Journal of the Hellenic Veterinary Medical Society

Vol 76, No 3 (2025)

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Z Sönmez, M Özdemir

doi: 10.12681/jhvms.39426

To cite this article:

Sönmez, Z., & Özdemir, M. (2025). Identification of polymorphisms in ELF5 and BMP2 genes and their impact on milk production in Brown Swiss cattle. *Journal of the Hellenic Veterinary Medical Society*, *76*(3), 9631–9644. https://doi.org/10.12681/jhvms.39426

J HELLENIC VET MED SOC 2025, 76 (3): 9631-9644
ΠΕΚΕ 2025, 76 (3): 9631-9644

Research article
Ερευνητικό άρθρο

Identification of polymorphisms in ELF5 and BMP2 genes and their impact on milk production in Brown Swiss cattle

Z. Sönmez,[1(*)] M. Özdemir[2]

[1]*Department of Agricultural Biotechnology, College of Agriculture, Ataturk University, Turkey.*

[2]*Atatürk University, Faculty of Agriculture, Department of Animal Science, 25240, Erzurum, TÜRKİYE*

ABSTRACT: Identifying genetic markers to comprehend the population structure and genetic foundation of cattle breeds, enhancing production and elevating yield quality, is vital in the realm of modern breeding technology. The aim of this study was to determine the genetic variations in E74-Like Factor 5 (ELF5), also known as Epithelial Specific ETS Factor 5 gene, and Bone Morphogenetic Protein 2 (BMP2), also known as Bone Mineral Density Protein 2 gene, and to correlate them with milk yield. The study's purpose was to define genetic markers in genes affecting milk yield performance in cattle. We used DNA samples from 90 of Brown Swiss cattle. Sequence analyses were applied to samples randomly selected after High-Resolution Melting analysis (HRMA), and polymorphic regions were identified. As a result of DNA sequences i) g.65825499 G/A transitions were determined for the intron 2 region of the ELF5 gene, ii) 5 different polymorphic regionsg.65826062 G/A, g.65825909 T/C, g.65826134 C/T, g.65826123 A/C and g. 65826138 T/C were identified for the exon 5 region of the ELF5 gene; iii) g.49551405 G/T, g.49551337 G/A, g.49551433 A/C, g.49551428 G/C and g.49551449 T/G polymorphic regions were identified for the exon 3 region of the Bone Morphogenetic Protein 2 (BMP2) gene. There was no relationship between the polymorphic regions in the exon 5 and the intron 2 region of ELF5 and exon 2 and exon 3 of BMP2 genes SNP polymorphisms and milk yield (P>0.05). Our results indicate that the polymorphisms identified in these genes can be used as molecular markers in modern breeding programmes to identify various traits, such as milk production and growth performance, in cattle breeding.

Keyword: ELF5, BMP2, SNP, milk yield, DNA sequencing.

Correspondence author:
Z. Sönmez,
Department of Agricultural Biotechnology, College of Agriculture, Ataturk University, Turkey
E-mail address: zeynepsonmez@atauni.edu.tr

Date of initial submission: **21-11-2024**
Date of acceptance: **29-3-2025**

INTRODUCTION

In recent years, advances in sequencing genomes and SNP data, along with improvements in genotype identification technologies, have enabled the creation of genetic association maps across the entire genome (Schlötterer et al., 2014). In line with these developments, Quantitative Trait Locus (QTL) maps, linkage maps and genome-wide association (GWAS) maps have been constructed (Drögemüller et al., 2001; Höglund et al., 2014; Hu et al., 2022).

Ririe, et al. (1999), using High-Resolution Melting Analysis (HRMA) and real-time Polymerase Chain Reaction (PCR) technologies, demonstrated that DNA melting curves could be determined by measuring the fluorescence characteristic of the SYBR Green I dye. The dye can bind to double-stranded DNA during the polymerase chain reaction. Ririe et al. (1999) defined HRMA as the shapes and positions of DNA melting curves that vary depending on several variables such as the difference in GC/AT ratio in the DNA content, product length, and the annealing temperatures of the primers.

The ELF5 protein factor gene also knowns as a AKA Epithelial-Specific ETS Factor 2 (ESE-2a), is located on the 15th chromosome in cattle and contains nine exons and seven introns. The ELF5 gene is a transcriptional activator that may play a role in regulating the later stages of keratinocyte terminal differentiation. ELF5 gene is involved in the differentiation of trophectoderm (TE) cells in mouse embryonic development as well as in the differentiation of embryonic TE cells in bovine breeds (Chen et al. 2010; Ozawa et al.2012; Hosseini et al.2015). ETS factor protein plays a crucial role in the regulation of various systems, including epithelial, haematopoietic, neuronal, endothelial and endocrine, through its trans-acting phosphoproteins, as well as in the formation of cell migrations during embryonic development, cell proliferation, differentiation and oncogenic transformation. (Moreau et al., 1996; Zhou et al., 2005; Tummala & Sinha 2006).

Bone Morphogenetic Protein (BMP) genes, which belong to the Transforming Growth Factor beta (TGF-β) superfamily (Glister et al., 2011), regulate various cellular processes, including embryogenesis, tissue development, and the differentiation of adult tissues, as well as the maintenance of their functions (Wang et al., 2014). BMPs exert their cellular effects through ligand-receptor interactions induces a signal transduction cascade, where the type II receptors (i.e., TGFBR2, ACVR2A, ACVR2B, BMPR2, and AMHR2) activate functionally related type I receptors (i.e., ACVRL1/ALK1, ACVR1/ALK2, BMPR1A/ALK3, ACVR1B/ALK4, TGFBR1/ALK5, BMPR1B/ALK6, and ACVR1C/ALK7) via phosphorylation (Valdecantos et al.2017) (Figure 1.).

BMP2 gene, located on the chromosome 13 in cattle, consisting of 3 exon regions and three intron regions and encoding approximately 395 amino acids. It affects embryonic, cartilage, and bone development and differentiation of tendons in addition to playing a reparative role in bone, cartilage, and tendon injuries (Moreau et al.,1996; Attisano et al.,2002; Lavery et al.,2008). Previous studies have demonstrated that the BMP2 gene is related to muscle area size, body length, and structure (Cruise et al., 2004; Park et al., 2010; Moura et al., 2013), body size, and muscle development (Xu et al., 2019; Lu et al., 2020) in beef cattle. The gene has also been shown to affect fat storage in the tail region of fat-tailed sheep breeds (Park et al., 2010; Baazaoui et al., 2021).

Brown Swiss cattle breed, native to Schwyz region in the north-east of Switzerland, belongs to the genus of *Bos Taurus Brachiceros* (Briggs, 1980).

