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Association of polymorphisms in the *CSN2*, *CSN3*, *LGB* and *LALBA* genes with milk production traits in Holstein cows raised in Turkey

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ABSTRACT. A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) test was performed on DNA samples extracted from blood samples of 189 Holstein Friesian cows to detect genotypic distribution of polymorphic markers in the bovine beta-casein (*CSN2*), kappa-casein (*CSN3*), beta-lactoglobulin (*LGB*) and alfa-lactalbumin (*LALBA*) genes responsible for milk production traits. Statistical analysis was carried out using least square methods of the general linear model (GLM) procedure. *CSN2* locus was significantly associated with the following traits: 305-day milk yield, days before peak milk production, fat percentage and protein yield. There was no significant effect of *CSN3*, *LGB* and *LALBA* markers on the traits analysed. These results suggested that *CSN2* marker may be evaluated in selection programmes regarding not only milk content but also milk production.

***Keywords*:** cattle, polymorphic marker, milk production, association

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INTRODUCTION

Advances in molecular technics, will ultimately provide researchers and breeders a better knowledge of the locations and effects of major loci, and evaluating of their associations with phenotypic traits that contribute to variation in quantitative traits (Spelman and Garrick, 1997). In this context, the use of genetic markers to increase the frequency of desired genotypes in herds, based on selection in breeding programmes, can be used to determine properties that are essential to achieve economical benefits in dairy cattle. The development of genotypic selection has enhanced the use of genome-wide dense marker data to reduce generation intervals depends mainly on the accuracy of genomic breeding values (Habier et al., 2010).

In recent years, genes associated with milk production traits have been identified and single nucleotide polymorphisms (SNPs) of many candidate genes have been specifically determined. On the one hand, some studies have focused on associations between phenotypes of cows and their corresponding genotypes and on the other hand, some of them evaluated the relationships between estimated breeding values of bulls and their respective genotypes (Boettcher et al., 2004). Bovine milk protein largely (± 90 % wt/wt) consists of six major milk proteins that are products of the α-LA, β-LG, αS1-CN, αS2-CN, β-CN, and κ-CN genes (Heck et al., 2009). Composition of bovine milk is highly affected by season, stage of lactation, feeding, and health status of the cow but is also is predominantly determined and regulated by genomic structure (Bobe et al., 1999). Therefore, candidate gene studies have examined the effects of the casein (*CN*), beta-lactoglobulin (*LGB*) and alfa-lactalbumin (*LALBA*) genes on milk production traits. Among them, *CN* genes, including β-CN and κ-CN, are located at chromosome 6 within a region of about 200 kb (Ferretti et al., 1990; Ikonen et al., 2001). The *CSN2* gene encodes the β-casein which is a major milk protein in most mammals and this gene is generally induced by lactogenic hormones bound to its promoter. In addition, the expression of the *CSN2* can be enhanced by signal transducers and activators of transcription (STAT) and glucocorticoid receptor (GR) (Lee et al., 2008). *CSN2* gene has 12 known genetic variants in the coding sequence of the gene including A, B, C (Aschaffenburg, 1961), A1, A2, A3 (Kiddy et al., 1966), D (Aschaffenburg et al., 1968), E (Voglino, 1972), F (Visser et al., 1991), X (Visser et al., 1995), G (Chin and Ng-Kwai-Hang, 1997), H (Han et al., 2000), I (Jann et al., 2002). The most common variants of β-casein in dairy cattle breeds are A1 and A2, while B is less common, and A3 and C are rare (Farrell et al. 2004; Keating et al. 2008). A1 variant differs from the A2 variant at position 67 where a histidine replaces a proline (Miluchova et al., 2009). Multiple genome scans have reported presence of significant quantitative trait loci (QTL) in this region that affect milk productions traits (Ikonen et al., 2001; Olsen et al., 2002; Viitala et al., 2003, Huang et al., 2012). The total size of the *CSN3* gene is about 13 kb and this gene has 12 genetic variants including A, B, B2, C, E, F, F1, G, H, I, A(1), and J (Kaminski, 1996; Prinzenberg et al., 1999; Sulimova et al., 2007; Sulimova et al., 1992). However, most of these variants are rare; they are found in only a few breeds and usually at low frequencies (Sulimova et al., 2007). A and B are the most common genetic variants in dairy cattle (Mitra et al., 1998). Variants A and B of the *CSN3* gene differ from each other in two amino acid substitutions: threonine (Thr)/isoleucine (Ile) at position 136 and aspartic acid (Asp)/alanine (Ala) at position 148 (Grosclaude et al., 1972). The *CSN3* gene encodes milk protein that is important for the structure and stability of casein micelles (Alexander et al., 1988). Alleles A and B of the gene, have been reported to be associated with milk yield, milk protein content and milk quality (Boettcher et al., 2004; Caroli et al., 2004; Kucerova et al., 2006). The *LGB* gene is located on bovine chromosome 11 and encodes the main protein of whey (Eggen and Fries, 1995). This gene has a total of 15 variants with alleles A and B being the most frequent (Matejicek et al., 2007). The mentioned variants differ by two amino acid substitutions in the polypeptide chains and 2 single nucleotide substitutions in the bovine *LGB*. In this context, variant A has aspartic acid (GAT) and valine (GTG) at positions 64 and 118, respectively, whereas variant B has glycine (GGT) and alanine (GCG) (Patel et al., 2008). Results of studies on the effect of *LGB* genotypes on milk production traits have been rather more consistent compared to studies of other milk protein polymorphisms. Thus, this gene was suggested as a genetic factor influencing mainly milk composition and milk quality and especially B allele was recognized as superior for milk quality in European cattle breeds. Allele A has been associated

