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Genotyping of the κ – *casein* gene by PCR – RFLP (*AcuI* digestion) and its relationship to milk yield traits in Anatolian buffaloes, Murrah buffaloes, and their crosses

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Abstract: The aim of this study was to investigate the polymorphism of the κ – *casein* gene (*kappa casein, CSN3*) in buffaloes and determine its effects on milk yield traits. Data were obtained from 2016 to 2019 from all animals in the ongoing Anatolian Buffalo Breeding Project. Genomic DNA was collected from a total of 209 buffaloes of different ages and sexes, namely 31 Murrah, 46 Anatolian buffaloes, and 132 of their crosses (Murrah × Anatolian). Genotyping of *CSN3* gene by polymerase chain reaction–restriction fragment length polymorphism (PCR – RFLP) technique with restriction enzyme (*Acu*I) revealed three genotypes: *AA* (0.689), *AB* (0.282), and *BB* (0.028), and the frequencies of *A* and *B* alleles were 0.83 and 0.17, respectively. A total of 113 milk yield data and 74 milk component analyses of dairy buffaloes were used to compare *CSN3* genotypes for milk yield traits. Statistical analysis showed that there was a significant difference in lactation milk yield between CSN3 genotypes: *BB*, 1560.3 ± 326.7 kg, *AB*, 1278.1 ± 111.3 kg, and *AA*, 1060.9 ± 85.6 kg ($p < 0.05$). However, the mean values of lactation length and milk components were not significant between genotypes ($p > 0.05$). These results suggest that these variations ($135^{ThrACC\,/\,IleATC}$ /$136^{ThrACC\,/\,ThrACT}$) in the buffalo *CSN3* gene may be used as genetic markers in dairy buffalo breeding programs.

Key words: Buffalo, milk yield, genetic polymorphism, kappa casein

1. Introduction

Domestic buffalo (*Bubalus bubalis*) is mainly native to Southeast Asian countries (India, Pakistan, and China), with 95% of the world's buffalo population living on this continent. Of the remaining population, 2.9% live in Africa, 0.02% in Europe, and 1.9% in the Americas [1]. The buffalo is a livestock that occurs mainly in tropical and subtropical countries with hot and humid climates and contributes significantly to global milk production at a lower cost than cattle.

The Anatolian buffalo, the only native water buffalo breed in Turkey, has evolved from the Mediterranean water buffalo, which is a subgroup of the Asian river buffalo and is found in most regions of the country [2]. The Murrah buffalo, known for its high milk yield and adaptability to environmental conditions, originated in Rohtak and Haryana and has spread to other parts of India and other countries [3].

In livestock production, it is important to increase milk yield and ensure that the composition of milk is as desirable as possible. Since milk components and milk yield are directly related, variations in genes thought to affect milk yield are studied to improve milk quality and quantity. Selection methods can be more easily determined when yield-related genes are used as markers, and breeding goals can be achieved more quickly by using these genes.

Because 80% of milk proteins are caseins, most studies of milk performance traits in livestock focus on casein genes. The protein content of buffalo milk is the same as in other animal species and consists of caseins (CN), *β – lactoglobulin* (*β – LG*), and *α – lactoalbumin* (*α – LA*). There are four types of casein in milk: *αs1 – casein* (*αs1 – CN*), *αs2 – casein* (*αs2 – CN*), *β – casein* (*β – CN*), and *κ – casein* (*κ – CN*). Each of these caseins is encoded by the *CSN1S1, CSN1S2, CSN2*, and *CSN3* genes, respectively [4, 5].

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It has been reported that 11 different types of κ – *CN* exist in cattle (*A, B, B2, C, D, E, F1, F2, G1, G2, H, I, J*), with alleles *A* and *B* being the most common [6]. The *CSN3* gene of buffaloes is located on chromosome 7 and consists of five exons. It highlights the role that genetic markers play in milk production traits in dairy breeds and can be used in breeding. It is responsible for about 12% of the total casein in buffalo milk. Therefore, the polymorphism of *CSN3* gene encoding κ–casein protein is of particular importance [4 –7]. A similar study was conducted in buffaloes in response to *CSN3* gene variations detected in cattle. A significant association was found between the *B* allele and milk yield and protein content [8]. In addition, the *B* allele was reported to be more abundant in buffaloes than in cattle, resulting in higher efficiency in the production of butter and cheese from buffalo milk [9]. As a result of these alleles, codons 136 and 148 in *CSN3* are altered, $136^{ThrACC}/148^{AspGAT}$ in the *A* allele, $136^{IleATC}/148^{AlaGCT}$ in the *B* allele, and the genotype *BB* produced more cheese and required less time to renette than the genotype *AA* in buffaloes [10, 11]. Some studies have reported the effects of *CSN1S1* and *CSN3* genes on milk yield and coagulation. Allelic combinations of *CSN1S1* and *CSN3* genes have been investigated for their effects on milk yield, milk composition, and coagulation traits, and differences between *CSN1S1 – CSN3* composite genotypes in milk yield, milk composition, and coagulation traits were found [12, 13].

