

Journal of the Hellenic Veterinary Medical Society

Vol 73, No 2 (2022)



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doi: [10.12681/jhvms.27018](https://doi.org/10.12681/jhvms.27018)

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To cite this article:

Şahin, Özcan, & Boztepe, S. (2022). The potential status of A1 and A2 variants of bovine beta-casein locus of some indigenous genetic resources reared in Turkey. *Journal of the Hellenic Veterinary Medical Society*, 73(2), 4211–4218. <https://doi.org/10.12681/jhvms.27018>

The potential status of A1 and A2 variants of bovine beta-casein locus of some indigenous genetic resources reared in Turkey

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ABSTRACT: The aim of this study was to determine the Beta-Casein gene (CSN2) polymorphism in four domestic cattle breed. The genomic DNA of 374 animals, including Anatolian Black (AB, n= 100), Eastern Anatolian Red (EAR, n= 100), Southern Anatolian Red (SAR, n= 87), Turkish Grey (TG, n= 87), were obtained, and C>A polymorphism in 67th amino acid in the 7th exons of β -casein gene was determined by *TaqI* enzyme with PCR-RFLP with method. The A1 allele frequency were determined in AB, EAR, SAR and TG as 0.200, 0.195, 0.190 and 0.201, respectively, while the A2 allele frequency were 0.800, 0.805, 0.810 and 0.799, respectively. A1 and A2 allele frequencies were generally calculated as 0.200 and 0.800, respectively. A1A1 genotypes in AB, EAR, SAR and TG breeds were 0.020, 0.070, 0.000 and 0.020, respectively. A1A1 genotypes were 0.360, 0.250, 0.380 and 0.360, respectively; A2A2 genotypes were determined as 0.620, 0.680, 0.620 and 0.620, respectively. The A1A1 genotype frequency has not been detected in the SAR breed. AB, EAR, SAR and TG cattle breeds were calculated as 0.360, 0.250, 0.379 and 0.356 for H_c ; 0.320, 0.314, 0.307 and 0.321 for H_e and 0.380, 0.320, 0.379 and 0.379 for PIC, respectively. EAR and SAR population were not found in Hardy-Weinberg equilibrium ($P < 0.05$). However, AB and TG populations were found in Hardy-Weinberg equilibrium in terms of β -casein gene ($P > 0.05$).

In conclusion, AB, EAR, SAR and TG cattle breeds having A2A2 genotype commonly reared in Turkey are satisfactory and could be used in selection programs as a domestic genetic resource for A2 milk production in the future.

Keywords: A1 Allele, A2 Allele, *Beta-Casein*, PCR-RFLP, Polymorphism.

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Date of initial submission: 12-05-2021
Date of acceptance: 01-08-2021

INTRODUCTION

During the last century in world, several indigenous breeds in livestock species underwent a consistent decline in numbers in order to meet the food demands such as especially milk and meat of the growing population, and some of these have endangered or extinct. Namely, the most significant threat to genetic diversity is the marginalization of traditional production systems and the associated local breeds, driven by the rapid spread of intensive livestock production systems (Signorelli et al., 2008; Aytekin et al., 2011; Fadhil et al., 2018; Aytekin et al., 2020). Actually, The reasons for depletion of native breeds includes crossbreeding with exotic breeds, economically less viable, loosing utility, reduction in herd size and the large scale mechanization of agricultural operation. The native breeds need to be conserved for genetic insurance in future, scientific study, as a part of our ecosystem, cultural and ethical requirements and for energy sources in future (Srivastava et al., 2019). For conservation of indigenous cattle breeds, genetic resources have been conserved in situ, ex situ in vivo and ex situ in vitro (Mapiye et al., 2019). The future strategy should be to combine genetic improvement and conservation. However, before recommending these programs, some clues about their sustainable use need to be identified for not only the endangered but also native breeds with distinctive characteristics (Signorelli et al., 2008).

