DESIGNNING SPECIFIC PRIMERS TO AMPLIFY INTRON 1 OF LEPTIN GENE IN HOLSTEIN – FRISIAN CROSSBRED CATTLE; SUGGESTIVE DIAGNOSTIC TOOL TO BREEDS' SELECTION

Mohammed Baqur S. Al-Shuhaib[1], Hafedh M. A. Al-Tayy[2], Sara R. Al-Lamy[3]
To whom it may correspond: baquralhilly_79@yahoo.com
[1 - 3] department of Animal Resources/College of Agriculture/University of Al-Qasim Green – Iraq

ABSTRACT

Holstein – Frisian (HF) cattle crossbred is the most important cattle breed in Iraq for both meat and milk production. The genetic studies of animal production related gene in this breed is very poor in this country. Therefore, a pair of specific primers was designed to amplify a highly polymorphic region in leptin gene, which is intron 1. Genomic DNA was extracted from the peripheral blood of 60 randomly selected local HF cattle. One pair of specific primers was designed using primer BLAST online software. The optimum annealing temperature of primers was determined using a couple of gradient PCR reactions. It was found that the optimum annealing temperature to amplify this intron 1 fragment in leptin gone as 66.9°C. In conclusion, it was found that the intron 1 fragment is a successful genetic candidate that can be used in the subsequent selection of the best breed of HF cattle.

Keywords: Holstein Frisian cattle, PCR, intron 1, leptin gene, designing

---

تصميم بوادئ متخصصة لتضخيم قطعة الانترون رقم واحد في جين اللبتين لابقار الهولشتاين – فريزيان المضرّبة: اداة تشخيصية مقترحة في انتخاب النسل

محمد باقر صاحب الشهيّب حافظ موسى الطائي سارة منير عباس اللامي
قسم الثروة الحيوانية / كلية الزراعة / جامعة القاسم الخضراء

الخلاصة:

تعد ابقار الهولشتاين – فريزيان المضرّبة هي النسل الحيواني الأكثر أهمية في العراق لانتاج كل من الحليب واللحم. ان الدراسات الوراثية للجينات ذات العلاقة بالانتاج الحيوانية في هذا النسل هي فقيرة جدا في هذا البلد. ولهذا السبب، تم تصميم زوج من البوادئ لتضخيم منطقة عالية التغاير في جين اللبتين، والتي تدعى بالانترون رقم واحد. تم عزل الـ DNA الجينومي من الدم المحيطي لستين بقرة هولشتاين – فريزيان محلية مختارة عشوائياً. تم تصميم زوج من البوادئ المتخصصة باستعمال برنامج الحاسوب primer BLAST المتوفرعلى شبكة الانترنت. تم تحديد حرارة التلدن المثالية باستخدام تفاعلين من الـ PCR المتدرج. وجد بان الحرارة المثالية للتلدن لتضخيم قطعة الانترون رقم 1 في جين اللبتين هي 66.9 درجة مئوية. وبالنتيجة، وجد بان قطعة الانترون رقم 1 هي مرشح وراثي ناجح والذي يمكن ان يستخدم في الانتقاء المستقبلي لافضل نسل لابقار الهولشتاين – فريزيان.

الكلمات المفتاحية: ابقار هولشتاين – فريزيان، تفاعل انزيم البلمرة المتسلسل ، انترون رقم واحد، جين اللبتين، تصميم

INTRODUCTION

The development of DNA markers and molecular biological techniques is fundamental tool for applications in animal breeding (Kale *et al*., 2014). During the last two decades, DNA markers gave more benefits of genetic improvement in animals' breeds (Arif & Khan, 2009).

