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Single nucleotide polymorphisms of GDF9 gene/exon 2 region and their associations with milk yield and milk content traits in Karakaş and Norduz sheep breeds

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Abstract: Karakaş and Norduz sheep have been adapted to the Lake Van region of Turkey for many years as being domestic genetic resources and meat, milk, and fleece traits are a reliable economic resource especially preferred by small family businesses around the area. In this study, the data set consisted of milk yield (MY) and milk content components; milk fat (MF), fat-free dry matter (FFDM), dry matter (DM), protein (Pro), lactose (Lac), pH, acidity H (aH), and lactic acid (Lac). Besides, Karakaş (n = 30) and Norduz (n = 26) sheep were chosen to investigate based on the SNP method. Correspondingly, genomic DNA from both breeds exon 2 of the GDF9 gene region was amplified, 815 base pairs (bp) in length, by means of PCR. Therefore, there were three noval SNPs detected in both breeds under investigation. Although SNP1 and 2 with genotypes of GG and AG had statistically significant impacts on both milk production and milk components ($p < 0.01$), SNP3 with genotypes of TT, TC had no significant effects on the milk characteristics in question.

Key words: GDF9 gene/exon 2 region, genetic resources, Karakaş and Norduz sheep, milk production and components, SNP

1. Introduction

The domestication and breeding of small ruminants are virtually as old as human history. As in many countries of the world, sheep farming activities are attractive for producers due to ease of management, care, efficient utilization of undersized grasslands, resistance to disease and harsh climatic conditions. Sheep *(Ovis aries)* is the most cultivated farm animal in different parts of the world, after goat, among livestock. There are approximately 1314 sheep breeds around the world [1]. Sheep are multipurpose animals that are raised for their meat, milk, fleece, skin, fur and fertilizer. Turkey is one of the world's most important sheep farming countries in the globe and there are about 37.28 million heads in almanac of 2019.[1] Essentially, Turkish sheep breeds are divided into two categories: thin- and fat-tailed breeds. The most common Turkish sheep breeds are fat-tailed breeds such as Akkaraman, Morkaraman, İvesi, Karakaş and Norduz [2].

The region of Lake Van basin with high altitude conditions is home to many native animal species such as Karakaş and Norduz sheep that are thought to be well adapted domestic gene sources around the region for many years. Besides, these sheep breeds are a reliable economic resource preferred by small family businesses in and around the region for sheep farming. Norduz and Karakaş sheep are categorized as a multipurpose, fat-tailed sheep and produced for milk, meat and fleece in the region [3–5].

Various molecular techniques and gene markers are used in genomic levels for molecular identification (phylogenetic) and genetic variation (polymorphism) analysis and studies. Over the past decades, different forms of genetic markers have been used in gene mapping attempts of plants, animals, and humans with polymerase chain reaction (PCR) techniques. These are briefly; restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), single-strand length polymorphism (SSLP), minisatellites and microsatellites, and single nucleotide polymorphisms

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(SNP). All of these marker technologies can be applied to the marker-assisted selection (MAS) in animal breeding programs [6–7][2].

Recently, worldwide studies in the molecular genetics area have shown that economically important traits in farm animals have been specified by means of the SNPs technique in genes for detecting functional or qualified genetic variations throughout the genomes. Milk yield and milk content traits are economically important traits in sheep, also known as complex traits, are controlled by a large number of unlinked genes with small impacts [8]. Growth differentiation factors (GDFs) are a subfamily of proteins associated with the transforming growth factor beta superfamily that has certain roles in development and female reproductive traits during early folliculogenesis [9,10]. Furthermore, the GDF9 gene, a candidate gene for high prolificacy in farm animals, conducting several key granulosa cell enzymes involved in cumulus expansion in female reproduction [11].

In addition, of the GDFs, the GDF9 is an oocyte-derived component, fundamental for folliculogenesis, oogenesis, and ovulation playing a crucial role in the fertility of female [12]. The gene encoding for the GDF9 is mapped on ovine chromosome 5 (OAR5) [13,14] and spans about 2.5 kbp included 2 exons. The two exon regions are interrupted by an intron with 1126 bp and coding a propeptide of 453 amino acids, with the mature peptide consisting of 135 amino acids [15]. Moreover, single nucleotide polymorphisms (SNPs) on sheep GDF9 have been revealed to influence fecundity traits such as ovulation rate and litter size in sheep. As a result, the GDF9 gene could serve as a genetic marker for the improvement of reproductive performance in sheep [16].

