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ALLELE AND GENOTYPE FREQUENCIES OF THE KAPPA-CASEIN (CSN3) LOCUS IN MACEDONIAN HOLSTEIN-FRIESIAN CATTLE

Nikola Adamov, Branko Atanasov, Ksenija Ilievska, Martin Nikolovski, Monika Dovenska, Vladimir Petkov, Toni Dovenski

Faculty of Veterinary Medicine - Skopje, Ss. Cyril and Methodius University in Skopje, 1000 Skopje, Republic of North Macedonia

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ABSTRACT

The bovine kappa-casein (κ-CN) is a phospho-protein with 169 amino acids encoded by the *CSN3* gene. The two most common gene variants in the HF breed are *CSN3*A* and *CSN3*B* while *CSN3*E* has been found with lower frequency. The aim of this study was to optimize a laboratory method for genotyping of these three alleles as well as to determine their genotype and allele frequencies in the HF cattle population in the Republic of North Macedonia. Genomic DNA was extracted from full blood from 250 cows. The target DNA sequence was amplified with newly designed pair of primers and the products were subjected to enzymatic restriction with *Hind*III and *Hae*III endonucleases. Genotype determination was achieved in all animals. The primers successfully amplified a fragment of 458 bp and the digestion of this fragment with both endonucleases enabled differentiation of five different genotypes with the following observed frequencies: AA (0.39), AB (0.29), BB (0.16), AE (0.10), and BE (0.06). The estimated allele frequencies were: *CSN3*A* (0.584), *CSN3*B* (0.336) and *CSN3*E* (0.08). The observed genotype frequencies differed significantly (P<0.01) from those that would be expected under HW equilibrium, while the fixation index (*F*=0.17) indicated moderate heterozygosity deficiency. Nevertheless, the *CSN3*B* allele was present with relatively high frequency which should be used to positively select for its carriers, since increasing its frequency could help to improve the rheological properties of the milk intended for cheese production.

Key words: *CSN3*, genetic polymorphism, Holstein-Friesian cattle, kappa-casein, milk protein

INTRODUCTION

The most important milk proteins are caseins which are produced by the mammary gland secretory cells. They constitute about 80% of the bovine milk proteins (1) and are divided into four main fractions: α_{s1}-CN, β-CN, α_{s2}-CN, and κ-CN.

The kappa-casein (κ-CN) fraction which constitutes around 12% of the total caseins found in the bovine milk (2) is a phospho-protein with 169 amino acid residues (3) located predominantly on casein micelle surface and is specific substrate of the chymosin which hydrolyses its amino acid chain at position Phe105- Met106 and yields insoluble para-κ-CN (amino acid 1-105) and soluble caseino-macropeptide-CMP (amino acid 106-169) (4, 5).

The bovine κ-CN is encoded by the *CSN3* gene located on BTA6 (6, 7, 8, 9). This gene is around 13 kb in length and is divided in transcription unit (5 exons and 4 introns) and 5' and 3' untranslated regions (10). The fourth exon which is 517 bp long (11) harbors all the 11 non-synonymous single-nucleotide substitutions which code 11 different variants of the mature κ-CN protein identified so far in the *Bos* genus (3): A, B, C, E, F^1, F^2, G^1, G^2, H, I and J. In a more recent review on milk protein polymorphism in cattle (12) it has been suggested two more alleles to be included to this list since they are non-synonymous mutations in this exon namely: *CSN3*B*^{2} and *CSN3*D*. In addition, one more synonymous nucleotide substitution has been identified, namely *CSN3*A1* of Damiani et al. (13) or *CSN3*A*^{1} of Prinzenberg et al. (14) which does not modify the correspondent amino acid.

Corresponding author: Asst. Prof. Nikola Adamov, PhD
E-mail address: adamovn@fvm.ukim.edu.mk
Present address: Faculty of Veterinary Medicine - Skopje, Ss. Cyril and Methodius University in Skopje, 1000 Skopje, Republic of North Macedonia
Phone: +389 2 324 0731; *Mobile:* +389 75 244 138

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The two most common protein variants in the HF breed are κ-CN A and κ-CN B (3, 14) while κ-CN E has been found with lower frequency in this breed (12). With the exception of the Jersey breed the variant κ-CN A is the most common among dairy cattle breeds (16, 17, 18). The κ-CN B protein variant differs from κ-CN A at two amino acid positions: The136 is substituted with Ile and Asp148 is exchanged with Ala. The κ-CN E variant differs from κ-CN A at amino acid position 155 where Ser is substituted with Gly (12). The *CSN3*B* allele is used as a genetic marker in dairy cattle breeding programs because the milk with κ-CN B protein variant has been shown to have better rheological properties such as shorter rennet coagulation time and higher yield during cheese production (1, 19, 20) when compared to milk with κ-CN A variant. Bovenhuis et al. (21) suggested that the favourable milk protein genotype κ-CN BB should be included in the criteria for selection of dairy cattle because of economic interest.