The indigenous breeds of cattle possess various unique characteristics, which makes them well adapted to the different environments. One of them is that they have the highest frequency of the A2 allele of bovine beta-casein locus worldwide, according to our recent literature knowledge. Milk is the main food source of the offspring in both human and animals. The structure and quality of milk is extremely important for growth, development and health of mammals. Approximately 80% of the protein in cow's milk components consists of casein and 20% whey proteins (Martien et al., 1994; Niki et al., 1994; Shah, 2000; Naik et al., 2013). However, some researchers reported whey proteins as approximately 14% (McLachlan, 2001; Roginski et al., 2003; Sharma et al., 2013). Casein proteins weigh approximately 18-25 kDa (Priyadarshini et al., 2018). The β -casein, one of the milk proteins, consists of a total of 209 amino acids, and alleles encoding the β -casein protein are localized on the 6th chromosome in the bovine genome (Rijnkels, 2002; Farrell et al., 2004; Jaiswal and Sarsavan, 2013). β -casein makes up 25-35% of the total milk protein (Eigel et

al., 1984; Roginski et al., 2003). The most common forms of β -casein in dairy cattle breeds are A1 and A2 (Kamiński et al., 2007; Caroli et al., 2009), the difference between A1 and A2 variants is the change of only one amino acid in the chain at position 67 of the β -casein amino acid peptide in exon 7. At position 67 of the β -casein, the A1 variant contains histidine and the A2 variant contains proline. The codon coding for the A2 variant at amino acid 67 of the-casein gene is CCT, and the codon for the A1 variant is CAT (Groves, 1969; Roginski et al., 2003). This milk containing the proline amino acid in the 67th position of the-casein gene is defined as the original A2 milk, while the milk containing histidine amino acid is called A1 milk. During digestion of A1 milk, due to the presence of the amino acid histidine, it causes the release of a seven amino acid polypeptide called Beta casomorphine-7 (BCM-7). Although β casein A1 variant is responsible for many diseases such as Type 1 diabetes, autism, schizophrenia and heart diseases, it has been reported that A2 variant does not cause such diseases (Sun et al., 1999; Woodford, 2007; Mishra et al., 2009; Sodhi et al., 2012). While the genotypes of cattle producing A1 milk are A1A1 and A1A2, the genotype of cattle producing A2 milk is A2A2.

The aim of this study is to reveal β -casein polymorphism of the AB, EAR, SAR and TG breeds reared in Turkey, and also its potential to produce A2 milk from existing animals and help to develop strategies in this area.

MATERIALS AND METHODS

Experimental animals and Sample collection

A total of 374 whole blood samples of the animals such as AB (n: 100), EAR (n: 100), SAR (n: 87) and TG (n: 87) cattle were used for CSN2 gene in this study.

Blood samples of AB breed from Ankara province, EAR breed from Erzurum province, SAR breed from Hatay and Şanlıurfa province and TG breed from Balıkesir province in Türkiye were collected from cattle farms. Whole blood samples from each animal were taken into vacutainer tubes containing Ethylenediaminetetra-acetic acid (EDTA) from the *vena jugularis* and stored at -20°C until DNA extraction analysis.

DNA extraction and PCR-RFLP method

Genomic DNA from whole blood was extracted according to Quick Gene DNA whole blood kit S according to the kit procedures (DB-S; KURABO, JAPAN). The RFLP-ACRS method was used to deter-

Table 1. The primer sequences PCR conditions of CSN2 gene

Gene	Primer sequences	Position	Reference
CSN2	F/5'CCTGCAGAATTCTAGTCTATCCCTTCCCTGGGCCCATCG-3' R/5'GAGTCGACTGCAGATTTTCAACATCAGTGAGAGTCAGGCCCTG-3'	Exon 7	Lien et al. (1992)

mine for the C>T polymorphism on exon 7 of CSN2 gene. The primer sequences were used to amplify a DNA fragment of 251 bp from exon 7 of CSN2 gene (Lien et al., 1992) (Table 1).