Several candidate markers were targeted on the level of DNA architecture, one of these candidate genes was leptin (Nassiry *et al*.,

ISSN 2072-3875

2007). Leptin word is derived from the Greek term *leptos*, which means "thin" (Liefers, 2004). Leptin was a 16 kD serum circulated protein, it plays a crucial role in the regulation of feed and energy metabolism of the body (Geary *et al*., 2003, Chilliard *et al*., 2005). However, it was known that leptin was considered as a hormone that regulate the body weight by maintaining the balance between food intake and energy expenditure through signaling to the brain the changes in stored energy levels (Zhou *et al*., 2009). In addition to the obvious role of leptin in controlling appetite, it has other roles in regulating growth, reproduction, body composition and immunity, therefore, the *LEP* gene may be a strong candidate gene for evaluation of genetic polymorphism (Agarwal *et al*., 2009). The size of *LEP* gene was estimated about 20kb and it consisted of three exons separated by two introns, the exons for *LEP* gene cover about 15kb of DNA genome, and the entire coding region of the exon II is separated from the exon III by 1.6kb of intron (Rock *et al*., 1996). Leptin could be considered as one of the best biological markers in mammals since it might play a significant role in "marker assisted selection" (Dubey *et al*., 2008). Accordingly, several techniques were employed to exploit the portions of this gene to detect the extent of polymorphisms in a way that could be used in routine labs, such as RFLP and SSCP (Orita *et al*., 1989).

The Iraqi Holstein cows that belong to one of the main native and commercial breeds' cows were considered as the most important origins for animal resources in Iraq. Holstein Friesian is a high-yielding dairy cow in temperate countries with good management of feeding and milking, HF cows can yield more than 9,000 kg/cow/305 day lactation period (Chandan *et al*., 2008). The milk lactose, fat and protein contents range from 4.6-4.8%, 3.8-4.9% and 3.0- 3.6%, respectively (Blowey & Edmondson, 2010; Akers, 2002). However, very little genetic data is currently available about each individual cattle. Thus, the aim of this study is to develop a successful genetic marker of the local HF cattle in this country. This is done by designing one pair of specific oligonucleotides in order to amplify small portion of the highly polymorphic intron1 inter-genetic region of leptin.

MATERIALS AND METHODS

Blood collection; blood samples were collected from 60 randomly selected cows that were reared in Al-Qadysia station. From each selected cow, about 2.5 ml were withdrawn from the jugular vein of each cow. The blood is placed in anticoagulation tubes and stored in -20°C until the next step.

Genomic DNA extraction; DNA was extracted manually from blood using Lahiri and Nurnbergers' method (1991) with several modifications. Briefly, 2.5 ml of blood was transferred into a 15 ml centrifuge tube. 10 ml of distilled water . was added and mixed well by inversion several times and centrifuged at 4400 rpm for 4 min at room temperature. The supernatant was discarded and this step was repeated. The supernatant was poured off and the pellet was resuspend in 1 ml of TNES lysis buffer (10Mm Tris-Cl, pH 7.7, 0.4 M NaCl, 2 mM EDTA, 0.5% SDS), and then – if necessary – the whole suspension was mixed thoroughly by pipetting back and forth several times. Once the suspension is homogenized the mixture was incubated for 5 min at 55°C. Only 0.30 ml of 6 M NaCl was added in the tube and mixed well. Centrifugation at 13500 rpm for 5 min was taken place in a microcentrifuge. The supernatant containing DNA was saved and the precipitated protein pellet was discarded at the bottom of the tube. Two volumes of 100% ethanol at room temperature were added to the supernatant and the tube was inverted several times until the DNA precipitates. The precipitated DNA strands was removed with a pipette and placed in a microcentrifuge tube containing 1 ml of ice-cold 70% ethanol. Centrifugation for 5 min at 13500 rpm at room temperature was performed. After discarding ethanol, the DNA was resuspeded in 0.5 ml of TE buffer at 65°C for 15 min. To ensure further dilution, this suspension was pipetted back and forth several times. The isolated DNA was

 ISSN 2072-3875

quantified by a nanodrop (Biodrop – UK). Results were obtained as "µg/ml" units. Absorbance was measured at 260nm. The purity of DNA samples was measured automatically by calculating ration 260/280. The quality and quantity of the isolated DNA was further checked by 0.8% agarose gel electrophoresis, and stained by ethidium bromide (0.5 µg/ml), and photographed by gel photodocumentation.

