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Genetic variation at the *OLR1*, *ANXA9*, *MYF5*, *LTF*, *IGF1*, *LGB*, *CSN3*, *PIT1*, *MBL1*, *CACNA2D1*, and *ABCG2* loci in Turkish Grey Steppe, Anatolian Black, and East Anatolian Red cattle

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Abstract: Native breeds are excellent sources of genetic variation. Anatolian native breeds are relatives of the first cattle domesticated and are ancestors of many European breeds. Therefore, this study aimed to evaluate the genetic variation of *OLR1*, *ANXA9*, *MYF5*, *LTF*, *IGF1*, *LGB*, *CSN3*, *PIT1*, *MBL1*, *CACNA2D1*, and *ABCG2* markers in Turkish Grey Steppe, Anatolian Black, and East Anatolian Red cattle. The analysis included 367 cattle and the genotyping was performed by the PCR-RFLP. Population genetics indices including heterozygosity, the number of effective alleles, the polymorphism information content, fixation index, and the level of possible variability realization, and moreover, the genetic diversity parameters including Shannon–Weaver diversity index and Simpson dominance index were estimated. Hardy–Weinberg equilibrium was evaluated based on the number of individuals per genotype. Native breeds exhibited admissible population genetics and diversity levels. There was no animal with the *MYF5*-AA, *IGF1*-CC, *LGB*-BB, *CSN3*-BB, *CACNA2D1*-GG, and *ABCG2*-AC genotypes in the Anatolian Black breed. The frequencies of the genotypes/alleles favorable for milk production traits were remarkably low in all breeds. These findings could provide useful information on the genetic variation of Anatolian native cattle and the genetic investigations of resistance and health traits in bovine breeding and genetics.

Key words: Cattle, genetic variability, native cattle, polymorphism, population genetics

1. Introduction

Genetic diversity is one of the most important biological wealth of a country and is needed to meet not only the current but also future demands regarding milk and meat from various livestock species. In this context, Turkey contributes to the world's animal genetic resources with six different native cattle breeds, including South Anatolian Red, South Anatolian Yellow, East Anatolian Red, Turkish Grey Steppe, Anatolian Black, and Zavot [1]. Among these native breeds, East Anatolian Red is raised in a limited area of the east part of Turkey including Erzurum, Kars, and Ardahan provinces, as a dual-purpose breed [1–3]. Turkish Grey Steppe cattle is one of the most important native animal genetic resources of Turkey which is characterized by remarkably high resistance to infections and/or infestations and adaptability for surviving under harsh conditions [4]. Another native cattle breed is Anatolian Black with its distinctive completely black body color. Although this breed is originated from Middle Anatolia, Anatolian Black is raised almost across all parts of the country [1]. Turkey is one of the leading countries in terms of cattle presence but import practices that have been applied intensively in recent years have had significant negative effects on cattle breeding. In native cattle, genetic characterization through the evaluation of variation in major genes is important for molecular examination of the balance between economically important quantitative traits and traits such as resistance to disease or environmental conditions. On the other hand, Anatolian native breeds should be given priority as a genetic resource stock considering their closeness to the domestication center and being relatives of the first cattle domesticates [3].

Several genetic markers have been identified in the bovine genome, particularly those that are effective in economically important quantitative traits. In this respect, bovine chromosome 5 (BTA5) harbors quantitative trait loci (QTL) that influence many characteristics in a very wide perspective from milk yield to growth and development [5]. This genomic region consists of popular genes in livestock studies, such as oxidized low-density lipoprotein receptor 1 (*OLR1*), myogenic factor 5 (*MYF5*), and insulin-like growth factor 1 (*IGF1*). On the other hand, casein kappa (*CSN3*) and ATP binding cassette subfamily

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G member 2 (*ABCG2*) markers have similar phenotypic effects and they are located on BTA6 [6–8]. Genetic variations of the bovine pituitary-specific transcription factor (*PIT1*) gene that was mapped to BTA1 have been reported to be associated with production performance related to the synthesis of certain proteins and hormones [9,10]. Many other polymorphisms within the annexin A9 (*ANXA9*), lactoferrin (*LTF*), beta-lactoglobulin (*LGB*), calcium voltage-gated channel auxiliary subunit alpha2delta 1 (*CACNA2D1*) have been identified as candidate markers for milk production traits and also for somatic cell scores and/or mastitis resistance [6,11–13]. Furthermore, mannose-binding lectin (*MBL*) is one of the most important constituents of the innate immune system in mammals, and accordingly, bovine *MBL1* has been shown to be an indicative genetic marker for mastitis resistance traits in dairy cattle [14].

