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Molecular characterization of Kappa-casein and β-lactoglobulin genes in Anatolian Black cattle and Holstein breeds

[*Caracterização molecular dos genes Kappa-casein e β-lactoglobulina em gado negro anatoliano e raças Holstein*]

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ABSTRACT

κ-Cn and β-lactoglobulin are important candidate genes associated with milk yield and milk protein content. The present investigation is carried out to determine the polymorphisms status of κ-Cn and β-lactoglobulin genes in Anatolian Black cattle and Holstein breeds. PCR-RFLP technique was used to determine Kappa-Casein and β-lactoglobulin polymorphisms in both cattle breeds. The allele frequency of Anatolian Black cattle in terms of κ-Cn and β-lactoglobulin genes were 0.50 (A) 0.50 (B) and 0.20 (A) 0.80 (B) respectively, whereas in Holstein were 0.29 (A) 0.71 (B) and 0.44 (A) 0.56 (B) respectively. The chi-square test showed that each cattle breed was in Hardy–Weinberg equilibrium (P>0.05).

Keywords: κ-Cn; β-lactoglobulin; Anatolian Black; Holstein; polymorphism

RESUMO

κ-Cn e β-lactoglobulina são importantes genes candidatos associados à produção de leite e ao teor de proteína do leite. A presente investigação é realizada para determinar o status de polimorfismos dos genes κ-Cn e β-lactoglobulina nas raças Anatolian Black cattle e Holstein. A técnica PCR-RFLP foi utilizada para determinar os polimorfismos Kappa-Casein e β-lactoglobulina em ambas as raças de gado. A freqüência dos alelos do gado negro anatólio em termos de κ-Cn e β-lactoglobulina foi de 0,50 (A) 0,50 (B) e 0,20 (A) 0,80 (B) respectivamente, enquanto que em Holstein foi de 0,29 (A) 0,71 (B) e 0,44 (A) 0,56 (B) respectivamente. O teste do qui-quadrado mostrou que cada raça bovina estava em equilíbrio de Hardy-Weinberg (P>0,05).

Palavras-chave: κ-Cn; β-lactoglobulina; Anatolian Black; Holstein; polimorfismo

INTRODUCTION

The Anatolian Black (Turkish: Yerli Kara), also known as Native Black Cattle, is a breed of cattle that originated in Anatolia, now Turkey. They are used in dairy production, meat production, and as draught animals on small farms. It is hardy, disease-resistant and tolerant of poor care, meager diet and adverse climate conditions. Females weigh 200–300 kg and males weigh 300–400 kg. Cows can produce up to 1000–1100 kg of milk per lactation. Their lactation period can last from 240–260 days. The milk has a 4-5% fat content (Ozdemir et al., 2009; Yilmaz and Wilson, 2012). Milk is a highly nutritious liquid formed in the mammary glands of mammals that contains essential nutrients that the body needs to perform vital functions and is the main ingredient for dairy products. Genes and the environment are essential factors affecting milk production (Aytekin and Boztepe 2013; Şahin *et al.*, 2018; Rachagani and Gupta 2008). Milk proteins consist of whey and casein proteins. Casein constitutes about (80%) of milk protein and consists of αs1‑casein, αs2‑casein, β‑casein, and κ‑casein, while whey protein constitutes about (20%) of milk proteins and consists of α‑lactalbumin, β‑lactoglobulin (β-lag), and other proteins (Čítek *et al.*, 2019). Many studies reported that β‑lactoglobulin and κ-Cn are candidate genes that affect milk production in different cattle breeds (Botaro *et al.*, 2009; Dogru *et al.*, 2008; Doosti *et al.*, 2011; Selvaggi *et al.*,

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2014; Singh *et al.,* 2015; Zhang *et al.,* 2006). Casein contains around 75–80% of the total milk proteins (Azevedo *et al.,* 2008; Bhat *et al.,* 2016; Riaz *et al.,* 2012).

κ-CN is a single chain polypeptide that contains 196 amino acids with a molecular weight of 19.2 kDa and constitutes about 12% of the bovine casein. The κ-Cn acts as a stabilizer to prevent the pressure of calcium on the casein to ensure that the micelles retain their colloidal state (Azevedo *et al.* 2008; Deb *et al.,* 2014). Bovine κ-CN is located at chromosome 6 (6/BTA 6q31-33) and includes 5 exons and 4 introns with a total DNA fragment of 200kb (Barłowska *et al.,* 2012). κ-CN contains fourteen polymorphic variants and the A and B variants are the most frequent variants within fourteen variants (Djedovic *et al.,* 2015). A variant differs from B variant in two amino acids substitution at positions 136 (threonine to isoleucine) and 148 (aspartic acid to alanine) (Azevedo *et al.* 2008; Alexander *et al.,* 1988). A variant is associated with milk yield and lower milk protein content; however, B variants are associated with higher fat percentage, milk protein contents and lower milk yield (Barbosa *et al.,* 2019).