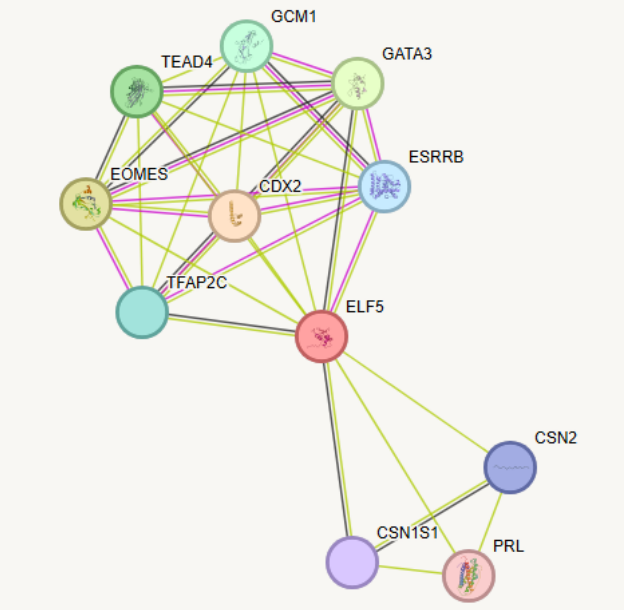


Figure 1. ELF5 signaling pathway: Red Cluster; Trophectodermal cell differentiation genes (CDX2, ELF5, EOMES, ESRB, GATA3, GCM1, TEAD4), Green Cluster; Milk protein genes; CSN1S1, PRL, CSN2, Blue Cluster; Transcription factor AP-2 gamma (TFAP2C) genes .

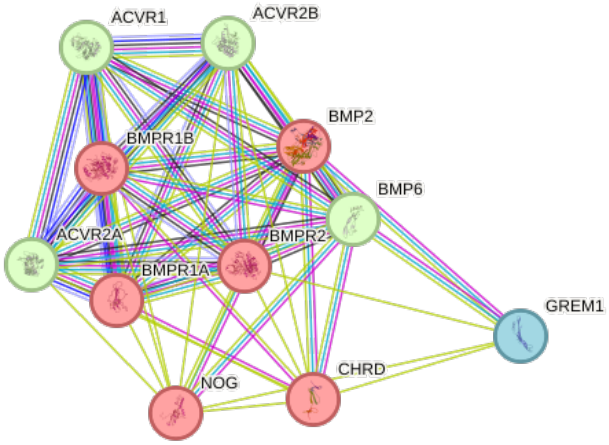


Figure 2. BMP2 signaling pathway: Red Cluster; type I receptors (BMP2, BMPR1A, BMPR1B, BMPR2, CHRD, NOG) TGFBR2, ACVR2A, ACVR2B, BMPR2, and AMHR2) Green Clusters: type II receptors (ACVRL1, ACVR2A BMPR1A/ALK3, ACVR1B/ALK4, TGFBR1/ALK5, BMPR1B/ALK6, and ACVR1C/ALK7) Blue Cluster: BMP antagonist genes; gremlin 1, DAN family.

Brown Swiss cattle demonstrate adaptability to diverse climates, management, and feeding regimes, which enables their successful rearing in various countries under different environmental conditions (Özhan, 2012).

This study aims to identify single nucleotide polymorphisms (SNPs) in the ELF5 and BMP2 genes, which are known to influence productivity traits in livestock, and to investigate their association with milk yield in Brown Swiss cattle. The findings will contribute to evaluating the potential use of these SNPs as markers in cattle breeding programs.

MATERIAL AND METHODS

Investigations on animals were carried out in compliance with the institutional animal use committee (27.10.2022 and decision number 257). Blood samples from genetically unrelated 90 Brown Swiss cows that had undergone three lactations, raised on a private farm in Erzurum, TÜRKİYE were used as material. DNA extraction from whole blood was carried out using the Purgene DNA kit following the guidelines provided by Gentra Systems of Minnesota, USA. Primers were designed to cover the exons and introns of the relevant gene using Primer3 plus software in line with the literature and based on the DNA sequences of the relevant gene regions in NCBI-GenBank (Table 1).

PCR conditions

100 ng of template DNA, 2.5 µL of dNTP mix (Sigma Aldrich, St. Louis, MO, USA, 0.25mM), 10 pmol/µL of each primer, 0.5 units of Taq DNA polymerase (Sigma Aldrich, St. Louis, MO, USA), 5 µL of 10x PCR Buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.01% gelatin), 1.5 µL of 0.25 mM MgCl2, and the final volume is made up to 25 µl with ddH2O.

We used 12.5 µL of Qiagen 2X HRM PCR master mix (Qiagen, Hilden, Germany), 0.7 µM of forward

Table 1. Primer sequences, product length and NCBI accession numbers used for ELF5 and BMP2 gene regions

Gene regions	(5'->3')	Product length (bp)	NCBI access numbers
	***ELF5* gene primers**		
Intron 2	F ACCCAGTGAGCCTTGTATGG R AAGCATCACTGCACAGAACG	492bp	NC_037342.1(65825262-65825757
Exon 5	F CGTTCTGTGCAGTGATGCTT R CCCAGTCTCCCCAAAGTGTA	500bp	NC_037342.1(65825735-65826235)
	***BMP2* gene primers**		
Exon 2	F GTCTTCTAGCGTTGCTGCTT R CCAGGTTCGGAAAGGTTCTTAC	480bp	NC_037340.1 49550050-49561304
Exon 3	F GG GTTGTGGGTGTCGTTAG R GCCAGAGTAACCTTCCATGTAG	467bp	NC_037340.1 (49551161-49551628)

primer, 0.7 µM of reverse primer and 100 ng of template DNA making the final volume of 25 µL with RNase-free water for HRMA PCR analysis. PCR amplification conditions were determined for each gene region using gradient PCR. To control amplification after PCR, base sizes and PCR product were visualized on a 2.5% agarose gel with ethidium bromide under UV light using a standard DNA marker (GeneAll Biotechnology Co., Ltd, Seoul, Korea).

PCR optimization conditions

PCR amplification conditions were determined separately for each gene and gene region. For all gene regions of the ELF5 and BMP2 genes, the initial denaturation temperature was established at 95°C for a duration of 5 minutes, followed by one cycle. The subsequent denaturation phase was also conducted at 95 °C for a period of 45 seconds. Extension cycles for ELF5 gene regions the exact extension times as 45 sec at 60 °C for the intron 2 (492 bp) and the exon 5 (500 bp) regions, and 58°C of ELF5 32 cycles. The primer binding temperature at 467 bp covering the exon 3 region of BMP2 gene was determined as 45 seconds at 60°C. The final extension temperatures were set at 72 °C for 5 min and one cycle in a T100 Thermal Cycler (Biorad, Hercules, California, USA) PCR device under the same conditions for all gene regions.