mostly with milk yield parameters (Strzalkowska et al., 2002; Matějíček et al., 2007; Czerniawska-Piatkowska et al., 2011). In addition, the AA genotype of *LGB* has been shown to have a significant effect on protein yield (Sabour et al., 1996; Lunden et al., 1997), whereas the BB genotype has been associated with fat content (Ron et al., 1994; Tsiaras et al., 2005; Czerniawska-Piatkowska et al., 2011). The bovine *LALBA* gene, mapped to chromosome 5, is considered a candidate gene for milk performance traits and technological properties of milk (Kaminski et al., 2002). *LALBA*, which encodes one of the major milk whey proteins, has two genetic variants namely A and B. Variant A differs from B by a glutamine (Gln) for arginine (Arg) substitution (Sashikanth and Yadav, 1998). Variations in the *LALBA* gene have been associated with lactose concentration, milk yield, protein yield, and fat yield (Bleck, 1993; Bleck and Bremel, 1993; Voelker et al., 1997; Bleck et al., 1998; Martins et al., 2008).

Among known genes that affect milk production, milk proteins have received considerable attention in recent years because of possible associations between the genotypes and economically important traits in dairy cattle (Peñagaricano and Khatib, 2012). It is important to note that studies on the significance of milk protein polymorphisms have been carried out by many authors and inconsistencies in the outcomes, however, have prevented clear conclusions from being made. Further genetic studies focusing on milk yield and quality are needed to achieve efficient selection procedures. Therefore, the aim of the study was to investigate the effects of *CSN2*, *CSN3*, *LGB* and *LALBA* genes on milk production traits in Holstein cows raised in Turkey.

MATERIALS AND METHODS

Animals and management

Data from 189 purebred Holstein cows randomly selected from a commercial herd located in South Marmara region (40° 15' 09.5" N and 28° 17' 59.9" E) were used in the present study. The study protocol was approved by the ethics committee of by the Uludag University Local Ethics Committee for Animal Research (Approval number: 2010-08/06). The animals were housed in three free-stall barns and fed with the same diets according to standard commercial practices. All cows were milked three times a day when allowed free access to milking parlors in which they were equipped with electronic devices that automatically recorded the quantity of milk. Milk yield for each cow at each milking was recorded using DeLaval's Alpro Herd Management System (DeLaval International, Tumba, Sweden). Lactation milk yield, 305-day milk yield, days before peak milk yield and peak milk yield were evaluated to test possible associations of *CSN2*, *CSN3*, *LGB* and *LALBA* gene polymorphisms with milk production traits. The phenotypic data set was defined based on milk production traits availability in a way that would describe a complete picture of a productive history for a cow for four lactations. 305-day milk yield was calculated for each cow based on total milk yield (Lucy et al., 1993). Milk samples, obtained thrice a day seperately, were analyzed monthly for fat protein, and lactose content by infrared analysis with a Bentley 2000 midinfrared instrument (Bentley Instruments, Chaska, MN, USA). Milk fat yield, lactose yield, protein yield and total milk solids were calculated individually based on the milk production levels obtained from the analysis.

Markers Used and Genotyping

In order to perform polymorphism analysis and genotyping, initially, DNA was isolated from 4 mL blood samples obtained from the vena jugularis of each cow and collected in K_3EDTA tubes (Vacutest Kima, SRL, Italy) by a phenol-chloroform method as described by Green and Sambrook (2012). The amount and purity measurements were carried out on a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All PCR reactions were carried out in a final volume of 50 μL. The reactions consisted of: 33.5 μL ddH_2O, 5 μL 10x buffer (100 mM Tris HCI at pH 8.3, 500 mM KCI, and 0.01% gelatin, Mg-free), 5 μL (25mM) $MgSO_4$, 1 μL dNTPs (2.5 mM), 2.5 U Taq DNA polymerase (Biomatik, Cambridge, Canada, A1003-500U, 5U/μL), 1 μL (0.025 μM) of each primer, and 3 μL template DNA (approximately 80 – 150 ng). Detailed information about the markers selected and the original citation for PCR-RFLP are shown in Table 1. Gene-specific primer sequences for *CSN2* locus were as follows: forward primer 5′-CCTTCTTTCCAGGATGAA CTCCAGG-3′ and reverse primer 5′-GAGTAAGAGGAGGGAT GTTTTGTGGGAGGCTCT-3′. For determining the *CSN3* genotypes, primers were used to amplify the 379 bp (10791-11170) fragment of the gene containing a part of exon 6 and intron 5.