β-lactoglobulin is the major whey protein of cattle milk and it is also present in many mammalian species, but it is absent in human milk (Crowther *et al.,* 2016; Sawyer and Kontopidis 2000; Xiang *et al.,* 2019). The bovine β-lactoglobulin gene is located at chromosome 11 and contains 7 exons and 6 introns with approximately 4.7kb (Riaz, 2013). Detected fifteen polymorphic variants of β-lactoglobulin gene have two most frequent variants A and B. The difference between A and B variants are two amino acid substitutions; Val118Ala (V118A) and Asp64Gly (D64G) (Aschaffenburg and Drewry 1955; Farrell Jr *et al.,* 2004; Martin *et al.,* 2013; Zaglool *et al.,* 2016). Several studies have described an association between A allele with higher milk production and higher milk protein, whereas B allele with higher fat content, increased cheese yields, total solids, and casein (Botaro *et al.,* 2008; Heydari *et al.,* 2009; Karimi *et al.,* 2009; Yang *et al.,* 2012).

This investigation aims to determine the genetic characterization of κ-Cn and β-lactoglobulin genes in Anatolian Black cattle and Holstein breeds.

MATERIAL AND METHODS

In the present study, a total of 200 animals (Anatolian Black cattle n= 100; Holstein Friesian n=100) belonging to two different breeds were used. Samples were collected from Ankara and Konya city/Turkey.

The blood was collected from the tail vein and stored at -20°C. Genomic DNA was extracted from whole blood by using the Quick Gene DNA whole blood kit S (DB-S) (KURABO, Japan). The primer sequences and PCR conditions are given in (Table 1). The PCR was achieved in a reaction volume of 10µL containing 1 µL DNA, 5µL of 2X Dream Taq Green PCR Master Mix (Thermo Scientific, USA), 0.30µL for each primer (10µ mol) (Macrogen, Turkey) and 3.4µL distilled water.

Table 1. The primer sequences, position, PCR fragment sizes and the annealing temperatures for two genes

Primer code	primer sequences	PCR bp	Sources	PCR conditions	RE
κ-Cn	F/5’-TGTGCTGAGTAGGTATCCTAGTTATGG-3’ R/5’GCGTTGTCTTCTTTGATGTCTCCTTAG-3’	453	Barroso *et al.,* (1998)	95°C 5m, 95°C 10s, 62°C 20s, 72°C 30s, 35 cycles 72°C 5m	*HinfI*
β-lactoglobulin	F/5’-CGCTCCCCACCCCGTCCTCACC-3’ R/5’-CCCGTCCCCAGTCACCCACAGG-3’	434	Zhao *et al.,* (1999)	94°C 3m, 94°C 30s, 60°C 40s, 72°C 1m, 39 cycles 72°C 10m	*HaeIII*

PCR products were digested with a fast digest enzyme (Thermo Scientific), which contains 5µL PCR product, 8.5µL distilled water, 1µL 10X buffer, and 0.5µL restriction enzyme (total of 15µL). Digestion products were separated on 3% agarose gel at 85 V for 50min, in 0.5X TBE buffer stained by ethidium bromide with used 100bp plus DNA marker (Vivantis, Malaysia). The results were checked under ultraviolet light.

The allele and genotype frequency of the genes and the Chi-square test χ^2 were calculated by popgen32 (ver.1.32).

RESULTS

A 453 bp PCR fragment of the κ-Cn gene was amplified and the PCR product was digested with *HinfI* restriction enzyme (Figure 1). Three genotypes (AA, AB and BB) were determined after digested by *HinfI* enzyme. The fragment lengths were 326 bp for genotype AA; 426 bp for genotype BB; 426 and 326 bp for genotype AB. Allele and genotype frequencies were 0.50(A) and 0.50(B); 0.30(AA), 0.40(AB) and 0.30(BB) in Anatolian Black. Whereas was 0.29(A) and 0.71(B); 0.50(AA), 0.41(AB) and 0.09(BB) in Holstein. χ^2 test showed that cattle breeds in Hardy–Weinberg equilibrium (P>0.05) (Table 2).