Different breeds of buffaloes from different countries were investigated for polymorphisms of the *CSN3* gene. The exon 4 region of *CSN3* in Bulgarian Murrah buffaloes was amplified and directly sequenced, and two SNPs were found [11]. Similar results were also obtained for exon 4 in Italian and Indian buffaloes [12, 14]. In Egyptian buffaloes, a region of 453 base pairs on exon 4 was examined by sequence analysis and found to contain two SNPs causing silent mutations in the peptide regions $135^{ThrACC}/^{IleATC}$ and $136^{ThrACC}/^{ACT}$. By RFLP with the other enzyme *Acu*I, three different genotypes were detected in the same region. Two SNPs are responsible for this difference, with the *A* allele causing a change in $135^{ThrACC}/136^{ThrACT}$, while the *B* allele causes a change in $135^{IleATC}/136^{ThrACT}$ [7, 15–17]. According to a study on the effects of this variation in Egyptian buffaloes, the *B* allele has a significant effect on total milk yield and milk composition [16].

Direct sequencing analysis of the *CSN3* CDS region of river and swamp buffalo in China revealed 5 amino acid changes, but only one was effective ($135^{Thr/Ile}$) [8]. *CSN3* in buffaloes is usually analyzed with restriction enzymes, which have also been shown to be effective in cattle. In native Indian buffalo breeds (South Kanara, Surti, Murrah), the restriction enzyme *Hinf*I was used to digest 350 bp of the 4th exon of *CSN3*. Based on RFLP results, all samples showed the homozygous genotype *BB*, and this region was monomorphic [8]. Using different restriction enzymes such as *Alu*I and *Hind*III, 400 and 280 bp of the same exon were analyzed in 115 lactating Murrah buffaloes, and all individuals belonging to the *BB* genotype were found to be monomorphic [18]. A PCR–RFLP analysis using *Hinf*I and *Hae*III restriction enzymes was performed in Anatolian buffaloes to analyze exon 4 and intron 4 (350 bp), resulting in monomorphic results [19, 20]. Similarly, all samples showed monomorphic results after amplification of a 556–bp region containing *CSN3* STS (*CSN3* sequence–tagged sites) and *Hind*III digests. However, sequence analysis of this region revealed a substitution in codon $135^{ThrACC}/^{IleATC}$ in some samples [21]. Another study in Anatolian buffaloes revealed that exon 4 was monomorphic after *Hinf*I and *Hind*III digests and that the genotypes *AB* and *BB* were detected after *Hind*III enzyme digests of exon 4 and intron 5 [22]. Moreover, many studies in Iranian and Pakistani buffaloes have shown similar monomorphic results when *Hinf*I enzyme was digested in the exon 4 region [23–25]. Despite the digestion of 350 bp of *Hinf*I enzyme, both *BB* and *AB* genotypes were found, and polymorphic structure in this region was detected in Indian river buffaloes [26]. When *CSN3* exon 4 was digested by *Hind*III in native Egyptian buffalo and cattle, the genotypes *AA* and *AB* were found in both species, with cattle having a high frequency (0.630) for the *A* allele and buffalo having a high frequency (0.875) for *B* allele [9].

The *CSN3* polymorphism was previously studied in cattle and buffalo, and the results of this study show that the restriction enzymes *Alu*I, *Hae*III, *Hinf*I, and *Hind*III and *CSN3* genotyping were successful in cattle, whereas monomorphic results were obtained in buffalo. This is due to the fact that the restriction sites for the *A* and *B* alleles are different in the two species [7]. Previous studies using PCR–RFLP in Anatolian buffaloes have shown that the exon 4 region of the *CSN3* gene is monomorphic with the *Hinf*I, *Hind*III, and *Hae*III restriction enzymes [19–22]. Therefore, the aim of this study was to determine buffaloes with a different restriction enzyme (*Acu*I/PCR–RFLP) in exon 4 of *CSN3* gene and relate it to milk yield traits.