Gene-specific primer sequences for *CSN3* locus were as follows: forward primer 5'-CACGTCACCCACACC CACATTTATC-3' and reverse primer 5'-TAATT-AGCCCATTTCGCCTTCTCTGT-3'. The bovine *LGB* A variant differs from the B variant by two amino acids (Asp and Val) at positions 64 and 118. The primers used for primer of a 247 bp fragment of the *LGB* gene with the following specific primer sequences: forward primer 5´-TGTGCTGGACACCGACTACAAAAA-3´ and reverse primer 5´-GCTCCCGGTATATGACCACCC TCT-3´. The bovine *LALBA* variant A differs from B by a single amino acid substitution of glutamine (Gln) for arginine (Arg) at position 10. Gene-specific primer sequences for amplifying a 309 bp fragment of *LALBA* locus were as follows: forward primer 5'-TTGGTTT-TACTGGCCTC TCTTGTCATC-3' and reverse primer 5'-TGAATTATGGGACAAAGCA AAATAGCAG-3'.

The DNA amplification reactions were performed in a Thermal Cycler (Palm Cycler GC1-96, Corbett Reseach) with thermal conditions as follows:

(a) *CSN2* locus: 95°C for 5 min, followed by 30 cycles of 95°C for 40 sec, 58°C for 1 min, and 72°C for 1.5 min, followed by a final extension step at 72°C for 10 min.

(b) *CSN3* locus: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final extension step at 72°C for 10 min.

(c) *LGB* locus: 95°C for 5 min, followed by 30 cycles of 94°C for 45 sec, 60°C for 1 min, and 72°C for 5 min, followed by a final extension step at 72°C for 5 min.

(d) *LALBA* locus: 95°C for 5 min, followed by 30 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec, followed by a final extension step at 72°C for 10 min.

Following PCR amplification, polymorphic sites were genotyped using Restriction Fragment Length Polymorphism (RFLP) analysis. PCR amplicons of the *CSN2*, *CSN3*, *LGB* and *LALBA* were subjected to restriction enzyme digestion with *Dde*I, *Hin*dIII, *Hae*III and *Msp*I restriction enzymes (Biomatik, Cambridge, Canada), respectively, according to the suppliers instructions. Briefly, enzyme-buffer mix was prepared by mixing 2 µL of restriction enzyme with 8 µL of the respective buffer. Reaction mix was prepared by mixing 10 µL PCR product with 2 µL of enzyme buffer mix. Volume was made up to 20 µL with autoclaved MilliQ water. Afterwards, these reaction mixtures were incubated at 37°C for 16 h. The digestion products were electrophoresed in 3 % agarose gels (Sigma Aldrich, Steinheim, Germany) containing ethidium bromide (0.4 µg/mL) under 85 – 90 V for about 1 h. Electrophoresis was performed in 1x TBE buffer (108 g Tris, 55 g boric acid, and 40 mL 0.5 M EDTA in 1,000 mL for 10x concentrated stock solution at pH 8). Visualisation of the gels was performed by a gel imaging system (DNr-Minilumi, DNR Bio-Imaging Systems, Israel). Genotypes were assigned by multiple operators in accordance with authors designations of observed patterns of RFLP bands.

Statistical Analysis

Frequency and distribution of genotypes and alleles were calculated by the standard procedure as described by Falconer and Mackay (1996). Hardy-Weinberg equilibrium (HWE) was tested as a genotyping quality control procedure using the χ^2 statistic, with expected frequencies derived from allele frequencies. All markers were evaluated on the basis of their population genetic indices including gene heterozygosity (He), effective allele numbers (Ne) and polymorphism information content (PIC) as described by Nei and Roychoudhury

Table 1. Detailed information about the markers selected, the original citation and RFLP method, as well as the amplicon sizes and annealing temperatures for PCR amplification.

Gene	NCBI Gene ID	Reference	PCR amplicon (bp)	Chromosomal Location	Allele	Annealing temperature (°C)	Enzyme for RFLP
CSN2	281099	Miluchova et al. (2009)	121	6q31	A1/A2	58	*Dde*I
CSN3	281728	Mitra et al. (1998)a	379	6q31	A/B	55	*Hind*III
LGB	280838	Strzalkowska et al. (2002)	247	11q28	A/B	60	*Hae*III
LALBA	281894	Mitra et al. (1998)b	309	5q21	A/B	60	*Msp*I

CSN2: beta-casein; *CSN3*: kapa-casein; *LGB*: beta-lactoglobulin; *LALBA*: alfa-lactalbumin

(1974) and Botstein et al. (1980). The general linear model (GLM) procedure of Minitab (Minitab Inc., State College, PA, USA, v17.1.0) was used to perform association analysis and Levene's test was used to test for homogeneity of the variances. Differences between the genotypes were evaluated using the following statistical model:

$$Y_{ijklmno} = \mu + W_i + X_j + Z_k + AG_l + BG_m + CG_n + DG_o + e_{ijklmno}$$

where: $Y_{ijklmno}$ = the studied traits; μ = the overall mean; W_i = the fixed effect of lactation season (i = autumn, winter, spring and summer); X_j = the fixed effect of service period (j = ≤50, 51-80, 81-110, 111-140, 141≤); Z_k = the fixed effect of lactation rank (k = 1, 2, 3, 4); AG_l = the fixed effect of the *CSN2* genotype (l = A1A1, A1A2, A2A2); BG_m = the fixed effect of the *CSN3* genotype (m = AA, AB, BB); CG_n = the fixed effect of the *LGB* genotype (n = AA, AB, BB); DG_o = the fixed effect of the *LALBA* genotype (o = BA, BB) and $e_{ijklmno}$ = the random residual effect.

