UDC 575.
https://doi.org/10.2298/GENSR2302655A
Original scientific article

THE EFFECTS OF *MC4R* AND *CACNA2D1* GENE POLYMORPHISMS ON CARCASS TRAITS AND MARBLING SCORE IN TURKISH NATIVE CATTLE BREEDS AND THEIR CROSSBREDS WITH THE HOLSTEIN-FRIESIANS

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Ardicli S. and F. Alpay (2023). *The effects of MC4R and CACNA2D1 gene polymorphisms on carcass traits and marbling score in Turkish native cattle breeds and their crossbreds with the Holstein-Friesians.* - Genetika, Vol 55, No.2, 655-672.

Carcass and beef quality traits are economically important traits and are expressed by multiple genes. The effects of the *MC4R* c.856C>G and *CACNA2D1* c.2027A>G polymorphisms on carcass and meat quality traits are limited. Therefore, this study aimed at evaluating the association of bovine *MC4R* and *CACNA2D1* markers with carcass characteristics and meat quality. A total of 102 cattle including Turkish Grey Steppe, East Anatolian Red, Zavot, and their F_1 crossbreds with the Holstein-Friesians were genotyped using the PCR-RFLP method. The phenotypic traits measured were slaughter weight, hot carcass weight, chilled carcass weight, dressing percentage, chilling loss, carcass fatness score, carcass pH_{24}, and marbling score. Statistical analyses were performed using linear mixed models in the entire cattle population and also from a breed-specific aspect. Population genetics and diversity indices were also estimated. Results revealed that the genetic markers in this study are reasonably informative for the studied cattle population and exhibit an intermediate genetic diversity. Concerning the *MC4R* c.856C>G polymorphism, there was no significant association with any of the traits analyzed, indicating that this *MC4R* c.856C>G is not a desirable marker for carcass traits and marbling. Here, we report a novel association between the *CACNA2D1* c.2027A>G

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polymorphism and marbling score. The GG genotype was characterized by higher marbling scores (P<0.05). Bovine *CACNA2D1* is located on BTA 4, which consists of important QTLs for marbling, and hence, the evaluation of genetic markers within this genomic region may reveal novel genetic associations through meat quality.

Keywords: association analysis , carcass traits, cattle, *MC4R*, *CACNA2D1*

INTRODUCTION

Native cattle breeds have low levels of production characteristics, but these breeds are essential elements of biodiversity. The eastern and southeastern Anatolian regions are the oldest domestication centers where many breeds originated and spread from Anatolia to the rest of the world (ÖZŞENSOY *et al*., 2019). Thus, Anatolian cattle breeds have contributed to creation of different breeds, especially in Europe. Another critical point is that native breeds have high resistance to various infections and exo/endoparasites and exhibit remarkable adaptability to harsh environmental conditions (SOYSAL and KÖK, 2006). The genetic analysis of Anatolian cattle breeds may provide essential clues about molecular mechanisms underlying non-production traits such as health and survival traits. On the other hand, variation in major genetic markers also allows for valuable interpretations of both production and non-production traits in cattle breeding (COBANOGLU and ARDICLI, 2022).

Melanocortin-4 receptor (*MC4R*) encodes a G-protein-coupled receptor which is highly expressed in the hypothalamus (SEONG *et al*., 2012; YEO *et al*., 1998). The molecular mechanisms related to MC4R signaling pathways contribute to appetite regulation through leptin mediation, feeding, and energy homeostasis (SEONG *et al*., 2012; ZHANG *et al*., 2009). The bovine *MC4R* gene has been mapped to BTA 24q27 (HAEGEMAN *et al*., 2001). It has one exon and one transcript with a length of 1,794 bp (ENSEMBL GENOME BROWSER, 2022). Many nucleotide alterations have been identified within the *MC4R* including nonsense, missense, and frameshift mutations which were particularly associated with serum triglyceride levels, fat metabolism, feed intake, energy expenditure, and weight gain/loss (PRIHANDINI *et al*., 2019; SEONG *et al*., 2012; ZHANG *et al*., 2009). Unsurprisingly, variations in this gene have been previously associated with live weight (LIU *et al*., 2010; SEONG *et al*., 2012), carcass weight, backfat thickness, marbling score (LIU *et al*., 2010), daily gain (ZHANG *et al*., 2009), carcass grade fat, lean meat yield, longissimus dorsi measurements (MCLEAN and SCHMUTZ, 2011), and even calf's birth body length (MAHARANI *et al*., 2018). All of these functional traits are directly or indirectly related to energy and fat metabolism in which the bovine *MC4R* gene plays a significant role. Another important gene potentially effective on meat production traits is the calcium channel, voltage-dependent, alpha-2/delta subunit 1 (*CACNA2D1*). Because this gene has been mapped to BTA 4q18 (BUITKAMP *et al.*, 2003) which is a genomic region for some crucial QTLs associated with average daily weight gain, carcass weight (CASAS *et al*., 2000), meat/bone ratios (ASHWELL *et al*., 2005; GUTIÉRREZ-GIL *et al*., 2009), feed intake and feed conversion ratio (SHERMAN *et al*., 2009), marbling (YOKOUCHI *et al*., 2009), *longissimus dorsi* area (TAKASUGA *et al*., 2007). *CACNA2D1* encodes a functional protein involved in voltage-dependent calcium channel complex and this gene has been reported to be an important candidate gene for carcass and meat quality traits in cattle selection programs.