2. Materials and methods

2.1. Animal material

All purebred and crossbred buffalo breeds of the "National Anatolian Buffalo Breeding Project" at Bandırma Sheep Research Institute were used for the study. A total of 209 buffaloes of different ages (1 month to 16 years) and sexes (57 males and 152 females), calves, heifers, young bulls, cows, and adult bulls were genotyped for the variation of *CSN3* gene. The number of genotyped purebred and crossbred buffaloes was determined to be 46 Anatolian buffaloes, 31 Murrah buffaloes, 50 F1, 48 G1, 30 G2, and 4 G3.

2.2. Feeding program for buffaloes

Throughout the facility, the entire herd is fed according to a feeding program that changes according to age and physiological phases. The group feeding program is calculated based on the daily milk yield of the dairy buffalo.

The buffalo's roughage needs are met at regular intervals from a variety of sources. Vetch hay and barley straw form the basis of the roughage source. In the fall and winter, corn silage is also fed. A mixture of sunflower seed meal, barley, and wheat is used as a concentrate. The content of the ration for 500 kg live weight, without milk yield, is 14.2 energy (ME in MCAL), 3.7 (kg) of total digestible nutrients (TDN), and 364 (g) of total crude protein (CP).

2.3. Milk performance traits and milk component analysis

A total of 113 milk yield records of dairy buffaloes from 2016, 2017, and 2018 were collected for comparison of milk yield traits of *CSN3* genotypes (83 *AA*, 28 *AB*, and 2 *BB*). Daily milk yield records (morning and evening) were used to determine the total milk yield and length of lactation period of buffaloes. To compare milk constituents (fat, nonfat dry matter, protein, lactose, minerals, density, and freezing point) between *CSN3* genotypes, 74 milk constituent analyzes were performed on the same buffalo from 2016, 2017, and 2018 (56 *AA*, 16 *AB*, and 2 *BB*). Milk samples collected monthly were analyzed using a milk analyzer (Funke Gerber® Milk Analyzer).

2.4. Blood collection and DNA isolation

Blood was collected from the jugular vein into 10 mL K3–EDTA tubes (anticoagulant) with sterile disposable needles and stored at –20 °C. After thawing the frozen blood samples, DNA isolation was performed using a commercial kit (Macharey Nagel®), and a DNA quantification device was used to measure the extracted DNA density.

2.5. PCR–RFLP analysis

DNA was isolated from blood samples and verified by 2% gel electrophoresis. A 453 bp fragment of exon 4 of the CSN3 gene was amplified using a T100™ thermal cycler with primers: F: 5′–TGTGCTGAGTAGGTATCCTAGTTATGG–3′; R: 5′–GCGTTGTCTTCTTTGATGTCTCCT – 3′ [7]. The PCR mix contained: 12.5 µL 2X Taq Master Mix (Vivantis®), 1 µL MgCl2 (3.5 mM), 1 µL Forward Primer (F), 1 µL Reverse Primer (R), 1 µL DNA sample, and 8.5 µL water (dd). The PCR cycle performed: Denaturation 2 min at 94 °C, 35 cycles at 94 °C 2 s, annealing 30 s at 63 °C, synthesis 30 s at 72 °C, and final extension 7 min at 72 °C. Amplicons were digested with *Acu*I restriction endonuclease enzyme (Thermo Scientific) for 30 min at 37 °C with the following mixture: 10 µL PCR product, 1 µL *Acu*I restriction enzyme, 2 µL 10X Fast Digest Buffer, 1.5 µL 20X SAM (0.2 mM), and added to 15.5 µL sterile distilled water for a total of 30 µL. After inactivation of the enzyme at 65 °C for 5 min, the digested samples were separated on a 3.0% agarose gel, stained with Syber Safe DNA Stain, and imaged using a gel imaging device.

2.6. Statistical analysis

Allele and genotype frequencies were determined using Popgene 1.32 software [27] and Hardy–Weinberg equilibrium was determined using the chi–square ($\chi 2$) test. *CSN3* genotypes were evaluated using the least squares means method (LSM) [28] for milk yield, lactation length, and milk component. The following statistical model was used for the analysis of milk yield traits;

$Yijklm = \mu + ai + bj + ck + dl + dm + eijklm$

Where;

Yijklm: vector of observed traits.

