# The cytosine/thymine polymorphism detected in the promoter region of the IGF-1 gene using the PCR-RFLP technique in certain local cattle breeds and the genetic structure of the studied population

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**Abstract**. In this study, the cytosine/thymine polymorphism was investigated in the promoter region of the IGF-1 gene (insulin-like growth factor 1 gene) in individuals of the Bălțată cu Negru Românească (BNR) cattle breed with low and high milk production. This research is important because IGF-1 is a polypeptide hormone that plays a crucial role in the development of bones and other tissues. Using the PCR-RFLP technique, two alleles, C and T, were detected in the studied populations. In both categories of individuals, the C allele showed a higher frequency than the T allele. The three identified genotypes were CC (uncut 249 bp), CT (249 bp, 223 bp, 26 bp), and TT (223 bp, 26 bp). In both individuals with high and low milk production, the C allele appeared with a higher frequency than the T allele in the population.
**Key Words**: IGF-1, polymorphism, molecular markers, cattle, genotype, enzyme, PCR-RFLP.

**Introduction**. In recent decades, concerns among cattle breeders regarding the increase in fertility have intensified due to recorded trends of decline in fertility. These past trends were caused by the intensified selection efforts aimed at genetic traits for milk production (Royal et al 2000; Lucy 2001; Grădinaru et al 2018).

A significant number of peptide hormones are involved in the expression of productive and reproductive traits in animals (Carșai et al 2005; Petrescu-Mag 2023; Shaarawy et al 2024). The IGF-1 gene (insulin-like growth factor 1 gene) is a candidate gene for marker-assisted selection strategies, and the single nucleotide polymorphism in the promoter region (IGF1/SnaBI) can be associated with production traits in several breeds of taurine (de la Rosa Reyna et al 2010). IGF-1 is considered to be one of the main mediators of the effects of energy balance on reproductive performance in post-calving dairy cows (Zulu et al 2002), due to the fact that circulating IGF-1 concentrations are strongly associated with energy balance (Pushpakumara et al 2003; Fenwick et al 2008), follicular growth (Gong 2002) and resumption of ovarian cyclicity (Konigsson et al 2008). The IGF-1 gene is considered a promising candidate gene for the identification of molecular markers with a role in predicting reproductive performance in dairy cows (Nicolini et al 2013) and a study was conducted to identify the IGF-1/SnaBI single nucleotide polymorphism (SNP) association with fertility, milk production and body traits in lactating Holstein-Friesian cows kept on pasture, and other studies reported a significant effect of IGF1/SnaBI SNP on calving interval (CFS) in primiparous cows from Holstein-Friesian breed (Nicolini et al 2013).

Molecular techniques for investigating animal productivity have evolved significantly (Coșier 2006; Coșier & Marian 2017; Yuca & Kopuzlu 2023; Ayele et al 2024). De la Rosa Reyna et al (2010), who studied single nucleotide polymorphism in the promoter region (IGF1/SnaBI), reported that it is associated with some production traits in Charolais and

Beefmaster taurine breeds. In another study conducted on an Indonesian cattle breed, Agung & Saputra (2022) concluded that the IGF-1 gene was polymorphic but exhibited low heterozygosity. As a result, it was not considered a suitable marker for assisted selection in Indonesian Sumba Ongole cattle.

Other studies on different breeds of cows, including Jabres, state that to confirm the effect of SNPs specific to the FSHR and IGF-1 genes a large sample of individuals is necessary (Hartanto et al 2023).

Gerasimov et al (2023), in a study carried out on individuals from the Kazakh White-Headed cattle breed, obtained significant differences in metabolic mid-weight (MMWT) between bulls with IGF-1TT and IGF-1CT genotypes, showing a 2.2 kg increase in heterozygous cattle ($p<0.05$). IGF-1CT heterozygous bulls differed by a higher dry matter intake (DMI) of 0.087 kg day$^{-1}$ ($p<0.05$) compared to IGF-1TT homozygotes. The carriers of the IGF-1TT genotype had the highest feed efficiency at 0.068 kg day$^{-1}$ ($p<0.05$) among the bulls.

