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Detection of the Bovine Leptin Gene rs29004488 Polymorphism by Introducing a New Restriction Enzyme Site For PCR-RFLP

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Abstract

Leptin is a hormone mainly synthesized in adipose tissue and plays an important role in the regulation of appetite, energy metabolism, body weight and reproductive functions. The rs29004488 polymorphism in the 2nd exon of the bovine leptin gene, which results in an amino acid change from arginine to cysteine, has been reported to be associated with economically important traits such as growth, carcass traits, milk yield and reproduction. In cattle, the leptin gene rs29004488 polymorphism can be detected by PCR-RFLP method using *Kpn2I* enzyme. However, this method has some limitations in terms of primer degradation. In this study, a new PCR-RFLP method was investigated to detect rs29004488 polymorphism of leptin gene in cattle. Using the created restriction site PCR (CRS-PCR) method, *PstI* enzyme recognition site was introduced into the PCR products, and rs29004488 polymorphism was successfully detected using newly designed primers and *PstI* enzyme. The obtained results were confirmed by DNA sequence analysis and comparison with *Kpn2I* enzyme cleavage results. This study demonstrates that the rs29004488 polymorphism can be detected by an efficient and low-cost method using CRS-PCR-RFLP and *PstI* enzyme.

Keywords: Bovine leptin gene, rs29004488, CRS-PCR, Kpn2I, PstI

1. Introduction

Leptin is a protein hormone synthesized primarily by adipose tissue. It plays an important role in regulating appetite, energy metabolism, body weight, and reproductive functions (Friedman and Halaas, 1998; Ingvartsen and Boisclair, 2001; Kadokawa et al., 2000; Zhang et al., 1994; Zieba et al., 2008). Leptin decreases body weight by both suppressing appetite and promoting energy expenditure (Morris and Rui, 2009). Leptin concentrations in dairy cows are high during late pregnancy. An increased body condition before calving provides energy storages to support milk production during lactation. During the early stages of lactation, when dairy cows are in a state of negative energy balance, fat storage during the dry period is crucial for sustaining high milk yields (Liefers et al., 2003; Buchanan et al., 2003). In cattle, the leptin gene (LEP) is located on chromosome 4 (4q32), consists of three exons and spans a length of 16,735 kb. The LEP gene encodes a protein of 167 amino acids, including a signal sequence of 21 amino acids (Pomp et al, 1997; Taniguchi et al, 2002; Giblin et al, 2010). In 2002, Buchanan et al. (2002), identified a cytosine (C) to thymine (T) substitution (rs29004488) in exon 2 of the leptin gene, that encodes the conversion of an arginine to a cysteine. They have shown that the rs29004488 (also known as g.1180C>T, g.1047C>T, g.73C>T, Arg25Cys, R25C, R4C and LEP/*Kpn2I*, Nugroho et al., 2021) polymorphism can be detected by PCR-RFLP using the *Kpn2I* enzyme. The authors suggested that the T allele might be the causative mutation, which could cause a partial loss of biological function by adding an extra cysteine to the protein. Subsequently, many studies were conducted to understand the effects of the rs29004488 polymorphism (Nugroho et al., 2021) and it was found to be associated with economically important traits, such as growth (Fathoni et al., 2019), carcass characteristics (Shin and Chung, 2007), milk yield (Fontanesi et al., 2014; Chessa et al., 2015) and reproductive traits (Chebel and Santos, 2011) in cattle. The LEP rs29004488 polymorphism in cattle can be detected using the primer pair designed by Buchanan et al. (2002) and the *Kpn2I* enzyme. However, this method has some limitations in terms of primer degradation. The aim of this study was to identify the leptin gene rs29004488 polymorphism associated with important yield traits in cattle using an efficient, stable and cost-effective approach.