Due to the fact that no research had been performed on the GDF9 gene and milk content properties together, it was aimed to investigate the exon 2 region of the GDF9 gene, assuming that it would make some contributions to the genetic literature. Thus, the objective of this research was to identify polymorphisms for the growth differentiation factor 9 (GDF9) gene/exon 2 region by sequencing and evaluate statistical associations between SNP-genotypes with milk yield and milk content traits of Karakaş and Norduz sheep.

2. Materials and methods

2.1. Animal selection, milk yield records and measurements

The animal material of this research was chosen randomly from Norduz and Karakaş sheep flocks in animal breeding research farm of the Faculty of Agriculture at Van Yüzüncü Yıl University (Van-YYU) as Karakaş (n = 30) and Norduz (n = 26). The data set used in this study was composed of milk yield (MY) and milk content traits; milk fat (MF), fat-free dry matter (FFDM), dry matter (DM), protein (Pro), lactose (Lac), pH, acidity H (aH), and lactic acid (LA). The milk controls were started 30 days after the first lambing occurred in the flocks. In every milk control, 50 mL of milk sample was taken from each ewe and kept at +4 °C for the milk component analyses.

2.2. Milk components analysis

Milk components analyses were performed at the milk laboratory of Food Engineering Department of Van-YYU using different methods such that: milk fat based on Gerber method, dry matter based on gravimetric method, protein based on Kjeldahl method, fat-free dry matter based on calculation method and lactic acid based on titration method [17, 18].

2.3. Molecular genetics analysis

The analyses were carried out in the Animal Biotechnology Laboratory of the Faculty of Agriculture at Harran University. Sheep genomic DNA was isolated from 3–5 mL peripheral sheep blood samples using the DNA isolation kit (Gene JET Whole Blood Genomic DNA Purification Mini Kit # K0781, Thermo Fisher Scientific, Waltham, MA, USA). Genomic region sequence information of Ovis aries breed (Texel chromosome 5; gi | 417531957: c41843517 - 41840787) from the GenBank[3] was primarily utilized for the design primers. Forward and reverse primers (5'- GAGCCAGAGTTTTCTAGCAAG -3' and 5'-ATCGGTGATTCACATGGACAGC - 3') were designed with Primer3Input (version 0.4.0) package software to include the exon 2 region based on the GDF9 gene sequence information that was 815 bp in length (Figure 1).

PCR reactions were prepared in 0.2 mL tubes on ice in the specified proportions and then the PCR conditions were placed on the PCR device (Boeco, UK). And the reactions were adjusted in a volume of 40 mL for each sample as follows; 4.0 µL 10 × PCR buffer, 3.5 µL $MgCl_2$ (25 mM), 1.0 µL dNTP mix (10 mM), 1 µL primer F (10 pmol), 1 µL primer R (10 pmol), 0.4 µL Taq polymerase (5 U), 1.5 µL template DNA and 27.6 µL nuclease-free ddH_2O. The thermal cycle was programmed as follows; 4 min initial denaturation at 95 °C followed by 30 cycles consisting of 45 s denaturation at 94 °C, 60 s primer binding at 57 °C, 1.5 min elongation at 72 °C, and 5 min for final elongation at 72 °C. A 100-bp ladder (Fermentas) was used as a marker to run the PCR yields. The DNA samples were then sequenced to perform the GDF 9 gene region of PCR amplification (İontek Gen Company, İstanbul, Turkey). The samples for gene sequencing were prepared as 40µL (20µL PCR product + 20µL ddH_2O).

[2] Hayes B. Statistical genomics. Lecture notes. University of Palermo, Palermo, Italy, 2006.