Even though domestic cattle breeds may not be ideal for production-oriented breeding, their genotypic variation offers significant insights into the genetic foundation of quantitative traits. Additionally, these breeds represent valuable components of biodiversity. As a result, conducting genetic studies on these breeds becomes crucial in safeguarding diversity at both the national and international levels. To date, SNPs of bovine *MC4R* and *CACNA2D1* genes have been associated with carcass characteristics and meat quality traits in various cattle breeds. However, the studies related to the effects of the c.856C>G and c.2027A>G polymorphisms in the *MC4R* and *CACNA2D1* genes, respectively, on carcass traits are rather limited. Moreover, the effects of these genes on beef yield and quality traits have not been reported in Anatolian native breeds, and therefore, were studied in the present study.

MATERIALS AND METHODS

Animals and sampling

A total of 102 bulls from a commercial farm were used in this study. The animals were randomly selected from three purebred native breeds (*n*=73) and their F_1 crossbreds with the Holstein-Friesians (*n*=29) including Turkish Grey Steppe, East Anatolian Red, and Zavot breeds. They were raised in semi-open free-stall barns with straw as bedding. All animals were subjected to the same environmental and feeding conditions according to the commercial farm's practices. Animals of similar weight and age were preferred. Cattle sent to slaughter due to health problems were excluded from the analysis. The average slaughter weight was 453±5.48 kg in the total cattle population.

The peripheral blood samples (~4 mL) were obtained using sterile K_3EDTA tubes (Vacutest Kima, SRL, Italy). The blood sampling was performed in the slaughterhouse and the samples were obtained from flowing blood during routine exsanguination. The procedures complied with the relevant national regulations and institutional policies for the care and use of animals. No invasive procedures were applied to animals for this study, and all sampling procedures were completed within the abattoir's routine arrangements. The blood samples were stored at -20°C until DNA isolation.

Carcass traits and meat quality analysis

All animals were slaughtered in the same abattoir according to the standard commercial routines. The carcass and meat quality traits measured were slaughter weight (SW), carcass weight (hot and chilled), dressing percentage (DP), chilling loss (CL), carcass fatness score (CFS), carcass pH, and marbling score (MS). The SW was recorded in the abattoir following a 12 h fasting. All cattle were slaughtered, exsanguinated, and suspended through the Achilles' tendons. Carcasses were electrically stimulated (60 V for 30s) and dressed according to the national regulations. Following non-carcass components were removed, hot carcass weight (HCW) was measured. The subcutaneous, kidney, and pelvic fat were included in HCW. Afterward, carcasses were chilled in a ventilated room for approximately 20 h at 4°C, and thus chilled carcass weight (CCW) was measured. DP was calculated based on both the HCW and CCW. CL was calculated by subtracting the HCW from the CCW. The CFS was determined based on a 1–5 classification as described by PIEDRAFITA *et al*., 2003. Ultimate pH was measured in the *m. longissimus thoracis*, between the 12^{th} and 13^{th} vertebra, using a portable digital pH

meter (Testo 205 pH-Temperatur-Messgerat, Lenzkirch, Germany) at 24 h postmortem. The device was calibrated to pH 4 and 7 using standard buffer solutions (Testo) at 25°C. Concerning MS, ten degrees of marbling were evaluated including 1=practically devoid, 2=traces, 3=slight, 4=small, 5=modest, 6=moderate, 7=slightly abundant, 8=moderately abundant, 9=abundant, 10=very abundant (MALHEIROS *et al*., 2020; AGRICULTURAL MARKETING SERVICE USDA, 2023). MS was visually graded regarding the cross-sectional area between the 12th and 13th ribs.

Genomic DNA extraction and the genotyping procedure

DNA was extracted from whole blood by the standard phenol-chloroform–isoamyl alcohol extraction protocol (GREEN and SAMBROOK, 2012). The amount and purity of the samples were measured using a NanoDrop 2000c (NanoDrop Technologies, Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). Samples with a concentration range of 45-90 ng/µL and the a 260/280 ratio of 1.6-1.9 were considered acceptable for further analyses.

In this study, g.59164671G>C polymorphism in the *MC4R* gene (c.856C>G) and g.38638971A>G polymorphism in the *CACNA2D1*gene (c.2027A>G) were evaluated (rs108968214 and rs448872602, respectively; ENSEMBL GENOME BROWSER, 2022). The locations of these two nonsynonymous alterations in the flanking sequence are shown in Figure 1.

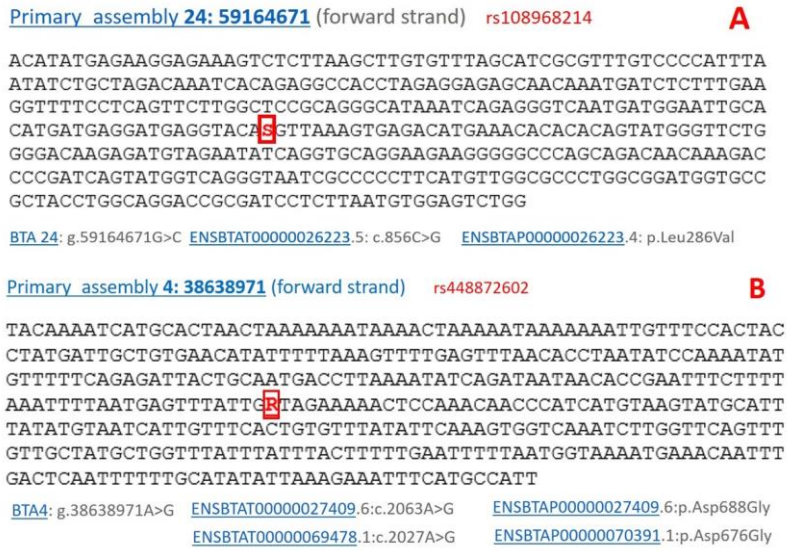

Figure 1. The locations of the polymorphisms studied in the 200 bp flanking sequence. (A) g.59164671G>C polymorphism (rs108968214) in the bovine *MC4R* gene (c.856C>G) (B) g.38638971A>G polymorphism (rs448872602) in the bovine *CACNA2D1*gene (c.2027A>G). The locations of the nucleotide changes in the corresponding transkripts and their amino acid alterations also were shown. The *MC4R* rs108968214 causes a leucine to valine (Leu286Val) whereas the *CACNA2D1* rs448872602 causes a Aspartic acid to Glycine (Asp688/676Gly) alteration (https://www.ensembl.org/index.html).