2. Materials and Methods

2.1. Primer design

Polymerase chain reaction (PCR) primers were designed on nucleotide sequence of the *Bos taurus* leptin (obese) gene (NCBI, Genbank, Accession #U50365) (Tellam, 1996) using the PrimerQuest online primer design tool (Integrated DNA Technologies, Inc., Coralville, IA, USA). The nucleotide sequences of the primers used for PCR analysis are shown in Table 1. Detection of the rs29004488 polymorphism of the leptin gene was initiated with the K_1 primers designed by Buchanan et al. in 2002. Although this method was initially successful in identifying genotypes, after a short time the problem of atypical band formation due to cleavage of the *Kpn2I* enzyme was encountered. K_2 primers were designed in which the forward primer was completely modified and new nucleotides were added to the 5' end of the reverse primer to increase the specificity of the primers. Although better performance in detecting the polymorphism was obtained with the K_2 primers, the same problem was encountered again after a short time. Investigations showed that the problem was temporarily resolved when the primers were resynthesized, but the same problem reoccurred after a short time. It was concluded that the problem was caused by degradation of the primers over a short period of time. To prevent primer degradation, another restriction enzyme was sought that could shift the mismatch from 1 base before the 3' end of the reverse

primer to 2 bases before. As a result, P primers were designed using the *PstI* enzyme to create a restriction site with a mismatch that occurs 2 bases before the 3' end of the reverse primer.

2.2.Sequence analysis

The nucleotide sequence of the rs29004488 polymorphism region of the leptin gene was analyzed for compatibility with restriction endonuclease (RE) recognition and cleavage sites for both alleles. The New England Biolabs (NEB) cutter program (https://nc3.neb.com/NEBcutter) was also used to analyze the restriction sites present in the polymorphism region. The primer binding regions based on different restriction enzymes (RE) used for the detection of the rs29004488 polymorphism within the leptin gene #U50365 between nucleotides 1020-1260 and the restriction recognition regions created with a mismatch at the 3' end of the reverse primer are shown in Figure 1.

Table 1. Information on the nucleotide sequences of the primers used for the PCR analysis

Name	Primer Sequence (5′→ 3′)[a]	Chromosomal positions of the primers[b]	Amplicon length	References
K_1	F: ATGCGCTGTGGACCCCTGTATC	BTA4: 93261984 - 93262005	94 bp	Buchanan et al., 2002
	R: TGGTGTCATCCTGGACCTTCC	BTA4: 93262077 - 93262057		
K_2	F: GACGATGTGCCACGTGTGGTTTCTTCTGTT	BTA4: 93261921 - 93261950	166 bp	modified version of Buchanan et al., 2002
	R: TGAGGGTTTTGGTGTCATCCTGGACCTTCC	BTA4: 93262086 - 93262057		
P	F: GTGCCACGTGTGGTTTCTTCTGTT	BTA4: 93261927 - 93261950	155 bp	This study
	R: GTTTTGGTGTCATCCTGGACCCTG	BTA4: 93262081 - 93262058		
S	F: TCAAACCAGACCTTGAAAGCC	BTA4: 93261663 - 93261683	798 bp	This study
	R: TTACCAGGCAGGAAGAATGCC	BTA4: 93262460 - 93262440		

[a]: In the reverse sequences underlined letter indicates the mismatched nucleotide. The underlined C replaces the natural base G in K1 and K2 primers and the natural base T in the P primer.
[b]: Genomic positions refer to the Bos taurus UMD 3.1 genome assembly, BTA: Bos taurus autosome.

2.3.PCR

PCR amplification of a 155 bp fragment of LEP exon 2 was performed in a total volume of 30 µL reaction mixture containing 3 µL of genomic DNA, 1 µL of of each primer (20 pmol/µL), 3 µL of dNTP (2.5 mM), 3 µL of $MgCl_2$ (25 mM), 3 µL of 10X *Taq* buffer with $(NH_4)_2SO_4$, 0.2 µL of *Taq* DNA polymerase (5 U/µL) and 16 µL of ddH2O. Thermal cycler condition included of an initial denaturation step at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s and the final extension step was continued for 5 min at 72°C.