The PCR reaction was performed in 10 µl reaction volume. The polymerase chain reaction comprised genomic DNA, 2 µmol L⁻¹ 5x HOT FIREPol® Blend Master Mix PCR Master Mix (5X; Solis BioDyne), 0.4 µmol L⁻¹ of each primer and 5.2 µmol L⁻¹ ddH2O in a 10 µl volume. The amplification was performed in a gradient thermal cycler (BIO RAD T100) using the following program: an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of 94 °C for 60 s, 63 °C for 60 s and 72 °C for 60 s. Final extension was at 72 °C for 10 min. The PCR products were digested with 0.6 U of *TaqI* fast digest restriction enzyme in 20 µl volume (Thermo Fisher Scientific). The restriction fragments were subjected to electrophoresis on 3% agarose with ethidium bromide gel in 0.5X TBE buffer and then visualized under UV light and

scored in a gel documentation system.

Statistical analysis

The Chi-square test whether the distribution of the genotype frequencies was in the Hardy-Weinberg equilibrium was carried out by using PopGene32 (ver.1.32) statistical program (Yeh et al., 1997). Polymorphism information content (PIC) was calculated for CSN2 according to Botstein et al. (1980) and Anderson et al. (1993).

RESULTS

β -casein Gene Exon 7 region *TaqI* Polymorphism in Domestic Breeds

In the present study, AB, EAR, SAR and TG breeds, a polymorphic structure has been determined with the *TaqI* restriction enzyme cut of the 251 bp region amplified with PCR in exon 7 region of the CSN2 gene. The PCR product and the genotype image resulting from the cutting are given in Figure 1

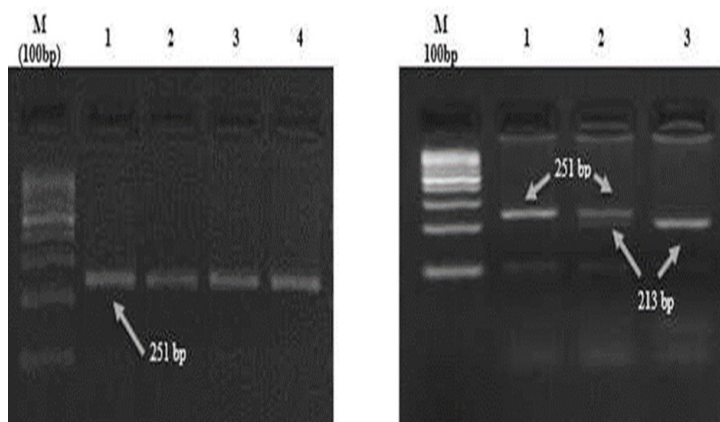


Figure 1. Agarose gel electrophoresis of PCR and digested products via *TaqI* restriction enzyme of CSN2 gene; M: 100bp Plus DNA Ladder (*Vivantis Technologies*), Left image Line 1-4: PCR cut products and Right image Line 1-3: PCR restriction cut the products, Line 1: A2A2: 251 bp, Line 2: A1A2: 251, 213 and 38 bp and Line 3: A1A1: 213 bp and 38 bp (38 bp cannot be seen on the gel)

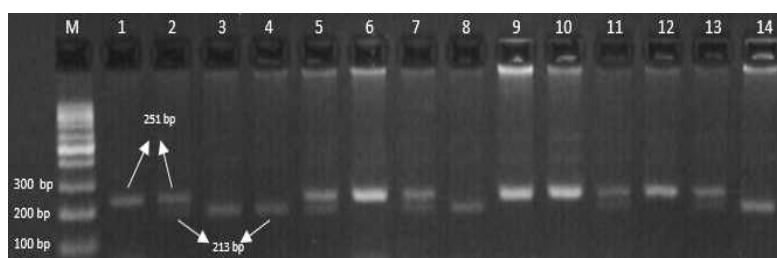


Figure 2. PCR products digested with *TaqI* on 3% agarose gel electrophoresis of CSN2 gene in AB, EAR, SAR and TG cattle breeds; Line 3,4,8 and 14: A1A1, Line 2,5,7,11 and 13: A1A2, Line 1,6,9,10 and 12: A2A2