The genotyping of the bovine *MC4R* and *CACNA2D1* polymorphisms was performed by PCR-RFLP. From genomic DNA, the PCR was used to amplify the 493-bp and 249-bp DNA fragments for polymorphisms in the bovine *MC4R* and *CACNA2D1* genes, respectively. Regarding the *MC4R*, the primer sequences are as follows: 5′-GTCGGGCGTCTTGTTCATC-3′ for forward and 5′-GCTTGTGTTTAGCATCGCGT-3′ for reverse primers. The PCR protocol for the *MC4R* was 94°C for 5 min, followed by 35 cycles of 30 s at 94°C, 58°C annealing for 30s, 72°C extension for 30s, and a final step of 10 min at 72°C. Concerning the *CACNA2D,* the primer pairs were as follows: 5′-GTTTCCACTACCTATGATTGC-3′ for forward and 5′-ACTGAACCAAGATTTGACCAC-3′ for reverse primers. The PCR protocol for the *CACNA2D1* was 95°C for 5 min, followed by 32 cycles of 94°C for 30 s, 54°C annealing for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. PCR was performed in a 50 µL total volume containing 2.50 µL genomic DNA, 5 µL of Mg^{2+}-free 10× buffer, 5 µL of $MgCl_2$ (50 mM), 1 µL of Taq DNA polymerase (Finnzymes Oy; Thermo Fisher Scientific, Inc., USA), 1 µL of each primer (forward and reverse), 1 µL (2.5 mM) of dNTPs (Bio Basic Inc., Markham, Ontario, Canada), and 33.50 µL of nuclease-free water (Thermo Fisher Scientific, #R0581). Corbett GC1-96 PalmCycler (Corbett Research, Australia) was used for both DNA amplification and restriction enzyme incubations. PCR amplifications were controlled by 2% (w/v) standard agarose gels (Sigma Aldrich, Steinheim, Germany) stained with ethidium bromide (Sigma Aldrich). The PCR products (15 µL) were digested by the *Hpy*CH4IV and *Hae*III restriction enzymes (New England BioLabs) at 37ºC overnight for the *MC4R* and *CACNA2D1*, respectively. Digested fragments were visualized in 3% agarose gel electrophoresis and the banding was evaluated by a gel imaging system (DNR-Minilumi, DNR Bio-Imaging Systems, Israel). Electrophoregram results were interpreted based on the papers by PRIHANDINI *et al*. (2019) and HOU *et al*. (2010) for the *MC4R* and *CACNA2D1*, respectively.

Statistical analysis

The gene and allele frequencies were estimated as described by FALCONER and MACKAY, 1996. The Hardy–Weinberg equilibrium (HWE) was tested for both locus by using Pearson's chi-square or Fisher's exact test based on the number of animals per genotype. Population genetics parameters, including gene heterozygosity (He), polymorphism information content (PIC), and the effective allele number (Ne) were calculated as described by NEI and ROYCHOUDHURY (1974) and BOTSTEIN *et al*. (1980) by using the following formulas:

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

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

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

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

$$F_{IS} = \frac{\text{Hthe} - \text{Hexp}}{\text{Hthe}}$$

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

The level of possible variability realization (*V*%) was calculated according to CROW and KIMURA (1970) as follows:

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

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 (ORTIZ-BURGOS, 2016).

Association analysis was performed by the least-squares method as applied in a general linear model (GLM) procedure of Minitab (MINITAB®, Pennsylvania, USA, v17.1.0). The selected statistical model based on the adjusted R^2 values was as follows:

$$Y_{ijklmnop} = \mu + S_i + W_j + MG_k + CG_l + IG_m + e_{ijklmnop}$$

where Y_{ijklm} = phenotypic traits; μ = the overall mean; S_i = the fixed effect of slaughter season (i = summer, autumn, and winter); W_j = the fixed effect of the slaughter age (j = 17-20 m); MG_k = the fixed effect of the *MC4R* genotypes (k = CC, CG, GG); CG_l = the fixed effect of the *CACNA2D1* genotypes (l = AA, AG, GG); IG_m = the fixed effect of the genotypic interaction (*MC4R* × *CACNA2D1*); and $e_{ijklmnop}$ = the random error.

Tukey's test was used for post hoc analysis.

RESULTS

Genotypic distribution and the genetic diversity

In the total population, all three possible genotypes were observed for both genes. Table 1 shows the genotypic and allelic frequencies of the SNP L286V of the bovine *MC4R* gene in the studied breeds and the total cattle population. The GG genotype has a remarkably low frequency in all examined breeds. Moreover, this genotype was absent in Zavot cattle. Unsurprisingly, the C allele was predominant in both breed-specific and total population evaluations. Concerning *CACNA2D1*, the GG genotype was absent in the Turkish Grey Steppe, East Anatolian Red, and Zavot breeds. However, this genotype was predominant in Holstein × native cattle crossbreds (Table 2). In Zavot cattle, the heterozygous genotype was fixed. In the entire population, 37 and 51 animals were genotyped as the AA and the AG, respectively. As shown in Table 2, this resulted in a considerably high frequency of the A allele (0.61).

Table 1. Genotype and allele frequencies of the SNP L286V of the bovine MC4R gene in different breeds and the total sample of animals.