With the HRM-PCR optimization conditions being different for all regions of ELF5 gene, and intron 2, 5, and 6 exons of BMP2, initial denaturation temperatures were determined as 95°C for 5 min, second denaturation temperatures as 95°C for 10 s, extension temperatures as 58 °C for 30 second for all regions of the ELF5 genes, 56°C for 30 s for BMP2 gene regions, and final denaturation temperatures as 72°C for 24 s with a total of 40 reaction cycles. With the same HRM conditions for all gene regions, the PCR of the samples were performed using EvaGreen intercalation dye. Correspondingly, HRM analyses of different gene regions were performed using the Rotor-Gene Q real-time PCR device (Qiagen, Hilden, Germany), increasing by 90 s for the 1st cycle and by 2 s for each step in the range of 61°C–95°C.

Evaluation of HRMA data: HRMA analysis of the samples were made with a Rotor-Gene Q Real-time PCR device. Samples were clustered in UNSUPERVISED mode using the Rotor-Gene ScreenClust HRM software program(Qiagen, Hilden, Germany). ScreenClust HRM software program collects samples in clusters according to HRM temperature differences and GC contents by Principal Component analysis.

DNA sequence analyses

90 Brown Swiss animals DNA samples, whose temperature profiles were determined according to the base sequence differences they contain by HRM-PCR analysis, were divided into clusters according to the principal component analysis (PCA) based on the temperature similarities in the Screen Clust HRM software program.

Sequence analysis was applied to the PCR products, collecting four random samples from each cluster for each gene region. Thus, instead of sequencing 90 PCR products for each gene and gene region, sequence analysis was applied to the PCR products we randomly selected from samples divided into different clusters. Sanger sequencing technique was carried out using outsourcing services (Stabvida, Caparica, Portugal) and the raw DNA sequence of each sample was obtained. Genetic variations in the samples were identified based on the DNA sequence data obtained from BioEdit 7.2.6 (Hall et al., 2011) and MEGA 7.0 (Kumar et al. 2016) programs. Gene and genotype numbers were identified by a direct counting method using FinchTV v1.5(https://digitalworldbiology.com/FinchTV) software. Gene protein interactions were determined using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) software (https://string-db.org/).

Statistical Analysis

Lactation milk yield, Daily milk yield, 305 days milk yield, Milking period, Peak Day Yield, Peak Milk Yield, Avg. Peak Milk Yield of Brown Swiss cattle were employed in the statistical association analysis. In the analysis of the data obtained, SPSS 22.0 packaged software was used based on the General Linear Model (Harvey, 1990). Association analyses were performed between the milk yield traits of each animal and the genotypes belonging to the identified gene regions.

Based on the yield characteristics examined in the research, the subsequent statistical model was employed.

Y_{ijkl}: $\mu+a_i+b_j+c_k+ e_{ijk}$

Where

Y_{ijkl} is any of the milk yield traits yield (Lactation milk yield, Daily milk yield, 305 days milk yield, Milking period, Peak Day Yield, Peak Milk Yield, Avg. Peak Milk Yield),

μ is the population average,

a_i is the i^{th} genotype effect,

bj is the effect of the j^{th} lactation order (j: 3; 1^{st} Lactation: 1, 2^{nd} Lactation: 2, 3^{rd} Lactation: 3),

c_k is the effect of l^{th} calving season (l:2; 1: winter-spring, 2: summer-autumn),

e_{ijk} is margin of error.

RESULTS

Figure 3 shows the sizes of the PCR products for the gene and gene regions to determine if amplification has occurred after PCR.

According to the PCA analysis performed by defining normalisation temperature ranges via the ScreenClust HRM software for the genes under investigation concerning the ELF5 gene, the intron 2 region was collected in 8 clusters, and the exon 5 region in 6 clusters. BMP2 gene exon 2 and 3 region were collected in 6 clusters (Figure 4).

In our study sequence analysis was utilized to certificate the HRM results as it is intended as the standard technique for SNPs discovery. PCA analysis was performed according to the GC contents, and base differences of the ELF5 and BMP2 gene regions using the ScreenClust HRM program. The samples were divided into clusters according to their PCA similarity rates. Four samples from each cluster, which ranged in size from four to eight, were chosen at random and sequenced.

The sequence analysis of approximately 40 PCR samples covering the intron 2 region of the ELF5 gene (NC_037342.1 from bp 65825262 to 65825757) with a length of 492 bp was performed. Polymorphism screening was carried out on the 33 samples that remained after samples with flawed sequence analysis from PCR products were excluded during sequencing. As a result of the study of the base sequences of 33 samples, g.65825499 G/A polymorphisms were determined (Figure 5).

PCR analysis with a length of 492 bp was performed in Brown Swiss cattle. After defining one different polymorphic region for the intron 2 region of the ELF5 gene, The ELF5 gene g.65825499 G/A polymorphic region, GG, GA, and AA genotype

