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Assessment of polymorphism on kappa-casein gene of Anatolian water buffalo breed using the PCR–RFLP method

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Abstract: Water buffalo breeding is practiced in various provinces of Turkey, and is also included in the National Breeding Project. It was aimed in this study to investigate polymorphisms on exon 4–intron 5 and exon 4 only of the kappa-casein (CSN3) gene for milk yields of water buffalo bred in Sivas Province. Blood samples were taken from 135 water buffaloes. The phenol/chloroform method for DNA extraction was conducted on collected blood. Polymerase chain reaction (PCR) was used to amplify CSN3-specific regions, and amplified products were run using 2% agarose gel electrophoresis (AGE). Various restriction endonucleases (RE) were utilized in respect to their protocols to investigate polymorphisms in amplified products. Following the RE digestion, mixtures were run in 2.5% AGE. It was observed that only the BB genotype was obtained in terms of Hinf I enzyme; however, the E allele (Hae III) was not obtained for the CSN3 gene. For the Hind III enzyme, only the BB genotype was obtained from exon 4 of the CSN3 gene, while both AB (27.41%) and BB (72.59%) genotypes were determined from exon 4–intron 5 of the CSN3 gene. No statistical difference was detected for genotypes of the restriction enzymes used in Anatolian water buffaloes ($P > 0.05$). In conclusion, the Hinf I/BB genotype, associated with higher milk protein amount and milk fat, was observed in Anatolian water buffaloes.

Key words: Anatolian buffalo, CSN3, milk protein, polymorphism, PCR–RFLP

1. Introduction

The water buffalo population of the world is reported to be 168 million; 95.83% (161 million) of this population is in Asia and 0.30% (500.000) is in Europe. India hosts approximately 56.5% (approximately 95 million) of the world's buffalo population and has the highest milk production (approximately 134 million tonnes) [1]. The reasons behind the gradual decrease in the water buffalo population in many countries (including Turkey) over the years include the preference for the highly efficient Holstein cattle breed for breeding rather than these lower-yield animals. Additionally, the prevalence of mechanization in farming and market-related issues for water buffalo products have played roles in the stated decrease [1].

There are 2 main water buffalo breeds from the Bovidae family: Asian (*Bubalus bubalus*) and African (*Syncerus caffer*) buffaloes. Both water buffalo breeds have 2 subspecies: Asian water buffaloes are classified into swamp and river buffalo subspecies, and African water buffaloes are classified into cape (*S.c. caffer*) and forest buffalo (*S.c. nanus*) subspecies [2]. All water buffalo breeds in Near East countries and Europe are river buffaloes. Buffaloes raised in Turkey are called Anatolian water buffaloes; they are raised in small tied or paddock herds (83% of which consist of 15 animals; 17% of 8 animals on average) [1].

While water buffaloes are raised for milk, meat, skin, and labor-related purposes in other countries, they are raised for their milk and meat in Turkey. Meat and milk of water buffaloes have specific properties [3]. Water buffaloes raised in Turkey are regionally called Dombay, Camız, Camış, and Kömüş; only Anatolian water buffaloes are raised in Turkey. The appearance of Anatolian water buffaloes is similar to the appearance of water buffaloes raised in Mesopotamia. Anatolian water buffaloes were subjected to artificial insemination with sperm brought from Italy in 2002 [1]. While the amount of milk obtained from water buffaloes in Turkey was 69.401 tonnes in 2017, this amount reached 75.742 tonnes in 2018, an increase of 9.1%. The share of milk obtained from water buffaloes in total milk production was 0.3% in both years [4].

While the native distribution areas of Anatolian water buffaloes in Turkey are reported as Balıkesir, Isparta, Manisa, and Tekirdağ provinces [5], they are now being raised in all regions of Turkey within the scope of the National Breeding Project. Anatolian water buffalo has a combined yield characteristic (meat and milk yield).

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Turkish bow and ney materials are made from its horn; cream and mozzarella cheese are made from its milk; soudjouk is made from its meat due to its colour [6].