Especially in farm animals, purely yield-oriented breeding strategy, meaningless and unorganized crossing studies based only on phenotypic evaluation, caused the disregard of native breeds, which are low in yield but are important constituents of national biodiversity. This situation has caused many breeds to come to the border of extinction and even extinct without genetic characterization. In recent years, as in many other countries, some research projects and studies have been carried out for the protection of native breeds, but these studies have not been carried out stably in Turkey. It is clearly seen that the existing information and genetic datasets are quite inadequate. For this reason, molecular genetic studies should be carried out to protect national gene resources in Turkey. On the other hand, native breeds are capable of surviving in poor environmental and nutritional conditions and are highly resistant to diseases. Genetic studies in these animals can also provide data for the concept of "lifetime productivity", which is a very popular approach, especially in cattle breeding. Concerning the recent literature, genetic studies on native cattle breeds in Turkey are generally conducted using a limited number of genetic markers and a small number of animals, with some exceptions. It is worth noting that the selected markers in the present study have been previously studied in various breeds, especially in dairy cattle with the main focus on milk production traits. However, the genetic knowledge on these markers in Turkish native cattle breeds is rather limited and even there are no studies performed for some markers. Notably, Turkish Grey Steppe, Anatolian Black, and East Anatolian Red cattle are the relatives of many cattle breeds in Europe and/or contributed to their development [15,16]. Therefore, in this study, genetic variation in 11 popular gene markers, including *OLR1*, *ANXA9*, *MYF5*, *LTF*, *IGF1*, *LGB*, *CSN3*, *PIT1*, *MBL1*, *CACNA2D1*, and *ABCG2*, were assessed in a relatively large number of cattle consisting of Turkish Grey Steppe, Anatolian Black, and East Anatolian Red cattle breeds. An additional aim was to determine the frequency of favorable/unfavorable genotypes and/or alleles for milk yield/quality and to discuss their potential influence on disease resistance and health traits of native cattle breeds regarding the abovementioned genetic markers.

2. Materials and methods

2.1. Animals, sampling, and DNA extraction

A total of 367 cattle belonging to three different native cattle breeds of Turkey were used as animal material in this study. The population consists of 137 Turkish Grey Steppe, 105 Anatolian Black, and 125 East Anatolian Red male cattle from four different farms located in the Marmara region of Turkey. From the *vena jugularis* of each animal, approximately 4 mL of peripheral blood sample was taken into K_3EDTA vacuum tubes (Vacuette, Greiner bio-one-Frickenhausen, Germany) to be used in DNA isolation. The blood samples were stored at –20 °C until the DNA isolation process. The present study was performed complied with the relevant national regulations and institutional policies for the care and use of animals (Approval Number: 2010/6-05). Within the scope of the study, blood samples were taken from the animals only once and no other invasive procedures were applied. Genomic DNA was extracted using a standard phenol/chloroform/isoamyl alcohol (25:24:1) method as demonstrated by Green and Sambrook [17] with some modifications applied by the authors. Quantity (ng/µL) and purity (260/280 absorbance ratio) of isolated DNA samples were determined using a NanoDrop 2000c microvolume spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Approximately 50–90 ng/µL of pure DNA with a 260/280 value between 1.7 and 1.9 was used for PCR. The high amount of DNA samples were diluted and stored at –20 °C or included in PCR in lower volumes. Samples outside the acceptable purity ranges were reisolated.

2.2. Genotyping

Genotyping of the selected polymorphisms in 11 genes (*OLR1*, *ANXA9*, *MYF5*, *LTF*, *IGF1*, *LGB*, *CSN3*, *PIT1*, *MBL1*, *CACNA2D1*, and *ABCG2*) was performed by PCR-RFLP. The details of the selected genetic markers were shown in Table 1. For each locus, primer sequences (from 5′ to 3′) and PCR conditions were represented in Table 2. PCR amplifications were performed in a total volume of 25 µL using 2.5–3 µL of total purified DNA, 1 µL of forward and reverse primers each (0.5 µM), 12.50 µL PCR master mix (OneTaq Quick-Load 2x MM, New England BioLabs (NEB) Inc., Ipswich, MA, USA, Cat# M0486S or Thermo scientific PCR master mix (2X), Cat# K0171, New York, USA), 8 µL autoclaved Milli-Q water (Millipore, Bedford, MA, USA). Primers were purchased from Macrogen

Table 1. A brief description of the genetic markers considered in this study.

Locus symbol	Chromosomal location	GenBank Acc. No.	SNP location	Functional significance	Allele
OLR1	5	NM_174132	3'UTR	-	A/C
ANXA9	3	AY785287	Exon V	H84R[1]	A/G
MYF5	5	M95684	Intron II	-	A/G
LTF	22	NM_180998.2*	Intron VI		A/B
IGF1	5	AF210383	5´UTR	-	C/T
LGB	11	X14710	Exon IV	V118A[2]	A/B
CSN3	6	AY380229.1	Exon VI	A148I[3]	A/B
*PIT1***	1	Y15995.1	Exon IV	#	A/B
MBL1	28	AC_000185.1	Intron I	-	G/A
CACNA2D1	4	GU586866.1	Exon XXV	D688G[4]	A/G
ABCG2	6	JQ398798.1	Exon XIV	Y581S[5]	A/C

SNP: single nucleotide polymorphism.
[1]Histidine to arginine at amino acid 84.
[2]Valine to alanine at amino acid 118.
[3]Alanine to isoleucine at amino acid 148
[4]Aspartic acid to glycine at amino acid 688.
[5]Tyrosine to serine at amino acid 581.
*Supports all introns SAMN03145413, SAMN03145414 [ECO: 0000348].
**Also designated as the *POU1F1*.
#Silent mutation.

Company (Seoul, South Korea) or Thermo Fisher Scientific (Wilmington, DE, USA). For PCR amplification, three thermal cyclers were used including MyGenie 96 thermal block (Bioneer Corporation, South Korea), Palm Cycler GC1-96 (Corbett Research, Australia), and T100 Thermal Cycler (Bio-Rad, USA). PCR products were controlled using 2% agarose gel electrophoresis (migration for 1 h at 100 V). Following the PCR amplification stage, PCR products were digested with the corresponding restriction endonucleases (Table 2) by the same thermal cyclers. In this context, 0.50 µL of restriction enzyme was directly added to the PCR products (15 µL) and subjected to the corresponding incubation process (according to the manufacturer's suggestions for each restriction enzyme). Restriction enzymes were purchased from NEB (Ipswich, MA, USA). Banding was evaluated using a gel documentation and analysis system (DNR MiniLumi Bio-Imaging Systems, Israel).

