

Primers used in this study designed by are search team led by Doctor S.C. Lifers in Wageningen University of the Netherlands and taken is and then taken from the article published by, Lee et al. (2005) with documentation number1 AJ571671 in NCBI database. Used primers’ sequences for the fragments 566 bp and 544 bp, respectively:

Leptin-F	5′-GGG GGA GGC GGA GAG GAG
Leptin-R	5′-TAC ATG GCC ACT AAA AAG GTT G-3′
Leptin-F	5′-TAG TAC AAT ATC CTT CCT TTC TT -3′
Leptin-R	5′-CCT GCC TTG ATG ATG GTG TGG- -3′

To survey the design accuracy, the primers compared with reference sequence using Nti Vector and Oligo Analyzer Soft-wares. After binding confident for 4 primers with model sequence, following primers were used.

2.4. Polymerase chain reaction (PCR)

For the fragment 566 bp, the PCR reaction in the volume25µl with temperature program 95°C for prior DNA denaturation for 300s, the secondary denaturation at 95° C for 60 s, at 59°C for primer binding for 60 s, at 72 ˚C for primerse longation for 60 s, and at 72° C for ultimate elongation for 600 s conducted. For the fragment 544 bp, a nealing temperature 61° C for 60 sis the most ideal condition for amplifying the planned primers.

2.5. Electrophoresis

1% agarose gel and a voltage of 80 V applied for 1 h, for observing PCR products. Staining gel was done by DNA Safe Stain. Amplified fragment under UV light was observed and taken photograph was recorded by Gel Doc. PCR-products for the fragments 566 bp and 544 bp illustrated in figures1and2, respectively.

3. Results and Discussion

Using Boom et al. method for extracting DNA showed the good prepotency of quality, quantity and time consuming. PCR products for sequencing conveyed to Takapoozist Company and then transferred to South Korea Bayounir Company, in order to determine the fragments' sequences. Sequence identifying of samples was performed with ABI 3730 sequencer. The sequences were submitted with Bio Edit and Chromas soft-wares and then compared with the template sequence by MEGA5 and Clustalw2 soft-wares. As any sequence is the same with template sequence, means there is no mutation. The sequence should be assessed in the aspect of mutation and effects-on-transcription if there are differences between planned- and template sequences. The mutations, point- and replacement (transition type) were appeared in the site 209 in the first fragment, and the site 56 in the second fragment. A point mutation (Transversion type) was observed in the site 363 in the first fragment and the site 217 in the second fragment. This type of mutation causes non-producing of programmed protein or changes the protein turnover. Also one Frame shift mutation type (deletion) was observed in the sites 66, 346, and 347 in the first fragment and the site 485 in the second fragment. The sequence and the Leptin gene pro-motor components were identified, by using Vector Nti Soft-ware. All of the samples were homozygotes in both fragments and no heterozygote state was appeared, these obtained results were pointed in occurred mutations out. The occurred mutations in mentioned sites, did not illustrate in any Boxes and pro-motor components. After studying the Leptin gene mutations and pro-motor components, the sequence of each sample was compared with other documented sequence, in the aspect of similarity per cent, this survey was conducted in the database NCBI. The BLAST result related to L21 sequence in database NCBI showed that 10 sequences have the most similarity with the planned sequence, the figure 5 illustrates. In the figure 5, the most similarity per cent related to the sequences 1 and 2. In the sequence 1 with the number AB070368.1, the overlap range between the sequences L21 with mentioned one, was 99 per cent. In the highest overlap per cent, 95 to 99 per cent similarity was observed, in the other samples. The BLAST result related to the second fragment was similar to the first fragment, and the highest similarity was appeared in the sequences 1 and 2 with the record numbers AB070368.1 and AJ571671.1. If the samples were so high, mutations probability would be more that could help to identify SNP chip.

Conclusion

The sequence and components of Leptin gene's promoter were identified. It demonstrated that there are no occurred-mutations of mentioned places in boxes and promoter's components, therefore; they have no effects upon boxes and promoter's components. Also, in this study, the sequences related to each sample was compared in the two fragments in the aspect of similarity with other documented sequences, that BLAST results showed that high percentage of similarity.

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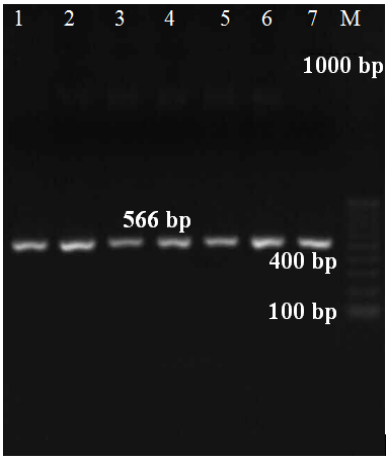


Figure 1:

PCR production 1% agarose gel, for a few samples of the fragment bp 566 (marker Size 100 bp)