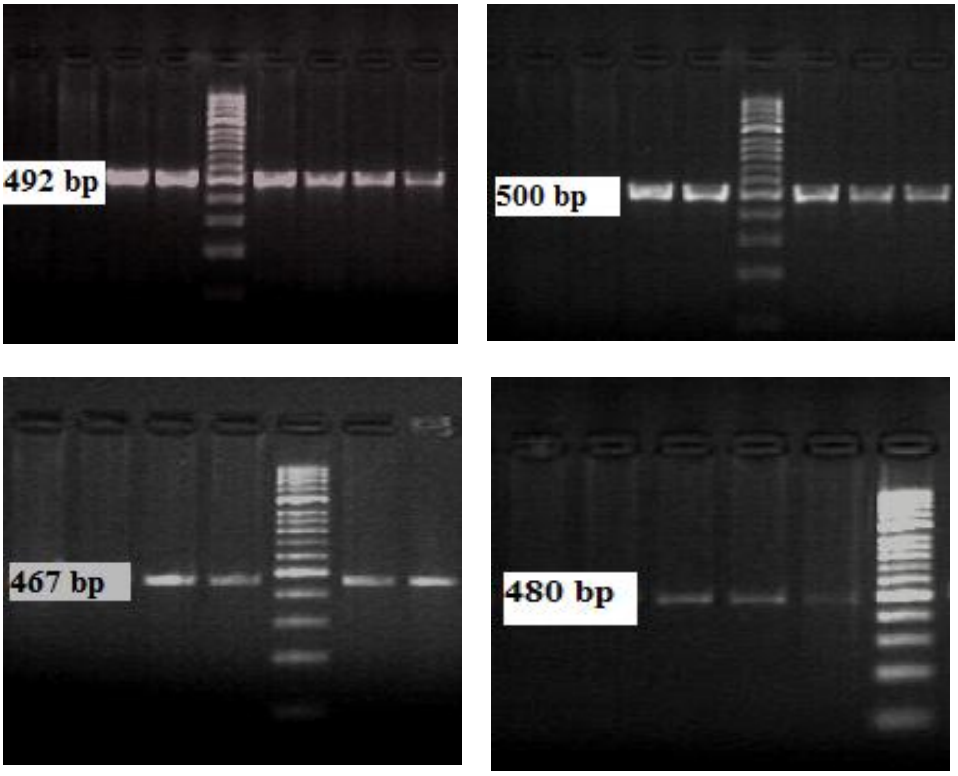


Figure 3. PCR results of the ELF5 and BMP2 gene regions; ELF5: 1st PCR amplicon pair (intron 2) 492 bp, 2nd PCR amplicon (exon 5) 500 bp, BMP2:1st PCR amplicon (exon 2) 467 bp, 2nd PCR amplicon 480 (exon 3) bp with Standard Genesta 100 bp DNA marker images, respectively, and all PCR product showing positive results for 96 Brown Swiss DNA samples.

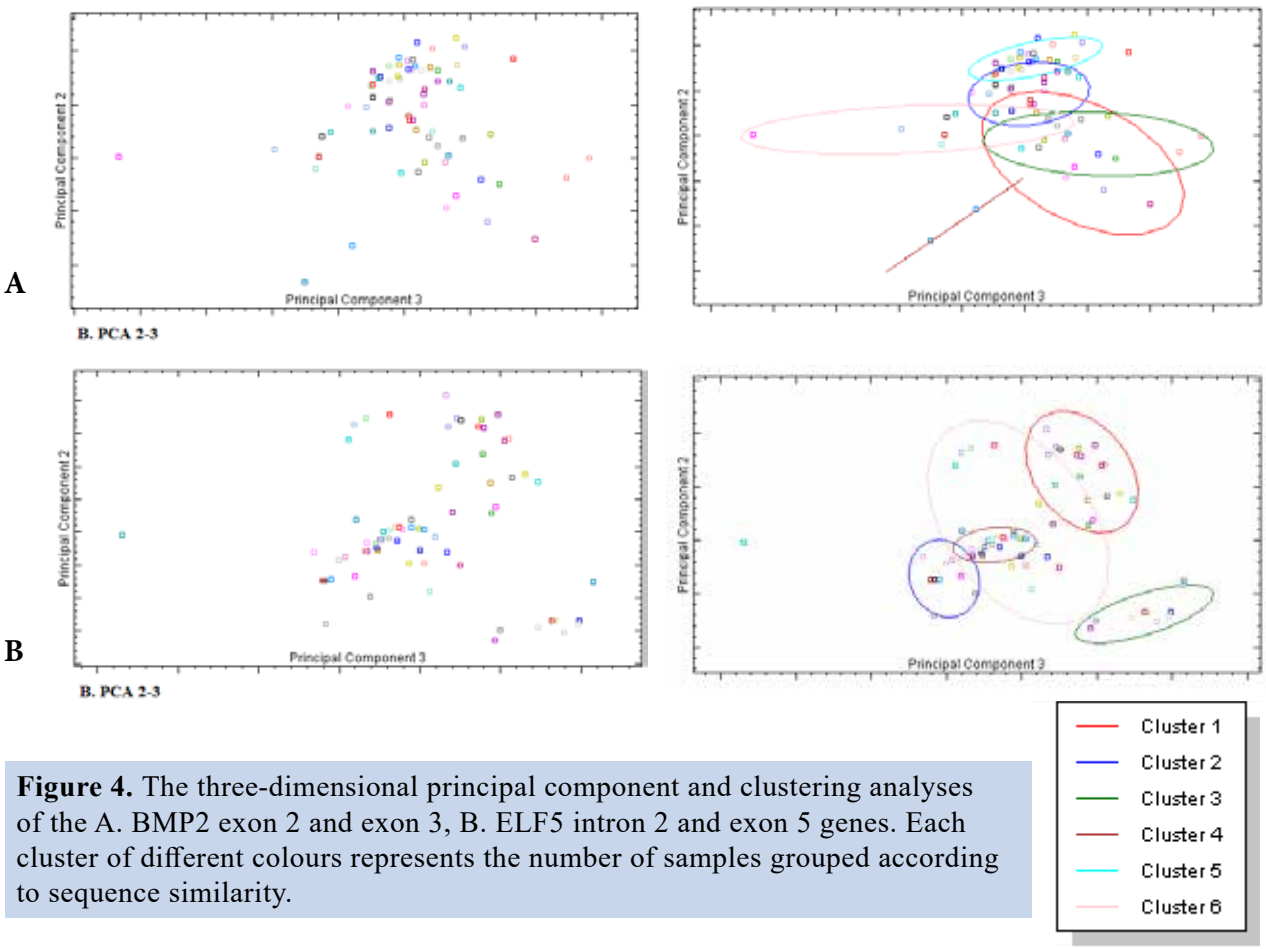
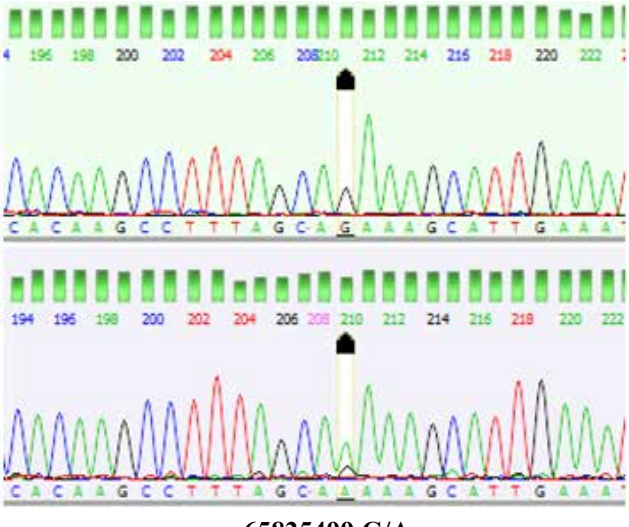


Figure 4. The three-dimensional principal component and clustering analyses of the A. BMP2 exon 2 and exon 3, B. ELF5 intron 2 and exon 5 genes. Each cluster of different colours represents the number of samples grouped according to sequence similarity.

frequencies were found to be 7.92%, 11.15%, and 3.92%, and G and A allele frequencies were determined as 0.59 and 0.41.



g.65825499 G/A

Figure 5. The Polymorphic sequence regions of the intron 2 region of the ELF5 gene g.65825499 G/A SNP is indicated by a dark arrow.