Six structural genes (alpha lactalbumin, beta lactoglobulin, and 4 casein genes) encode more than 95% of the proteins in ruminant milk. The 4 casein genes are located on the 6th chromosome in goats and cattle, and on the 7th chromosome in buffalo breeds, and have an effect on milk protein yield [7]. Kappa-casein (CSN3) contains 12% of the total casein proteins and consists of a total of 5 exons, but the majority of the gene coding is located in the exon 4 region of the gene [8,9]. Although the 6 alleles of the CSN3 gene are known, the most common ones are alleles A and B. Moreover, it has been reported that alleles C and E have also been detected [7,10,11]. It has been reported that while allele A is associated with low milk protein content but high milk yield, allele B is responsible for high milk fat and protein content and high milk quality but low milk yield. The genotype BB is responsible for genetic diversity related to high protein content [7,8,12]. In addition, it has been reported that allele E has a negative effect on the quality of milk protein [12]. There are differences in the 136th and 148th amino acid residues between the 2 variants (alleles A and B) of the CSN3 gene. In allele A, the 136th amino acid is threonine and the 148th amino acid is aspartic acid, while in allele B, the respective amino acid sequences are isoleucine and alanine [9,11]. While alleles A and B are identified by using Hind III and Hinf I restriction enzymes (REs), it is reported that these enzymes cannot be used for allele E; Hae III RE must be used to identify this allele [10].

It has been stated that the allele B variant of the CSN3 gene was obtained at a higher frequency than other alleles, and the frequency of genotype BB was observed more often in the progeny test studies. Therefore, bulls with the genotypes AB and BB have been recommended for use in commercial breeding, especially in artificial insemination procedures [11,13]. Hence, animals having allele B of the CSN3 gene should be given priority in molecular breeding of dairy breeds [8]. The importance of molecular studies related to yield has been accelerated. In one study, it was reported that CSN3 locus polymorphism could be investigated quickly and conveniently through the PCR–RFLP technique without the need for obtaining milk samples from lactating females [7].

A limited number of PRL [14,15], GH and GHR [16], and B-LG and CSN3 [17,18] polymorphism studies have been conducted on Anatolian water buffaloes raised in Turkey. However, more polymorphism studies are needed on Anatolian water buffaloes. The aim of this study was to investigate the CSN3 gene polymorphisms related to milk yield in Anatolian water buffaloes raised within the scope of the National Breeding Project in Sivas through the PCR–RFLP technique.

2. Materials and methods

In the sampling studies conducted on the water buffaloes used in the study, a total of 135 animals were randomly selected from 81 establishments in 27 villages of 8 districts of Sivas Province; all were outbred and born in 2014 and 2015. Blood samples were collected from the animals when they were 1–2 years of age; at the time of blood sampling, all of the animals were clinically healthy.

The standard phenol/chloroform method was used for DNA isolation from the blood samples [19]. To calculate the purity levels of the isolated DNA samples, optical densities (OD) of the samples at 260-nm and 280-nm wavelengths were calculated using a nano spectrophotometer. The DNA samples obtained were also run using 0.6% agarose gel, was observed in a gel imaging system under UV light.

The DNA samples were amplified through the polymerase chain reaction (PCR) method using the primer pairs synthesized for CSN3 (Table 1). PCR mixtures were prepared in 15-µL volumes. Each reaction was prepared as previously described [15]. A touchdown PCR protocol was used for amplified PCR mixtures [20]. Touchdown PCR protocol, digestion of RE, and fragments obtained from digestion were performed as previously described [15].

Table 2 shows the gene regions used to detect CSN3 polymorphisms, PCR fragment sizes, REs used, allele genotypes to be detected, and from which regions these genotypes were obtained.

For the product obtained as a result of the amplification of the 379 base pair (bp) region of the CSN3 gene containing exon 4 and a part of the intron 5 region, Hinf I, Hind III, and Taq I REs were used for the detection of alleles A and B; Hae III RE was used for the detection of allele E.

Genotypic structures and allele frequencies of the study samples were figured through gene counting. Polymorphism differences were computed using a chi-square test in the Microsoft Excel program.

The ethics committee report was obtained from Cumhuriyet University Animal Experiments Local Ethics Committee, with permission dated 19.06.2014 and numbered 65202830/122.