Table 2. Genotype and allele frequencies of some domestic cattle breeds

Breeds	N	Allele Frequencies		Genotype Frequencies (n)			χ^2	H _o	H _e	PIC
		A1	A2	A1A1 (n)	A1A2 (n)	A2A2 (n)				
Anatolian Black	100	0.200	0.800	0.020 (2)	0.360 (36)	0.620 (62)	1.563*	0.360	0.320	0.380
Eastern Anatolian Red	100	0.195	0.805	0.070 (7)	0.250 (25)	0.680 (68)	4.149	0.250	0.314	0.320
Southern Anatolian Red	87	0.190	0.810	0.000 (0)	0.380 (33)	0.620 (54)	4.766	0.379	0.307	0.379
Turkish Grey	87	0.201	0.799	0.020 (2)	0.360 (31)	0.620 (54)	1.029*	0.356	0.321	0.379
Overall	374	0.197	0.803	0.030 (11)	0.330 (125)	0.640 (238)	1.272*	0.334	0.316	0.364

N: Number of experimental cows, χ^2 (HWE): Hardy-Weinberg equilibrium χ^2 value, H_o: Observed heterozygosity, H_e: Expected heterozygosity; PIC: Polymorphism information content, * P>0.05

and Figure 2.

The allele and genotype frequencies obtained through genotyping of *TaqI* polymorphism CSN2 gene in exon 7 region of Turkey domestic cattle breeds are shown in Table 2.

As can be seen from Table 2, A1 allele frequencies were found as 0.200, 0.195, 0.190 and 0.201 in AB, EAR, SAR and TG breeds, respectively. A2 allele frequencies were determined as 0.800, 0.805, 0.810 and 0.799, respectively. The breed with the highest A2 allele frequency was the SAR. As a result of the analysis made by considering the some domestic cattle breeds, A1 and A2 allele frequencies were generally calculated as 0.197 and 0.803 in overall, respectively.

The frequencies of A1A1 genotypes in AB, EAR, SAR and TG breeds were 0.020, 0.070, 0.000 and 0.020, respectively; A1A2 genotypes were 0.360, 0.250, 0.380 and 0.360, respectively; A2A2 genotypes were determined as 0.620, 0.680, 0.620 and 0.620, respectively. Accordingly, the highest A2A2 genotype frequency was found in EAR (0.680). The A1A1 genotype frequency has not been detected in the SAR breed.

EAR and SAR populations could not be found in Hardy-Weinberg equilibrium in terms of β -casein locus (P < 0.05). However, AB and TG populations were found in Hardy-Weinberg equilibrium in terms of β -casein locus (P > 0.05). AB, EAR, SAR and TG cattle breeds were calculated as 0.360, 0.250, 0.379 and 0.356 for H_o; 0.320, 0.314, 0.307 and 0.321 for H_e and 0.380, 0.320, 0.379 and 0.379 for PIC, respectively.

DISCUSSION

Polymorphisms in literature related to the allele and genotype frequencies of β -casein gene are given in Table 3.

In the present study, the mean value of A2 allele in all the animals was 0.803 and ranged from 0.799 (TG) to 0.810 (SAR). The mean value of A2A2 genotype was 0.640 in all the animals and ranged from 0.620 (AB, SAR and TG) to 0.680 (EAR). A1 and A2 allele frequencies were found in the range of 0.190 to 0.201 and 0.799 and 0.810 in AB, EAR, SAR and TG breeds, respectively. In overall, A1 and A2 allele frequencies were found as 0.197 and 0.803, respectively.

Dinç (2009) found that A1 and A2 allele frequencies were 0.125 and 0.781 in the AB breed, 0.080 and 0.880 in the EAR breed, 0.117 and 0.766 in the SAR breed, and 0.426 and 0.544 in the TG breed in Turkish indigenous cattle breeds. A1A1, A1A2 and A2A2 genotype frequencies were 0.062, 0.125 and 0.625, 0.00, 0.160 and 0.760, 0.00, 0.233 and 0.534 and 0.235, 0.382 and 0.324 in AB, EAR, SAR and TG breeds, respectively. While A1 and A2 allele frequencies in AB, EAR and SAR breeds in the present study were found to be compatible with Dinç (2009) study, the A1 allele frequency value in TG breed was found to be lower from Dinç (2009). However, the A2 allele frequency value was found to be higher in the current study.