A total of 5 different polymorphic regions, including g.65826062 G/A, g.65825909 T/C, g.65826134 C/T, g.65826123 A/C and g. 65826138 T/C were identified for ELF5 exon 5. After sequence analysis, it was observed that g.65825909 T/C transition caused Alanine782-Alanine (Ala782Ala) amino acid exchange with GCT-GCC codon differentiation, g.65826062 G/A transition caused GGG-GGA Glycine 833-Glycine (Gly833Gly) amino acid exchange, and g.65826134 C>T transition caused CAC-CAT Histidine857-Histidine (His857His) amino acid exchange, g.65826123 A>C transversion caused AGC-CGC Serine854-Arginine (Ser854Arg) amino acid exchange, and g. 65826138 T>C transition caused TGG-CGG Tryptophan859-Arginine (Trp859Arg) amino acid exchange (Figure 6).

In the range of ARS-UCD1.2 NC_037340.1 (49551161-49551628), the exon 3 region of the BMP2 gene with a product length of 467, and g.49551405 G/T, g.49551337 G/A, g.49551433 A/C, g.49551428 G/C, and g.49551449 T/G polymorphic regions were found (Figure. 7).

Polymorphic bases identified for the exon 3 of

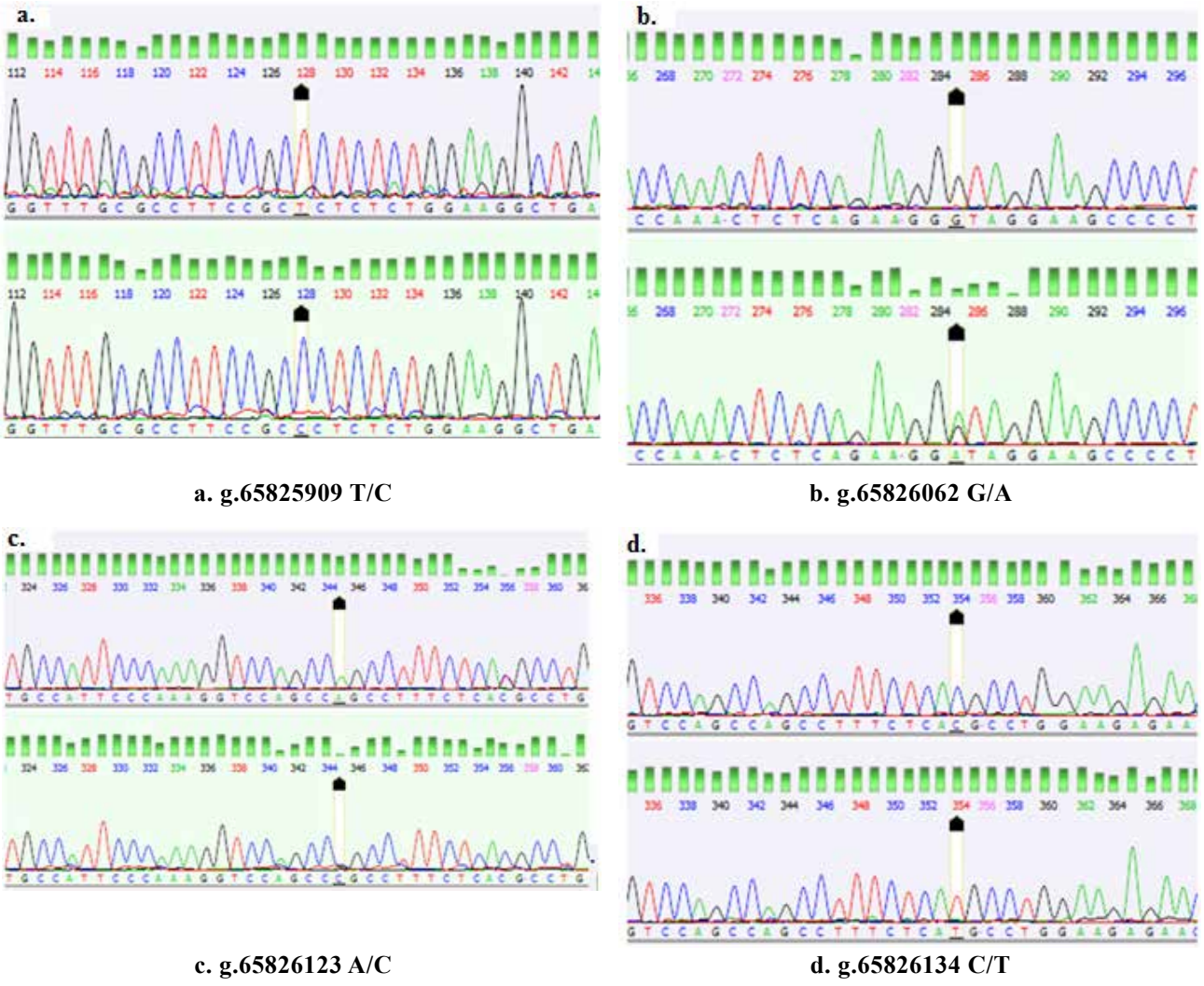
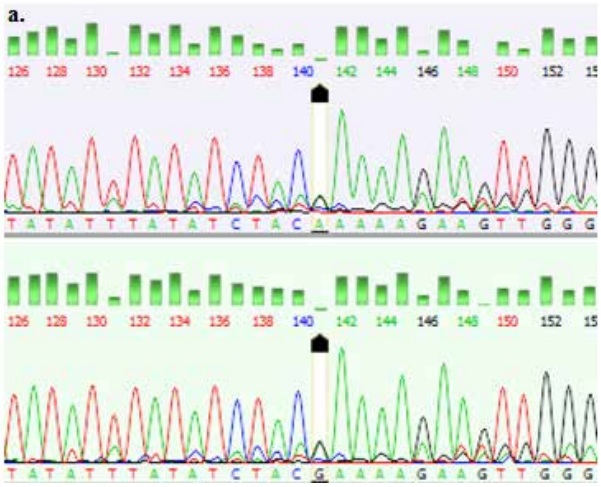


Figure 6. The SNPs identified in exon 5 of the ELF5 gene, according to the NCBI reference sequence (NC_037342.1, 65825262bp to 65825757bp), were compared to sequences obtained via Brown Swiss PCR. The identified SNPs are as: a.g.65825909 T/C, b. g.65826062 G/A, c. g.65826123 A/C, d. g.65826134 C/T, e. g.65826138 T/C. Each SNP is marked with a bold arrow in the respective sequence.