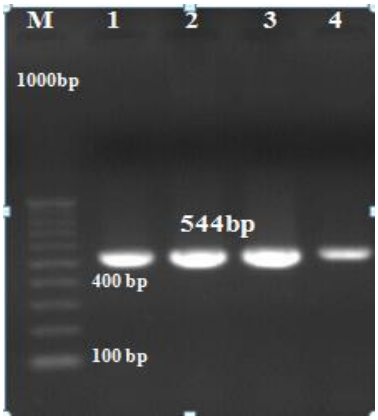
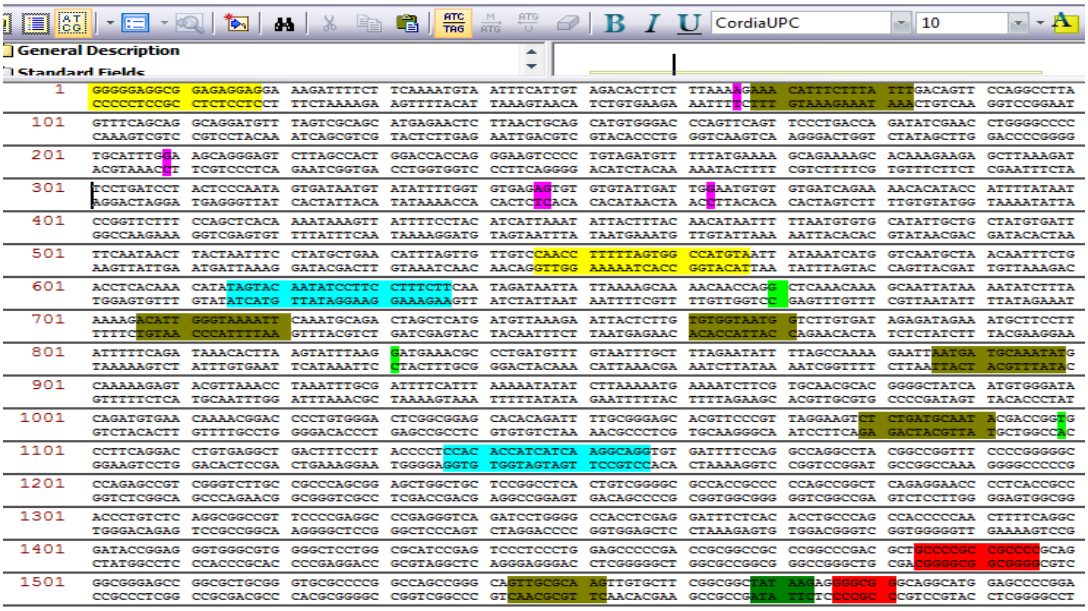


Figure 2:

PCR products on 1% agarose gel, for a few samples of the fragment bp 566 (marker Size 100 bp)



Position 1-18: Forward Primer 1 binding site

Position 546-567: Reverse Primer 1 binding site
primers sitefragment 566 bp

Position66: Identified change; Homozygous (A- Deletion) in all Cattle
mutationsfragment 566 bp

Position 209: Identified change; Homozygous (G>A) in all Cattle
primers sitefragment 544bp

Position 346: Identified change; Homozygous (A- Deletion) in all Cattle
mutationsfragment 544 bp

Position 347: Identified change; Homozygous (G- Deletion) in all Cattle
C/EBP

Position 363: Identified change; Homozygous (Singl A- insertion)
regulatoryfactorSp1

Position 404: Identified change; Homozygous (G>C) in all Cattle TATA box

Position 615-637: Forward Primer 2 binding site

Position 1137-1157: Reverse Primer 2 binding site

Position 56: Identified change; Homozygous (G>A) in all Cattle

Position 217: Identified change; Homozygous (G>C) in all Cattle

Position 485: Identified change; Homozygous (T- Deletion) in all Cattle

Position 68-83: C/EBP AAACATTTCTTTATTT

Position 706-720: C/EBP ACATTGGGTAAAATT

Position 761-771: C/EBP TGTGGTAATGG

Position 886-899: C/EBP AATGATGCAAATAT

Position 1079-1091: C/EBP CTCTGATGCAATA

Position 1543-1552: C/EBP GTTGCGCAAG

Position 1484-1496: SP1 GCCCCGCCGCCCC

Position 1576-1581: SP1 GGGCGG

Position 1568-1573: ATAAG sig

Figure 3:

The Leptin gene sequence and components

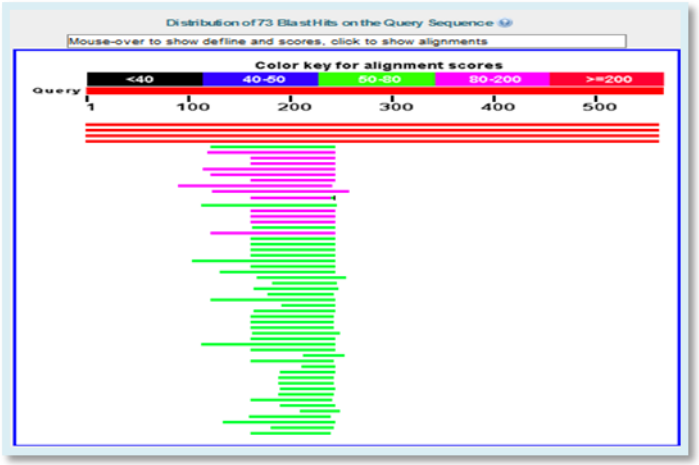
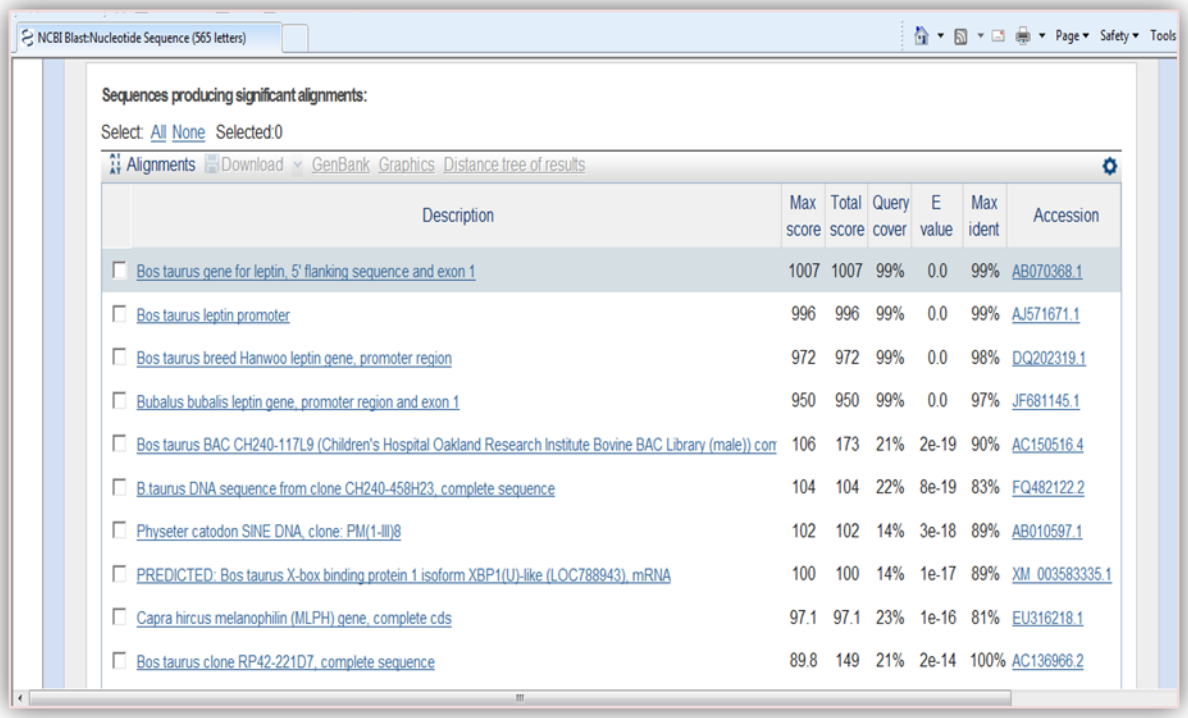


Figure 4:

Comparison of overlap range between the samples L21, the sequence related to the fragment 566 bp and other recorded sequences in database NCBI



Description	Max score	Total score	Query cover	E value	Max ident	Accession
Bos taurus gene for leptin, 5' flanking sequence and exon 1	1007	1007	99%	0.0	99%	AB070368.1
Bos taurus leptin promoter	996	996	99%	0.0	99%	AJ571671.1
Bos taurus breed Hanwoo leptin gene, promoter region	972	972	99%	0.0	98%	DQ202319.1
Bubalus bubalis leptin gene, promoter region and exon 1	950	950	99%	0.0	97%	JF681145.1
Bos taurus BAC CH240-117L9 (Children's Hospital Oakland Research Institute Bovine BAC Library (male)) cor	106	173	21%	2e-19	90%	AC150516.4
B.taurus DNA sequence from clone CH240-458H23, complete sequence	104	104	22%	8e-19	83%	FQ482122.2
Physeter catodon SINE DNA, clone: PM(1-III)8	102	102	14%	3e-18	89%	AB010597.1
PREDICTED: Bos taurus X-box binding protein 1 isoform XBP1(U)-like (LOC788943), mRNA	100	100	14%	1e-17	89%	XM_003583335.1
Capra hircus melanophilin (MLPH) gene, complete cds	97.1	97.1	23%	1e-16	81%	EU316218.1
Bos taurus clone RP42-221D7, complete sequence	89.8	149	21%	2e-14	100%	AC136966.2

Figure 5:

Comparison of similarity range between the sample L21, the sequence related to the fragment 566 bp with 10 recorded sequences in database NCBI.