2.3. Genotypic data analysis

Estimation of allele and genotype frequencies and the testing for departure from Hardy–Weinberg equilibrium (HWE) were performed by using the Cervus (v3.0) software. In this study, population genetics data was analyzed to determine population genetics indices and the genetic diversity among the selected cattle breeds. In this context, population genetics indices including heterozygosity (He), the number of effective alleles (Ne), and the polymorphism information content (PIC) were calculated using the formulae previously described by Nei and Roychoudhury [18] and Botstein et al. [19] presented as follows:

$$He = 1 - \sum_{i=1}^{n} P_i^2$$

$$Ne = 1/\sum_{i=1}^{n} P_i^2$$

$$PIC = 1 - (\sum_{i=1}^{n} P_i^2) - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2,$$

where P_i is the ith allele frequency, n was the allele number.

The fixation index (F_{IS}) was estimated as follows:

$$Fis = \frac{\text{Hthe} - \text{Hexp}}{\text{Hthe}},$$

where H_{the} is theoretical heterozygosity, H_{exp} is the experimental heterozygosity.

The level of possible variability realization (V%) was estimated based on Crow et al. [20] as follows:

Table 2. Primer sequences (from 5′ to 3′), PCR conditions, and restriction enzymes used for genotyping the polymorphisms in this study.

Gene	Primer sequence (5′→3′)	PCR conditions	Restriction enzyme
OLR1	F: TCCCTAACTTGTTCCAAGTCCT R: CTCTACAATGCCTAGAAGAAAGC[1]	94°C 5′ (94°C 30s, 62°C 30s, 72°C 40s) 30 cycles, 72°C 5′	*Pst*I
ANXA9	F: TCCCAGACCTTGTCATFTCC R: CTCCTGGGAATCAGTGTGGT[2]	95°C 3′ (94°C 45s, 55°C 45s, 72°C 45s) 30 cycles, 72° C 5′	*Nla*III
MYF5	F: ACAGCGTCTACTGTCCTGATG R: CGTGGTATATACTAAGGACAC[3]	94°C 4′ (94°C 30s, 58°C 1′, 72°C 1′) 38 cycles, 72° C 4′	*Taq*I
LTF	F: GCCTCATGACAACTCCCACAC R: CAGGTTGACACATCGGTTGAC[4]	95°C 4′ (95°C 1′, 58°C 1′, 72°C 1′) 30 cycles, 72° C 10′	*Eco*RI
IGF1	F: ATTACAAAGCTGCCTGCCCC R: ACCTTACCCGTATGAAAGGAATATACGT[5]	94°C 5′ (94 °C 1′, 64°C 1′, 72°C 1′) 31 cycles, 72°C 5′	*Sna*BI
LGB	F: TGTGCTGGACACCGACTACAAAAAG R: GCTCCCGGTATATGACCACCCTCT[6]	95°C 5′ (94°C 1.5′, 58°C 1′, 72°C 2′) 30 cycles, 72°C 10′	*Hae*III
CSN3	F: CACGTCACCCACACCCACATTTATC R: TAATTAGCCCATTTCGCCTTCTCTGT[7]	95°C 5′ (95°C 1′, 55°C 1′, 72°C 1′) 30 cycles, 72°C 10′	*Hin*dIII
PIT1	F: ACTCGCTATTACACAATAGGAGAGCCT R: TCCTGCCAACTCCTCACCTCCC[8]	94°C 5′ (94°C 30s, 62°C 30s, 72°C 30s) 30 cycles, 72°C 5′	*Hin*fI
MBL1	F: ACCTTGGGTCACCTGCAACAG R: GGTAGTTTAGGCAGCCCTAAAGC[9]	94°C 5′ (94°C 30s, 62.5°C 30s, 72°C 30s) 35 cycles, 72°C 8′	*Ava*II
CACNA2D1	F: GTTTCCACTACCTATGATTGC R: ACTGAACCAAGATTTGACCAC[10]	95°C 5′ (94°C 30s, 54°C 30s, 72°C 30s) 32 cycles, 72°C 10′	*Hae*III
ABCG2	F: AACAGCCTCAGCTCCAGAGAGATAT R: CGGTGAAGATAAGGAGAACATACT[11]	95°C 5′ (95°C 45s, 57.6°C 40s, 72°C 45s) 35 cycles, 72°C 5′	*Pst*I

[1]Komisarek and Dorynek [37]; [2]Kulig et al. [13]; [3]Ardicli et al. [5]; [4]Wojdak-Maksymiec et al. [33]; [5] Siadkowska et al. [28]; [6]Curi et al. [6]; [7]Soyudal et al. [8]; [8]Ozdemir [38]; [9]Yuan et al. [14]; [10]Zheng et al. [11]; [11]Sharma et al. [7].

$$V\% = \frac{1-E}{1-\frac{1}{N}} \times 100,$$

where E is the expected homozygosity, N is the number of individuals in a population regarding a particular locus.

The Shannon–Weaver diversity index (H') was calculated as follows:

$$H' = -\sum_{n=1}^{n} P_i^2 \ln P_i,$$

where P_i is the proportion of each species/taxa/allele in the population, and ln is the natural logarithm [21].