PCR design; It was decided to amplify a portion DNA segment that covers almost of intron 1 in the leptin gene was designed using the primer BLAST online software (www.ncbi.nlm.nih.gov). The genbank accession number of *Bos taurus* (AJ512638.1) is used to design leptin gene intron 1 primers. Several lengths of PCR primers were obtained online. Based on several primers parameter, one of them is used and selected in the subsequent PCR reaction. The length of the designed primers was 256 bp.

PCR analysis; One pair of the oligonucleotide primers, which have the following sequences (forward: 5′-CAGGGGGAGTTTTTAGCGGT-3′ and reverse: 5′-ACGAGATTTGCTGTCTGCCA -3′), was chosen from the cattle LEP gene sequence (GenBank Acc. No. AJ512638.1) to amplify 256 bp sequence of leptin gene, intron 1. PCR reaction was performed using AccuPower PCR premix (Cat # K-2012, Bioneer - Korea). Each 20µl of PCR premix was contained 1 U of Top DNA polymerase, 250 µM of dNTPs, 10 mM of Tris-HCl (pH 9.0), 30 mM of KCl, and 1.5 mM of MgCl2. The reaction mixture was completed with 10 pmol of each primer and 50 ng of genomic DNA. The optimum annealing temperatures were determined empirically using gradient PCR (mastercycler-nexus, Eppendorf – Germany). The amplification was began by initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 49 – 66.9 °C for 30 sec, and elongation at 72°C for 30 sec, and was concluded with a final extension at 72°C for 5 min. After performing PCR thermocycling, the qualities of PCR products were tested by 1.5% agarose gel electrophoresis and stained by ethidium bromide (0.5 µg/ml), and photographed by gel photodocumentation. Once the optimum annealing temperature of the chosen primers' pair is selected, PCR reaction is applied on all 60 samples of cows.

RESULTS AND DISCUSSION

This is the first study that designed specific oligonucleotides to amplify intron 1 of leptin gene of local HF *Bos taurus*, and there is no previous study designed such sequences. The gradient PCR must be performed to identify the optimum temperature of annealing these primers to the template. Accordingly, two gradient PCR experiments were used. The first one (49 – 62) °C is failed to pick proper temperature for the annealing of these primers (Fig. 1,A). While, the second gradient PCR (62 – 66.9) °C was found to be successful in picking up the proper annealing temperature, which is 66.9°C (Fig. 1, B). However, as long the temperature of primers' annealing with its template target sequence is high as it means on the high specificity that primers have in their amplification of their target sequence (McPherson & Meller, 2000).