Saleh et al (2024) made associations between IGF-1 and GH polymorphisms and milk production and composition, reproductive performance in a herd of 1000 Holstein–Friesian dairy cattle. They employed digestion of the 249 bp fragment of IGF1-SnaBI using the RFLP technique, identifying two alleles with frequencies T (0.59) and C (0.41), and three genotypes with frequencies TT (0.52), TC (0.39), and CC (0.09). Sequencing analysis of the IGF-1 gene revealed a polymorphism at nucleotide position 472 (C>T). The study concluded that genetic variation in the studied genes can be applied in selecting animals for superior milk production, composition, and reproductive performance in Holstein-Friesian dairy cattle under subtropical conditions. Furthermore, IGF-1 can be used as a genetic marker (Saleh et al 2024). The aim of this study was to investigate the cytosine/thymine polymorphism in the promoter region of the IGF-1 gene (insulin-like growth factor 1 gene) in individuals of the Bălțată cu Negru Românească (BNR) cattle breed with low and high milk production.

**Material and Method**. In this study, blood samples were taken from 60 bulls of the Bălțată cu Negru Românească (BNR) breed, originating from cows with low milk production, and from 60 bulls originating from cows with high milk production, from different farms in Romania over the past few years.

For DNA extraction from blood samples, the protocol from the Fast to Tissue PCR kit (Fermentas) was modified. Thus, 200 μL of whole blood from each sample were transferred into a sterile 1.5 mL Eppendorf tube. 600 μL of NE solution per sample (containing NaCl + EDTA, pH=7) were added to each sample. The tubes were then centrifuged at 14000 g for 30 sec., the supernatant being removed each time, and the washing was repeated two times. After the final washing, the supernatant was removed, 110 μL of the lysis solution from the Fast to Tissue PCR kit (Fermentas) was added to the formed sediment (containing the nucleated white figured elements).

For genotyping at the IGF-1 locus, a 249 bp fragment was amplified from the gene promoter region, where a cytosine/thymine substitution is located, which characterizes the two C and T alleles from this locus. This region is particularly important for gene expression. To highlight these two alleles, the primers chosen for genotyping were specific primers:

IGF1-F: ATTACAAAGCTGCCTGCCCC

IGF1-R: ACCTTACCCGTATGAAAGGAATATACGT

Both primers located in a region of the IGF-1 gene promoter, important in gene expression. Amplification was performed using the 2X MyTaq Red Mix kit (Bioline), 100 μg of genomic DNA extracted from individuals with low milk production and high milk production and 10 picomoles of each primer with the following amplification conditions: 1 cycle at 94°C /3 min., followed by 35 cycles at 94°C/30 sec., 58°C/30 sec., 72°C/60 sec., and a final extension cycle at 72°C/3 minutes.

Amplification products were analyzed by migration in 2% agarose gel, containing 1X SybrSafe (Invitrogen). Electrophoresis was performed in TBE 1X buffer (pH=8.5), at a constant voltage of 65 V for 2 hours. The gel was analyzed using the Molecular Imager Gel Doc XR System image acquisition system (BioRad Laboratories).

**Results and Discussion**. The results regarding the amplification of the coding regions of the IGF-1 gene were highlighted by the PCR-RFLP technique with the discovery of a cytosine/thymine type polymorphism located in the promoter region of the IGF-1 gene, a particularly important region for the expression of the IGF-1 gene. Genotyping at the IGF-1 locus a of the taurine individuals studied from the BNR breed (with low milk production and with high milk production) was possible due to the existence of a cytosine/thymine type mutation located in the restriction site of the SnaBI enzyme on the fragment of 249 bp amplified from the IGF-1 gene promoter region.

Following the digestion of this 249 bp fragment, three restriction profiles (electrophoretic) corresponding to 3 genotypes TT, CT and CC and two alleles T and C were highlighted. The 249 bp fragment amplified from the C allele does not present any restriction site for the SnaBI enzyme. The substitution of a cytosine (present in the C allele) with a thymine (present in the A allele), located on the amplified fragment, creates a restriction site for this enzyme, following the restriction resulting in two fragments of 223 bp and 26 bp respectively, characteristic of the T allele. In both categories of individuals studied, all 3 possible genotypes were observed, namely: CC which correspond to a single undigested fragment of 249 bp; TT corresponding to fragments of 223 bp; and CT - 26 bp, showing all three fragments. The 26 bp fragment cannot be visualized in the gel due to its very small size. The most representative agarose gel is illustrated in Figure 1.