µ: Overall mean

ai: Fixed effect of genotype *CSN3* (*i: AA, AB*, and *BB*)

bj: Fixed effect of milking year (*j:* 2016, 2017, and 2018).

ck: Fixed effect of breed and crossbreed (*k:* A, M, F1, G1, G2, G3).

dl: Fixed effect of age (*l:* 3–10 years)

dm: Fixed effect of lactation number (*m:* 1–7)

eijklm: Residual error.

3. Results

Figure 1 shows the gel electrophoresis image of the raw DNA samples obtained from 209 buffaloes. After digestion with *Acu*I restriction enzymes, RFLP analysis was performed to determine the length of the bands.

CSN3 genotypes were determined from the band images and RFLP results shown in Figure 2. An uncut single band (453 bp) as *AA*, three bands (453, 339, and 114 bp) as *AB*, and two bands (339 and 114 bp) were described as *BB* genotypes.

In the analysis of PCR–RFLP, the difference between *A* and *B* alleles is the presence of two SNPs previously reported (C/T). The *A* allele is $135^{ThrACC}/136^{ThrACC}$, whereas the *B* allele is $135^{IleATC}/136^{ThrACT}$. Accordingly, the presence of the *B* allele leads to amino acid substitutions [7].

Table 1 shows the detected genotypes in *CSN3*, alleles, and genotype frequencies of buffaloes and 146 *AA*, 55 *AB*, and 8 *BB* genotypes, with genotype frequencies of 0.69, 0.26, and 0.038, respectively. Based on the chi–square (1.0098) and P value (0.3139), the population is in Hardy–Weinberg equilibrium, because $p > 0.05$.

In Table 2, the comparison of lactation milk yield between genotypes was evaluated by multifactorial analysis of variance with LSM. The mean values of genotypes for lactation milk yield were obtained as follows: *AA* 1060.9 ± 85.6 kg, *AB* 1278.1 ± 111.3 kg, and *BB* 1560.3 ± 326.7 kg, with significant differences between means ($p < 0.05$). Genotype *BB* had the highest milk yield, followed by genotypes *AB* and *AA*. LSM values for the lactation length

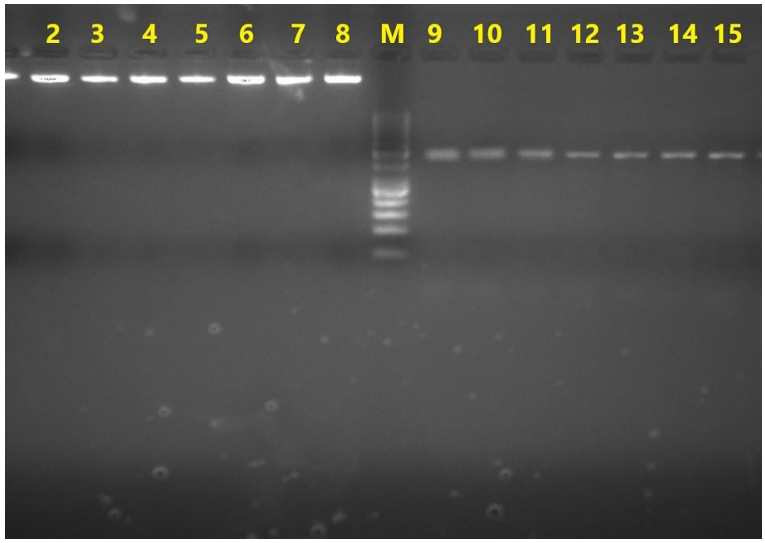

Figure 1. Gel image of buffalo genomic DNA (lanes: 1–8) and *CSN3* exon 4 PCR products 453 bp (lines 9–16), M: marker, 50 bp.