ISSN 2072-3875

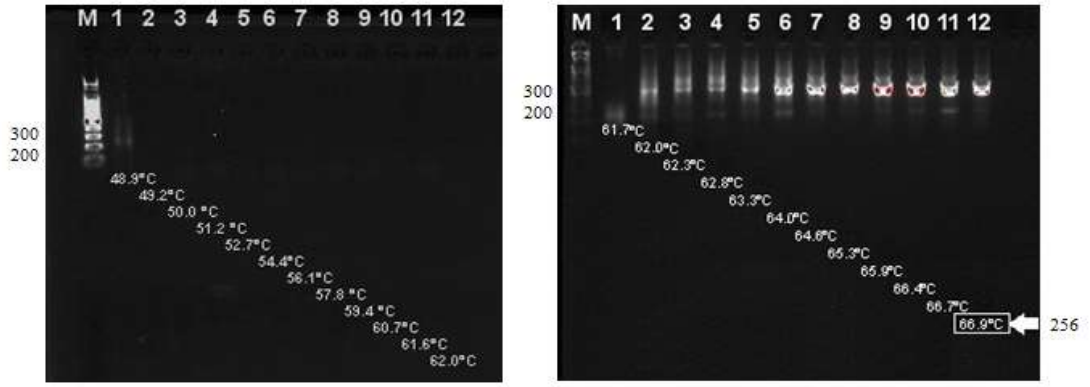

Fig (1): Agarose gel electrophoresis of leptin gene, intron 1 gradient PCR fragments. (A): gradient PCR (49°C – 62°C) applied on LEP, intron 1 to identify the optimum primer annealing temperature. M; refers to DNA size marker lane 1 into lane 12 refers to the variable gradient annealing temperature applied. B): gradient PCR (62°C – 67°C) applied on LEP, intron 1 to identify the optimum primer annealing temperature. M; refers to DNA size marker lane 1 into lane 12 refers to the gradient annealing temperature applied. The square bound the number 66.9°C (lane 12) refers to the optimum PCR annealing temperature of leptin gene, intron 1 PCR fragments. Electrophoresis conditions: Agarose concentration 1.5%, power applied: 135V (7V / cm), time of run: 45 min. staining method; (0.5µg/ml) ethidium bromide.

Each PCR amplicon before its being subsequently applied for any post-PCR its purity should be confirmed. Only one clean and sharp bands can be used for the subsequent post-PCR reactions. This notion must be relied on since the non-obvious PCR bands will reduce the sensitivity of post-PCR reactions. In this work, sharp and obvious bands were found after performing electrophoresis (Fig. 2). It was found that the leptin gene amplified DNA samples that numbered 3, 49, 52, and 60 were given non-efficient amplicons (Fig. 2). Therefore, out of 60 amplified genomic DNA samples only 4 samples (0.66%) were not given a satisfied amplicons. This, in turn, gives an obvious clue about the efficiency of this pair of primers in the amplification of the targeted intron 1. However, it must be noted that not all the observed bands for all samples have the same precise location concerning the shape of band and its density (Fig. 2). This is attributed into several reasons; such as the differences of quality DNA samples among each others wit respect to the presence of some salts that may amper Taq polymerase in its action, or other unknown factors. However, there is some ambiguities in the results of PCR amplicons that might be attributed into the presence of some variation in the highly variable intron region of leptin gene.