The aim of this study was to optimize laboratory method for genotyping of the most common κ-CN variants in the HF cattle population in the Republic of North Macedonia as well as to determine the genotype and allele frequency at this locus. We focused on the *CSN3*A, B* and *E* alleles because they have been reported in the literature as the most frequent variants in different dairy cattle populations.

MATERIAL AND METHODS

DNA extraction and quantification

Genomic DNA was extracted from blood obtained by venipuncture of the jugular or the coccygeal vein from 250 cows, selected randomly from five cattle farms in the Republic of North Macedonia. The blood was drawn in vacutainers with anticoagulant (EDTA) and was stored at +4°C until extraction. The DNA was extracted from blood using two different methods: i) Phenol-Clorophorm-Isoamil alcohol followed by ethanol precipitation, and ii) with commercial DNA extraction kit. The amount and the purity of the extracted DNA was determined with spectrophotometer.

PCR amplification of the CSN3 locus

The primes used for amplification (KCN-F: GGTCACCTGCCCAAATTCTTCAA and KCN-R: AGCCCATTTCGCCTTCTCTGT) were designed using the Primer Premier software (Premier Biosoft International) based on GenBank sequence X14908.1. (10). The coefficients of hairpin formation, self-dimerization and creation of cross-dimers as well as the primer's optimal annealing temperature necessary to design the reaction conditions for the thermo-cycling protocol, were determined with the same software. These primers amplified a region of 458 bp of the 4th exon of the bovine *CSN3* gene. Part of this nucleotide sequence with the primer annealing positions and the restriction endonucleases cleavage sites are shown in Fig. 1.

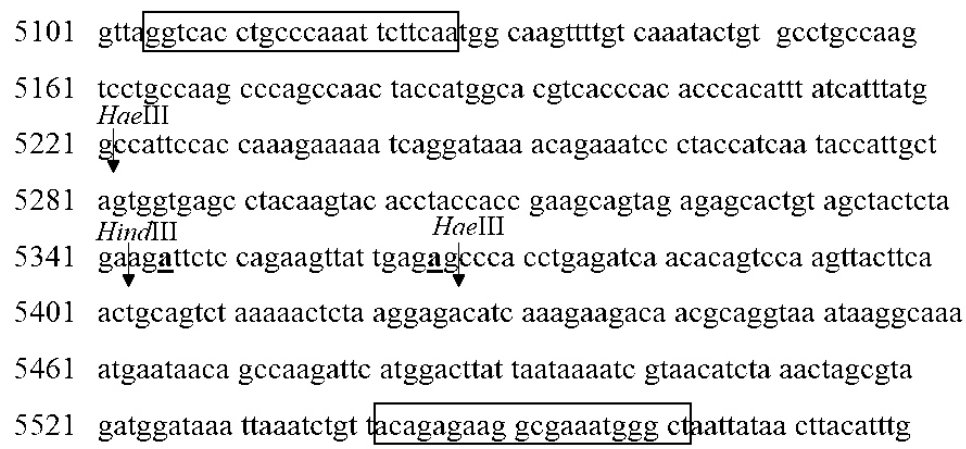

Figure 1. Part of the nucleotide sequence GenBank X14908.1 that corresponds to *CSN3*A* allele. The primer binding sites are denoted with boxes, the restriction sites of the enzymes are marked with arrows and the positions of the two nucleotide substitutes that create additional restriction sites for alleles *CSN3*B* or *CSN3*E* are shown with bold underlined letters

The amplifications were prepared in total volume of 20 µl containing 1 X PCR buffer, 200 µM dNTP, 2.0 mM $MgCl_2$, 0.6 U DNA Polymerase, 0.2 µM of each oligonucleotide and 40-50 ng genomic DNA. The following thermal protocol was applied: initial denaturation of 95°C/5 min. then the *Taq* DNA Polymerase was added followed by 35 cycles of 94°C/45 sec., 56°C/45 sec., 72°C/1 min., and final elongation step of 72°C/5 min. on Biometra TPersonal Thermocycler (Biometra GmbH, Germany). The amplified DNA fragments were checked by staining with ethidium bromide on 1.5 % (w/v) agar-gel followed by visualization on a UV transiluminator (Figure 2). A 100 bp DNA ladder was lined up as molecular size marker.