3. Results

The region of the CSN3 gene, which contains exon 4 (280 bp) and exon 4–a part of intron 5 (379 bp), was amplified through PCR for the detection of the alleles A, B (Taq I, Hind III, and Hinf I), and E (Hae III) (Figure 1). These PCR products were investigated in terms of polymorphisms by using different REs (Figures 2 and 3). According to the polymorphism obtained, the observed and expected allele genotypes and frequencies were calculated (Table 3).

As a result of the study, no allele E could be detected in any sample in the exon 4–intron 5 region from Hae III restriction enzyme digestion. In the same region, only

Table 1. Primer and restriction enzyme list used in the study.

Locus	Primer sequence **(5' –> 3')**	PCR (bp)	Restriction enzyme	Reference
CSN3 Exon 4–intron 5	F: CACGTCACCCACACCCACATTTATC R: TAATTAGCCCATTTCGCCTTCTCTGT	379	Hind III Hinf I Taq I Hae III	[11]
CSN3 Exon 4	F: CACGTCACCCACACCCACATTTATC R: CGTTGTCTTCTTTGATGTCTCC	280	Hind III	

F: Forward; R: Reverse; bp: base pair.

Table 2. Kappa-casein (CSN3) genotypes.

Gene	PCR (bp)	Restriction enzyme	AA (bp)	AB (bp)	BB (bp)	EE (bp)
Exon 4–intron 5	379	Hind III Hinf I Taq I Hae III	379 156, 132, 91 379 -	379, 223, 156 288, 156, 132, 91 379, 256, 123 -	223, 156 288, 91 256, 123 -	 225, 145, 67
Exon 4	280	Hind III	280	280, 180, 100	180, 100	

bp: base pair.

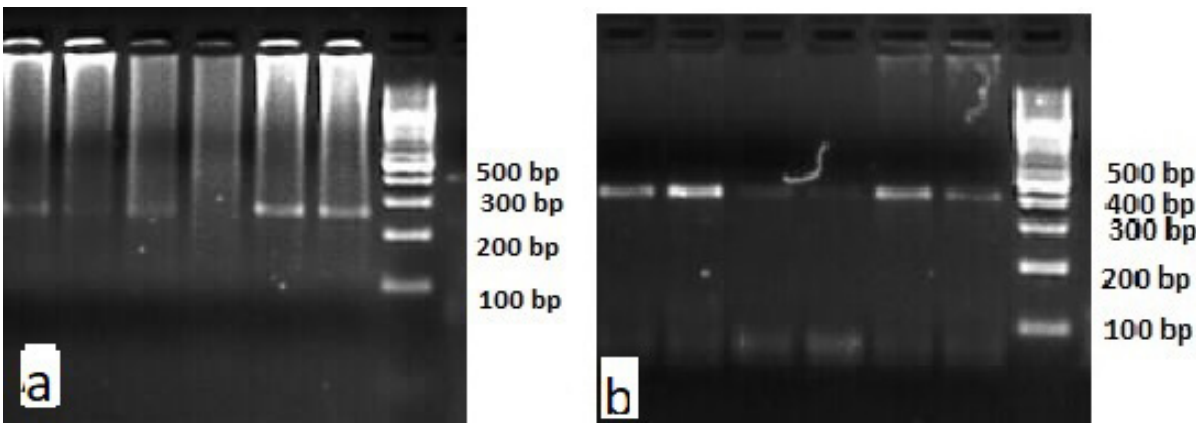

Figure 1. PCR result of the exon 4–intron 5 region of the kappa-casein gene in Anatolian water buffalo. A: 280-bp band; M: 100-bp DNA ladder. B: 379-bp band; M: 100-bp DNA ladder. bp: base pair.

genotype BB was found as a result of Hinf I restriction enzyme digestion, and genotype AA was found from Taq I restriction enzyme digestion. As for Hind III restriction enzyme digestion, only genotype BB was detected in the exon 4 region, but the genotypes AB (27.41%) and BB (72.59%) were obtained from the exon 4–intron 5 region. Furthermore, although the level of allele A was found to be 14% using Hind III enzyme digestion, no statistical significance was found in terms of the 4 enzymes in all samples ($P > 0.05$).