 ISSN 2072-3875

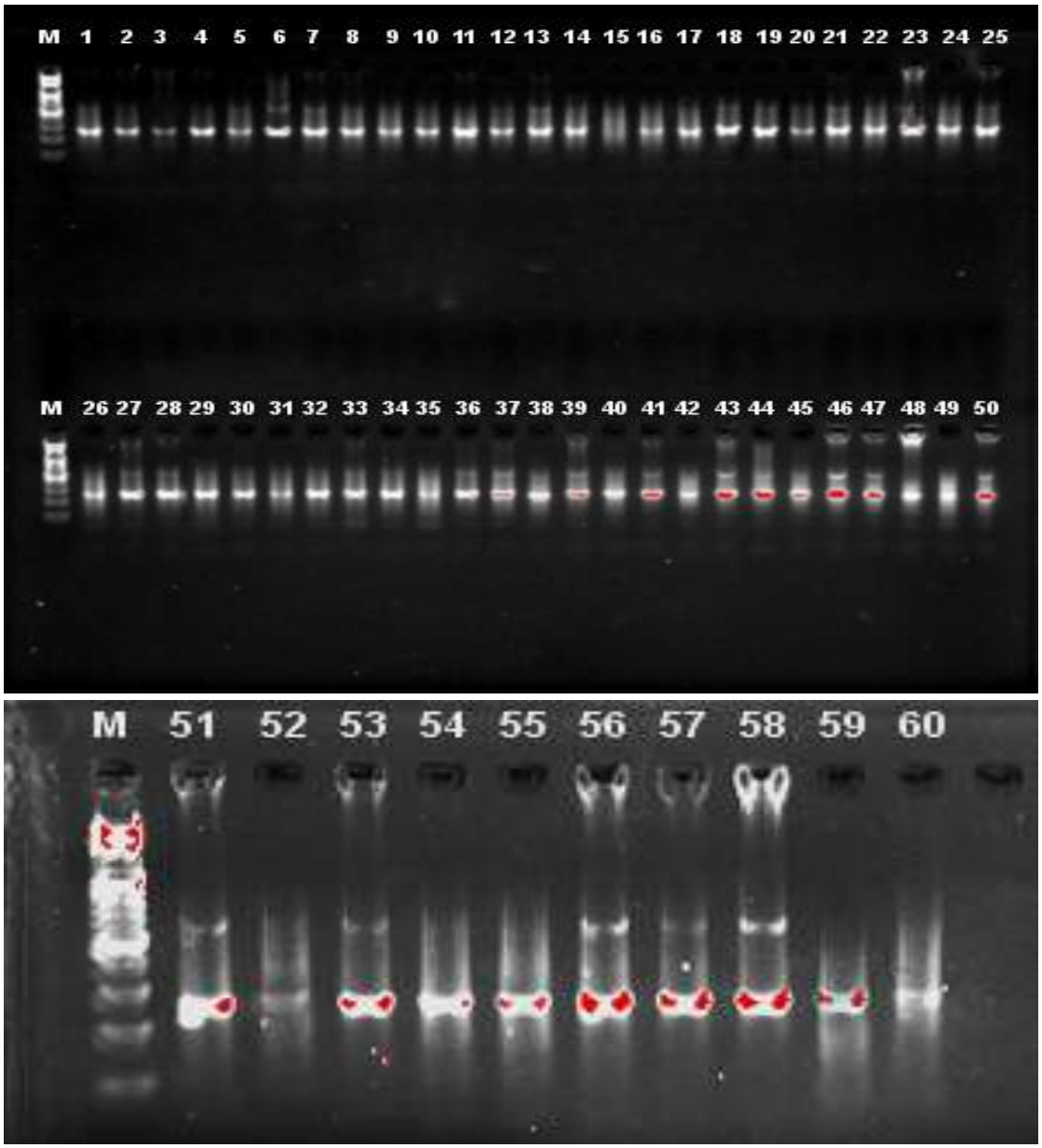

Fig (2): Agarose gel electrophoresis of leptin gene, intron 1 PCR fragments. M; refers to DNA size marker lane 1 into lane 60 refers to leptin gene, intron 1 PCR fragments patterns. Electrophoresis conditions: Agarose concentration 1.5%, power applied: 135V (7V / cm), time of run: 45 min. staining method; (0.5µg/ml) ethidium bromide.

The presence of such variation might belong into the main changes that HF cattle submitted to during many years of genetic drift, in which many mutations were replaced the original NCBI reference sequences. However, many factors might interact in the modulating the DNA sequences that possibly repsonsible about the possible modifications in the final phenotypic manifestation of the studied animals. accordingly, the main meat and milk production traits that is correlatd with leptin gene is affected too. Although, no comparable standards were available in this local breed, the results of this study in Iraqi were strongly suggest the very high polymorphisms gained as a result of the uncontrolled circumstances that these cattle are exposed to. However, the polymorphism obtained was highly dependable on the type of the breed used. Although the revealed genotypes might be highly variable, the range of the obtained genotypes was between "two" into "ten" different polymorphisms (Radhi *et al*., 2015).

 ISSN 2072-3875

In the present study, specific primers PCR design of 60 Holstein cows from the middle Euphrates region of Iraq were performed on one intron portion on LEP and to serve this task. However, to find the evolutionary relationships among close populations, leptin is a suitable and informative marker system (Hashemi *et al*., 2011).