2.4.RFLP

Digestion of PCR products with restriction enzymes was performed in a total volume of 30 µL. The digestion mixture contained 25 µL PCR product, 2.5 µL 10X buffer, 0.5 U of the respective restriction enzyme (10 U/µL), and ddH2O to a final volume of 30 µL. Digestion was performed at 37°C for *PstI* enzymes, and 55°C for *Kpn2I* enzyme, each for 16 hours. The digested DNA fragments were separated on 3.5% agarose gel electrophoresis, stained with EtBr, and visualized under UV light. The number and sizes of the resulting fragments corresponding to different genotypes for rs29004488 polymorphism in the exon 2 region of the leptin gene after digestion with *Kpn2I* and *PstI* enzymes are shown in Table 2.

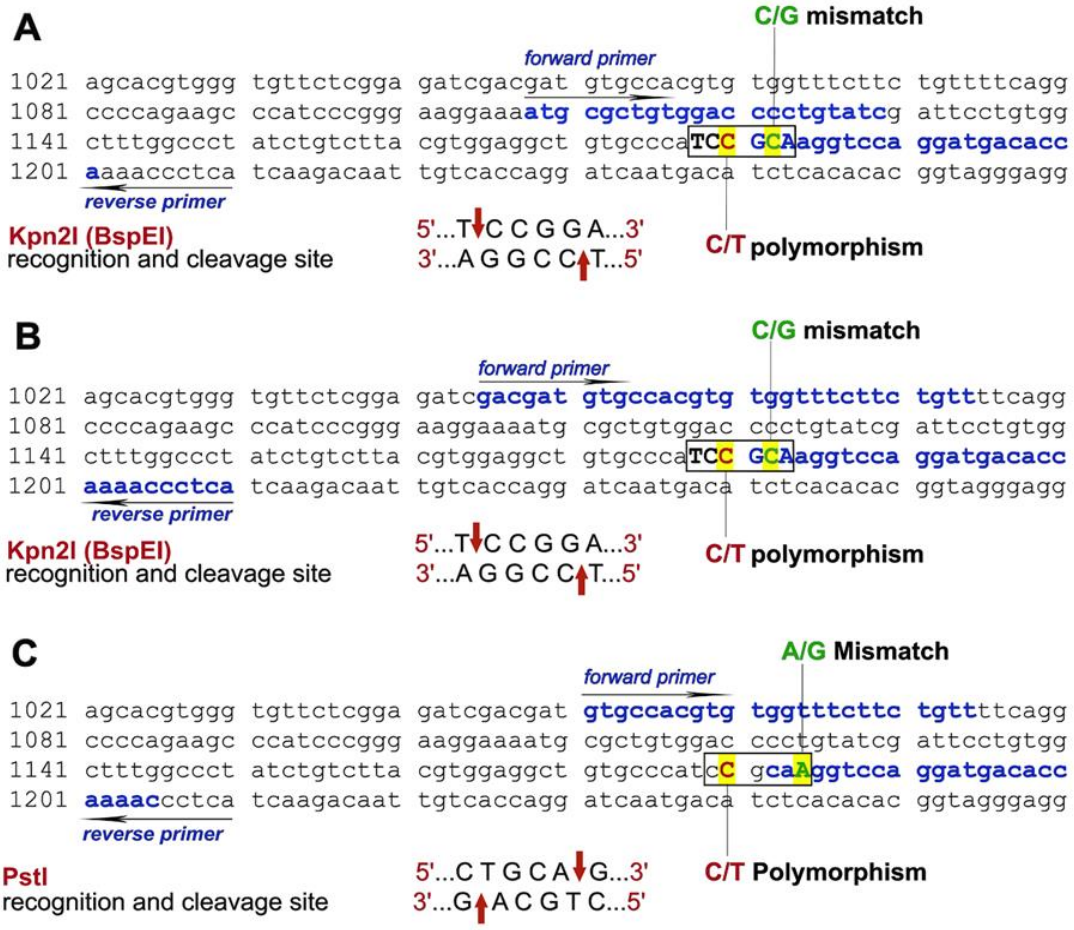

Figure 1. Detection of rs29004488 polymorphism by different primers and restriction endonucleases **A.** K_1 primers and *Kpn2I* enzyme, **B.** K_2 primers and *Kpn2I* enzyme, **C.** P primers and *PstI* enzyme