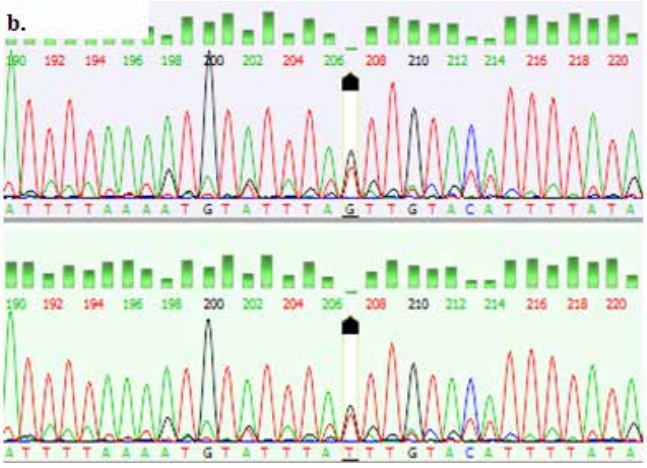
the BMP2 gene caused codon changes and resulted in the coding of different amino acids. Identified polymorphic bases, codon changes, and amino acid differences were the amino acid exchanges of threonine 3429-threonine (Thr3429Thr) and g.49551433 as a result of g.49551337 G/A transition ACG-ACA, A>C CCA-CCC proline3461-proline (Pro3461Pro), g.49551405 G/T AGT-ATT serine-isoleucine (Ser-3452Ile), g.49551428 G/C GCC-CCC proline-alanine (Pro3460Ala), and TGG-GGG tryptophan3467-glycine (Trp3467Gly) as a result of g.49551449 T>G transversions, respectively.

Concerning the exon 2 region NC_037340.1: 49550050-49561304 sequence of the BMP2 gene, the sequence analysis of the 480 bp DNA sequence amplified by PCR was performed, and no sequence showing polymorphism was found in 23 DNA samples.

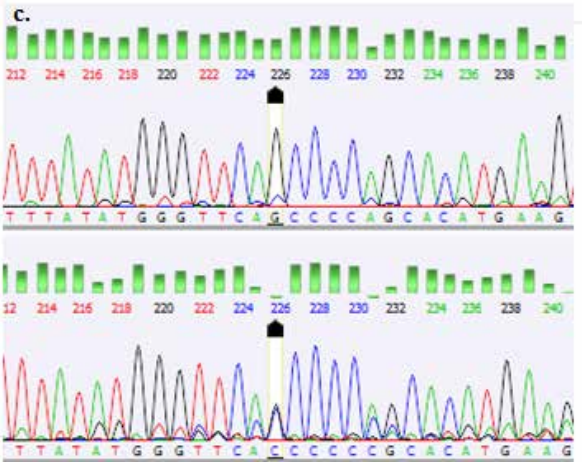
According to the results of the analysis of variance between sources of variation, the g.65825499 G/A polymorphic regions identified in the intron 2 region of the ELF5 gene and real milk yield, 305-day milk yield, daily milk yield, lactation period, peak milk yield, average peak milk yield, and peak day



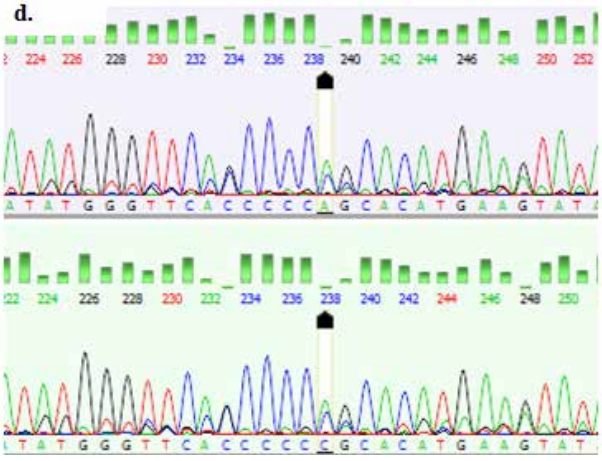
a. g.49551337 G/A



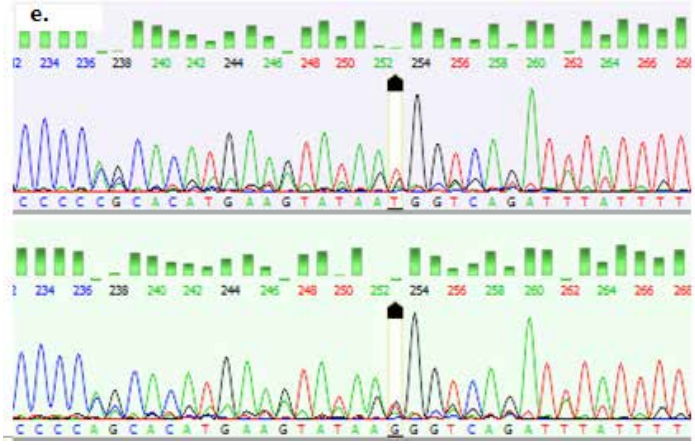
b. g.49551405 G/T



c. g.49551428 G/C



d. g.49551433 A/C



e. g.49551449 T/G

Figure 7. NC_037340.1 (49551161-49551628), the exon 3 region of the BMP2 gene with a Product length of 467 bp g.49551405 G/T, g.49551337 G/A, g.49551433 A/C, g.49551428 G/C, and g.49551449 T/G polymorphic SNPs, Each polymorphic base is indicated by a dark mark.

yields, it was revealed that there was no statistically significant relationship was found between the polymorphic sequences, intron 2 g.65825499 G/A, exon 5region of the ELF5 gene and 3. Exon BMP2 polymorphic regions and other sources of milk yield variations in the Brown Swiss cattle (P>0.05) (Table 2 and 3)

DISCUSSION

The NCBI dbSNP database was checked to compare the similarities of the g.65825499 G/A polymorphisms that we identified on the intron 2 region of the ELF5 gene in Bos taurus breeds. According to the NCBI data, g.65825499 G/A SNP region, ID-Chr15: g.65825495T>A (RefSNP-rs475815172) and ID-Chr15: g.65825496 C>G (RefSNP-rs438011969) SNPs were reported by the Cofactor-Genomics company (2013).

The g.65826134 C>T polymorphism in the exon 5region of the ELF5 gene and SNP ID-15: g65826134 C>T (RefSNP-rs444548435), especially in the Holstein-Friesian and Fleckvieh breed, were previously identified. These findings align with the whole-genome sequencing study of 234 bulls by Daetwyler, *et al.* (2014), which facilitated the mapping of monogenic and complex traits in cattle; the region closest to the exon 5region g.65825909 T/C polymorphism of the ELF5 gene (determined by us in Brown Swiss cattle, ID Chr15:g.65825915 A>C (RefSNP-rs451761759) and the region closest to the g.65826062 G/A polymorphism were identified within the Cofactor-Genomics project (2013) by Daetwyler *et al.* (2013). According to the NCBI dbSNP data, Cofactor-Genomics-identified ID-Chr15: g.65825915 A>C (RefSNP-rs451761759) and ID-Chr15: g.65825922 T>C (RefSNP-rs472070278) same SNPs were detected at the g.65825909 T/C SNP that we determined for the exon 5region of the ELF5 gene.