As to genotype frequencies, the A1A1 (0.000) genotype frequency value in the SAR breed in the current study are compatible with the A1A1 (0.000) genotype frequency value in the SAR breed in the Dinç (2009). In addition, in the study of Dinç (2009), the A2A2 genotype frequencies such as 0.625 in AB, 0.760 in EAR and 0.534 in SAR were found to be compatible with this study results as 0.620 in AB, 0.680 in EAR and 0.620 in SAR cattle breeds, except 0.324 (Dinç, 2009) and 0.620 (present study) in TG breed.

Although there are breed differences, considering the domestic breeds in different literatures, A1 and A2 allele frequencies were found as 0.20-0.80 in Vechur breed (Muhammed and Stephen, 2012) and 0.20-0.80

Table 3. The allele and genotype frequencies of β -casein gene

References	Breeds/Cross breeds	Country	N	β -Casein					H_c
				Allele Frequencies		Genotype Frequencies			
				A1	A2	A1A1 (n)	A1A2 (n)	A2A2 (n)	
Dinç (2009)	Turkish Grey	Turkey	34	0.426	0.544	0.235	0.382	0.324	-
	Eastern Anatolian Red		25	0.080	0.880	0.00	0.160	0.760	-
	Anatolian Black		16	0.125	0.781	0.062	0.125	0.625	-
	Southern Anatolian Red		30	0.117	0.766	0.00	0.233	0.534	-
	Turkish Holstein		22	0.523	0.454	0.182	0.682	0.091	-
	Holstein Candidate Bulls		18	0.278	0.722	0.00	0.556	0.444	-
Muhammed and Stephen (2012)	Vechur	India	72	0.20	0.80	0 (0)	0.34 (29)	0.66 (43)	0.322*
	Crossbred cattle		100	0.46	0.54	0.32 (32)	0.28 (28)	0.40 (40)	0.497*
	Kasargode		14	0.39	0.61	0 (0)	0.79 (11)	0.21 (3)	0.477*
Sodhi et al. (2012)	Holstein	India	51	0.441	0.559	0.216 (11)	0.451 (23)	0.333 (17)	0.493*
	Jersey		40	0.325	0.675	0.025 (1)	0.600 (24)	0.375 (15)	0.439*
	Crossbred		89	0.298	0.702	0.101 (9)	0.393 (35)	0.506 (45)	0.418*
Ganguly et al. (2013)	Ongole Indian Zebu	India	38	0.06	0.94	0.00	0.11	0.89	-
	Frieswal HFxSahiwal Crossbred Heifers		124	0.32	0.68	0.12	0.40	0.48	-
	Frieswal HFxSahiwal Crossbred bulls		48	0.44	0.56	0.23	0.42	0.35	-
Navyashree (2014)	Malnad Gidda	India	40	0	1	0 (0)	0 (0)	1 (40)	-
	Holstein Friesian Crossbred		40	0.21	0.79	0.13 (5)	0.18 (7)	0.69 (28)	0.335*
Zepeda et al. (2015)	Mexican Jersey Cattle	Mexican	401	0.22	0.69	0.06	0.30	0.50	-
	Mexican Jersey Sires		52	0.12	0.86	0.02	0.20	0.73	-
Dar et al. (2018)	Garhwal Badri cattle	India	42	0.12	0.88	0	0.24	0.76	-
	Kumaon Badri cattle		48	0.13	0.87	0	0.25	0.75	-
Jawane et al. (2018)	Zebu Dangi	India	31	0	1	0	0	1	-
	Holstein Friesian crossbreds 75%		15	0.13	0.86	0.06	0.13	0.81	-
	Holstein Friesian crossbreds 62.5%		17	0.03	0.97	0	0.06	0.94	-
	Overall Zebu x HF crossbreds		32	0.08	0.92	0.03	0.09	0.88	-
Pandey et al. (2018)	Sahiwal	India	50	0.15	0.85	0 (0)	0.30 (16)	0.70 (34)	0.269*
	Holstein Friesian Crossbred		50	0.32	0.68	0 (0)	0.64 (32)	0.36 (18)	0.435*
Sodhi et al. (2018)	Ladakhi cattle	India	85	0.10	0.90	0	0.21	0.79	-
Firouzamandi et al. (2018)	Holstein	Iran	40	0.5250	0.4750	7.5	90.00	2.5	-
	Sarabi		38	0.5131	0.4868	15.78	71.05	13.15	-
	Gaja Native Breeds		13	0.5000	0.5000	0.00	100	0.00	-
Cieślińska et al. (2019)	Polish Red cows	Poland	177	0.63	0.37	36.7 (65)	52.5 (93)	10.7 (19)	0.466
	Polish Red bulls		24	0.42	0.58	12.5 (3)	58.3 (14)	29.2 (7)	0.486
Srinivas et al. (2019)	Deoni	India	12	0.29	0.71	0	0.58	0.42	-
	Sahiwal		12	0.37	0.63	0	0.75	0.25	-
	Malnad Gidda		10	0.20	0.80	0	0.40	0.60	-
	Holstein Friesian cross		12	0.50	0.50	0	1	0	-
Sebastiani et al. (2020)	Holstein Friesian	Italy	1629	0.3039	0.6065	0.988	0.3579	0.3696	-