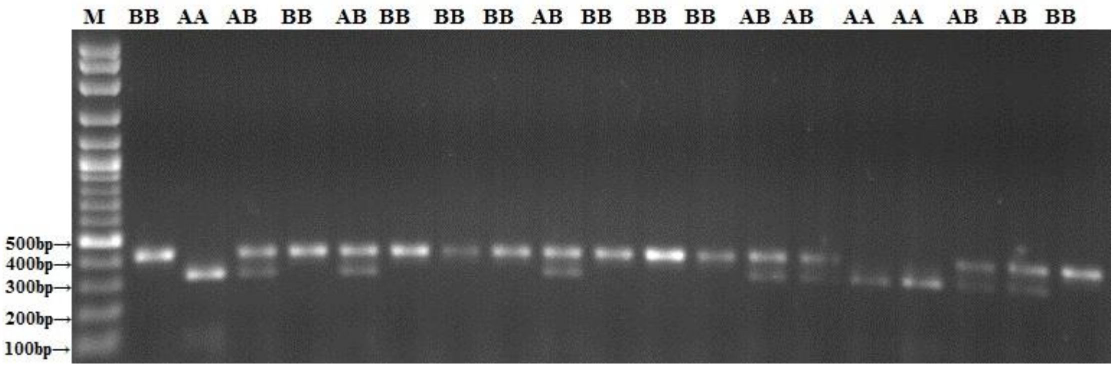

Figure 1. Agarose gel electrophoresis of digested products of κ-Cn gene with *HinfI* restriction enzyme, 326 bp for AA genotype; 426 bp for BB genotype; 426 and 326 bp for AB genotype.

Table 2. Anatolian Black cattle and Holstein genotypic distribution of the κ-Cn and β-lactoglobulin

Cattle breed	N	Allele Frequency		Genotype frequency			χ^2
		A	B	AA	AB	BB	
Allele and genotype frequencies for κ-Cn gene							
Anatolian Black	100	0.50	0.50	0.30	0.40	0.30	2
Holstein	100	0.29	0.71	0.50	0.41	0.09	4.84
Allele and genotype frequencies for β-lactoglobulin gene							
Anatolian Black	100	0.20	0.80	0.020	0.36	0.62	0.78
Holstein	100	0.44	0.56	0.26	0.36	0.38	3.63

x^2;test of Hardy-Weingberg equilibrium; (P>0.05)

A 434 bp PCR fragment of the β-lactoglobulin gene was amplified and the PCR product was digested with *HaeIII* restriction enzyme (Figure 2). Three genotypes (AA, AB and BB) were identified after digested by *HaeIII* enzyme. The fragment lengths were 300, 113 and 21bp for AA genotype; 300, 226, 113 and 75bp for AB genotype; 226, 113 and 75 bp for BB genotype. Allele and genotype frequency was 0.20(A) and 0.80(B); 0.02(AA), 0.36(AB) and 0.62(BB) in Anatolian Black. While was 0.44(A) and 0.56(B); 0.26(AA), 0.36(AB) and 0.38(BB) in Holstein. X^2 test showed that cattle breeds in Hardy–Weinberg equilibrium (P>0.05) (Table 2).

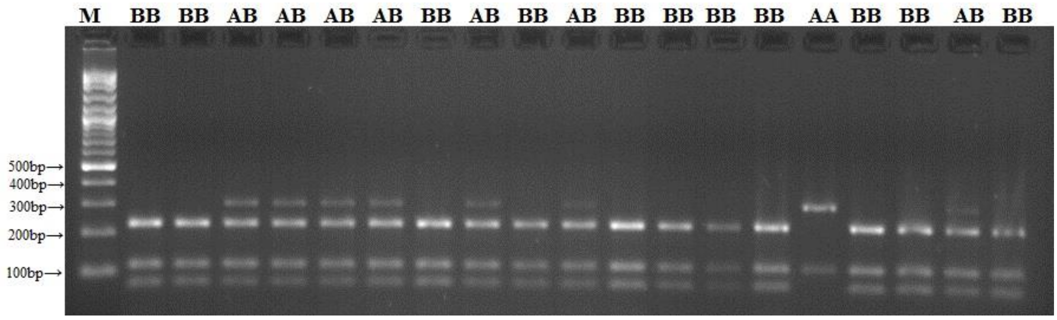

Figure 2. Agarose gel electrophoresis of digested products of β-lactoglobulin gene with *HaeIII* restriction enzyme, 300, 113 and 21bp for AA genotype; 300, 226, 113 and 75bp for AB genotype; 226, 113 and 75bp for BB genotype.