Simpson dominance index (D_2) was estimated as follows:

$$D_2 = 1/\sum_{n=1}^{n} P_i^2,$$

where P_i is the proportion of individuals belonging to species/taxa/allele i. [22].

3. Results

Genotyping results showed that at least two genotypes were observed in all genetic markers and breeds, except for the *CSN3* in East Anatolian Red and the *MBL1* in Anatolian Black cattle (Table 3). In this context, all East Anatolian Red and Anatolian Black were genotyped as the AA and the GG for the *CSN3* and *MBL1* markers, respectively. Minor allele frequencies ranged from 0.0571 to 0.4781 for the remaining 9 genes. The heterozygous genotype was predominant in *OLR1* and *CACNA2D1* for all three breeds. The breed with the highest rate of absence of certain genotypes was Anatolian Black. *MYF5* AA, *IGF1* CC, *LGB* BB, *CSN3* BB, *CACNA2D1* GG, and *ABCG2* AC genotype frequencies were 0 in this cattle breed. In addition, the *OLR1* CC genotype in Turkish Grey Steppe

Table 3. Genotypic and allelic frequencies of the studied gene loci in breed-specific evaluation. The symbols, 0 and +, indicate the corresponding alleles for each locus.

Locus	Allele		Breed	Number of cattle per genotype			Genotypic frequency (%)			Allelic frequency	
	0	+		00	0+	++	00	0+	++	0	+
OLR1	A	C	TGS	56	81	0	40.88	59.12	0	0.7044	0.2956
			AB	42	49	14	40.00	46.67	13.33	0.6333	0.3667
			EAR	22	69	34	17.60	55.20	27.20	0.4520	0.5480
ANXA9	A	G	TGS	8	83	46	5.84	60.58	33.58	0.3613	0.6387
			AB	30	59	16	28.57	56.19	15.24	0.5667	0.4333
			EAR	0	57	68	0	45.60	54.40	0.2280	0.7720
MYF5	A	G	TGS	14	65	58	10.22	47.45	42.34	0.3394	0.6606
			AB	0	50	55	0	47.62	52.38	0.2381	0.7619
			EAR	61	28	36	48.80	22.40	28.80	0.6000	0.4000
LTF	A	B	TGS	58	71	8	42.34	51.83	5.83	0.6825	0.3175
			AB	35	59	11	33.33	56.19	10.48	0.6143	0.3857
			EAR	65	52	8	52.00	41.60	6.40	0.7280	0.2720
IGF1	C	T	TGS	7	64	66	5.11	46.72	48.17	0.2847	0.7153
			AB	0	54	51	0	51.43	48.57	0.2571	0.7429
			EAR	5	29	91	4.00	23.20	72.80	0.1560	0.8440
LGB	A	B	TGS	34	75	28	24.82	54.74	20.44	0.5219	0.4781
			AB	37	68	0	35.24	64.76	0	0.6762	0.3238
			EAR	22	93	10	17.60	74.40	8.00	0.5480	0.4520
CSN3	A	B	TGS	107	25	5	78.10	18.25	3.65	0.8723	0.1277
			AB	87	18	0	82.86	17.14	0	0.9143	0.0857
			EAR	125	0	0	100	0	0	1	0
PIT1	A	B	TGS	34	58	45	24.81	42.34	32.85	0.4599	0.5401
			AB	19	40	46	18.10	38.09	43.81	0.3714	0.6286
			EAR	10	93	22	8.00	74.40	17.60	0.4520	0.5480
MBL1	A	G	TGS	15	46	76	10.95	33.58	55.47	0.2774	0.7226
			AB	0	0	105	0	0	100	0	1
			EAR	10	35	80	8.00	28.00	64.00	0.2200	0.7800
CACNA2D1	A	G	TGS	52	64	21	37.96	46.72	15.32	0.6131	0.3869
			AB	44	61	0	41.91	58.09	0	0.7095	0.2905
			EAR	40	67	18	32.00	53.60	14.40	0.5880	0.4120
ABCG2	A	C	TGS	90	17	30	65.69	12.41	21.90	0.7190	0.2810
			AB	99	0	6	94.29	0	5.71	0.9429	0.0571
			EAR	82	6	37	65.60	4.80	29.60	0.6800	0.3200

OLR1: oxidized low-density lipoprotein receptor 1, *ANXA9*: annexin A9, *MYF5*: myogenic factor 5, *LTF*: lactoferrin, *IGF1*: insulin-like growth factor 1, *LGB*: beta-lactoglobulin, *CSN3*: casein kappa, *PIT1*: pituitary specific transcription factor, *MBL*: mannose-binding lectin, *CACNA2D1*: calcium voltage-gated channel auxiliary subunit alpha2-delta 1, *ABCG2*: ATP binding cassette subfamily G member 2, TGS: Turkish Grey Steppe; AB: Anatolian Black; EAR: East Anatolian Red.

and *ANXA9* AA and *CSN3* BB genotypes in East Anatolian Red were absent. As shown in Table 3, the highest allele frequency was observed for the *ABCG2* gene in Anatolian Black cattle. This marker was also characterized by the most unbalanced genotypic distribution in Turkish native cattle breeds in this study.