Breed	Individuals	Genotype frequencies (%)*			Allele frequencies	
		CC	CG	GG	C	G
Turkish Grey Steppe	28	28.57 (8)	57.14 (16)	14.29 (4)	0.5714	0.4286
East Anatolian Red	36	36.11 (13)	55.56 (20)	8.33 (3)	0.6389	0.3611
Zavot	9	55.56 (5)	44.44 (4)	0	0.7778	0.2222
Holstein × Native cattle crossbreds	29	68.97 (20)	27.59 (8)	3.44 (1)	0.8276	0.1724
Total	102	45.10 (46)	47.06 (48)	7.84 (8)	0.6863	0.3137

*The number of animals per genotype is presented in parentheses.

Table 2. Genotype and allele frequencies of the SNP A526745G of the bovine CACNA2D1 gene in different breeds and the total sample of animals.

Breed	Individuals	Genotype frequencies (%)*			Allele frequencies	
		AA	AG	GG	A	G
Turkish Grey Steppe	28	50.00 (14)	50.00 (14)	0	0.7500	0.2500
East Anatolian Red	36	38.89 (14)	61.11 (22)	0	0.6944	0.3056
Zavot	9	0	100.00 (9)	0	0.5000	0.5000
Holstein × Native cattle crossbreds	29	31.03 (9)	20.69 (6)	48.28 (14)	0.4138	0.5862
Total	102	36.28 (37)	50.00 (51)	13.73 (14)	0.6127	0.3873

*The number of animals per genotype is presented in parentheses.

Table 3 shows the HWE testing, He, PIC, Ne, Fıs, *V*%, and *H´* results for the *MC4R* L286V polymorphism in the selected breeds. There was a deviation from the HWE in the Zavot cattle and Holstein × Native cattle crossbreds $P<0.05$. The highest gene heterozygosity was observed in Turkish Grey Steppe cattle (0.4898). The PIC ranged from 0.2446 to 0.3699, and the Ne ranged from 1.3931 to 1.9616. A negative value of the Fıs was observed only in the entire population evaluation. Turkish Grey Steppe breed exhibited the highest values in the *V*% and the *H´* (0.4541 and 0.9557, respectively). The only incompatibility with the HWE was seen in crossbreds for the bovine *CACNA2D1* (Table 4). The low levels of population genetics parameters were observed in Turkish Grey Steppe cattle compared to other breeds studied. Regarding Zavot cattle, all of the animals were heterozygous genotype carriers that resulted in a

high level of Fıs (0.82). The *V*% values ranged from 0.3393 to 0.4549. The highest level of diversity was observed in the crossbreds (*H´*=1.041).

Table 3. Genetic diversity at the bovine MC4R gene in different breeds and the total sample of animals (n=102).

Breed	HWE test	He	PIC	Ne	Fıs	*V*%	*H´*
Turkish Grey Steppe	Equilibrium	0.4898	0.3699	1.9616	0.6733	0.4541	0.9557
East Anatolian Red	Equilibrium	0.4614	0.3550	1.8546	0.5665	0.4336	0.9014
Zavot	Disequilibrium*	0.3457	0.2859	1.5225	0.8842	0.2346	0.6870
Holstein × Native cattle crossbreds	Disequilibrium*	0.2854	0.2446	1.3931	0.7197	0.2509	0.7276
Total	Equilibrium	0.4306	0.3379	1.7476	-0.1147	0.4208	0.9135

HWE: Hardy-Weinberg equilibrium; He: gene heterozygosity; PIC: polymorphism information content; Ne: effective allele number; F_{IS}: fixation index; *V*%: level of possible variability realization; *H´*: the Shannon-Weaver diversity index. **P*<0.05

Table 4. Genetic diversity at the bovine CACNA2D1 gene in different breeds and the total sample of animals (n=102).

Breed	HWE test	He	PIC	Ne	Fıs	*V*%	*H´*
Turkish Grey Steppe	Equilibrium	0.3750	0.3047	1.6000	0.6267	0.3393	0.6931
East Anatolian Red	Equilibrium	0.4244	0.3344	1.7476	0.4816	0.3966	0.6682
Zavot	Equilibrium	0.5000	0.3750	2.0000	0.8200	0.3889	-[1]
Holstein × Native cattle crossbreds	Disequilibrium*	0.4851	0.3675	1.9372	0.8763	0.4506	1.041
Total	Equilibrium	0.4746	0.3620	1.9077	-0.0746	0.4549	0.9870

HWE: Hardy-Weinberg equilibrium; He: gene heterozygosity; PIC: polymorphism information content; Ne: effective allele number; F_{IS}: fixation index; *V*%: level of possible variability realization; *H´*: the Shannon-Weaver diversity index. [1]The population is fixed. **P*<0.05

Association analysis between the SNPs and the phenotypic traits

ANOVA results have indicated a novel effect of the A526745G polymorphism of the bovine *CACNA2D1* gene on the marbling score (*P*<0.05). Individuals with the GG genotype had a higher marbling than those with the AA and AG genotypes in the total cattle population (Table 5). On the other hand, Tables 6-8 show the association analysis results for the breed-specific evaluation. The significant association between the *CACNA2D1* gene and marbling score was evident in Turkish Grey Steppe cattle (*P*<0.05). Heterozygous animals had higher means for marbling scores, but it is worth noting that the GG genotype was absent in Turkish Greys (Table 6). The association between the *CACNA2D1* genotypes and chilling loss indicated a tendency (*P*<0.1). The animals with the AA genotype seemed to lose more weight compared to the heterozygous animals (Table 7). In the present study, remarkable differences in the SW and carcass weights among the genotypes were observed in both breed-specific evaluation and the entire cattle population. But these differences were not substantiated in the statistical analysis.