The diversity data generated for native Iraqi cows breeds may be utilized for characterizing the genetic relationships with other countries as well. According to our knowledge, no genetic information is currently available to compare different bovine populations from Iraq. Although, we have used only one breed, the present study may be regarded as the beginning of attempts to understand the genetic diversity of local cattle breeds in Iraq. In cattle, analysis of allelic variation of leptin loci can potentially be used to evaluate temporal changes in genetic diversity and our analysis showed that breeds can be differentiated using leptin variability. The present study was the first attempt for the identification of *LEP* (intron 1) gene targeting in Iraq HF crossbred cows.

The variations of LEP gene have been characterized in several domestic animals around the world, but in Iraq, these characterizations haven't been applied in Iraqi Holshtein bovine till now. These possible data might be attributed to the highly genetic variability for this breed in Iraq. These data were concomitant with the fact of the increasing of genetic diversity as a result of the increasing of number of alleles (Arranz *et al*., 2001). These results might be referred to the presence of a wide ratio of hybridization, selection, or genetic diversity for Holstein cows.

Also, no genetic data is currently available to compare different sheep populations in Iraq at least in terms of their PCR or post-PCR experiments. As long as the utilization of leptin is a suitable and informative genetic marker system to find the evolutionary relationship among closed and inbred population (Aslaminejad *et al*., 2010), it's possible to apply this promising marker on other breeds as well as Holstein in Iraq. Eventually, in addition to Holstein breed, further investigations including more Iraqi native cattle, since it will be useful to clarify their origin and any possible genetic relationships among these important Iraqi breed using the same *LEP* based PCR-SSCP technique. Nevertheless, further studies are needed to explain the DNA polymorphism and to obtain more precise description about these cases in terms of DNA sequencing studies. It is advisable to apply more markers, in addition to the applied markers, in this breed and some other native breeds in order to uncover their genetic relationships.

However, though several successful primers were designed in several regions of leptin gene in cattle around the world (Buchanan *et al*., 2003, Liefers, 2004, Tanpure *et al*., 2012), this study is focused on the feasibility of designing PCR primers based on the same NCBI reference sequences. This is performed to serve two main reasons. The first one is correlated with the intron DNA segment itself. Though a portion of this segment is amplified specifically in several animal production reports (Dubey *et al*., 2014), only small portion of this region is exploited in this aspect, while the most of this region wasn't covered. This study is covered almost all intron 1 region to explore correlation between leptin gene and many productive traits to further detect any unknown mutation that might not be detected using only a small portion of this segment. Add to that, the possible altered genetic nature of the local HF cattle is very attractive for many animal breeder to focus directly on the genetic level of each HF crossbred cattle instead of the focusing on the classical studying of animal breeding in this country.

In conclusion, the designing of a pair of specific primers in Holstein cows indicates that the designed amplicons can be used as a selective criterion to improve productive traits genetically.

REFERENCES

Agarwal R., Rout P.K., and Singh S.K., 2009. Leptin: a biomolecule for enhancing livestock productivity. Indian J. Biotechnol, 8: 169 – 176.

Akers RM, 2002. Lactation and mammary gland. Iowa: Wiley-Blackwell.

Arif IA, Khan HA. 2009. Molecular markers for biodiversity analysis of wildlife animals: a brief review. Animal Biodiversity and Conservation 32.1.

Arranz JJ, Bayon Y, San Plimitivo F: Differentiation among Spanish sheep breeds using microsatellites. Genet. Sel. E., 2001, 33 (5): 529-542.

Aslaminejad AA, Nassiry MR, Farajollahi H, Mahdavi M, Abbasi H, Javadmanesh A: Polymorphism in Exon 3 of Leptin Gene in Iranian Native Cattle Breeds. J. Appl. Anim. Res 2010 ,37: 159-162.

Blowey RW, Edmondson PW. 2010. Mastitis control in dairy herds. 2nd. ed: CABI.

Buchanan, F.C., Kessel Van, A.G., Waldner, C., Christensen, D.A., Laarveld, B. and Schmutz, S.M., 2003. Hot topic: an association between a leptin single nucleotide polymorphism and milk and protein yield. J. Dairy Sci. 86, 3164–3166.