DISCUSSION

The A and B variants of the κ-CN gene are more frequent than other variants. The A variant is associated with milk yield and lower milk protein content; whereas, the B variant with higher fat percentages, milk protein contents, and lower milk yields. The genotype frequency for the κ-CN gene in the Anatolian Black cattle were 0.30 (AA), 0.40 (AB), and 0.30 (BB), with allele frequencies 0.50 for A and 0.50 for B. In contrast, the genotype frequency in Holstein breeds were 0.50 (AA), 0.41 (AB), and 0.09 (BB), with an allele frequency 0.29 for A and 0.71 for B.

Zhang *et al.* (2006) showed two genotypes AA and AB with genotype and allele frequency as 0.67, 0.32; 0.83(A) and 0.16(B). Whereas, no significant association was observed between polymorphism in the κ-CN gene and milk traits in Chinese Holstein. Rohallah *et al.*, (2007) detected allele and genotype frequency as 0.63(A), 0.36(B); 0.40(AA), 0.47(AB), and 0.12(BB) in the Sistani cattle breed. Azevedo *et al.* (2008) identified three genotypes AA, AB, and BB with genotype frequencies 0.45, 0.50 and 0.05; 0.74 0.25 and 0.01; 0.67 0.30 and 0.03 respectively in Sindhi, Guzerat Sires and Nellore. In contrast the allele frequencies of A and B for three cattle breeds were 0.70, 0.30; 0.86, 0.14 and 0.82, 0.18 respectively. Two genotypes AA and AB were identified in Gyr sires and Gyr x Holstein F1, with genotype and allele frequencies 0.88 and 0.12; 0.64 and 0.26; 0.94, 0.06, and 0.82 0.13 respectively. Riaz *et al.* (2012) revealed two genotypes (AA and AB) in the Cholistani, Sahiwal and Red Sindhi cattle breeds with frequencies of 0.80, 0.20; 0.39, 0.61; 0.71, 0.29 respectively, while the A and B allele frequency were 0.69, 0.90; 0.86, 0.31; 0.10, 0.14 respectively. In the study of Farhadi *et al.,* (2014) two alleles were observed in Najdi cattle breeds, A and B, with frequencies of 0.54 and 0.46. Also, genotypes AA, AB, and BB with frequencies of 0.35, 0.37, and 0.27, respectively. Djedovic *et al.* (2015) showed three genotypes in Simmental, Crossbreds and Busha with genotype and allele frequency 0.42(AA), 0.47(BB) and 0.9(AB); 0.75(AA), 25.0(BB) and 0.0(AB); 0.41(AA), 0.50(BB) and 0.8(AB); 0.66(A), 0.33; 0.87(A), 0.12; 0.66(A), 0.33. In the same study, determining the effect of κ-CN genotype on the milk yield and fat milk yield ($P<0.05$), however, did not found any effect on the milk fat content ($P>0.05$). Maletić *et al.,* (2016) showed the genotype frequency for AA, AB, and BB as 0.31, 0.52, and 0.15, while the A and B allele frequencies as 0.57 0.42 in Holstein. However, the genotype frequencies of AA and AB were 0.44 and 0.55, while the A and B allele frequencies were 0.72 and 0.27 in Busha cattle. Barbosa *et al.* (2019) showed that the genotype AA, BB, and AB frequency was 0.73, 0.25, and 0.02, while the allele frequency of (A and B) was 0.86 and 0.14. Also, no association showed between κ-CN polymorphisms and milk yield at 305 days in Girolando cattle. The B allele frequency in the Holstein breeds was higher compared to the Anatolian black breeds. The results showed that Anatolian black breeds have a higher frequency than the Holstein breeds in the A allele. The x^2 test showed that two cattle breeds were in Hardy–Weinberg equilibrium ($P>0.05$). When comparing our results with the results of previous studies, it was shown that Anatolia black agreed with Najdi cattle (Farhadi *et al.* 2014). Holstein showed higher B allele frequency than previous studies. Several studies confirm an association between the β-lactoglobulin gene's polymorphism and the milk's cheese yield and chemical composition