Moreover, there was no significant association between the *MC4R* genotypes and any of the carcass and meat quality traits analyzed in this study.

Table 5. Least-squares means observed in this study for the MC4R and CACNA2D1 genotype effects on phenotypic traits in the total cattle population (n= 102). The data were expressed as least-squares means and their corresponding standard errors (mean±SE).

Genotypes	*MC4R*			*CACNA2D1*			Significance
	CC	CG	GG	AA	AG	GG	
Slaughter weight, kg	447.52±11.82	448.01±11.33	473.55±20.61	458.03±11.52	464.25±14.50	446.70±17.68	NS
Hot carcass weight, kg	237.73±6.71	236.40±6.45	249.81±11.70	242.47±6.54	245.10±8.24	236.41±10.17	NS
Dressing percentage[1], %	53.29±0.74	52.90±0.71	52.77±1.29	53.10±0.72	52.75±0.91	53.11±1.11	NS
Chilled carcass weight, kg	233.98±6.59	232.28±6.33	245.21±11.50	238.16±6.42	240.72±8.09	232.57±9.88	NS
Dressing percentage[2], %	52.46±0.71	51.96±0.68	51.78±1.23	52.17±0.69	51.79±0.86	52.25±1.06	NS
Chilling loss, kg	3.75±0.33	4.12±0.32	4.60±0.58	4.31±0.32	4.39±0.41	3.78±0.49	NS
Carcass fatness score, 1-5	2.61±0.25	2.37±0.24	2.78±0.43	2.42±0.24	2.79±0.30	2.55±0.37	NS
Carcass pH	5.54±0.04	5.52±0.04	5.56±0.08	5.53±0.04	5.56±0.05	5.53±0.06	NS
Marbling score, 1-10	3.01±0.19	2.66±0.19	2.79±0.39	2.49±0.19[b]	2.71±0.22[ab]	3.27±0.29[a]	$P<0.05$

[a,b]Means with different superscripts are significantly different.

[1]dressing percentage based on hot carcass weight; [2]dressing percentage based on chilled carcass weight.

Table 6. Least-squares means observed in this study for the MC4R and CACNA2D1 genotype effects on phenotypic traits in Turkish Grey Steppe cattle (n= 28). The data were expressed as least-squares means and their corresponding standard errors (mean±SE).

Genotypes	*MC4R*			*CACNA2D1**		Significance
	CC	CG	GG	AA	AG	
Slaughter weight, kg	491.50±32.21	485.32±20.87	517.72±39.82	491.80±25.21	504.65±27.92	NS
Hot carcass weight, kg	272.11±15.42	266.51±9.97	279.30±19.10	267.12±12.10	278.14±13.42	NS
Dressing percentage[1], %	54.60±1.86	54.01±1.20	53.81±2.31	54.38±1.46	55.23±1.62	NS
Chilled carcass weight, kg	266.30±15.60	261.60±10.11	274.37±19.33	262.19±12.24	272.73±13.51	NS
Dressing percentage[2], %	54.44±1.82	53.93±1.18	52.73±2.26	53.36±1.43	54.04±1.58	NS
Chilling loss, kg	5.78±0.76	4.89±0.49	5.01±0.94	5.03±0.60	5.42±0.66	NS
Carcass fatness score, 1-5	1.56±0.78	1.94±0.50	2.41±0.96	1.93±0.61	2.02±0.68	NS
Carcass pH	5.76±0.14	5.75±0.09	5.62±0.17	5.72±0.11	5.69±0.12	NS
Marbling score, 1-10	2.64±0.46	3.02±0.29	3.02± 0.56	2.62± 0.36[b]	3.17± 0.39[a]	$P<0.05$

[a,b]Means with different superscripts are significantly different.

[1]Dressing percentage based on hot carcass weight; [2]Dressing percentage based on chilled carcass weight.

*The GG genotype was absent.

Table 7. Least-squares means observed in this study for the MC4R and CACNA2D1 genotype effects on phenotypic traits in East Anatolian Red cattle (n= 36). The data were expressed as least-squares means and their corresponding standard errors (mean±SE).

Genotypes	*MC4R*			*CACNA2D1**		Significance
	CC	CG	GG	AA	AG	
Slaughter weight, kg	402.91±10.50	403.80±6.79	423.61±12.80	409.00±10.17	411.18±8.41	NS
Hot carcass weight, kg	209.56±4.96	214.82±3.22	223.42±6.05	218.39±4.80	213.47±3.98	NS
Dressing percentage[1], %	52.07±1.09	53.22±0.71	52.74±1.33	53.41±1.05	51.94±0.87	NS
Chilled carcass weight, kg	206.61±4.98	211.07±3.23	219.40±6.08	214.40±4.82	210.32±4.00	NS
Dressing percentage[2], %	51.34±1.05	52.29±0.68	51.81±1.29	52.43±1.02	51.19±0.85	NS
Chilling loss, kg	3.95±0.34	3.75±0.22	4.02±0.42	4.99±0.33	4.15±0.27	*P*<0.1[3]
Carcass fatness score, 1-5	2.56±0.45	2.00±0.29	2.30±0.54	1.93±0.43	2.64±0.39	NS
Carcass pH	5.53±0.05	5.56±0.03	5.68±0.06	5.54±0.04	5.63±0.03	NS
Marbling score, 1-10	2.41±0.25	2.32±0.16	2.60±0.31	2.47±0.24	2.42±0.20	NS

[1]Dressing percentage based on hot carcass weight; [2]Dressing percentage based on chilled carcass weight; [3]Tendency for the *CACNA2D1* (*P*<0.1). *The GG genotype was absent.