Post hoc contrasts were conducted with Tukey's multiple comparison test ($P < 0.05$).

RESULTS

We have amplified the 121 bp fragment for the *CSN2* gene (Fig 1). Digestion of the PCR product with the *Dde*I nuclease resulted in two bands (121 bp and 86 bp) for heterozygote genotype (A1A2) and a single band (86 bp) for the A2A2 genotype. The DNA amplified from A1A1 animals remained undigested (121 bp) with the corresponding restriction enzyme (Fig 2). The amplified fragment (379 bp) of the *CSN3* gene (Fig 3) showed three genotypes including AA, AB and BB by digestion with the restriction enzyme *Hin*dIII and the AB genotype was cleaved into three bands of 379 bp, 225 bp and 154 bp, while AA genotype remained undigested. The BB genotype was characterized by fragment sizes of 225 bp and 154 bp (Fig 4). The electrophoresis pattern of PCR amplification for the *LGB* gene is shown in Fig 5. The cleavage of a 247 bp PCR product by *Hae*III yielded two fragments of 148 bp and 99 bp and was diagnostic for the AA genotype in the *LGB* assay. Heterozygote genotype was cleaved into three bands of 148 bp, 99 bp and 74 bp. Besides the DNA amplified from BB animals was characterized by fragment sizes of 99 bp and 74 bp (Fig 6). The electrophoresis pattern of PCR amplification for the *LALBA* gene is shown in Fig 7. Digestion of 309 bp amplified fragment was performed by using *Msp*I restriction enzyme. Typing *LALBA* allelic variation by PCR-RFLP showed a separation of two different genotypes including BB and AB. Hence, the AA genotype, which forms a single band of 309 bp, was not present in this study. Restriction digests of BB and AB animals yielded two (220 and 89 bp) and three (309, 220 and 89 bp) fragments, respectively (Fig 8).

The genotypic and allelic frequencies, population genetic indices including He, Ne and PIC and compatibility with the HWE are shown in Table 2. Results

Table 2. Allele and genotype frequencies of polymorphisms in *CSN2, CSN3, LGB and LALBA* genes, population genetic indices (He, Ne, PIC) and compatibility with the Hardy-Weinberg equilibrium.

	CSN2			***CSN3***			***LGB***			***LALBA***		
Genotypes	A1A1	A1A2	A2A2	AA	AB	BB	AA	AB	BB	AA	AB	BB
N	30	106	53	7	50	132	35	101	53	0	3	186
%	15.88	56.08	28.04	3.70	26.46	69.84	18.52	53.44	28.04	0	1.59	98.41
Allele Frequency	A1		A2	A		B	A		B	A		B
	0.439		0.561	0.169		0.831	0.452		0.548	0.008		0.992
He		0.4925			0.2809			0.4953			0.0158	
Ne		1.9707			1.3906			1.9817			1.0161	
PIC		0.3713			0.2414			0.3727			0.0157	
χ2(HWE)		3.63			0.67			1.17			0.01	
P*		0.06			0.41			0.28			0.91	

N: number of experimental cows, MAF: minor allele frequency, He: heterozygozity, Ne: effective allele number, PIC: polymorphism information content, χ^2(HWE): Hardy-Weinberg equilibrium χ^2 value, * P>0.05 – not consistent with HWE

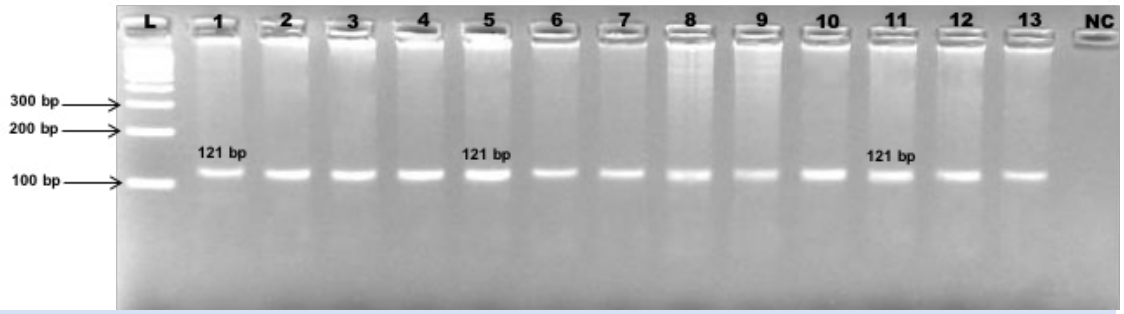

Figure 1 The electrophoresis pattern of PCR amplification for the bovine CSN2 gene (L: DNA Ladder-amplicon length 100–1500 bp; NC: Negative control).