4. Discussion

The prime objective for dairy production is to find economical and feasible ways to increase milk production [21]. The utilization of polymorphic genes as genetic molecular markers is of particular importance as a selection principle in the determination of selection methods, especially in livestock. For this purpose, milk fat and its composition are important criteria in the selection of breeding animals. Regarding milk yield and quality, primary milk proteins include casein proteins

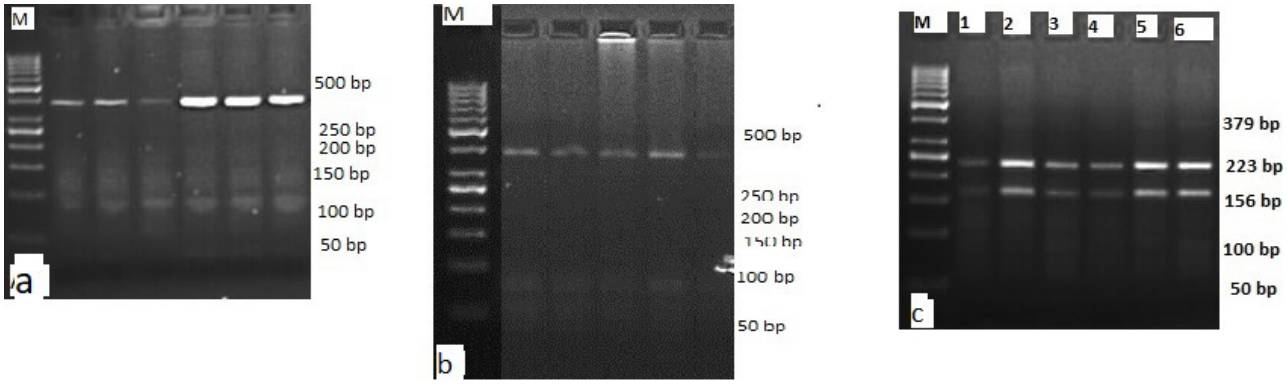

Figure 2. Enzyme digested result of the exon 4–intron 5 region of the kappa-casein gene in Anatolian water buffalo. A: Hae III restriction enzyme; M: 50-bp DNA ladder. B: Taq I restriction enzyme; samples with AA genotype, M: 50-bp DNA ladder. C: Hind III restriction enzyme; 1, 3, 4: BB genotype, 2, 5, 6: AB genotype, M: 50-bp DNA ladder. bp: base pair.

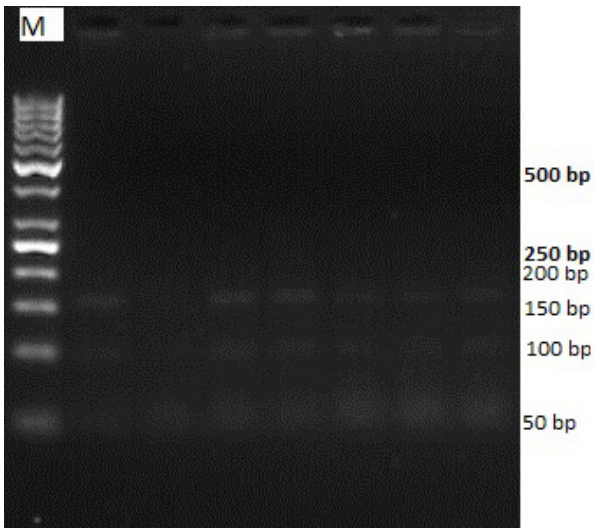

Figure 3. Hind III restriction enzyme digested result of the exon 4 region of the kappa-casein gene in Anatolian water buffalo. M: 50-bp DNA ladder; 1–7: BB genotype; bp: base pair.

(alfas1, alfas2, beta, and kappa-casein); the CSN3 protein is the most important one of these. The CSN3 locus has been reported to affect milk protein quality and the milk production framework [22]. Genotype polymorphisms of this gene have been studied in many countries [8,11,13,23–26].