Genotyping of the amplified products

In order to detect the three alleles and their combinations of genotypes, Restriction Fragment Length Polymorphism (RFLP) analysis was carried out with two different restriction endonucleases. Initially, each PCR product was digested with the *Hind*III enzyme (Thermo Scientific) which enables distinction of *CSN3*A* or *CSN3*E* allele carriers from those that are carriers of the *CSN3*B* allele. This enzyme did not enable distinction of the *CSN3*A* from *CSN3*E* allele since it has the same cleavage site in both alleles and consequently it yields same restriction fragment lengths from both alleles. xConsequently, those samples where the variant *CSN3*A* was detected with this enzyme (genotypes classified as AA and AB), were further digested with the *Hae*III enzyme in a separate reaction in order to enable distinction of *CSN3*A* from *CSN3*E* variant (since the *CSN3*E* allele has two cleavage sites for this enzyme and yields three fragments, while the *CSN3*A* variant has only one cleavage site and yields two fragments) as shown in Fig. 1.

In this study GenBank sequence X14908.1 was used as a reference sequence to design the primers and to predict the restriction patterns. In this sequence the following nucleotide positions were used to differentiate the three *CSN3* alleles:

a) the primers amplified the region between the nucleotide positions 5105 and 5562;

b) between nucleotides 5221 and 5222 there is a cleavage site for *Hae*III (GG/CC) in all three studied *CSN3* alleles;

c) at nucleotide position 5345 the transversion A→C in the *CSN3*B* allele (Asp148Ala) creates cleavage site for *Hind*III (A/AGCTT) while the other two alleles remain undigested at this position;

d) at nucleotide position 5365 the transition A→G in the *CSN3*E* allele (Ser155Gly) creates additional cleavage site for *Hae*III (GG/CC) while the other two alleles remain undigested at this position.

For each genotype, the expected fragment sizes after digestion with both enzymes are shown in Table 1.

Table 1. Expected fragment sizes (in bp) corresponding to different CSN3 genotypes after digestion of a 458 bp PCR product with two restriction enzymes

Genotype	*Hind*III	*Hae*III
AA	458	341, 117
AB	458, 238, 220	341, 117
BB	238, 220	341, 117
AE	458	341, 196, 145, 117
BE	458, 238, 220	341, 196, 145, 117
EE	458	196, 145, 117

The digestion reactions were prepared in total volume of 20 µl containing 2 µl 10X Buffer, 8 µl PCR product, 1-2 µl restriction enzyme, and 8-9 µl ddH_2O. The incubations were carried out at 37°C for a period of 3 h. Digested products were analysed using electrophoresis on 2.5% agarose gel stained with ethidium bromide. Band patterns were visualized via UV transilluminator photo documentation system (Fig. 3).

Statistical analysis

The observed number of animals for each of the five detected genotypes was calculated by direct counting.

The frequencies of each of the three alleles were estimated by allele counting method (22) by adding twice the number of homozygotes to the number of heterozygotes that possess the allele and divide this sum by twice the number of animals in the sample or:

$$p = f(\mathrm{A}) = \frac{2n_{AA} + n_{AB} + n_{AE}}{2N}$$

$$q = f(\mathrm{B}) = \frac{2n_{BB} + n_{AB} + n_{BE}}{2N}$$

$$r = f(\mathrm{E}) = \frac{2n_{EE} + n_{AE} + n_{BE}}{2N}$$

where:

n is the number of animals possessing the genotype, and

N is the number of animals in the sample.

These estimated allele frequencies *p*, *q*, and *r* were used to calculate the expected number of animals for each genotype as follows:

AA = N x p^2, AB = N x 2*pq*, BB = N x q^2, AE = N x 2*pr*, BE = N x 2*qr* and EE = N x r^2.