HWE testing results are shown in Table 4. Deviations from HWE were observed for *OLR1*, *ANXA9*, *LTF*, *CSN3*, and *ABCG2* in Turkish Grey Steppe cattle. Moreover, the genotypic distributions of *MYF5*, *LGB*, *PIT1*, *MBL1*, and *ABCG2* in East Anatolian Red and *LGB*, *CSN3*, and *ABCG2* do not meet the HWE requirements. Ho was higher than 0.50 for all the genes and the breeds studied. He value ranges of 0.2228–0.4990, 0.1077–0.4911, and 0.2633–0.4954 were observed in Turkish Grey Steppe, Anatolian Black, and East Anatolian Red breeds, respectively. Ne approached 2.00 in *OLR1*, *ANXA9*, *LGB*, and *PIT1* genes (>0.95). The vast majority of the PIC values were higher than 0.32 (Table 4). Negative values of F_{IS} were estimated, except for the *MYF5*, *PIT1*, and *ABCG2* genes. The *V*% values ranged from 0.1471 to 0.48917. Concerning genetic diversity, the highest *H′* (1.08) and D_2 (2.91) values were estimated for the *PIT1* marker in Turkish Grey Steppe cattle.

Deviation from HWE was observed in all markers, except the *PIT1* gene, regarding the total population studied (n = 367). In this evaluation, in which breed-specific discrimination is ignored, heterozygous genotype was found to be predominant in the *OLR1*, *ANXA9*, *LGB*, *PIT1*, and *CACNA2D1* markers (Table 5). Notably, 319 animals were genotyped as the AA in bovine *CSN3,* and accordingly, the largest frequency difference between the two alleles (0.8556) was observed in this gene. Two alleles and three genotypes in each genetic marker were observed in the evaluation of the total cattle population (Table 5). Figure shows the levels of population genetics indices and the genetic diversity parameters in the total population. In this context, the lowest He was observed for the *CSN3* (0.1340) and this resulted in also the lowest genetic diversity, including *H′* (0.4316) and D_2 (1.301). The *PIT1* marker had the highest Ne (approximately 1.96) and PIC values (approximately 0.37).

4. Discussion

In many countries, especially in European countries, national native breeds are given great importance and genetic analyzes have been carried out using these cattle breeds. The oldest of the centers in which cattle were first domesticated contain the eastern and southeastern Anatolian regions. Previously published archeology and genetics papers have demonstrated that most animal breeds originated and spread from these regions to the rest of the world, especially from Anatolia to Europe [23,24]. For this reason, Anatolian cattle breeds are very important not only for Turkey but also for Europe. The genetic investigations in Anatolian-originated breeds can provide crucial clues for the population genetics and diversity dynamics of various cattle breeds because of their closeness to domestication centers. On the other hand, these breeds are generally characterized by a high tolerance for extremely harsh environmental climatic conditions and are highly resistant to various infections and infestations [25]. This makes them highly resistant to widespread diseases that cause serious economic losses in dairy cattle, such as mastitis and laminitis. Investigation of genetic variation in major genetic markers with proven effects on economically important traits in native breeds may contribute to the understanding of the genetic background of complex traits, such as disease resistance. In this study, we aimed to analyze the genetic variation in such important genes including *OLR1*, *ANXA9*, *MYF5*, *LTF*, *IGF1*, *LGB*, *CSN3*, *PIT1*, *MBL1*, *CACNA2D1*, and *ABCG2* in Turkish Grey Steppe, Anatolian Black, and East Anatolian Red cattle. These genes were chosen because of their previously confirmed effectiveness on milk production traits (both yield and quality). Furthermore, they are strong candidates for mastitis resistance through somatic cell scores. This is critical data that provide the corresponding genetic variance in native breeds which exhibit low production levels but high resistance to harsh conditions and diseases. Purely high production-oriented cattle breeding has allowed the frequency of favorable genotypes and alleles for high milk production to increase over the years primarily by phenotypic selection (through an indirect manner) and then by genomic selection in dairy herds. It is well known that the genetic relationship between milk yield and many health traits in dairy cows is antagonistic [26]. It can be postulated that native breeds that were not subject to high selection pressure may carry the unfavorable genotypes and/or alleles for milk production resulting in good adaptability to environmental conditions and superior health traits. Furthermore, health or resistance traits can correlate with each other via positive genetic correlations. To give an example, Heringstad et al. [26] indicated that selection against clinical mastitis is expected to result in some genetic improvement of resistance to the other diseases. These positive or negative correlations among phenotypic traits and the corresponding genetic variation may be one of the major factors leading to superior health and adaptability traits in native cattle breeds. In the present study, this interpretation was partially corroborated in certain Turkish native cattle breeds. BTA5 is one of the indicative genomic regions to harbor QTLs associated with milk components, especially for fat properties. Major genes including *OLR1*, *MYF5*, and *IGF1* were mapped

Table 4. Genotypic, allelic frequencies (%), and population genetic indices in the studied gene loci.