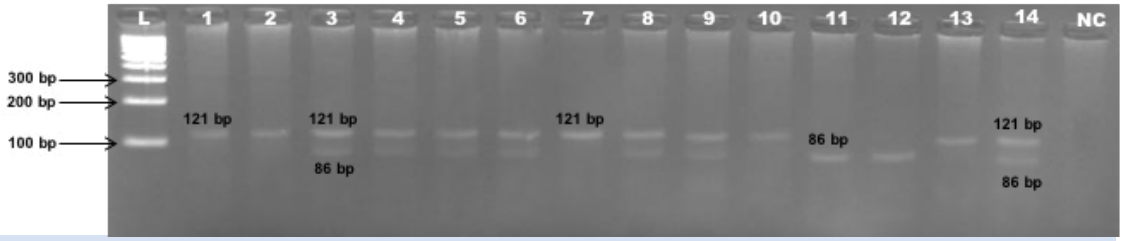

Figure 2 The electrophoresis pattern of restriction enzyme digestion of PCR product with DdeI for the bovine CSN2 gene (L: DNA Ladder-amplicon length 100–1500 bp; Lanes 1, 2, 7, 10 and 13: A1A1; Lanes 3-6, 8, 9 and 14: A1A2; Lanes 11 and 12: A2A2; NC: Negative control).

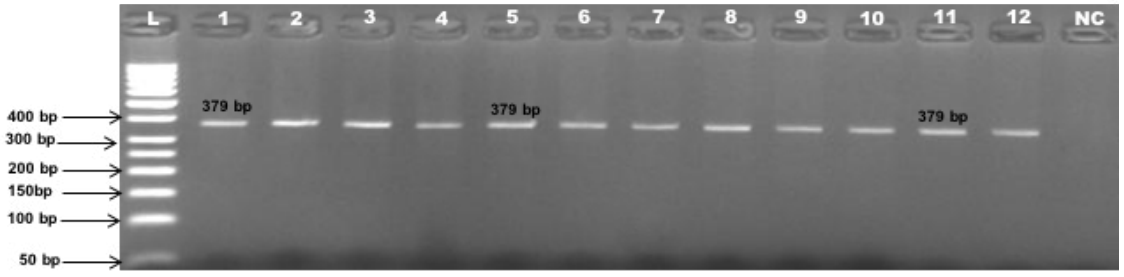

Figure 3 The electrophoresis pattern of PCR amplification for the bovine CSN3 gene (L: DNA Ladder-amplicon length 50–1000 bp; NC: Negative control).

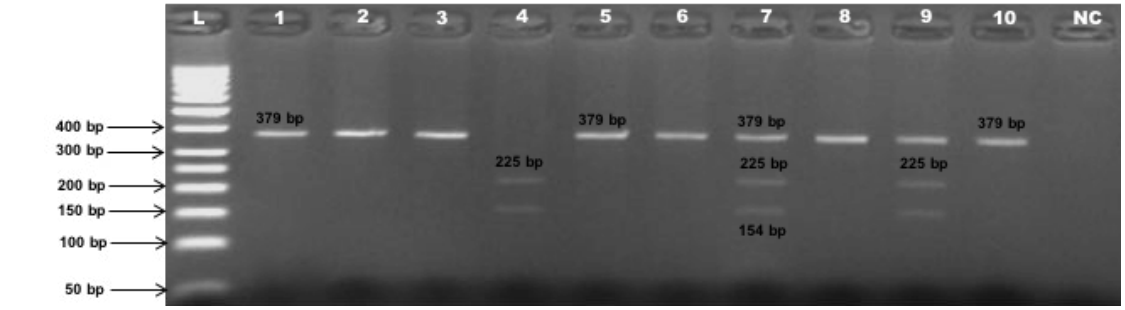

Figure 4 The electrophoresis pattern of restriction enzyme digestion of PCR product with HindIII for the bovine CSN3 gene (L: DNA Ladder-amplicon length 50–1000 bp; Lanes 1-3, 5, 6, 8 and 10: AA; Lanes 7 and 9: AB; Lane 4: BB; NC: Negative control).

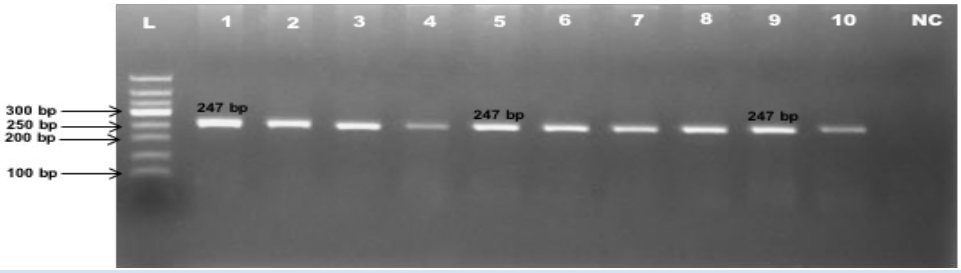

Figure 5 The electrophoresis pattern of PCR amplification for the bovine LGB gene (L: DNA Ladder-amplicon length 50–600 bp; NC: Negative control).