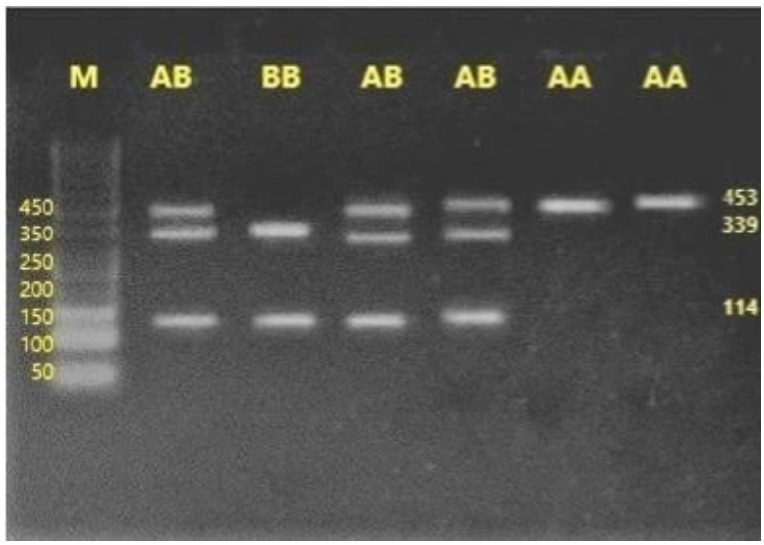

Figure 2. Gel image of buffalo *CSN3* exon 4 PCR–RFLP analysis with *Acu*I enzyme genotypes; lines: 5 and 6 (*AA* genotype, 453 uncut fragment), line 4 (*BB* genotype, two bands: 339 and 114 bp) and lines 1, 3, and 5 (*AB* genotype, three bands: 453, 339 and 114 bp) M: marker 50 bp.

period were: *AA* 249.9 ± 13.8 days, *AB* 259.9 ± 17.9 days, and *BB* 313.8 ± 52.6 days, but no significant differences between genotypes ($p > 0.05$).

The *CSN3* genotypes of milk are shown in Table 3 with their LSMs for fat, nonfat dry matter, protein, lactose, density, freezing point (–), and minerals. There was no significant difference in milk components between genotypes ($p > 0.05$).

4. Discussion

An essential component of genetic progress is the identification and sustainable use of indigenous breeds. To improve sustainability, genes must be identified that affect economic efficiency traits important for food consumption, such as milk and meat. With the increase in molecular genetic studies, breeding programs are taking advantage of the quantitative trait loci identified. It is well

Table 1. Allele and genotype frequencies of buffalo *CSN3* variants.

Breed	N	GF			AF		Exp. Homo.	Exp. Het.	χ^2	P value
		AA	AB	BB	A	B				
A	46	0.696	0.282	0.021	0.837	0.163	0.7241	0.2729	0.0287	0.8655
M	31	0.645	0.258	0.097	0.774	0.226	0.6446	0.3554	2.4737	0.1157
F1	50	0.740	0.220	0.040	0.850	0.150	0.7424	0.2576	1.1306	0.2877
G1	48	0.646	0.333	0.021	0.813	0.187	0.6921	0.3079	0.3441	0.5575
G2	30	0.733	0.233	0.034	0.850	0.150	0.7407	0.2593	0.3342	0.5632
G3	4	1.000	0.000	0.000	1.000	0.000	1.0000	0.0000	–	–
Overall	209	0.699	0.263	0.038	0.830	0.170	0.7173	0.2827	1.0098	0.3149

GF: Genotype frequency, AF: Allele frequency, Exp. Homo.: Expected homozygosity, Exp. Het.: Expected heterozygosity, A: Anatolian buffalo, M: Murrah buffalo, F1, G1, G2, G3: Croses (Murrah × Anatolian), χ^2: Chi – square, P: Probability

Table 2. Least squares mean values of lactation milk yield and lactation length of buffalo *CSN3* genotypes.

Trait/efficiency	Unit	LSM* ± SE			P-value
		AA (N = 83)	AB (N = 28)	BB (N = 2)	
Lactation milk yield	kg	1060.9 ± 85.6^{b}	1278.1 ± 111.3^{a}	1560.3 ± 326.7^{ab}	0.0445
Lactation length	days	249.9 ± 13.8	259.9 ± 17.9	313.8 ± 52.6	0.4104

LSM*: Least Squares Means, SE: Standard error, P: Probability, $^{a, b, ab}$: Differences in mean values, indicated by different letters in each row or column, are statistically significant.

Table 3. Least squares mean values of milk components of buffalo *CSN3* genotypes.