The probability of Hardy-Weinberg equilibrium associated with the observed genotype frequencies was calculated by the Chi-squared (χ^2) goodness-of-fit test (23) as follows:

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

where:

χ^2: Chi-squared test statistic

O: Observed number of genotypes

E: Expected number of genotypes

The χ^2 test statistic had $k - 1 - m$ degrees of freedom, where *k* is the number of genotypes and *m* is the number of independent allele frequencies estimated from the data (24).

To determine the level of departure from the HW expectations in the studied population, the average expected heterozygosity (*He)* or Nei's gene diversity was calculated by adding up the expected frequencies of each possible homozygous genotype and subtracting this sum from one (23) or:

$$He = 1 - \sum_{i=1}^{k} p_i^2$$

where:

k is the number of alleles at the locus,

p_i^2 is the expected genotype frequency of homozygotes based on allele frequencies, and

$\sum_{i=1}^{k}$ indicates summation of the frequencies of the *k* homozygous genotypes.

The observed heterozygosity (*Ho*) was calculated by adding the frequencies of the three observed heterozygous genotypes or *Ho* = f(AB) + f(AE) + f(BE) (23). From these data the fixation index - *F* was calculated as follows:

$$F = \frac{He - Ho}{He}$$

where *He* is the H-W expected frequency of heterozygotes based on estimated allele frequencies and *Ho* is the observed frequency of heterozygotes.

RESULTS

The primers KCN-F and KCN-R that were designed in this study successfully amplified a fragment of 458 bp in length as illustrated in Fig. 2.

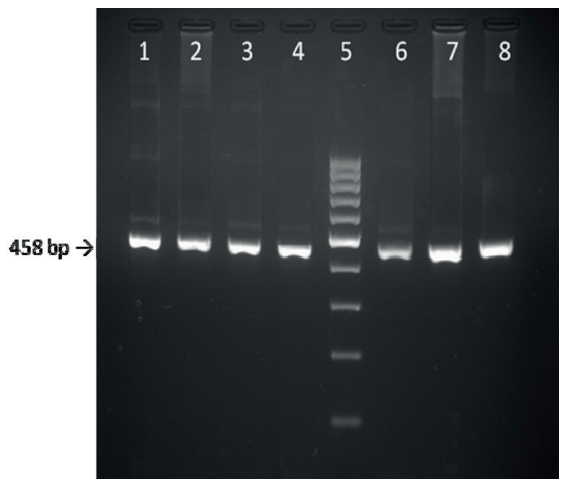

Figure 2. Representative agarose gel showing PCR amplification of 458 bp fragment of the bovine *CSN3* gene. Lanes 1-4 and 6-8: 458 bp fragment, lane 5: GeneRuler 100 bp DNA ladder (Thermo Scientific)

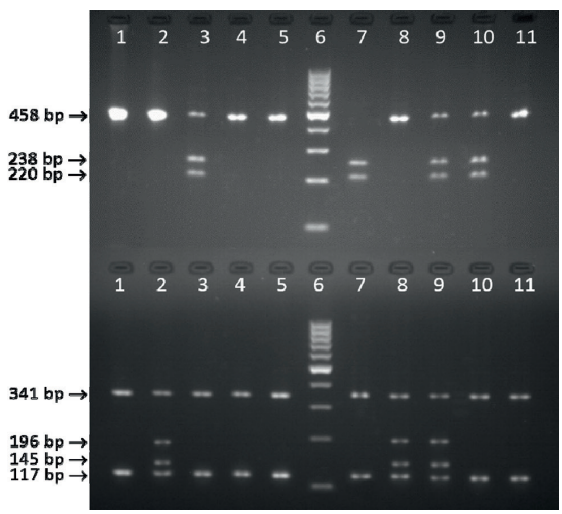

Figure 3. Digestion patterns of exon 4 of the bovine CSN3 gene. Lanes 1, 4, 5 and 11- genotype AA; lanes 3 and 10 - genotype AB, lane 7 - genotype BB; lanes 2 and 8 - genotype AE; lane 9 - genotype BE; lane 6 - GeneRuler 100 bp DNA ladder (Thermo Scientific). The upper half of the agarose gel represents digestion with *Hind*III and on the lower half the same samples in the same order are digested with *Hae*III.