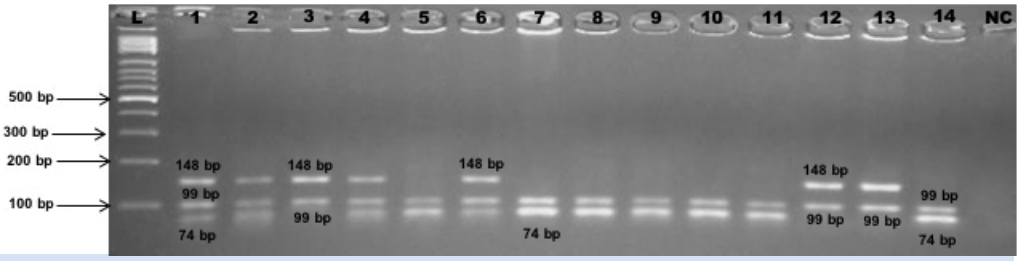

Figure 6 The electrophoresis pattern of restriction enzyme digestion of PCR product with HaeIII for the bovine LGB gene (L: DNA Ladder-amplicon length 100–1500 bp; Lanes 3, 12 and 13: AA; Lanes 1, 2, 4 and 6: AB; Lanes 5, 7-11 and 14: BB; NC: Negative control).

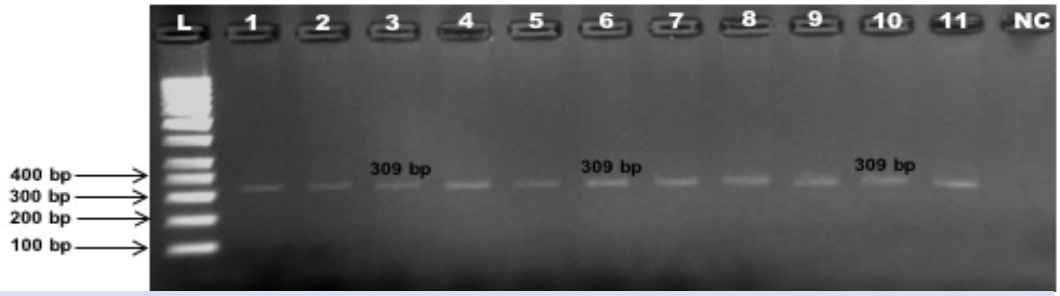

Figure 7 The electrophoresis pattern of PCR amplification for the bovine LALBA gene (L: DNA Ladder-amplicon length 100–1500 bp; NC: Negative control).

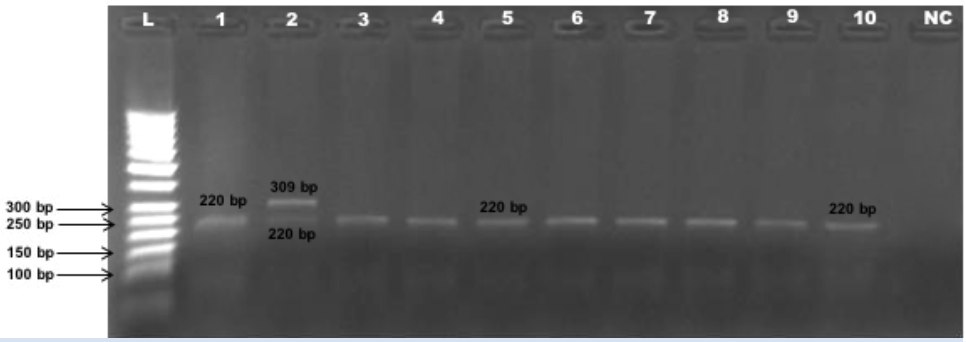

Figure 8 The electrophoresis pattern of restriction enzyme digestion of PCR product with MspI for the bovine LALBA gene (L: DNA Ladder-amplicon length 50–1000 bp; Lane 2: AB; Lanes 1, 3-10: BB; NC: Negative control).
Note: The AA genotype was not present in this study.

Table 3. Effects of *CSN2, CSN3, LGB and LALBA* gene polymorphisms on milk production traits in Holstein cows.