Gene locus	Breed	χ^2 (HWE)[1]	*P* (HWE)	He[2]	Ne	PIC	F_{IS}	*V*%	H′	D_2[3]
OLR1	Turkish Grey Steppe	-	0.0399*	0.4164	1.7241	0.3297	–0.9452	0.4091	0.6764	1.9490
	Anatolian Black	0.0024	0.9609	0.4645	1.8734	0.3566	–0.0548	0.4549	0.9908	2.5660
	East Anatolian Red	1.6321	0.2014	0.4954	1.9802	0.3727	–0.3928	0.4874	0.9879	2.4700
ANXA9	Turkish Grey Steppe	13.3934	0.0002***	0.4615	1.8546	0.3550	–0.7984	0.4542	0.8359	2.0860
	Anatolian Black	2.1818	0.1396	0.4911	1.9616	0.3705	–0.2013	0.4815	0.9685	2.4100
	East Anatolian Red	-	0.3711	0.3520	1.5485	0.2901	–0.6193	0.3440	0.0871	1.0360
MYF5	Turkish Grey Steppe	0.4615	0.4969	0.4484	1.8142	0.3479	–0.4495	0.4411	0.9507	2.4360
	Anatolian Black	-	0.6964	0.3628	1.5743	0.2970	–0.3782	0.3532	0.0965	1.0410
	East Anatolian Red	35.5555	0.0000***	0.4800	1.9231	0.3648	0.4166	0.4720	1.0440	2.7310
LTF	Turkish Grey Steppe	5.2507	0.0219*	0.4334	1.7705	0.3395	–0.6382	0.4261	0.8704	2.2360
	Anatolian Black	3.6231	0.0569	0.4739	1.9077	0.3616	–0.2397	0.4643	0.9264	2.3130
	East Anatolian Red	0.3177	0.5729	0.3960	1.6507	0.3176	–0.3131	0.3880	0.8808	2.2570
IGF1	Turkish Grey Steppe	2.9621	0.0852	0.4073	1.7001	0.3243	–0.5713	0.4000	0.8593	2.2280
	Anatolian Black	-	0.8453	0.3820	1.6255	0.3090	–0.4136	0.3724	0.6927	2.0180
	East Anatolian Red	1.7692	0.1834	0.2633	1.3676	0.2287	–0.1014	0.2553	0.6988	0.6988
LGB	Turkish Grey Steppe	1.2888	0.2562	0.4990	1.9968	0.3745	–0.5030	0.4917	1	2.5080
	Anatolian Black	-	0.0032**	0.4379	1.7705	0.3420	–0.5528	0.4284	0.6489	1.8550
	East Anatolian Red	31.4805	0.0000***	0.4954	1.9802	0.3727	–0.8772	0.4874	0.7278	1.7020
CSN3	Turkish Grey Steppe	4.4938	0.0340*	0.2228	1.2923	0.1980	–0.1220	0.2155	0.6243	1.5580
	Anatolian Black	-	0.0000***	0.1567	1.1959	0.1444	–0.1486	0.1471	0.4581	1.4020
	East Anatolian Red	-	NA[4]	-	-	-	-	-	-	-
PIT1	Turkish Grey Steppe	2.9923	0.0836	0.4968	1.9873	0.3734	–0.1674	0.4895	1.0750	2.9080
	Anatolian Black	3.5606	0.0591	0.4669	1.8734	0.3579	0.1432	0.4574	1.0390	2.7490
	East Anatolian Red	31.4805	0.0000***	0.4954	1.9802	0.3727	–0.8772	0.4874	0.7278	1.7020
MBL1	Turkish Grey Steppe	3.6138	0.0573	0.4009	1.6756	0.3205	–0.1474	0.3936	0.9355	2.3350
	Anatolian Black	-	NA[5]	-	-	-	-	-	-	-
	East Anatolian Red	4.2388	0.0395*	0.3432	1.5225	0.2843	–0.0198	0.3352	0.8441	2.0390
CACNA2D1	Turkish Grey Steppe	0.0319	0.8581	0.4744	1.9077	0.3619	–0.3491	0.4671	1.0110	2.6231
	Anatolian Black	-	0.1179	0.4122	1.7001	0.3273	–0.4798	0.4027	0.6800	1.9670
	East Anatolian Red	1.4116	0.2347	0.4845	1.9372	0.3671	-0.3828	0.4765	0.9779	2.4650
ABCG2	Turkish Grey Steppe	65.7801	0.0000***	0.4041	1.6756	0.3224	0.5793	0.3968	0.8675	2.0360
	Anatolian Black	-	0.0000***	0.1077	1.1271	0.1019	1′[6]	0.0982	0.2190	1.1220
	East Anatolian Red	98.9471	0.0000***	0.4352	1.7705	0.3405	0.8621	0.4272	0.7827	1.9370

OLR1: oxidized low-density lipoprotein receptor 1; *ANXA9*: annexin A9; *MYF5*: myogenic factor 5; *LTF*: lactoferrin; *IGF1*: insulin-like growth factor 1; *LGB*: beta-lactoglobulin; *CSN3*: casein kappa; *PIT1*: pituitary specific transcription factor; *MBL*: mannose-binding lectin; *CACNA2D1*: calcium voltage-gated channel auxiliary subunit alpha2delta 1; *ABCG2:* ATP binding cassette subfamily G member 2; χ2 (HWE): Hardy–Weinberg equilibrium χ2 value; He: gene heterozygosity; Ne: effective allele number; PIC: polymorphism information content; F_{IS}: fixation index; *V*%: level of possible variability realization; H′: the Shannon–Weaver diversity index; D_2: the Simpson dominance index.

*p < 0.01; **p < 0.01; ***p < 0.001 – not consistent with HWE.

[1]Fisher's exact test was used to evaluate the HWE because of the low number of individuals per genotype (genotype counts below 5).

[2]In diallelic loci, 1 - theoretical heterozygosity (H_{the}) = locus homozygosity (Ho).

[3]Reciprocal Simpson index values were calculated.

[4]Not applicable. The population is fixed and all East Anatolian Red cattle had the AA genotype for the *CSN3* locus. Hence, the population genetics and diversity parameters cannot be estimated.

[5]Not applicable. The population is fixed and all Anatolian Black cattle had the GG genotype for the *MBL1* locus. Hence, the population genetics and diversity parameters cannot be estimated.