2.5.DNA sequencing

DNA sequence analysis was performed to verify genotypes identified for the *PstI* enzyme. For this purpose, a new S-primer pair was designed covering the entire exon 2 region of the leptin gene (Table 1). The randomly selected 6 samples representing different band profiles (genotypes) due to *PstI* cleavage were sequenced by the Sanger method. The DNA sequence analysis of the PCR products was performed by a Sanger sequencing service purchased from a private company using the forward S primer.

Table 2. Fragment sizes for different genotypes of the rs29004488 polymorphism after cleavage with *Kpn2I* and *Pst*I enzymes

Genotype	94 bp[1*]/ *Kpn2I*	166 bp[2] / *Kpn2I*	155 bp[3] / *Pst*I
CC	75+19	138+28	155
CT	94+75+19	166+138+28	155+133+22
TT	94	166	133+22

[1]: K1, [2]: K2, [3]: P primers, [*]: Buchanan et al., 2002

3. Results

In this study, the leptin gene rs29004488 polymorphism was successfully detected in cattle using created restriction-site PCR-RFLP with *PstI* enzyme. PCR amplification yielded a product of a 155 bp fragment. As shown in Figure 2-B, after digestion with *PstI* enzyme, the homozygous C allele exhibited a single uncut band of 155 bp, the homozygous T allele exhibited a single band of 133 bp, and the heterozygous samples exhibited 2 bands of 155 and 133 bp, respectively. The 22 bp fragment resulting from the cleavage in the homozygous T allel and in the heterozygous samples is not visible in the agarose gel (Figure 2-B).