	TMY (kg)	305-dMY (kg)	DBP (days)	PMY (kg)	MFY (kg)	MFC (%)	MPY (kg)	MPC (%)	MLY (kg)	MLC (%)	TMS (kg)	MSC (%)
CSN2												
A1A1	8389	8235^{b}	55.83^{a*}	33.90	293.60	3.58^{a}	261.70^{b}	3.25	401.20	4.93	994.20	12.13
A1A2	8412	8147^{b}	41.71^{ab*}	33.93	274.00	3.36^{ab}	256.60^{b}	3.19	396.00	4.86	980.30	11.99
A2A2	9155	8969^{a}	34.89^{b*}	35.31	283.00	3.20^{b}	284.70^{a}	3.22	430.20	4.84	1049.80	11.80
CSN3												
AA	8513	8430	43.16	32.30	283.60	3.40	274.20	3.36	403.60	4.89	1004.60	12.10
AB	8927	8633	41.98	35.03	287.80	3.33	271.70	3.16	419.30	4.86	1029.30	11.91
BB	8517	8287	47.30	35.82	279.20	3.39	257.10	3.14	404.40	4.88	990.30	11.92
LGB												
AA	8942	8814	39.15	34.50	292.60	3.41	274.40	3.19	422.70	4.88	1040.80	11.98
AB	8694	8497	46.63	34.88	286.60	3.39	268.40	3.22	410.20	4.85	1014.20	11.95
BB	8320	8039	46.65	33.76	271.30	3.33	260.20	3.24	394.50	4.90	969.30	11.99
LALBA												
BA	7881	7389^{1}	34.43	34.66	262.30	3.44	253.20	3.36	370.70	4.90	925.00	12.13
BB	9424	9511^{1}	53.87	34.10	304.70	3.31	282.20	3.08	447.60	4.85	1091.20	11.81
PSE	773.42	662.54	10.90	2.07	30.98	0.23	21.95	0.10	37.27	0.07	88.83	0.29

TMY: total milk yield, 305-dMY: 305-day milk yield, DBP: days before peak milk production, PMY: peak milk yield, MFY: milk fat yield, MFC: milk fat content, MPY: milk protein yield, MPC: milk protein content, MLY: milk lactose yield, MLC: milk lactose content, TMS: total milk solids, MSC: milk solid content, PSE: Pooled Standard error

^{a,b} Different superscripts within a column indicate significant difference (P<0.05)

* represents significance level at P<0.01

^{1} represents a tendency P = 0.085

indicated that the population were determined to be compatible for *CSN2*, *CSN3*, *LGB* and *LALBA* genotypes in the Hardy-Weinberg equilibrium. The minor allele frequencies ranged from 0.008 to 0.452 and all markers in the present study were polymorphic except for *LALBA*, according to the classification reported by Menezes et al. (2006), because the frequency of the BB genotype at the *LALBA* locus was extremely high (98.41 %). The evaluation of population genetic parameters indicated that He values ranged from 0.0158 to 0.4953, PIC values ranged from 0.0157 to 0.3727 and Ne values ranged from 1.0161 to 1.9817.

Least squares means and pooled standard errors for *CSN2*, *CSN3*, *LGB* and *LALBA* genotype effects on milk production traits are shown in Table 3. The results indicated that *CSN2* had significant effects on 305-day milk yield (P<0.05), days before peak milk production (P<0.01). A2A2 genotype was associated with higher 305-day milk yield and fewer days before peak compared to alternative genotypes. In addition *CSN2* showed associations with milk content. Significant effect of the A1A1 genotype on fat percentage were found (P<0.05). Moreover, A2A2 genotype was associated with higher protein yield (P<0.05). There was no significant effects of the *CSN3*, *LGB* and *LALBA* genotypes on milk production traits in the current study. However, a tendency was observed (P = 0.085) for *LALBA* genotypes to be associated with 305-day milk yield, as shown in Table 3.

DISCUSSION

In the present study, the effects of *CSN2*, *CSN3*, *LGB* and *LALBA* genes on milk yield and content were evaluated in Holstein cows. These genes were chosen because they have been shown to be strongly associated with milk production traits in various cattle breeds. The population-based analyses showed a consistence with HWE for all markers. However, an unbalanced genotypic distribution was observed for *LALBA* resulting in low genetic variabilities of He, Ne and PIC compared

to other markers. Botstein et al. (1980) reported that a marker with a PIC value higher than 0.5 is considered to be very informative, whereas values between 0.25 and 0.5 are mildly informative, and values lower than 0.25 are low informative. According to this classification the *CSN2* and *LGB* markers were mildly informative whereas the *CSN3* and *LALBA* markers were low informative. It is worth noting that the major allele frequencies of *CSN3* and *LALBA* were extremely high, 0.831 and 0.992 respectively. Low values of population genetic parameters including He, Ne and PIC may be explained by high level of inbreeding or high selection pressure.