The polymorphic regions identified for the BMP2 gene with a product length of 467 containing the exon 3 region were checked for SNPs presented in the NCBI dbSNP database. According to the NCBI database, no SNP was identified in the same region with the g.49551337 G/A single-nucleotide polymorphism for the BMP2 gene, but ID-Chr13:g.49551369 C>A (RefSNP-rs478378586) SNP was identified by Cofactor-Genomics company (2013) at the nearest 32 bp length. The g.49551405 G/T SNP region, which we identified for the exon 3BMP2 gene in the Brown Swiss cattle breed, G>T SNP, was previously identified in the SNP ID-Chr13: g.49551407 (RefSNP-rs470388497) region in the dbSNP database. ID-Chr13:g.49551421 G>A (RefSNP-rs466516722) SNP at a seven bp distance and Chr13:g.49551435 A>G (RefSNP-rs434357845) SNP at a 14 bp distance were detected in the same region borders as the g.49551428 G/C SNP region. In the NCBI database, ID-Chr13: g.49551435 A>G (RefSNP-rs434357845) and ID-Chr13:g.49551437 A>C (RefSNP-rs451359294) SNPs were identified in the same region as the g.49551433 A/C SNP and the ID-Chr13:g.49551442 G>T (RefSNP-rs471472555) single-nucleotide polymorphism at a seven bp distance.

A genome-level association study of the BMP2 gene on feed intake quality and growth data in beef cattle reported that this gene was associated with muscle area size, body length and structure (Seabury et al., 2017), it controlled body size and muscle development in the same manner (Xu et al., 2019), was effective in fat storage in the tail region of fat-tailed sheep breeds (Baazaoui et al., 2021), and the amount of fat stored in the tail and the polymorphic regions detected in the BMP2 gene were associated with fat storage in the tail in sheep with different tail types (Lu et al., 2020). In our study, the polymorphic sequences that we identified for the BMP2 gene were not associated with milk yield records, but it was estimated that the polymorphic sequences that we determined in this gene might affect growth and bone development in dairy cattle as well as in beef cattle.

The ETS-related transcription factor ELF-5 gene is involved in the regulation of the later stages of terminal differentiation of keratinocytes and in the release of transcriptional activators. The ELF5 gene proteins coexpression with Alpha-S1-casein (CSN1S1), which plays a critical role in facilitating the transport of calcium phosphate in milk, Prolactin (PRL) which regulates lactation by acting on the mammary gland, and the antioxidant peptide (CSN2) genes which essential for determining the surface properties of casein micelles (Biswas et al .2022, Hornbachner et al.2021, Sun et al.2021; Szklarczyk et al. 2019) (Figure 8).

ELF5 gene expression plays a role in mammary gland development, milk yield, regulation of the synthesis of milk proteins such as casein in the mammary glands, and regulation of milk secretion during the lactation cycle in livestock. ELF5 gene also plays a role in growth during early embryogenesis, development of the mammary glands during pregnancy and

Table 2. ELF5 intron 2 and exon 5 region least squares mean (X), standard errors(S_x) and relationship analysis SNP variation with milk yields of Brown Swiss

Variation Sources			N	Lactation milk yield	Daily milk yield	305 days milk yield	Milking period	Peak Day Yield	Peak Milk Yield	Avg. Peak Milk Yield
				$X \pm S_x$	$X \pm S_x$	$X \pm S_x$	$X \pm S_x$	$X \pm S_x$	$X \pm S_x$	$X \pm S_x$
ELF5 intron 2	g.65825499G/A	G	10	4031,21±656,00	16,74±1,44	4893,98±423,81	243,16±36,03	36,03±17,64	394,46±241,45	15,76±2,51
		A	23	4523,00±695,65	16,88±1,53	5031,89±449,43	256,13±38,21	18,09±18,71	537,10±256,04	17,02±2,66
	Total		33	4277,10±560,20	16,81±1,23	4962,94±361,92	249,64±30,77	27,06±15,07	465,78±206,19	16,39±2,14
		p		0,223	0,657	0,455	0,479	0,024	0,547	0,232
		C	25	4102,1±721,8	16,4±1,6	4948,8±390,2	265,6±50,3	93,6±19,1	764,9±233,5	13,5±3,3
	g.65825909T/C	T	8	2456,0±1008,2	14,7±2,2	4152,6±545,0	173,6±70,2	46,6±26,6	314,6±326,1	16,3±4,6
		p		0,091	0,408	0,128	0,169	0,07	0,149	0,520
		G	24	4437,8±1157,7	17,5±2,5	5202,7±625,8	259,3±80,6	47,3±30,6	440,9±374,5	21,1±5,3
	g.65826062G/A	A	9	2120,3±917,5	13,6±2,0	3898,6±496,0	179,9±63,9	93,0±24,2	638,6±296,8	8,7±4,2
		p		0,13	0,242	0,116	0,446	0,253	0,681	0,081
		T	25	3559,1±870,7	16,0±1,9	4665,2±470,7	237,1±60,6	91,4±23,0	504,9±281,6	13,9±4,0
	g.65826138T/C	C	8	2999,1±922,7	15,1±2,0	4436,2±498,8	202,1±64,3	48,9±24,4	574,6±298,5	15,9±4,3
		p		0,582	0,662	0,677	0,621	0,125	0,832	0,683
		A	27	2921,2±769,4	14,6±1,7	4329,9±415,9	206,2±53,6	48,1±20,3	724,4±248,9	16,5±3,5
ELF5 exon 5	g.65826123A/C	C	6	3637,0±1092,8	16,5±2,4	4771,4±590,7	233,0±76,1	92,2±28,9	355,1±353,5	13,2±5,0
		p		0,546	0,482	0,491	0,745	0,167	0,339	0,545
		C	27	2362,6±838,5	13,8±1,8	3959,9±453,3	184,2±58,4	80,8±22,2	592,3±271,2	10,1±3,9
	g.65826134C/T	T	6	4195,6±1316,3	17,3±2,9	5141,4±711,6	255,0±91,7	59,5±34,8	487,2±425,8	19,7±6,1
		p		0,274	0,333	0,195	0,540	0,628	0,844	0,214
	Total		33	3846,8±302,2	16,0±0,7	4639,0±168,6	236,1±19,7	64,0±8,9	684,3±95,3	15,1±1,3