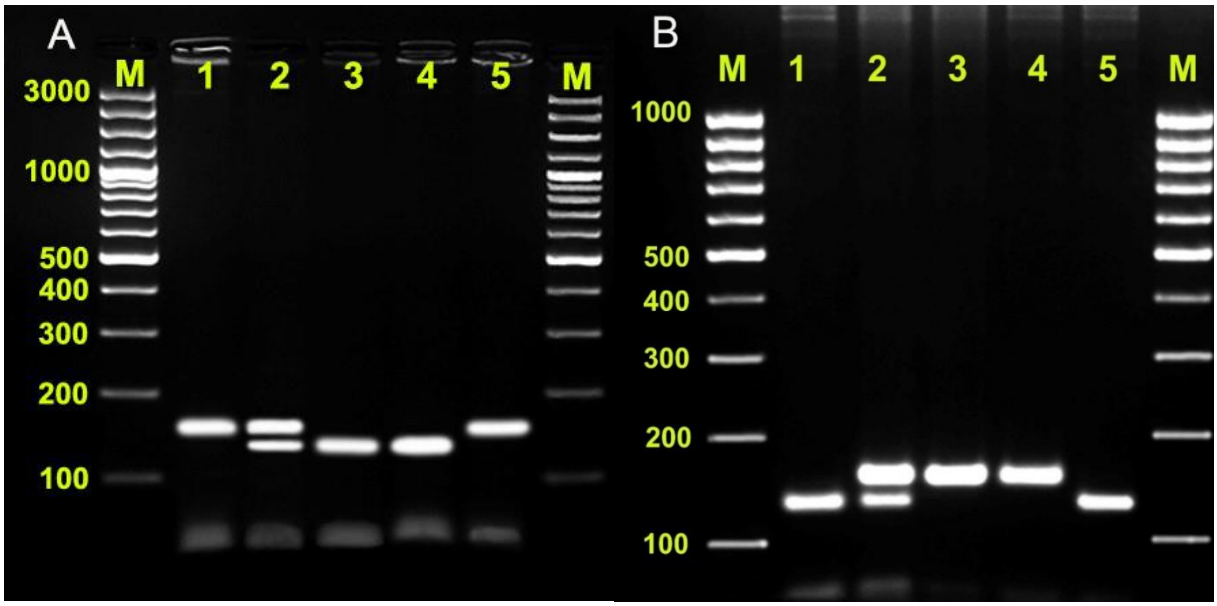

Figure 2. Restriction patterns of PCR products digestion with Kpn2I (A) and PstI (B) enzymes, respectively. Lane M: marker (100 bp); 1, 5: TT; 2: CT; 3, 4: CC genotypes

The results of samples digested with *PstI* enzyme were verified by comparison with *Kpn2I* enzyme digestion. The genotypes detected from the digestion results of both enzymes showed 100% compatibility (Figure 2-A and B). In addition, a total of 6 samples (2 samples representing each genotype) randomly selected from the samples genotyped for the rs29004488 polymorphism by *PstI* enzyme were confirmed by DNA sequence analysis. The results of the DNA sequence analysis of the CC, CT and TT genotypes for *PstI* enzyme are shown in Figure 3.

Table 3. Recognition sites and prices of respective endonucleases

Endonuclease	Recognition sequence	€/1.000 units
PstI	CTGCA/G	7,2[a]
BspEI(Kpn2I)[1]	T/CCGGA	63[b]
AciI	CCGC(-3/-1)	315[d]
HpyCH4V	TG/CA	630[c]

[1]*BspEI* is an isoschizomer of *Kpn2I*. Enzyme prices were determined by calculating the prices corresponding to 1.000 U of the closest quantities available for sale as follows; [a]: 5.000 U, [b]: 10.000 U, [c]: 500 U, [d]:1.000 U were calculated from the sales prices.

The NEB cutter program to separate different alleles of the Letin gene rs29004488 polymorphism showed that the "CCGC" sequence observed in the C allele in the polymorphism region can be cleaved by the enzyme *AciI* and the "TGCA" sequence observed in the T allele can be cleaved by the enzyme *HpyCH4V*. However, the cost of the *AciI* and *HpyCH4V* enzymes is considerably higher. The prices of the four restriction enzymes obtained from the NEB GmbH website (*https://www.neb-online.de/en*) are shown in Table 3. According to Table 3, the cost of the *PstI* enzyme is approximately 11.43% of the price of the *BspEI* (*Kpn2I*) enzyme.