The *CSN2* gene encodes milk protein that is important for the structure of casein micelles (Kucerova et al., 2006) and there are many studies about the association of *CSN2* genotypes with milk production traits in the literature (Ng-Kwai-Hang et al., 1984; Boettcher et al., 2004; Miluchova et al., 2009). However, the effects of alleles within the CN cluster determined by different authors in different breeds are sometimes conflicting (Caroli et al., 2009). In the current study, three genotypes (A1A1, A1A2, A2A2) of *CSN2* locus were determined in the genotyped animals and significant differences between the genotypes of this locus were found for 305-day milk yield, days before peak milk production, fat percentage and protein yield. Animals with the A2A2 genotype had the highest milk yield (+734 kg and +822 kg higher than A1A1 and A1A2, respectively) and protein yield (+23 kg and +28.1 kg higher than A1A1 and A1A2, respectively) and in addition they reached the peak faster (20.94 days and 6.82 days earlier than A1A1 and A1A2, respectively) compared to alternative variants. Besides, the present results indicated that the genotype A1A1 was associated with the highest milk fat percentage (+0.38 % and +0.22 % higher than A2A2 and A1A2, respectively). Similarly, Freyer et al. (1999) reported that the genotype A1A1 is superior regarding fat and protein percentage. Consistent with our results, Heck et al. (2009) also found that cows with the A1 allele had a lower protein yield than cows with the A2 allele, resulting from decreased milk production. Kucerova et al. (2006) reported that significant differences between the genotypes of *CSN2* locus were found in breeding values for milk yield, protein yield, fat yield and fat content. However, their results indicated that, conversely, the genotype A1A1 was associated with the highest breeding value for milk yield and the breeding values of animals with genotype A2A2 were negative for yield parameters but positive for content parameters compared to the animals with genotype A1A1. Comin et al. (2008) also found that *CSN2* locus was associated with milk and protein yields. As mentioned above, results of the studies about associations between *CN* genes and milk production traits are often inconsistent (Zepeda-Batista et al., 2017). On the one hand, in most cases, the reasons for this situation may be due to differences in population size, breed of cows, genotypic distribution, methods of expressing traits, the power of statistical models considering other factors such as age of cow, parity number, season, stage of lactation, health status, and effects of other genetic variants (Ng-Kwai-Hang et al., 1990). On the other hand, *CSN1S1* and *CSN2* are also located on chromosome 6, within a region of about 200 kb that includes *CSN3* (Ferretti et al., 1990). This closeness in physical location makes it difficult to separate the effects of different *CN* genes (Boettcher et al., 2004). Hence, because of the tight linkage between the two loci, the composite genotypes, or haplotypes, may provide more adequate outcomes than the single-locus genotypes before considering them in marker-assisted selection (Comin et al., 2008). Similar to *CSN2*, the *CSN3* gene encodes milk protein that plays an important role in providing an essential structure and stability of casein micelles (Alexander et al., 1988). However, the *CSN3* genotypes had no significant effect on milk production traits in this study, which is in agreement with previous studies reported by Aleandri et al. (1990), Davoli et al. (1990), Ikonen et al. (1999) and Comin et al. (2008). Moreover, the present results indicated that, this marker was not associated with milk content traits, which is consistent with the results reported by Comin et al. (2008). A possible explanation for the result could partially depend on the unbalanced genotypic distribution observed in this study. Besides, as mentioned above, tight linkage between *CN* genes on bovine chromosome 6 should be considered to perform an adequate evaluation of the association of this marker with phenotypic traits.

Although there were evident differences in milk yield with different *LGB* genotypes, they were not significant in the present study. The association between the *LGB* and breeding values for milk production parameters

has been investigated by several authors. Kucerova et al. (2006) reported that non-significant differences were observed in the average breeding values of animals with different *LGB* genotypes. Conversely, Kaminski et al. (2002) reported the relation between *LGB* genotypes and breeding values for protein yield. Czerniawska-Piatkowska et al. (2011) found that cows with the *LGB* AA genotype were characterized by the highest milk yield in all lactations and cows with the *LGB* BB genotype was associated with the highest milk fat yield and content for the three lactations. Neamt et al. (2017) reported that the AB genotype was associated with a higher milk production and the BB genotype was associated with a higher fat percentage in milk compared with the other two genotypes. Neither did Litwinczuk et al. (2003) and Micinski et al. (2008) find any association between *LGB* genotypes and milk production traits. The *LGB* gene encodes mainly whey protein and this gene has been shown to be effective on formation of physicochemical properties of milk. However, the results are not always concurrent and sometimes they are even contradictory. This justifies the necessity of continuing the research on the utilization of this marker in evaluating the genetic basis of milk production traits (Czerniawska-Piatkowska et al., 2011). The results indicated that there was no significant relationship between *LALBA* marker and any of the selected traits in the present study. However, there was a tendency for 305-day milk yield and animals with the BB genotype seemed to have higher milk production. Only two genotypes, BA and BB, for locus *LALBA* were detected in the genotyped animals, and moreover, there were only three animals with genotype BA in the examined group of animals. Hence, futher genetic studies should be performed to confirm the present results and to draw conclusions.

CONCLUDING REMARKS

The significant effects of *CSN2* genotypes, A1A1 and A2A2, was detected in the observed population of Holstein breed raised in Turkey. The genotype A1A1 affected fat content in milk, whereas, the genotype A2A2 was associated with 305-day milk yield, days before peak milk production and protein yield. The findings contribute to a better understanding of the role of the *CSN2* locus in dairy breeds and help with the application of the information to breeding process and effective selection procedures.

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CONFLICT OF INTEREST STATEMENT

None of the authors of this article has any conflict of interest.

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