(Barłowska *et al.* 2012). The current study identified three genotypes (AA, AB, and BB) for β-lactoglobulin in Anatolian black and Holstein breeds. The genotype frequency was 0.30, 0.40 and 0.30; 0.50, 0.41 and 0.09 respectively. The allele frequency (A and B) was 0.20 and 0.80; 0.44 and 0.56, respectively. Rachagani and Gupta (2008) observed the genotype frequency in two cattle breeds (Sahiwal and Tharparkar) were 0.03(AA), 0.27(AB), 0.69(BB); 0.02(AA), 0.73(AB), 0.24(BB), and allele frequency were 0.17(A) and 0.83(B); 0.39(A) and 0.61(B). Zhang *et al.* (2006) showed two alleles, A and B, in Chinese Holstein breeds with frequencies of 0.27 for A and 0.72 for B, and showed that the β-lactoglobulin gene effect on the fat and protein percentage was significant. Karimi *et al.* (2009) determined two genotypes AB and BB, in Najdi cattle breeds with frequency 0.17 and 0.82, respectively. The frequency of A and B were 0.08 and 0.91, respectively. Zaglool *et al.* (2016) reported a higher B allele 0.64 than A allele 0.36 in Holstein breeds and found that the genotype AA had more milk yield and protein, while the BB genotype recorded higher fat. Čítek *et al.* (2019) calculated allele frequency as 0.472 for A and 0.528 for B in Czech dairy cattle. Ferreira *et al.,* (2019) noted the presence of A and B alleles in crossbred dairy cattle with the frequencies ranging from 0.40 to 0.60. Pavlova *et al.,* (2019) identified two alleles in the Yakutian cattle breed: the frequency ranging from 0.20 to 0.61 for the A allele and 0.38 to 0.80 for the B allele. Also noted that animals with the BB genotype had the highest fat content of milk in Simmental and Kholmogory. In Anatolian black and Holstein breeds, the B allele showed higher frequencies than the A allele. Our results in terms of genotype and allele frequencies of β-lactoglobulin are consistent with previous studies' results.

Table 3. Distribution of Allele Frequency in κ-CN gene and β-lactoglobulin at some cattle breeds

	κ-CN gene		
References	Breed	Allele frequency	
Curi *et al.,* (2005)	Canchim	0.77(A)	0.23(B)
Zhang *et al.* (2006)	Chinese Holstein	0.83(A)	0.17(B)
Rohallah *et al.* (2007)	Sistani cattle	0.63(A)	0.37(B)
Sulimova *et al.,* (2007)	Kalmyk	0.68(A)	0.32(B)
Azevedo *et al.* (2008)	Sindhi	0.70(A)	0.30(B)
	Gyr	0.94(A)	0.06(B)
	Guzerat	0.86(A)	0.14(B)
	Nellore	0.82(A)	0.18(B)
Riaz *et al.* (2012)	Sahiwal	0.69(A)	0.31(B)
	Cholistani	0.90(A)	0.10(B)
	Red Sindhi	0.86(A)	0.14(B)
Farhadi *et al.* (2014)	Najdi cattle	0.54(A)	0.46(B)
Deb *et al.* (2014)	Frieswal heifers	0.79(A)	0.21(B)
Djedovic *et al.* (2015)	Simmental	0.67(A)	0.33(B)
	Crossbreds	0.87(A)	0.13(B)
	Busha	0.67(A)	0.33(B)
Lukač *et al.,* (2015)	Holstein	0.88(A)	0.12(B)
Maletić *et al.* (2016)	Holstein	0.57(A)	0.43(B)
	Busha	0.72(A)	0.28(B)
Ozdemir *et al.,* (2018)	Holstein	0.82(A)	0.18(B)
Tyulkin *et al.,* (2018)	Black Motley	0.77(A)	0.23(B)
Barbosa *et al.* (2019)	Girolando	0.86(A)	0.14(B)
β-lactoglobulin			
References	Breed	Allele frequency	
Zhang *et al.* (2006)	Chinese Holstein	0.27(A)	0.73(B)
Rachagani and Gupta (2008)	Sahiwal	0.17(A)	0.83(B)
	Tharparkar	0.39(A)	0.61(B)
Karimi *et al.* (2009)	Najdi cattle	0.09(A)	0.91(B)
Zaglool *et al.* (2016)	Holstein	0.36(A)	0.64(B)
Čítek *et al.* (2019)	Czech	0.47(A)	0.53(B)
Ferreira *et al.* (2019)	Crossbred	0.40(A)	0.60(B)
Pavlova *et al.* (2019)	Kholmogory	0.61(A)	0.39(B)
	Simmental Austrian selection	0.42(A)	0.58(B)
	Simmental local selection	0.29(A)	0.71(B)
Barbosa *et al.* (2019)	Girolando	0.52(A)	0.48(B)

CONCLUSIONS

This study determined a higher B Allele frequency in terms of κ-CN and β-lactoglobulin in Anatolian black and Holstein. B Allele can be used as a genetic marker in breeding programs to improve milk traits in Anatolian black and Holstein.

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