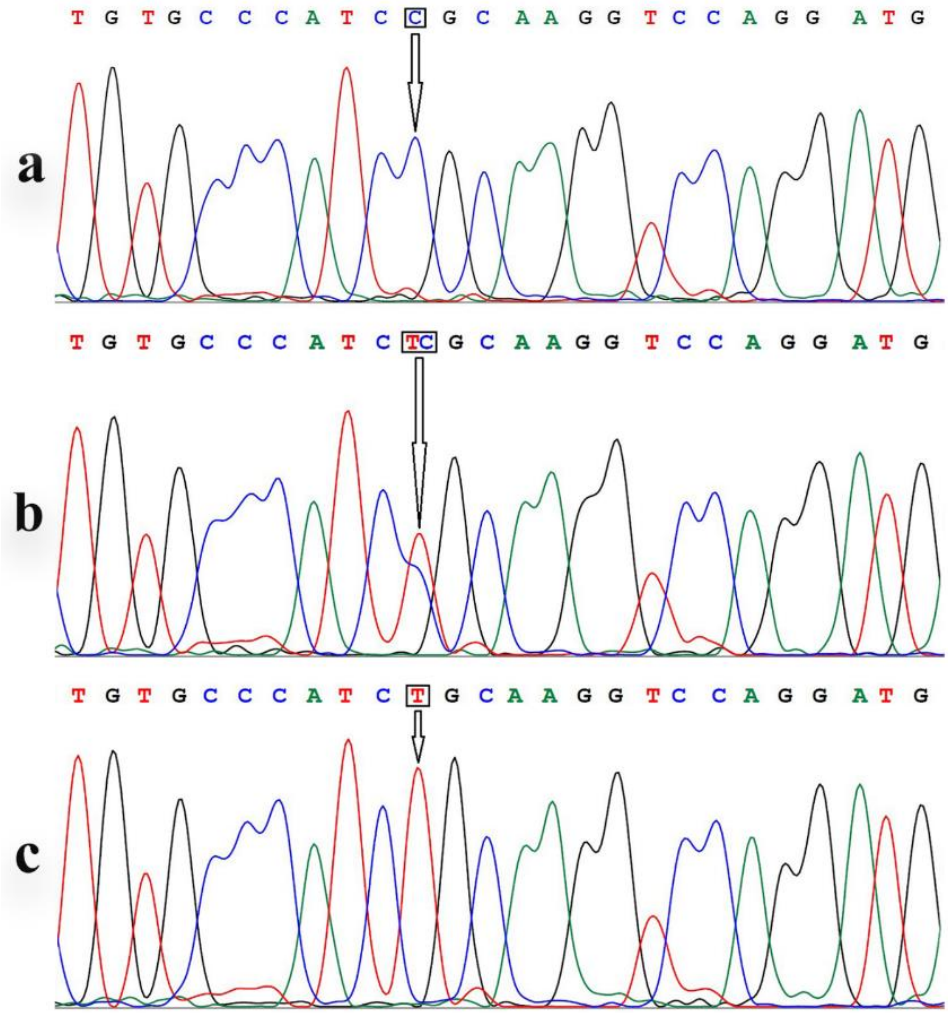

Figure 3. Examples of DNA sequencing of PCR product of LEP gene. Three figures representing the genotype of **a.** CC, **b.** CT, and **c.** TT are shown, and the bases representing rs29004488 polymorphism sites are indicated by arrows and are located within squares.

4. Discussion

In cattle, leptin gene rs29004488 polymorphism is detected by PCR-RFLP method using primers designed by Buchanan et al. (2002) and *Kpn2I* enzyme. However, this method has some limitations in terms of primer degradation. With the use of the *PstI* enzyme as a new approach in the detection of the polymorphism, the mismatch that should be made one base before the 3' end of the reverse primer for the *Kpn2I* enzyme, was shifted to 2 bases before, preventing the problem of primer degradation and providing a new opportunity for more economical detection of the rs29004488 polymorphism. In this study, a new *PstI* restriction enzyme site was introduced into the amplification products to detect the rs29004488 polymorphism of the leptin gene in cattle.

The use of the *PstI* enzyme saves approximately 88.57% of the enzyme cost compared to the *BspEI* (*Kpn2I*) enzyme (Table 3). This is important for the use of the leptin rs29004488 polymorphism, which is associated with economically important yield traits in cattle breeding, in developing countries and in large herds, and for its inclusion in breeding programs.

5. Conclusion

In conclusion, we present an efficient and cost-effective technique for the analysis of the bovine leptin rs29004488 polymorphism. This technique can be widely used in future studies investigating the effects of leptin gene polymorphisms on yield traits and in genotypic selection applications in cattle.

Declaration of Author Contributions

MG, MB and HY designed and conducted the study. MG designed the primers and performed the DNA sequencing, MG and EO performed the PCR-RFLP analysis and gel electrophoresis. MG, MB, and HY drafted the manuscript. All authors approved the final version of the article.

Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

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Ethical Committee Approval

This study was approved by the Animal Experiments Ethics Committee of the Veterinary Control and Research Institute of Elazig, Turkey (07/01-01).

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