Table 8. Least-squares means observed in this study for the MC4R and CACNA2D1 genotype effects on phenotypic traits in Holstein × Native cattle crossbreds (n= 29). The data were expressed as least-squares means and their corresponding standard errors (mean±SE).

Genotypes	*MC4R**		*CACNA2D1*			Significance
	CC	CG	AA	AG	GG	
Slaughter weight, kg	463.22±20.10	470.21±25.40	477.74±22.91	467.93±33.30	454.50±16.90	NS
Hot carcass weight, kg	240.71±13.70	239.44±17.32	243.50±15.60	239.91±22.70	236.70±11.50	NS
Dressing percentage[1], %	51.61±1.40	50.52±1.76	50.78±1.59	50.45±2.31	51.95±1.17	NS
Chilled carcass weight, kg	235.80±13.42	234.81±16.90	238.91±15.30	235.22±22.21	231.90±11.20	NS
Dressing percentage[2], %	50.54±1.34	49.56±1.69	49.81±1.53	49.45±2.22	50.89±1.13	NS
Chilling loss, kg	4.93±0.50	4.54±0.63	4.65±0.57	4.78±0.83	4.76±0.42	NS
Carcass fatness score, 1-5	2.96±0.33	3.13±0.41	2.88±0.37	3.39±0.54	2.86±0.27	NS
Carcass pH	5.59±0.06	5.48±0.07	5.46±0.06	5.63±0.09	5.53±0.04	NS
Marbling score, 1-10	3.27±0.32	2.92±0.40	2.86±0.36	3.40±0.52	3.02±0.27	NS

[1]Dressing percentage based on hot carcass weight; [2]Dressing percentage based on chilled carcass weight.
*There was only one animal genotyped as the GG and hence it was not considered in the association analysis

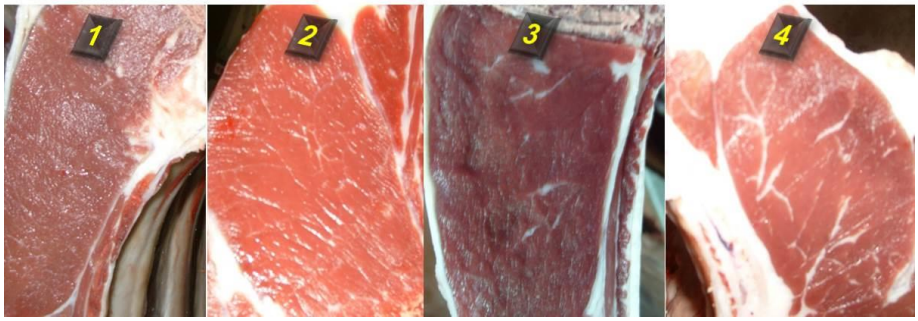

Figure 2. Examples of different marbling levels in this study. Marbling score was evaluated using a scale that represents ten degrees of marbling (1=practically devoid, 2=traces, 3=slight, 4=small, 5=modest, 6=moderate, 7=slightly abundant, 8=moderately abundant, 9=abundant, 10=very abundant). A maximum of four levels of marbling were observed.

DISCUSSION

Genetic variability

HWE testing provides valuable information on population substructure and selection dynamics (ARDICLI *et al*., 2019). Present results show that the genotypic distribution was not compatible with the HWE for the *MC4R* gene in the Zavot breed. Moreover, deviations from the HWE were observed in Holstein crossbreds for both studied genetic markers. In the entire population (n=102), the genotype frequencies were compatible with the HWE in the *MC4R* and *CACNA2D1* genes. In the analysis of population genetics, parameters such as He, PIC, and Ne provide important clues in the evaluation of genotypic distributions along with the informativeness and concurrency of the selected genetic markers for the studied population. For instance, He exhibits inbreeding levels, whereas Ne represents the effectiveness of loci allele impact in populations (ARDICLI *et al*., 2019; TRAKOVICKÁ *et al*., 2013). In this context, the highest He and Ne values for the *MC4R* marker were observed in the Turkish Grey Steppe cattle. Further, East Anatolian cattle show admissible results of He and Ne for this genetic marker, while the unbalanced genotypic distribution causes low genetic variability in Holstein crossbreds. Concerning the *CACNA2D1* marker, the highest He and Ne values were observed in Zavot cattle. However, it is important to note that, the number of individuals is rather limited for this breed. In the entire population, values of 1.90< and ~0.48, for Ne and He, respectively, were estimated that indicated an acceptable variability.

One of the most decisive parameters in population genetics is the PIC. This index precisely demonstrates the usefulness of a genetic marker in the studied population that is directly related to the marker's level of polymorphism (MACHADO *et al*., 2003). The commonly used PIC classification suggested by BOTSTEIN *et al*. (1980) is as follows: highly informative: PIC>0.50, reasonably informative: 0.25<PIC<0.50, and slightly informative polymorphism: PIC<0.25. Based on this classification, the *MC4R* marker is reasonably informative for the studied population and breed-specific evaluation, except for the Holstein crossbreds (Table 3). The g.38638971A>G of the *CACNA2D1* polymorphism was found to be a reasonably informative genetic marker for all breeds. The absence or very low frequencies of the GG genotype for both markers resulted in mediocre results for the Fıs and *V*%, especially in the breed-specific evaluation compared to the total cattle population. The highest values of the *H′* for the *MC4R* and *CACNA2D1* markers were observed in Turkish Grey Steppe and Holstein × native cattle crossbreds, respectively. Genetic variability results are directly associated with population characteristics in relation to selection process dynamics and inbreeding levels (ARDICLI *et al*., 2019; CROW and KIMURA, 1970; NEI and ROYCHOUDHURY, 1974). Here, it should be noted that native breeds are generally not subjected to high selection pressure for economically important production traits. On the other hand, these breeds are not preferred in high production-oriented breeding systems and therefore the number of purebred individuals is gradually decreasing (SOYSAL and KOK, 2006; COBANOGLU and ARDICLI, 2022). Hence, the population genetics parameters and diversity indices, which are directly related to the genotype/allele frequencies, may vary widely among different native breeds and even different populations of the same breed.