Trait/efficiency	Unit	LSM* ± SE			P value
		AA (N = 56)	AB(N = 16)	BB(N = 2)	
Fat	%	7.06 ± 0.32	7.06 ± 0.38	5.68 ± 0.86	0.2373
Nonfat DM	%	10.67 ± 0.15	10.71 ± 0.18	10.24 ± 0.40	0.4895
Total protein	%	4.01 ± 0.05	3.99 ± 0.06	3.79 ± 0.13	0.2375
Lactose	%	5.93 ± 0.08	5.96 ± 0.09	5.66 ± 0.21	0.3440
Density	g/mL	1.02 ± 0.00	1.03 ± 0.00	1.02 ± 0.00	0.5792
Freezing point (–)	°C	0.42 ± 0.03	0.43 ± 0.04	0.43 ± 0.09	0.9010
Mineral	%	0.25 ± 0.02	0.27 ± 0.02	0.25 ± 0.05	0.7139

LSM*: Least squares mean, SE: Standard error, P: Probability, DM: Dry matter

known that milk yield and quality are influenced by casein proteins, which account for 80% of milk proteins [29].

The *κ – CN* gene and its genetic polymorphisms, which are also the subject of this study, have been extensively studied in cattle and buffalo breeds in many geographic regions because of their effects on milk yield and quality [22]. In the past, numerous studies have been conducted to determine how variations in *CSN3* affect milk yield in cattle. In recent years, similar studies have been conducted in buffaloes, but there have been few studies correlating

CSN3 variations with milk yield traits. Therefore, this study is important because it contributes to further research on the *CSN3 B* allele in buffaloes.

In the present study, the *CSN3 B* allele and the genotypes *AA, AB*, and *BB*, identified by RFLP analysis of the buffalo exon 4 region (453 bp), also showed the presence of two SNPs (C/T) reported in some previous studies [7, 15–17].

In the present study, a Buffalo–specific *Acu*I enzyme restriction site (CTGAAGN16∧) was detected in exon 4 of the *CSN3* gene, as previously reported by El Nahas et al. [7]. As a result of RFLP analysis by digestion with *Acu*I enzyme, two different alleles (*A* and *B*) were detected in a 453 bp long region in *CSN3* exon 4. According to the results of gel electrophoresis, the frequency of the *B* allele was lower (0.17), whereas the *A* allele had a higher frequency (0.83). The frequencies of the homozygous *AA*, heterozygous *AB*, and homozygous *BB* genotypes were 0.699, 0.263, and 0.038, respectively. In a study of Egyptian buffaloes, after *Acu*I digestion in a 453 bp region in *CSN3* exon 4, allele frequencies *A* and *B* were 0.57 and 0.43, respectively, and genotype frequencies were *AA* 0.29, *AB* 0.65, and *BB* 0.06 [7]. In a similar study using the same method in Egyptian buffalo, allele frequencies of *A* and *B* were reported to be 0.58 and 0.42, respectively, and genotype frequencies were reported to be 17% for *AA* and 83% for *AB* [17].

The polymorphic structure detected in this study by *Acu*I digestion suggests that there are different *CSN3* genotypes in this herd and that selection in this direction may be occurring. The differences between *CSN3* genotypes in lactation milk yield were considerable, and it was found that there was a significant difference between buffaloes with the *B* allele and buffaloes with the *A* allele. The lactation milk yield of the genotypes was: *BB* 1560.3 ± 326.7, *AB* 1278.1 ± 111.3, and *AA* 1060.9 ± 85.6 ($p < 0.05$). In a similar study on Egyptian buffaloes, it was found that LSM of genotypes *AB* 1632.62 ± 67.66 and *AA* 1412.63 ± 116.52 were significant for milk yield [16]. The results of the present study indicate that the frequency of the *B* allele in this herd is low. This suggests that selection to increase the frequency of the *B* allele could lead to an improvement in milk yield.

Previous studies have reported that variations in the CSN3 gene affect milk composition and cheese traits in buffaloes [8, 9, 11–13]. When the mean values of milk components of CSN3 genotypes were compared in the present study, it was found that the difference between LSMs was not significant. Similar results were found in a study of Egyptian buffaloes in which the composition of milk was analyzed by fat, lactose, total dry matter, nonfat dry matter, ash, and humidity [16].

In buffalo breeding, the desired level of performance can be achieved faster if marker-assisted selection methods are used in addition to classical breeding methods. Therefore, it is important to generalize molecular genetic methods and investigate mutations in genes that affect yield. Using a previously defined variation in the *CSN3* gene, which is closely associated with milk yield traits. If other mutations in the *CSN3* gene are also studied, it will be easier to evaluate the effects of this variation ($135^{IleATC}/136^{ThrACT}$) on milk yield. In view of this, the results of this study will make an important contribution to ongoing breeding projects aimed at improving milk yield traits in Anatolian buffaloes.

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Conflict of interest

The authors declare that they have no competing interests.

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