[3] http://www.ncbi.nlm.nih.gov

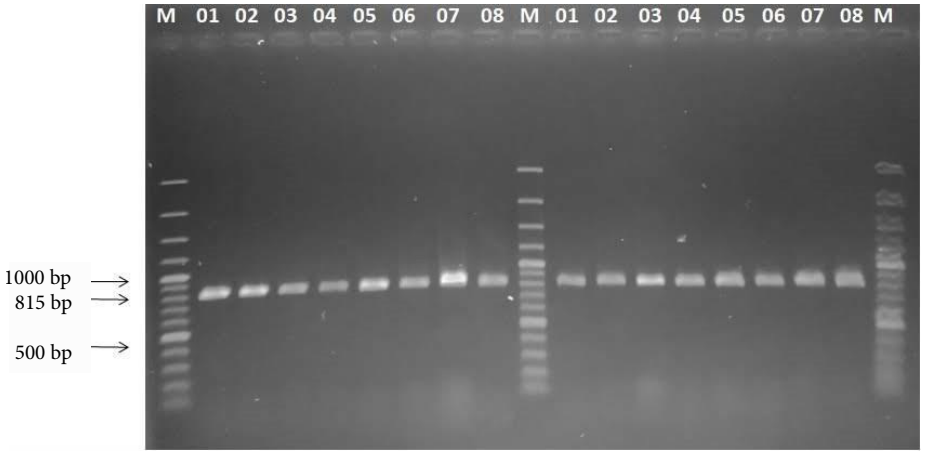

Figure 1. Gel photo of PCR yields (815 bp in length) of exon 2 region of GDF9 gene.

2.4. Sequence analysis

All yielded PCR products of the exon 2 region of the GDF9 gene were sent to İontek Gen Company for getting sequenced, and sequence analyses were performed using Chromas software (version 2.3).

2.5. Statistical analysis

The effects of environmental factors on milk yield and components were estimated by GLM procedure in SPSS 9.0 package program. The data were adjusted for body weight, age and birth type of ewes prior to final analyses and the final model included breeds, SNP genotypes and breed x SNP-genotype interactions as fixed effects. The final model was:

$y_{ijk} = \mu + b_i + g_j + bg_{ij} + e_{ijk}$,

where is the adjusted value of trait of interest, is the overall mean, is the fixed effect of ith breed, is the fixed effect of jth SNP-genotype, is the fixed effect of interaction between breed and SNP, and is the random error term. The analyses were carried out for each SNP separately and the Tukey test was used to compare the differences in group means.

2.6. Hardy–Weinberg equilibrium (HWE) analysis

In order to figure out allele and genotype frequencies and observed (Ho) and expected (He) heterozygosity, and HWE were calculated using an online software program[4].

3. Results

The PCR products of 815 bp, the part of exon 2 region of the GDF9 gene were obtained from all samples in both breeds (Figure 1).

3.1. Sequence analysis

Three novel single nucleotide polymorphisms (SNPs) were observed in both breeds after genomic DNA sequence analyses. As for the heterologous of SNPs, SNP1 (A/G), SNP2 (A/G) and SNP3 (T/C) were determined. Depending upon the sequence information of exon 2 of the GDF9 gene, all SNPs structurally displayed transition mutations.

3.2. Hardy-Weinberg equilibrium (HWE)

Allele and genotype frequencies and observed (Ho) and expected (He) heterozygosity values of these three novel SNPs are given in Table 1. While SNP1 and SNP2 (A/G) showed polymorphisms in both sheep breeds, SNP3 (T/C) displayed a polymorphism structure. In addition, neither the AA genotypes of SNP1 and 2 nor the CC genotype of SNP3 were observed in Karakaş and Norduz sheep. The highest (96%) of the G allele and the lowest (4%) values of the A allele in SNP1 were observed in Norduz sheep, while the highest (96%) of the G allele and the lowest (4%) of the A allele in SNP2 were found in Karakaş sheep. Similarly, Norduz sheep had the highest value (96%) of the T allele and the lowest (4%) values of the C allele in SNP3. Considering the genotype frequencies, the highest (92.3%) value of the GG genotype in SNP1 was observed in Norduz sheep, while the highest value of the GG genotype in SNP2 was found as 93.3% in Karakaş sheep. Furthermore, the TT genotypic value of SNP3 was found to be the highest (92.3%) in Norduz sheep. Eventually, Hardy–Weinburg equilibrium was detected even though two genotypes (AA and CC) of SNPs were absent in both flocks of breeds ($p < 0.05$, Table 1).