It has been reported that Hind III and Hinf I REs should be used for alleles A and B of the CSN3 gene region, while Hae III RE should be used to determine allele E [10]. It is also reported that the allele B variant of the CSN3 gene has been obtained at a higher rate and higher frequency than other alleles, and that genotype BB has been higher in frequency in progeny testing. Bulls with genotypes AB and BB are recommended to be used in commercial breeding, especially in artificial insemination programs [11,13]. In addition, it is stated that animals having allele B of the CSN3 gene should be given priority in the molecular breeding of dairy breeds [8]. In this study, Hind III, Hinf I, Taq I, and Hae III REs were used to determine alleles A, B, and E of the CSN3 gene, and genotype BB was found to be the highest in frequency.

Although the frequency of alleles C and E has been reported to be less than 10% in water buffaloes, allele E was not detected in a previous study [11]. Similarly, no allele E was detected in this study.

As a result of the investigation of Hinf I polymorphism of the CSN3 exon 4 region in water buffaloes, only the genotype BB was detected in Egypt [13,27], India [7], China [25], and Pakistan [24]. In another study conducted on Indian buffalo breeds, genotype BB was detected at the rate of 98.4% and genotype AB was detected at the rate of 1.6% [23]. In a study conducted on Anatolian water buffaloes, only genotype BB and allele B were observed in all samples for CSN3 gene with Hinf I restriction enzyme [17]. Similarly, in this study, only genotype BB was detected as a result of Hinf I RE digestion in the CSN3 region of Anatolian water buffaloes.

As in studies conducted in different countries, only genotype BB was determined in terms of Hind III polymorphism in the exon 4 region of the CSN3 gene [9,11,13,26–28]. In a study conducted on Anatolian water buffaloes, only genotype BB and allele B were observed in all samples [18]. In another study conducted on Egyptian buffaloes and cattle, both genotypes BB and AB were determined; however, allele B was found to have a high frequency (0.88) [8]. In this study, 2 genotypes, BB (72.59%) and AB (27.41%), were detected as a result of Hind III RE digestion in the exon 4–intron 5 region; however, no statistical significance was found ($P > 0.05$).

In another study examining only the polymorphism of the exon 4 region of the CSN3 gene, only genotype BB

Table 3. Genotype and allele frequencies, their importance, and chi-square analysis of genotypes of the kappa-casein (CSN3) gene in Anatolian water buffalo.

Gene	n	Genotype frequency				Allele frequency			χ2	P-value (df = 1)
		AA	AB	BB	EE	A	B	E		
		O (E)	O (E)	O (E)	O (E)					
Exon 4–intron 5 (Hae III)	135	-	-	-	-	-	-	0	-	-
Exon 4–intron 5 (Hinf I)	135	0	0	135 (135)	-	-	1	-	0.00	1.0 ns
Exon 4–intron 5 (Hind III)	135	0 (2.54)	37 (31.93)	98 (100.54)	-	0.14	0.86	-	3.404	0.065 ns
Exon 4–intron 5 (Taq I)	100	100 (100)	0	0	-	1	0	-	0.00	1.0 ns
Exon 4 (Hind III)	135	0	0	135 (135)	-	0	1	-	0.00	1.0 ns

O: observed genotype; E: expected genotype; n: sample count; df: degree of freedom; χ2: chi-square value; ns: P > 0.05.

was determined as a result of Hind III RE digestion [13]. Similar to these results, genotype BB was detected in all samples, and polymorphism could not be detected in this study examining the same region.

In conclusion, it has been stated in the literature that for the CSN3 gene, higher milk yield coupled with lower milk protein content was related to the presence of the A allele; however, the presence of the B allele has been reported to be related to higher milk fat, protein content, and higher milk quality coupled with lower milk yield. In addition, genotype BB is responsible for genetic diversity related to high protein content [7,8,12]. It can be stated that the allele B variant of the CSN3 gene was obtained at a higher frequency than other alleles, and the frequency of genotype BB was higher in the progeny test studies. Therefore, it is suggested that bulls with the genotypes BB and AB be used in commercial breeding, especially in artificial insemination programs [11,13]. Furthermore, animals having allele B of the CSN3 gene should be given importance in molecular breeding [8]. In light of this information, since only the genotype BB and the allele B were obtained in Anatolian water buffaloes in this study, it is possible to state that these water buffaloes have high milk quality as well as high milk protein and fat content.

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