Table 2. Observed and expected genotype frequencies and estimated allele frequencies

Genotype	Observed count	Observed frequency	Expected frequency under HWE	Estimated allele frequency
AA	97	97 / 250 = 0.388	$p^2 = 0.341$	f (A)=p=(2x97+98) /500 = 0.584
AB	73	73 / 250 = 0.292	2pq = 0.392	
BB	40	40 / 250 = 0.16	$q^2 = 0.113$	f (B)=q=(2x40+88) /500 = 0.336
AE	25	25 / 250 = 0.1	2pr = 0.093	
BE	15	15 / 250 = 0.06	2qr = 0.053	f (E) = r = (2x0+40) /500 = 0.08
EE	0	0 / 250 = 0	$r^2 = 0.006$	

Table 3. Observed and expected genotype counts and their χ^2 differences

Genotype	Observed number of animals	Expected number of animals under HWE	χ^2 statistic
AA	97	85.26	1.616
AB	73	98.11	6.426
BB	40	28.23	4.907
AE	25	23.36	0.115
BE	15	13.44	0.181
EE	0	1.6	1.6
Total	250	250	14.845 ($P < 0.01$)

The PCR products were further digested with *Hind*III and *Hae*III enzymes. Considering the information of both digestions in terms of number and sizes of the obtained fragments (Table 1), it was straightforward to identify 5 different genotypes as shown in Fig. 3. The genotype EE was not detected in the studied population.

Observed genotype counts and frequencies as well as estimated allele frequencies are shown in Table 2.

From the estimated allele frequencies, the number of animals which under HWE would be expected for each genotype was calculated as shown in Table 3.

Since the critical value of $\chi^2_{0.01,3} = 11.345$ it can be concluded that the H-W expected genotype frequencies are not present in the studied population.

The expected (*He*) and the observed (*Ho*) heterozygosity were calculated as follows:

He = 1 - (0.341 + 0.113 + 0.006) = 0.54, and

Ho = 0.29 + 0.1 + 0.06 = 0.45

The fixation index (*F*) was calculated as:

$$F = \frac{0.54 - 0.45}{0.54} = \frac{0.09}{0.54} = 0.17$$

This value indicated moderate (17%) heterozygosity deficiency relative to HW expectations.

DISCUSSION

One of the major effects of the milk protein polymorphism on cattle traits with economic interest is their influence on milk renneting capability and yield during cheese production. The κ-CN fraction, located mostly on casein micelle surface is the specific substrate of the chymosin, the hydrolytic enzyme that has the crucial role in initial phase of the cheese production - the rennet formation (25).

It has been reported that milk with *CSN3*BB* genotype had significantly higher casein content (26) and better milk rennet coagulation properties in terms of shorter rennet clotting time, higher curd firmness and higher cheese yield (20-32). These differences are related to the micelle size and the glycosylation degree of the coded protein (33).

The primers that were designed in this study successfully amplified a fragment of 458 bp from the fourth exon of the bovine *CSN3* gene that included

the nucleotide substitutions that differentiate the three investigated alleles. Genotype determination was achieved in all animals of the investigated population. For RFLP genotyping of the six genotypes, it was necessary each PCR product to be digested with the *Hin*dIII enzyme which enabled distinction of *CSN3*A* and *CSN3*B* alleles, but could not make distinction between alleles *CSN3*A* and *CSN3**E. For that purpose, it was necessary to further digest those samples carrying the allele *CSN*3 A* (genotypes AA and AB) with *Hae*III which has one cleavage site for the alleles *CSN*3 A* and *CSN*3 B* and two cleavage sites for allele *CSN*3 E*.

Similar approaches for *CSN3* genotyping have also been used previously with different restriction enzymes (1, 34-43).

In this study, the *CSN3*A* allele was found to be more commonly distributed (0.584) than the *CSN3*B* allele (0.336), while the *CSN3*E* allele was observed with lowest frequency (0.08). These results are in accordance with the previously published studies in which in HF cattle population in different countries the *CSN3*A* allele (genotypes AA and AB) have been more frequently observed than the *CSN3*B* allele (genotype BB) as summarized in Table 4.

With the exception of the Jersey cattle (26, 45, 52) and the Brown Swiss cattle (26) in which the *CSN3*B* allele has been reported to be more common, the *CSN3*A* variant tends to be predominant in most dairy breeds (16-18).

Moreover, some less-common *CSN3* alleles might affect milk rheological properties. For instance, Erhardt et al. (53) reported that in the Pinzgauer breed the *CSN3*G* allele had negative effect on milk coagulation properties. Similarly, Caroli et al. (54) and Jensen et al. (25) detected a negative effect of *CSN3*E* on milk coagulation properties in the Italian and Danish Holstein-Friesian populations, respectively.