** P<0.001, * P<0.05

Table 3. BMP2 exon 3 least squares mean (X), standard errors(Sx) and BMP2 exon 3 region g.49551405 G/T, g.49551337 G/A, g.49551433 A/C, g.49551428 G/C, and g.49551449 T/G polymorphic SNPs and Brown cattle breed milk yield association analysis

Variation Source			Lactation milk yield	Daily milk yield	305 days milk yield	Milking period	Peak Day Yield	Peak Milk Yield	Avg. Peak Milk Yield
		N	$X \pm S_x$	$X \pm S_x$	$X \pm S_x$	$X \pm S_x$	$X \pm S_x$	$X \pm S_x$	$X \pm S_x$
g.49551337G/A	A	5	4836,1±988,4	16,4±3,5	4791,6±983,3	323,5±75,9	114,8±34,4	1353,0±346,3	10,2±5,4
	G	27	3434,4±727,0	18,6±2,6	5138,1±723,2	180,7±55,9	14,6±25,3	675,9±254,7	24,4±4,0
	p	32	0,373	0,695	0,823	0,241	0,077	0,224	0,109
g.49551405G/T	G	17	4099,5±397,6	16,5±1,4	4909,5±395,5	262,4±30,5	77,0±13,9	917,8±139,3	15,2±2,2
	T	15	4171,0±616,7	18,5±2,2	5020,3±613,5	241,8±47,4	52,4±21,5	1111,2±216,0	19,4±3,4
	p	32	0,913	0,380	0,865	0,683	0,289	0,404	0,255
g.49551428G/C	G	8	4061,7±625,8	19,0±2,2	5218,4±622,5	231,0±48,1	44,0±21,8	819,4±247,8	19,5±3,4
	C	24	4208,7±356,7	15,9±1,3	4711,4±354,8	273,2±27,4	85,4±12,4	1239,5±254,6	15,1±2,0
	p	32	0,814	0,171	0,418	0,384	0,07	0,241	0,208
g.49551433A/C	A	21	4040,5±536,3	17,0±1,9	4783,4±533,5	251,0±41,2	53,3±18,7	923,0±187,9	17,5±3,0
	C	11	4229,9±624,2	18,0±2,2	5146,4±620,9	253,1±48,0	76,1±21,7	1105,9±218,6	17,1±3,4
	p	32	0,823	0,757	0,0667	0,974	0,443	0,539	0,942
g.49551449T/G	T	26	4989,9±891,1	18,2±3,2	5251,7±886,4	303,4±68,5	100,4±31,0	1232,7±312,2	12,2±4,9
	G	6	3280,5±813,3	16,8±2,9	4678,1±809,0	200,7±62,5	29,0±28,3	796,2±284,9	22,3±4,5
	p	32	0,270	0,799	0,705	0,385	0,189	0,417	0,235
Total		32	4135,2±405,8	17,5±1,4	4964,9±403,7	252,07±31,2	64,69±14,1	1014,46±142,2	17,3±2,2

** P<0.001, * P<0.05

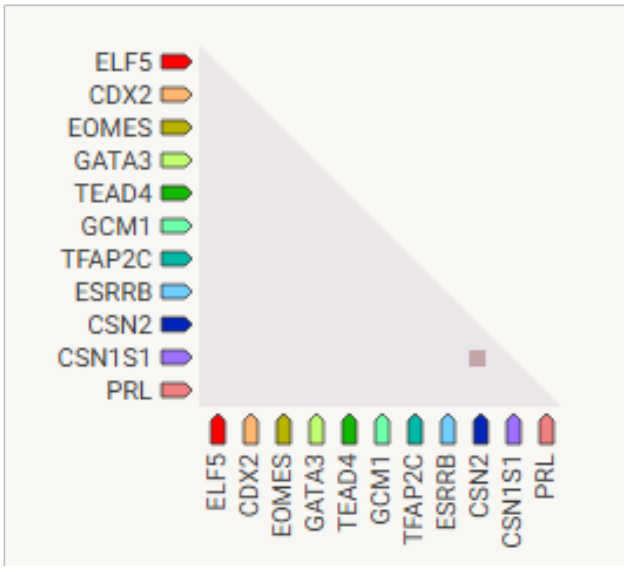


Figure 8. ELF5 gene coexpression in Bos taurus derived from GEO microarray expression data and STRING ((Search Tool for the Retrieval of Interacting Genes/Proteins)) network.

lactation, and maintaining amino acid and glucose balance during milk secretion (Wang et al., 2019; Dai et al., 2018; Xia et al., 2017; Guo et al., 2017). ELF5 gene is involved in the release of colostrum in yaks (Bos grunniens) as well as cattle breeds and in the synthesis of milk proteins in the mammary gland during lactation (Xia et al.2018; Bai et al.2014; Choi et al. 2009). We propose that ELF5 gene, which is co-expressed with genes associated with milk yield such as CSN1, CSN2 and PRL and used as markers in livestock, may be a potential marker gene in cattle breeds. In the association analysis of the ELF5 gene with the few available yield records, it was determined that there was a significant relationship between the g.65825359 C/T polymorphic region determined for the intron 2 region and the peak day yield values ($P<0.05$). It is thought that the effect may be significant in association analyses with the polymorphic regions that we determined for this gene and especially with the yield records in a higher number of individuals related to milk yield and development and size of the mammary glands.

Conclusions

Consequently, we have demonstrated in this study the identification of polymorphisms in ELF5 and BMP2 genes and validated the existence of others that have previously been reported in published works or online databases. Our analyses made it possible to analyse many samples at low cost using HRM and Sanger sequencing techniques.

It is thought that the polymorphic sequences that we detected in the exon regions of the ELF5 and BMP2 genes, known to affect yield and performance data in cattle breeds, may affect traits such as growth, development, and milk yield, and therefore, they can be used as markers in future studies.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

Conceptualization, M.O and Z.S. methodology, M.O and Z.S., software, M.O and Z.S. formal analysis, M.O and Z.S., investigation, M.O and Z.S., data curation, M.O and Z.S.,writing original draft preparation M.O and Z.S., All authors have read and agreed to the published version of the manuscript.

Acknowledgements

This study was supported by Ataturk University Scientific Research Projects Fund (Project no: 2015/399). We gratefully thank Ataturk University SRP foundation and Animal Science Genetic Laboratories of Atatürk University.

This study is an excerpt from the doctoral thesis titled “Determining some molecular marker polymorphisms that affect yield traits in cattle and associating them with yield”, was conducted in 2017. Atatürk University Graduate School of Natural and Applied Sciences Doctoral Thesis, ERZURUM.

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