[6]The experimental heterozygosity is 0.

Table 5. Allele and genotype frequencies for the 11 markers in the total sample of cattle (n = 367). The symbols, 0 and +, indicate the corresponding alleles for each locus.

Gene locus	Allele		Number of cattle per genotype			Genotypic frequency (%)			Allelic frequency		χ^2 (HWE)
	0	+	00	0+	++	00	0+	++	0	+	
OLR1	A	C	120	199	48	32.69	54.23	13.08	0.5981	0.4019	6.0015*
ANXA9	A	G	38	199	130	10.35	54.23	35.42	0.3746	0.6254	9.0678**
MYF5	A	G	75	143	149	20.44	38.97	40.59	0.3992	0.6008	12.9274***
LTF	A	B	158	182	27	43.06	49.59	7.35	0.6786	0.3214	6.8529**
IGF1	C	T	12	147	208	3.27	40.06	56.67	0.2330	0.7670	5.3511*
LGB	A	B	93	236	38	25.34	64.31	10.35	0.5750	0.4250	36.5664***
CSN3	A	B	319	43	5	86.92	11.72	1.36	0.9278	0.0722	5.7837*
PIT1	A	B	63	191	113	17.17	52.04	30.79	0.4319	0.5681	1.3458
MBL1	A	G	25	81	261	6.81	22.07	71.12	0.1785	0.8216	22.4539***
CACNA2D1	A	G	136	192	39	37.06	52.32	10.62	0.6322	0.3678	5.7255*
ABCG2	A	C	271	23	73	73.84	6.27	19.89	0.7698	0.2303	248.6989***

OLR1: oxidized low-density lipoprotein receptor 1; *ANXA9*: annexin A9; *MYF5*: myogenic factor 5; *LTF*: lactoferrin; *IGF1*: insulin-like growth factor 1; *LGB*: beta-lactoglobulin; *CSN3*: casein kappa; *PIT1*: pituitary specific transcription factor; *MBL*: mannose-binding lectin; *CACNA2D1*: calcium voltage-gated channel auxiliary subunit alpha2delta 1; *ABCG2:* ATP binding cassette subfamily G member 2; χ2 (HWE): Hardy–Weinberg equilibrium χ2 value; He: gene heterozygosity; Ne: effective allele number; PIC: polymorphism information content; F_{IS}: fixation index; *V*%: level of possible variability realization; H′: the Shannon–Weaver diversity index; D_2: the Simpson dominance index.
*p < 0.01; **p < 0.01; ***p < 0.001 – not consistent with HWE.

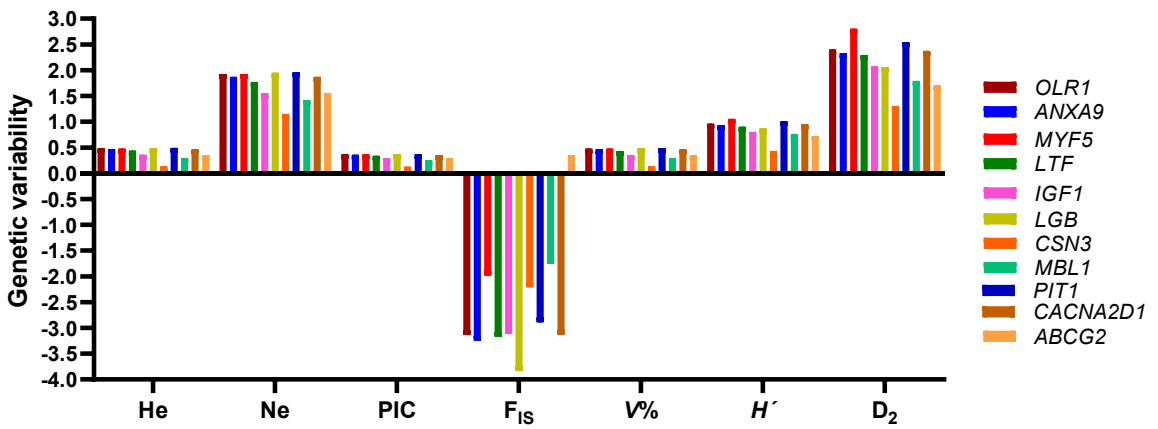

Figure. Genetic variability demonstrated by population genetics indices and genetic diversity parameters in the total sample of cattle (n = 367). He: gene heterozygosity; Ne: effective allele number; PIC: polymorphism information content; F_{IS}: fixation index; *V*%: level of possible variability realization; H′: the Shannon–Weaver diversity index; D_2: the Simpson dominance index.

to this chromosome. Concerning *OLR1*, the C allele was associated with a significant increase in milk fat production and fat percentage [27]. In this study, the frequencies of the CC genotype and C allele were remarkably low in both breed-specific and total population evaluation, except for East Anatolian Red cattle. Another gene located on BTA5, *IGF1*, was shown to be a strong candidate for many economically important traits. The C472T marker in this gene was associated with feed conversion for growth and the CC was the favorable genotype in this trait [28] and some other milk production traits. Among 367 cattle, only 12 animals were genotyped as the CC in this study.