*: values calculated from n.

in Malnad Gidda breed (Srinivas et al., 2019). To our knowledge of the recent literature on different indigenous breed, A2 allele frequencies were determined as 0.61 in Kasargode breed (Muhammed and Stephen, 2012), 0.94 in Ongole Indian Zebu (Ganguly et al., 2013), 0.88 in Garhwal Badri cattle and 0.87 in Kumaon Badri cattle (Dar et al., 2018), 0.85 in Sahiwal cattle (Pandey et al., 2018), 0.90 in Ladakhi cattle (Sodhi et al., 2018), 0.71 in Deoni, 0.63 in Sahiwal and 0.80 in Malnad Gidda cattle (Srinivas et al., 2019). As a result, it can be stated that the A2 allele frequency is high in these breeds in general, except for the Sarabi (Firouzamandi et al., 2018) and Polish Red (Cieślińska et al., 2019) breeds, and it is compatible with the current study results.

Mishra et al. (2009) stated that the A1 β -casein gene is less prevalent in cow milk of indigenous breeds, while the A2 allelic variant in cow milk is predominant in Indian Zebu cattle breeds with the highest frequency of 0.987. Navyashree (2014) reported that all animals in the Malnad Gidda cattle breed have the A2 allele. Similarly, Jawane et al. (2018) stated that there is no A1 allele in the Zebu Dangi breed. So, these breeds have great potential in terms of both A2 milk production and breeding programs. In the literature, the A1 allele ranges from 0.3039 (Sebastiani et al., 2020) to 0.5250 (Firouzamandi et al., 2018) in Holstein cattle and 0.22 (Zepeda et al., 2015) to 0.325 (Sodhi et al., 2012) in Jersey cattle. As for as A2 allele concerned, it ranges from 0.4750 (Firouzamandi et al., 2018) to 0.6065 (Sebastiani et al., 2020) in Holstein cattle and 0.675 (Sodhi et al., 2012) to 0.69 (Zepeda et al., 2015) in Jersey cattle.