3.3. The effect of SNPs on milk yield and milk content traits

Least square means and corresponding standard errors of milk yield and milk content traits in terms of SNP-genotypes (SNP1 and SNP2) and breeds are given in Tables 2. The effect of SNP3 was found to be nonsignificant on any of the traits in this study, thus, no result for SNP3 was presented in this manuscript. On the other hand, it

[4] www.oege.org/software/hwe-mr-calc.shtml

Table 1. Allele and genotype frequencies of SNPs observed (Ho) and expected (He) heterozygosity, and Hardy–Weinberg equilibrium test (χ2 p-values) for the loci of exon 2 region of GDF9 gene.

SNP ID	Breed	Genotype	G, T allele frequency %	A, C allele frequency %	Observed frequency (Ho) %	Expected frequency (He) %	χ2-test p-value
SNP1	KAR	GG n = 25	0.92	0.08	83.3	84.7	0.619
		AG n = 5			16.7	15.3	
	NOR	GG n = 24	0.96	0.04	92.3	92.5	0.838
		AG n = 2			7.7	7.4	
SNP2	KAR	GG n = 28	0.96	0.04	93.3	93.4	0.8502
		AG n = 2			6.7	6.4	
	NOR	GG n = 23	0.94	0.06	88.5	88.8	0.7549
		AG n = 3			11.5	10.9	
SNP3	KAR	TT n = 27	0.95	0.05	90.0	90.3	0.7731
		TC n = 3			10.0	9.5	
	NOR	TT n = 24	0.96	0.04	92.3	92.5	0.838
		TC n = 2			7.7	7.4	

*: $p < 0.05$.
KAR: Karakaş sheep, NOR: Norduz sheep, χ2-test: chi-square test.

was determined that the effects of genotypes (GG, AG) of SNP1 and (GG, GA) of SNP2 were estimated statistically significant in all milk content traits except milk yield and milk fat ($p < 0.05$, $p < 0.01$).

4. Discussion

Based on QTL mapping studies in sheep genome, Maddox et al. [19] mapped the chromosomal location of the GDF9 region, inferring fecundity gene, of chromosome 5 (OAR5) using 81 informative microsatellite markers selected from the sheep linkage map. Although most of the current research studies for the GDF9 gene in the sheep genome have been usually emphasized reproduction and related traits, the research attempts on the GDF9 gene regarding the milk yield and milk content traits are rare.

Nicol et al. [20] conducted a research study to characterize a novel mutation in growth differentiation factor 9 (GDF9) in Icelandic Thoka sheep genome using hormone assays of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) along with immunohistochemistry methods. They concluded that a single base change (A1279C) resulting in a nonconservative amino acid change (S109R) in the C-terminus of the mature GDF9 protein was identified and this mutation was to be associated with increased fecundity in heterozygous ewes and infertility in homozygotes. In addition, Liandris et al. [21] caried out a research to detect SNPs and their effects of genotypes of the GDF9 and BMP15 genes on litter size in two Greek dairy sheep breeds using PCR-RFLP technique. Their results displayed that G1 and G8 mutations in the GDF9 gene were detected as statistically significant in the highly prolific breed of Chios. Similarly, Bahrami et al. [22] with using PCR-RFLP method, aimed to analyze mutations in exon 1 region of the GDF9 gene associated with twining rate in Tajikistan sheep breed Hisari. The results of this study indicated that there were genetic polymorphisms

Table 2. Least square means and standart errors of milk content by breed and SNP1 and SNP2 genotypes.