Genetic marker associations

Improvement in carcass and meat quality traits play major roles in evaluating the profitability of beef cattle. In many countries, in addition to beef-specific cattle, males from native cattle breeds and their crosses with dairy breeds have a substantial share in meat production. Native cattle breeds still play an important role in cattle breeding in many regions, such as Europe, the Middle East, and Asia, due to their high adaptability to the environmental conditions in which they are raised, ability to withstand low-quality nutritional conditions and resistance to infections. The candidate gene approach reveals a more effective understanding of the genetic background of economically important quantitative traits and it provides a better comparison between high and low-yielding individuals (HOU *et al*., 2010; NOGUERA *et al*., 2003 ARDICLI *et al*., 2022). Although the polygenic inheritance makes the genetic evaluation of quantitative traits more complex than expected, the identification of novel associations and their widespread use in selection programs ensure the selection of superior individuals more accurately compared to conventional breeding practices. In this study, we analyzed the effects of polymorphisms in the *MC4R* and *CACNA2D1* genes on some carcass and meat quality traits in Turkish native cattle breeds and their crossbreds with the Holstein-Friesians. These genes were selected because both of them involve in biochemical mechanisms related to weight gain, feed intake, and energy expenditure, and thus, they are strong candidates for the carcass, and growth traits, along with meat quality traits (HOU *et al*., 2010; SEONG *et al*., 2012; RAJAWAT *et al*., 2022; ROCHA *et al*., 2023).

The central melanocortin system plays a major role in appetite and energy expenditure regulation in mammals. In this system, several molecular interactions through energy homeostasis get involved via neuropeptides released from pro-opiomelanocortin, agouti-related peptide neurons, or many other related factors (BUTLER, 2006; FATHI *et al*., 2018). The *MC4R* gene encodes a protein that belongs to the trans-membrane G-protein coupled receptor family and it is one of the considerable genes in the genetic mechanisms associated with feeding behaviors and energy balance (CALTON *et al*., 2009). Thus, *MC4R* is a strong candidate for the economically important quantitative traits such as growth, fatness, and carcass characteristics in beef cattle (MCLEAN and SCHMUTZ, 2011; SEONG *et al*., 2012). In this study, considerable differences were evident among the three genotypes of the bovine *MC4R* including SW, HCW, and CCW (Table 5) in the entire population. Animals with the GG genotype had +26.03 kg and +25.54 kg higher SW compared to CC and the heterozygous animals, respectively. The GG animals had also remarkably higher carcass weights than the alternative genotypes. These interpretations are valid for the breed-specific evaluation indicating that the GG animals are heavier (Tables 6 and 7), except for the Holstein crossbreds (The GG genotype frequency is 0). However, these associations were not substantiated in Tukey's multiple comparison test. There were no significant differences in the DP, CL, CFS, carcass pH, and MS (P<0.05) in both the entire cattle population and the breed-specific evaluation. The *MC4R* gene has been reported to be a candidate gene for carcass traits and the SNPs have been shown to be potentially valuable genetic markers for economic traits in beef cattle (MCLEAN and SCHMUTZ, 2011; SEONG *et al*., 2012). Although some suggestive associations were observed, this study shows that the *MC4R* L286V polymorphism may not be a powerful genetic marker for the carcass and meat quality

traits in the studied Turkish cattle breeds. But further investigations with larger cattle populations or different SNPs are needed to draw precise conclusions.

Intramuscular fat deposition is a highly decisive parameter in defining meat quality (LIM *et al*., 2013). It is well-known that body weight and growth traits relate to meat quality traits. In this respect, the development of adiposes in combination with declining muscle growth influences the formation of the desired marbling in beef via the interactions through fat development, connective tissue, or blood vessels (HOCQUETTE *et al*., 2010; LIM *et al*., 2013). Although the genetic background and regulation mechanisms of beef marbling have not been fully understood, some genomic locations have been proposed as important candidates. For instance, BTA 4, 6-10, 14, 20, and 21 have been shown to be significant QTL sources (MIZOGUCHI *et al*., 2006; MIZOSHITA *et al*., 2004; TAKASUGA *et al*., 2007). More specifically, YOKOUCHI *et al*. (2009) reported that the marbling QTL on BTA 4 is located in the 3.7-Mb region at around 46 cM. The corresponding genomic region, where the bovine *CACNA2D1* gene is located, seems to be a distinctive hot spot for marbling evaluation. Therefore, it can be likely expected that the *CACNA2D1* gene is effective in bovine marbling. In this study, this potential association was confirmed by observing the significant differences in marbling scores among the *CACNA2D1* g.38638971A>G genotypes. Although generally low MS results were obtained as shown in Figure 2, the GG genotype was characterized by the highest level of marbling (3.27±0.29) in the entire population. The GG genotyped animals had +0.78 and 0.56 higher MS than the AA and AG animals, respectively. It is important to note that this significant association was observed only for the Turkish Grey Steppe cattle in the breed-specific evaluation (Tables 6-8). Meat quality characteristics are highly affected by breed characteristics. Hence, different results obtained in the association analysis for MS can be interpreted as common circumstances. On the other hand, the GG genotype was absent in Turkish Grey Steppe and East Anatolian Red cattle. Nevertheless, it can be postulated that the A allele has a negative effect on MS. On the contrary, this effect of the *CACNA2D1* marker was not reported by HOU *et al*. (2010) in a commercial cattle population composed of Simmental, Angus, Hereford, Charolais, Limousin, and some Chinese local breeds (Qinchuan, Luxi, and Jinnan). But these researchers have reported that this SNP at position 526745 of the *CACNA2D1* gene was significantly associated with carcass weight, dressing percentage, meat percentage, and backfat thickness. Although a tendency was observed for chilling loss (P=0.069) in East Anatolian Red breed, no significant associations were found between the *CACNA2D1* and carcass traits in this study. It is worth noting that this gene is located within the genomic region of the QTLs for average daily weight gain and carcass weight (CASAS *et al*., 2000) and thereby a possible region for the corresponding carcass traits. Thus, the bovine *CACNA2D1* g.38638971A>G needs further investigations.