In this study, the average genetic diversity (H_e) was 0.316. When it comes to heterozygosity within breed, this value is appropriate for the selection of the desired A2 allele in these breeds for A2 milk production, as they have values close to the maximum heterozygous level of 0.50. The heterozygosity and PIC values are a useful descriptive measure of the polymorphism of a marker locus, and also considered as a parameter of the variation (Aytekin et al., 2011). When attention is paid to heterozygosity, H_e values reported as 0.322 in Vechur breed (Muhammed and Stephen, 2012), 0.335 in Holstein Friesian crossbreed (Navyashree, 2014) and 0.269/0.435 in Sahiwal/Holstein Friesian crossbreed (Pandey et al., 2018). PIC, another measure used to assess the quality of a marker, values were found as 0.364 in overall. Bolstein et al. (1980) stated that PIC values were between 0.25 and

0.50 are moderate informative. Although only CSN2 marker was used in the current study, it provided good information about A1/A2 variation. When examining the literature on the reason for the difference in allele and genotype frequencies from population to population may be due to reasons such as selection, chance, migration and mutation. Genetic diversity is wealth in a population. However, in order to eliminate some health concerns in the CSN2 gene (Şahin et al., 2018), it is desired that the A2 allele be dominant in the population or selection in this direction in herd management trend in recent years.

According to Turkish Statistical Institute statistics, the total number of animals milked from culture breed, crossbreed and domestic breeds in 2018 is 6 337 907 heads and the amount of raw milk produced is around 20 036 877 tons. While the amount of milk produced from culture breed animals is 12 301 080 tons, the amount of milk produced from domestic breeds is 778 082 tons. In other words, 61% of the total raw milk produced is obtained from culture breed cattle, and approximately 4% is obtained from domestic cattle (Anonymous, 2020). In the present study, the average A2A2 genotype frequency of 374 domestic head cattle used as animal material is around 64%. This means that 778 082 tons \times 0.64 = 497 972 tons of raw milk produced from domestic breeds is produced as A2 milk. However, such a genotype definition cannot be made throughout the country and there is no such thing as A2 milk production. Since all the milk obtained is mixed, A1 milk obtained from cows with A1A1 and A1A2 genotype and A2 milk obtained from A2A2 genotype cows are unwittingly mixed and marketed. Therefore, all of the 497 972 tons of milk produced can be defined as A1 milk.

The strategies of transition to A2 milk according to the probability and sex of offspring that may occur in the mating of animals of different genotypes are summarized. Firstly, in case there are stages to be applied according to the genotypes of the male offspring to be obtained in selection, animals with A1A1 and A1A2 genotypes cannot be used for selection in the next generation that can be sent to slaughter at the slaughter age or after birth. If it has A2A2 genotype, it can be used in mating program in selection. Secondly, in case there are stages to be applied according to the genotypes of the female offspring to be obtained in selection, Based on the research results showing that BCM-7 is ineffective in the production process until the production of A2 milk from A1 milk obtained

from female animals with A1A1 and A1A2 genotype, these milks can be used for yoghurt and some cheeses (De Noni and Cattaneo, 2010; Nguyen et al., 2015). When the transition phase is completed, Animals with A1A1 genotypes cannot be used for selection in the next generation that can be sent to slaughter at the slaughter age. Animals with the A1A2 genotype can be left in herd to maintain the size of the herd until the goal is reached. In other words, the selection of animals with A2A2 genotype will lead directly to success in achieving the goal. Especially, this strategy will prevent the large dairy industry and breeders from being victimized during the transition to A2 milk.

CONCLUSION

The high frequency of A2A2 genotype in indigenous breeds is important in terms of showing that A2 milk production potential is high in these breeds. Considering that A2A2 genotypes also will be found in culture and culture crossbreds bred in Turkey, it can be said that there is A2 milk production poten-

tial with indigenous breeds with high frequencies and them. But, the disadvantages here is the low milk yield potential of indigenous breeds. Moreover, by making use of the available indigenous gene resources, creating populations that have such characteristics as resistance to diseases and adverse climate changes would be of great importance. As a result, AB, EAR, SAR and TG cattle breeds having A2A2 genotype commonly reared in Turkey are satisfactory and could be used in selection programs as a domestic genetic resource for A2 milk production in the future.

ACKNOWLEDGMENTS

We thank The Scientific Research Project Office of Selcuk University for supporting this study with no.19101001 project. This work was produced from PhD thesis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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