Traits	Breeds	SNP1	Mean ± SEM	Source of V.			SNP2	Mean ± SEM	Source of V.		
				B	G	BG			B	G	BG
MY (mL)	KAR	GG	615.2 ± 55.95	NS	NS	NS	GG	603.2 ± 52.85	NS	NS	NS
		AG	592.0 ± 125.12				GA	725.0 ± 197.78			
	NOR	GG	681.3 ± 57.11				GG	646.8 ± 59.63			
		AG	480.0 ± 197.83				GA	770.0 ± 139.85			
MF (%)	KAR	GG	5.2 ± 0.22	NS	NS	NS	GG	5.2 ± 0.21	NS	NS	NS
		AG	5.4 ± 0.50				GA	4.5±0.78			
	NOR	GG	5.1 ± 0.23				GG	5.0 ± 0.23			
		AG	4.5 ± 0.79				GA	4.9±0.55			
FFDM (%)	KAR	GG	12.3±*0.11*[a]	**	**	**	GG	12.2 ± 0.11[a]	NS	NS	**
		AG	12.3 ± 0.24[a]				GA	12.5 ± 0.41[a]			
	NOR	GG	12.4 ± 0.11[a]				GG	12.4 ± 0.12[a]			
		AG	10.5 ± 0.38[b]				GA	11.3 ± 0.29[b]			
DM (%)	KAR	GG	17.5 ± 0.23[a]	*	*	*	GG	17.5 ± 0.23	NS	NS	NS
		AG	17.6 ± 0.52[a]				GA	17.1 ± 0.87			
	NOR	GG	17.5 ± 0.24[a]				GG	17.5 ± 0.26			
		AG	15.0 ± 0.83[b]				GA	16.3 ± 0.61			
Pro (%)	KAR	GG	6.7 ± 0.09[a]	**	**	**	GG	6.6 ± 0.09[a]	NS	NS	**
		AG	6.7 ± 0.20[a]				GA	6.8 ± 0.34[a]			
	NOR	GG	6.8 ± 0.09[a]				GG	6.8 ± 0.10[a]			
		AG	5.2 ± 0.32[b]				GA	5.9 ± 0.24[b]			
Lac (%)	KAR	GG	4.5 ± 0.01[a]	**	**	**	GG	4.5 ± 0.01[a]	NS	NS	**
		AG	4.5 ± 0.02[a]				GA	4.6 ± 0.03[a]			
	NOR	GG	4.6 ± 0.01[a]				GG	4.6 ± 0.01[a]			
		AG	4.4 ± 0.03[b]				GA	4.5 ± 0.02[b]			
pH	KAR	GG	6.5 ± 0.03[a]	**	*	**	GG	6.5 ± 0.03[b]	NS	**	NS
		AG	6.5 ± 0.07[a]				GA	6.7 ± 0.11[a]			
	NOR	GG	6.5 ± 0.03[a]				GG	6.5 ± 0.03[b]			
		AG	6.9 ± 0.11[b]				GA	6.8 ± 0.08[a]			
aH	KAR	GG	6.0 ± 0.06[a]	**	**	**	GG	6.0 ± 0.06[a]	NS	**	NS
		AG	6.1 ± 0.15[a]				GA	5.7 ± 0.25[b]			
	NOR	GG	6.0 ± 0.07[a]				GG	6.0 ± 0.07[a]			
		AG	4.9 ± 0.23[b]				GA	5.3 ± 0.17[b]			
La (%)	KAR	GG	0.3 ± 0.01[a]	**	**	**	GG	0.3 ± 0.01[a]	NS	**	NS
		AG	0.3 ± 0.01[a]				GA	0.3 ± 0.01[a]			
	NOR	GG	0.3 ± 0.01[a]				GG	0.3 ± 0.01[a]			
		AG	0.2 ± 0.01[b]				GA	0.2 ± 0.01[b]			

*: $p < 0.05$ **: $p < 0.01$ NS: no significance.

[a,b]: Means with different superscript within trait are significantly different.

MY: milk yield, MF: milk fat, FFDM: fat-free dry matter, DM: dry matter, Pro: protein, Lac: lactose, pH: pH, aH: acidity H, La: lactic asid, B: breed, G: SNP-genotypy, BG: interaction between breed and SNP, KAR: Karakaş sheep, NOR: Norduz sheep, SEM: standard error of mean.