It is worth noting that the majority of the previously published studies are concerned about discriminating *CSN3*A* and *CSN3*B* alleles in different cattle populations, while only a few of them deal with detection of other alleles of the *CSN3* locus such as Pacheco Contreras et al. (2), Barroso et al. (55).

The frequency for the *CSN3*E* allele of 0.08 found in this study is identical with the results for this allele reported by Jann et al. (56) and lower than Boetcher et al. (57) who reported a frequency of 0.32. Soria et al. (43), in the Argentinian Holstein population reported a frequency of 50 % AA, 40 % AB and 10% AE κ-CN genotypes.

Table 4. Relative distribution of the *CSN3* alleles and genotypes in different HF populations

Country	Number of animals	Alleles (%)		Genotypes (%)			Reference
		A	B	AA	AB	BB	
Macedonia	172	72,7	27,3	54,66	36,04	9,3	Tanaskovska et al., 2016
China	203	69	31	55,1	28,6	16,3	Ren et al., 2011
	398	86	14	73,7	24,6	1,7	Ju et al., 2011
Iran	247	59	41	18	82	-	Doosti et al., 2011
	102	83	17	68,63	28,43	2,94	Toorchi et al., 2006
Slovakia	210	83,33	16,67	69,52	27,62	2,86	Miluchova et al., 2018
Canada	1714	-	-	60,09	37,22	2,69	Ng-Kwai-Hang et al., 1991
Croatia	130	76,4	23,6	62,86	27,14	10,00	Ivankovic et al., 2011
Egypt	20	-	-	85	15	-	Galila et al., 2008
Argentina	360	76	24	-	-	-	Bonvillani et al., 2000
Romania	20	65	35	40	50	10	Ilie et al., 2007
Serbia	420	51	49	25	52	23	Lukac et al., 2013
Indonesia	342	64	36	31	65	4	Anggraeni et al., 2010
Russia	72	83	17	68,9	28,2	2,9	Alipanah et al., 2005
Poland	304	83	17	71	23	6	Sitkowska et al., 2009

In our opinion, besides detecting variants *CSN3*A* and *B*, it is important to genotype at least for those alleles of this locus that were reported to have negative effects on some milk properties in different cattle populations (25, 53, 54). Furthermore, it would also be more informative approach, whenever possible, to use direct sequencing of the *CSN3* gene such as Schlieben et al. (58) or Chen et al. (59) since this method enables discovery of new nucleotide variations while PCR-RFLP analysis is limited only to those that have already been reported.

Although κ-CN is the most important factor in the renneting process, interactions with other milk protein variants have to be considered. For instance, Comin et al. (60) reported that *CSN3* and *CSN2* are strongly associated with milk coagulation traits and milk and protein yields, respectively, and concluded that for coagulation time and curd firmness, the best composite genotypes were those with at least one *B* allele at both loci.In addition, because positive correlations have also been demonstrated between β-LG BB genotype and higher cheese yield and casein number (reviewed by Buchberger and Dovc; 20) these authors conclude that κ-CN B and β-LG B are the most advantageous variants with respect to milk's cheese making ability, and they propose that due to the tight linkage that exists between the casein loci, a more extensive study of their haplotype effects is needed.

In this study a departure from the HW equilibrium and moderate heterozygosity deficiency was observed for the investigated genotypes of the *CSN3* locus. This could be due to genetic drift as a result of finite population size, population subdivision or due to non-random mating or inbreeding.

CONCLUSION

The primers designed in this study successfully amplified a 458 bp fragment of the fourth exon of the bovine *CSN3* gene which harbours the nucleotide variations among the three *CSN3* alleles *A*, *B*, and *E*. In the studied population five out of six possible genotypes were identified and departure from the HW equilibrium was observed. Also, a moderate heterozygote deficiency was detected. The allele *CSN3*A* was the most commonly distributed followed by the *CSN3*B* while the *CSN3*E* allele was observed with the lowest frequency. Nevertheless, the *CSN3*B* allele was present with relatively high frequency which should be used to positively select for its carrier animals, since increasing its frequency could help to improve the rheological properties of the milk intended for cheese production.

CONFLICT OF INTEREST

The authors declared that they have no potential conflict of interest with respect to the authorship and/or publication of this article.

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