Chandan RC, Kalira a, Shah NP. 2008. Daily processing and quality assurance. Wiley - Blackwell.

Chilliard, Y., Deavaud, C., and Bonnet, M., 2005. Leptin expression in ruminants: nutritional and physiological regulations in relation with energy metabolism. Domest. Animal Endocrinol 29: 3 -22.

Chandan, R.C.; Kilara, A. and Shah, N.P. (2008). *Dairy processing & quality assurance*: Wiley-Blackwell

Dubey, P.P., Sharma, A., Gour, D.S., Prashant, J.A., Mukhopadhyay, C.S., Singh, A., Joshi, B.K., and Kumar, D., 2008. Leptin gene polymorphism in Indian Sahiwal cattle by single strand conformation polymorphism (SSCP). South African Journal of Animal Science 38 (2): 131-135.

Dubey, P.K., Singh, S., Yadav, N.K., Kathiravan, P., Mishra, B.P., and Kataria, R.S., 2014. PCR-SSCP and Sequence Analysis of Leptin Gene Reveals Novel Polymorphism in Intron 1 and Allele Fixation in Exon 2 of Indian Buffaloes (Bubalus bublalis). Indian J Biotechnol. 13, 47 – 51.

Geary, T.W., McFadin, E.L., MacNeil, D.M., Grings, E.E., Short, R.E., Funston, R.N., and Keisler, D.H., 2003. Leptin as a predictor of carcass composition in beef cattle. J. Anim. Sci 81: 1-8.

Hashemi A, Mardani K, Farhadian M, Ashrafi I, Ranjbari M: Allelic polymorphism of Makoei sheep leptin gene identified by polymerase chain reaction and single strand conformation polymorphism. Afr. J. Biotechnol 2011, 10: 17903-17906.

Kale, D.S., Yadav, B.R., and Prasad, I., 2014. DNA Polymorphisms at Candidate Gene Loci and Their Relation with Milk Production Traits in Murrah Buffalo (Bubalus bubalis). Iranian Journal of Applied Animal Sci 4: 39-43.

Lahiri, D.K., and Nurnberger, J.I., 1991. A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. Nucl. Acids Res. 19, 5444.

Liefers, S. Physiology and genetics of leptin in perparturient dairy cows. 2004, Wageningen University. Ph. D. Thesis.

McPherson MJ, Meller. PCR, the basics from background into bench. BIOS Scientific Publisher Limited, 2000, UK.

Nassiry, M.R., Heravi Moussavi, A., Alashawkany, A.R., and Ghovati, S., 2007. Leptin Gene Polymorphism in Iranian Native Golpayegani and Taleshi Cows. Pakist. J. Biol. Sci 2007, 10: 3738-3741.

Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., and Sekiya, T., 1989. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc. Natl. Acad. Sci. USA 86: 2766-2770.

Radhi AH, Al-Khauzai ALD, Al-Shuhaib MB, 2015. Rapid PCR-SSCP screening of Awassi sheep using a short fragment (260bp) of leptin gene. trans Clin Bio 3 (1): 1 – 6.

Rock, F.L., Altmann, S.W., Heek, M.V., Kastelein, R.A., and Bazan, J.F., 1996. The leptin haemopoietic cytokine fold is stabilized by an intrachain disulfide bond. Horm Metab Res 28: 649-652.

Tanpure T., Dubey PK, Kathiravan P., Mishra BP., Niranjan SK., Kataria RS., Singh KP. 2012. PCR-SSCP analysis of leptin gene and its association with milk production traits in river buffalo (Bubalus bubalis). Trop Anim Health Prod 44:1587–1592.

Zhou, H., Hickford, J.G.H., and Gong, H., 2009. Identification of allelic polymorphism in the ovine leptin gene. Mol, Biotech 41: 22-25.

ISSN 2072-3875

 ISSN 2072-3875