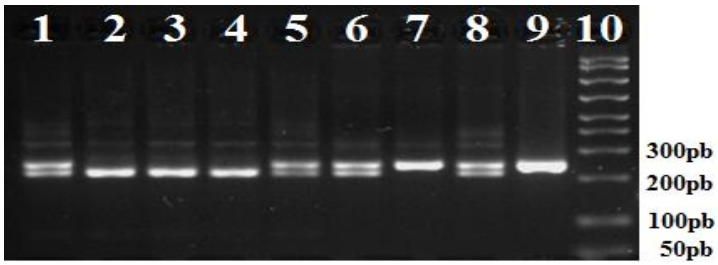


Figure 1. A representative gel with electrophoretic profile highlighting the SnaBI type polymorphism, located in the promoter region of the IGF-1 gene. Lane 1, 5, 6 and 8 - CT genotypes; lane 2, 3 and 4 - TT genotypes; lane 7 - CC genotype; lane 9 - undigested 249 bp PCR product; lane 10 - Ladder DNA Ampli SizeTM (BioRad Laboratories).

Following the centralization of the experimental data, the frequencies of alleles and genotypes were calculated both in individuals with high milk production and in those with low milk production.

After calculating the gene and genotype frequencies in individuals with high milk production from the BNR breed, the following genotype frequencies were obtained: CC genotype - 0.273, CT genotype - 0.545, and TT genotype - 0.182. The gene frequencies were as follows: gene C - 0.545 and gene T - 0.455 (Table 1).

Table 1

Genotype and allele frequencies of the IGF-1 gene in high milk production BNR individuals

| *Genotype* | *Frequency* | *Allele* | *Frequency* |
|---|---|---|---|
| CC | 0.273 | C | 0.545 |
| CT | 0.545 | T | 0.455 |
| TT | 0.182 | | |

After calculating the frequency of the gene and genotypes in individuals with low milk production from the BNR breed, the following genotype frequencies were obtained: CC genotype - 0.600, CT genotype - 0.400, and TT genotype - 0. The gene frequencies were as follows: gene C - 0.800 and gene T - 0.200 (Table 2).

Table 2
Genotype and allele frequencies of the IGF-1 gene in low milk production BNR individuals

| *Genotype* | *Frequency* | *Allele* | *Frequency* |
|---|---|---|---|
| CC | 0.600 | C | 0.800 |
| CT | 0.400 | T | 0.200 |
| TT | 0.000 | | |

Saleh et al (2024) conducted a recent study on the associations between IGF-1 polymorphisms and milk production, milk composition, and reproductive performance in a Holstein-Friesian dairy cattle herd. They used the RFLP technique to digest the 249 bp IGF1-SnaBI fragment. Similar to our findings, they reported two alleles with frequencies of T (0.59) and C (0.41), and three genotypes with frequencies of TT (0.52), TC (0.39), and CC (0.09). The analysis of the IGF-1 gene revealed polymorphism at position 472 (C>T), leading to the conclusion that genetic variation in the studied genes may be utilized in selecting animals for superior milk production, milk composition, and reproductive performance in Holstein-Friesian cattle under subtropical conditions. It was concluded that IGF-1 can be used as a genetic marker, a finding also confirmed by our study presented here. Comparing the gene frequencies in BNR individuals, the frequency of the C allele was higher in both high milk producers (C: 0.545) and low milk producers (C: 0.800).

**Conclusions**. In the present study, the PCR-RFLP technique proved to be convenient to detect IGF-1 gene polymorphism. In the studied population of BNR bulls, all three genotypes (CC, CT, TT) were detected in individuals with high milk production, and two genotypes were detected in individuals with low milk production (CC, CT). We can conclude that in individuals with high milk production, the heterozygous genotype appears with a higher frequency in the studied population (CT: 0.545), while in individuals with low milk production, the homozygous genotype CC appears with a higher frequency in the population (CC: 0.600) and the TT genotype is missing. In all individuals, the C and T alleles were present and in both categories of individuals we can conclude that the C allele has a higher frequency compared to the T allele. The frequency of the CC genotype, which was 0.273 in individuals with high milk production and 0.600 in those with low milk production, is higher than what has been reported by other authors in the literature.

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**Conflict of interest**. The author declares no conflict of interest.

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