Moreover, there was no CC genotype carrier in Anatolian Black cattle. In the *CSN3* gene (BTA6), the B allele of the A148I marker has been shown to be favorable for the processing properties of milk by Kucerova et al. [29] and high breeding value for protein content associated with the BB genotype. The frequencies of the B allele and the BB genotype were rather low in all breeds in this study. Furthermore, genotype AA (the frequency in this study: approximately 0.93) was associated with the low average breeding value for milk yield [29]. Another gene in BTA6 is *ABCG2* which affects fat and protein percentages. Olsen et al. [30] reported that the C allele of *ABCG2* Y581S has an extremely negative effect on fat and protein percentages and a positive effect on milk yield. This allele was found to be very low in both breed-specific and total population evaluation (Tables 3 and 5). The GG genotype in the *ANXA9* H84R marker was associated with higher fat milk content compared to AA and heterozygous animals [31]. Regarding the silent mutation in exon 5 of the bovine *PIT1*, Viorica [32] reported that there was a significant association between allele A and better milk performance in Simmental cattle. Cows with the BB genotype of the *LGB* were associated with a higher milk fat yield and percentage [6,8]. All the aforementioned genotypes related to many important milk production traits had prominently low frequencies in this study. On the other hand, Wojdak-Maksymiec et al. [33] reported that the lowest somatic cell score was found in cows with the AA genotype of *LTF* which indicates a potentially positive effect on mastitis resistance. The frequency of the AA genotype was approximately 43% and it was the predominant genotype in East Anatolian Red cattle. Moreover, the frequency of the A allele was approximately 0.68 in this study. LTF stimulates the immune system and serves as a natural antioxidant. It also plays an important role in the regulation of macrophages, lymphocytes, and neutrophil functions [33]. Accordingly, *LTF* may be considered as an indicative gene for disease resistance in native cattle breeds. Here, it should be noted that some genetic studies are contrary to our interpretations, and hence, further investigations at genome-wide levels should be performed to provide more confidential data on the genetic background of comparison between production and health traits. Moreover, allelic distributions are known to vary between breeds and even between different populations of the same breed [5]. This can also lead to conflicting results in the literature.

Population genetic parameters enable the evaluation of the variation and understanding of the determining power of the selected markers in the population. Thus, estimation of these parameters provides genetics researchers to evaluate the levels of selection pressure and eventual inbreeding. Compared to culture breeds, native cattle breeds are generally not subject to intense selection for economically important quantitative traits. This leads to the observation of admissible population genetic parameters in these breeds. This interpretation was substantiated in this study with some exceptions. The low genetic variability and unbalanced genotypic distribution in *CSN3* and *IGF1* genes resulted in inadequate levels of population genetic indices in all breeds. The vast majority of the genetic markers studied in this study exhibited PIC values higher than 0.30 and Ne values higher than 1.70 in breed-specific evaluation (Table 5). Consistent results were also observed in the whole population (Figure).

Although He and Ne express the effectiveness of loci allele impact in populations, PIC values are the most common indices to determine the extent of the polymorphism of a marker [5]. The classification of PIC has been widely performed using Botstein et al. [19] suggestions. In this context, PIC levels are categorized as follows: highly informative polymorphism with levels of PIC > 0.50, moderately informative polymorphism with levels of 0.25 < PIC < 0.50, and low informative polymorphism with levels of PIC<0.25. In this study, all of the markers were moderately informative polymorphism, except the *CSN3*. Furthermore, Ne values approached 2.00 in *OLR1*, *MYF5*, *LGB*, and *PIT1* markers in the total cattle population. Along with the population genetics indices, diversity levels are important indicators of analytical population dynamics. Although many parameters have been defined, the most common ones are H' and D_2. In this context, H' (also known as Shannon's index or Shannon entropy) can be utilized to describe variation at multiple levels of genetic organization from SNPs, through whole species or larger taxonomic units to ecosystems [34]. Despite the fact this parameter is negatively influenced when the sample size is small, it provides a more sensitive and informative evaluation compared to heterozygosity or the plain number of alleles [35]. Hence, Shannon's index is still widely used in genetics studies. In this study, admissible levels of diversity were estimated with some exceptions. On one hand, Turkish native cattle breeds are not suitable for high production breeding, and thus, the number of purebred individuals is constantly decreasing (despite the conservation efforts). On the other hand, these breeds have been raised with no (or very low) selection for quantitative traits which makes them able to maintain the genetic variability. This also enables them to adapt and survive in the harsh environmental conditions of the Middle, East, and South Anatolian regions [36].

This study presents the genetic variation results of 11 genetic markers, which are very popular in genetics studies on dairy cattle, in Turkish Grey Steppe, Anatolian Black, and East Anatolian Red cattle. It is worth noting that population genetics and genetic diversity of these genes in Anatolian cattle breeds are scrutinized for the first time. At

the same time, the frequencies of the desired genotypes of these markers in native breeds are also discussed. To the best of our knowledge, *ANXA9* and *CACNA2D1* genes were evaluated in Turkish native cattle for the first time. Notably, the present study is the first of its kind in native breeds which established comparative and comprehensive data in which population genetics and diversity parameters are evaluated together. Such knowledge is critical because as mentioned earlier Anatolian breeds are the ancestors of many cattle breeds in Europe [3]. Notably, unconscious crossbreeding and importation have resulted in a decrease or loss of diversity in native cattle breeds without genetic characterization [2]. This resulted in difficulties in finding purebred individuals. Moreover, these breeds are raised mostly in extensive or close to extensive conditions without data recording. Hence, we have used previously published papers to discuss the relationship between the genotypic/allele frequencies and the corresponding phenotypes. Taken altogether, the present results may be informative not only for further studies on the genetic variation of Anatolian native cattle breeds but also for the genetic investigations of resistance and health traits in dairy cattle.

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