To the best of our knowledge, this is the first study reporting a possible association with the *CACNA2D1* g.38638971A>G genotypes and bovine marbling. As mentioned above, BTA 4 consists of important genomic regions for marbling, and hence, focusing on the genetic markers/QTLs may reveal significant clues in understanding the genetic basis of bovine marbling. As PLATTER *et al*. (2005) suggested, MS influences both the probability that consumers will purchase and the price they are willing to pay for strip loin steaks. Therefore, identifying the genetic markers associated with MS and assessing the possibilities of using these

markers in selection practices have a very significant economic benefit as it affects consumer choices and retail prices.

CONCLUSION

This paper focuses on the association of two nonsynonymous alterations located in the bovine *MC4R* and *CACNA2D1* genes with the carcass characteristics and meat quality in Anatolian cattle breeds. The *MC4R* showed no significant associations in any of the traits analyzed. Here, we present a novel effect of the bovine *CACNA2D1* g.38638971A>G polymorphism on the marbling score. In this respect, the GG genotype exhibited more desirable marbling levels. Concerning BTA4: 38,152,730-38,673,177, where the bovine *CACNA2D1* gene is located, seems to be a potentially decisive genomic region for marbling. Evaluating of the effects of genetic markers in this genomic region may reveal novel associations through meat quality. Consequently, the *MC4R* c.856C>G polymorphism did not show any significant association with the analyzed traits, suggesting it is not a suitable marker for carcass traits and marbling. However, the *CACNA2D1* c.2027A>G polymorphism exhibited a novel association, with the GG genotype showing higher marbling scores. It should be noted that, nevertheless, the genotype-phenotype relationships observed in this study may only be applicable to the selected breeds. Alternative genotypic associations could be observed in different cattle breeds due to the genetic background and presence/absence of extensive selective pressures.

ACKNOWLEDGMENTS

The results of this study were presented on IV. International Agricultural, Biological & Life Science Conference, Edirne, Turkiye, 29 - 31 August 2022.

Received, October 21st, 2022
Accepted June 12th, 2023

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EFEKTI POLIMORFIZMA GENA MC4R I CACNA2D1 NA SVOJSTVA TRUPA I KVALITET MESA KOD TURSKIH RASA GOVEDA I NJIHOVIH UKRŠTANJA SA HOLŠTAJN-FRIZIJSKOM RASOM

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Izvod

Osobine trupa i kvalitet govedine su ekonomski važne osobine, čija je ekspresija pod uticajem više gena. Efekti polimorfizama MC4R c.856C>G i CACNA2D1 c.2027A>G na osobine kvaliteta trupa i mesa su ograničeni. Stoga je ova studija imala za cilj da proceni povezanost goveđih markera MC4R i CACNA2D1 sa karakteristikama trupa i kvalitetom mesa. A total of 102 cattle including Turkish Grey Steppe, East Anatolian Red, Zavot, and their F 1 crossbreds with the Holstein-Friesians were genotyped using the PCR-RFLP method. The phenotypic traits measured were slaughter weight, hot carcass weight, chilled carcass weight, dressing percentage, chilling loss, carcass fatness score, carcass pH 24 , and marbling score. Statistical analyses were performed using linear mixed models in the entire cattle population and also from a breed-specific aspect. Ukupno 102 goveda uključujući Tursku sivu stepsku, istočnoanadolsku crvenu, zavot i njihova F1 ukrštanja sa holštajn-frizijskom rasom su genotipizirana korišćenjem PCR-RFLP metode. Izmerene fenotipske osobine bile su masa prilikom klanja, težina vrućeg trupa, težina ohlađenog trupa, procenat kože, gubitak na hlađenju, ocena debljine trupa, pH 24 trupa. Statističke analize su vršene korišćenjem linearnih mešovitih modela u celokupnoj populaciji goveda, a takođe i sa aspekta specifičnog za rasu. Takođe je procenjena genetika populacija i indeksi diverziteta. Rezultati su otkrili da su genetski markeri u ovoj studiji prilično informativni za proučavanu populaciju goveda i da pokazuju srednju genetsku raznolikost. Što se tiče polimorfizma MC4R c.856C>G, nije bilo značajne povezanosti sa bilo kojom od analiziranih osobina, što ukazuje da ovaj MC4R c.856C>G nije poželjan marker za osobine trupa i kvalitet mesa. Ovde je prikazana nova povezanost između CACNA2D1 c.2027A>G polimorfizma i ocene kvaliteta. Genotip GG je bio okarakterisan višim vrednostima (P<0,05). Goveđi CACNA2D1 se nalazi na BTA 4, koji se sastoji od važnih QTL-ova za kvalitet mesa, i stoga, procena genetskih markera unutar ovog genomskog regiona može otkriti nove genetske asocijacije kroz kvalitet mesa.

Primljeno 21.X.2022.
Odobreno 12. VI 2023.