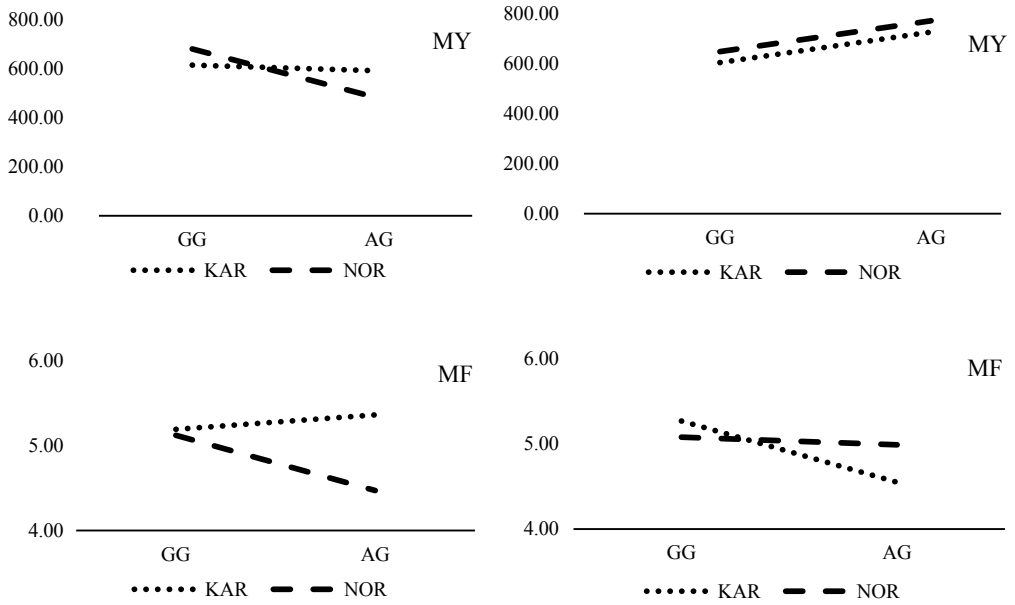

Figure 2. Milk yield (MY) and milk contents by breeds (KAR: Karakaş sheep and NOR: Norduz sheep) and SNP genotypes (SNP1 genotype is on the left column and SNP2 genotypes is on the right column).

and mutations in the gene loci GDF9. Moreover, Khodabakhshzadeh et al. [23] investigated polymorphisms using PCR-SSCP techique and found 3 mutations (SNP) (443, 477 and 721 positions) in the region of exon 2 of the GDF9 gene in the Kermani sheep. Furthermore, El Ficky et al. [25] studied to investigate polymorphisms in the exon 1 region of the GDF9 gene concerning lambing rate and litter size in Egyptian sheep based on PCR-RFLP method. This research brought a conclusion that sequence analysis and diversity of polymorphisms in the GDF9 gene (exon 1) had a novel base substitution (A–T) for detection of Fec^G mutations serving as a molecular marker for twinning.

In regard to growth traits in sheep, Jawasreh and Ismail [26] performed a research to determine the correlations among prolactin gene (PRG), GDF-9, and calpastatin (CAG) genes polymorphism with growth traits in Awassi lambs. Their results showed that there were no associations between any of the preweaning growth traits and GDF9. With respect to the milk yield and milk content traits, Al- Khuzai and Ahmad [27] conducted a study to determine the genotypes for GDF9 gene/exon-1 and the associations between these genotypes with some productive and reproductive traits of Awassi sheep with PCR-RFLP method. Their results revealed that there were no significant differences between genotypes of exon 1 of the GDF9 in total milk production and lactation period. It was implied that the formed proteins from gene expression of GDF9 influenced on milk production and lactation period, and they might also affect on other traits in animals. Throughout their research, it was also concluded that there were significant differences between genotypes in percentage of fat and solid nonfat in milk composition due to the effect of genotype of exon 1 of the GDF9 gene.

Based on the sequence information of exon 2 of the GDF9 gene, we have identified in this research study three novel single nucleotide polymorphisms (SNP1, 2, and 3, respectively): g. 146A > G, g. 419A > G and g. 464T > C. All SNPs structurally displayed transition mutations that SNP1 and 2 were with purine to purine (A ↔ G) substitutions whereas SNP3 was with pyrimidine to pyrimidine (T ↔ C) changes as point mutations. In addition, the GG genotypes of SNP1 and 2 and the CC genotype of SNP3 could not be observed in both sheep breeds. The possible reasons might be that these genotypes could have either a fatal effect in animals or absences in both sheep flocks depending upon other random environmental conditions such as diseases, sales of animals, etc. In order to reach more precise scientific evidence on this issue, genotypes that are observed less or not at all in the populations should be investigated by increasing the number of animal size.

As for the statistical analyses performed using a generalized linear model (GLM) in this study, SNP1 and 2 revealed some statistically extreme significant evidence among milk component traits. The effect of SNP1 and its interaction with the breed (BXG) had no significant effects on milk yield (MY) and milk fat (MF) but had a highly significant effect ($p < 0.01$) on the other traits in the analyses. In addition, it was determined that the genotypes of GG and AG of SNP1 in Karakaş sheep and the only GG genotype of SNP1 in Norduz sheep had increasing effects whereas SNP1 (AG) in Norduz sheep had decreasing effects on the levels of milk content: FFDM, Pro, Lac, pH, aH and

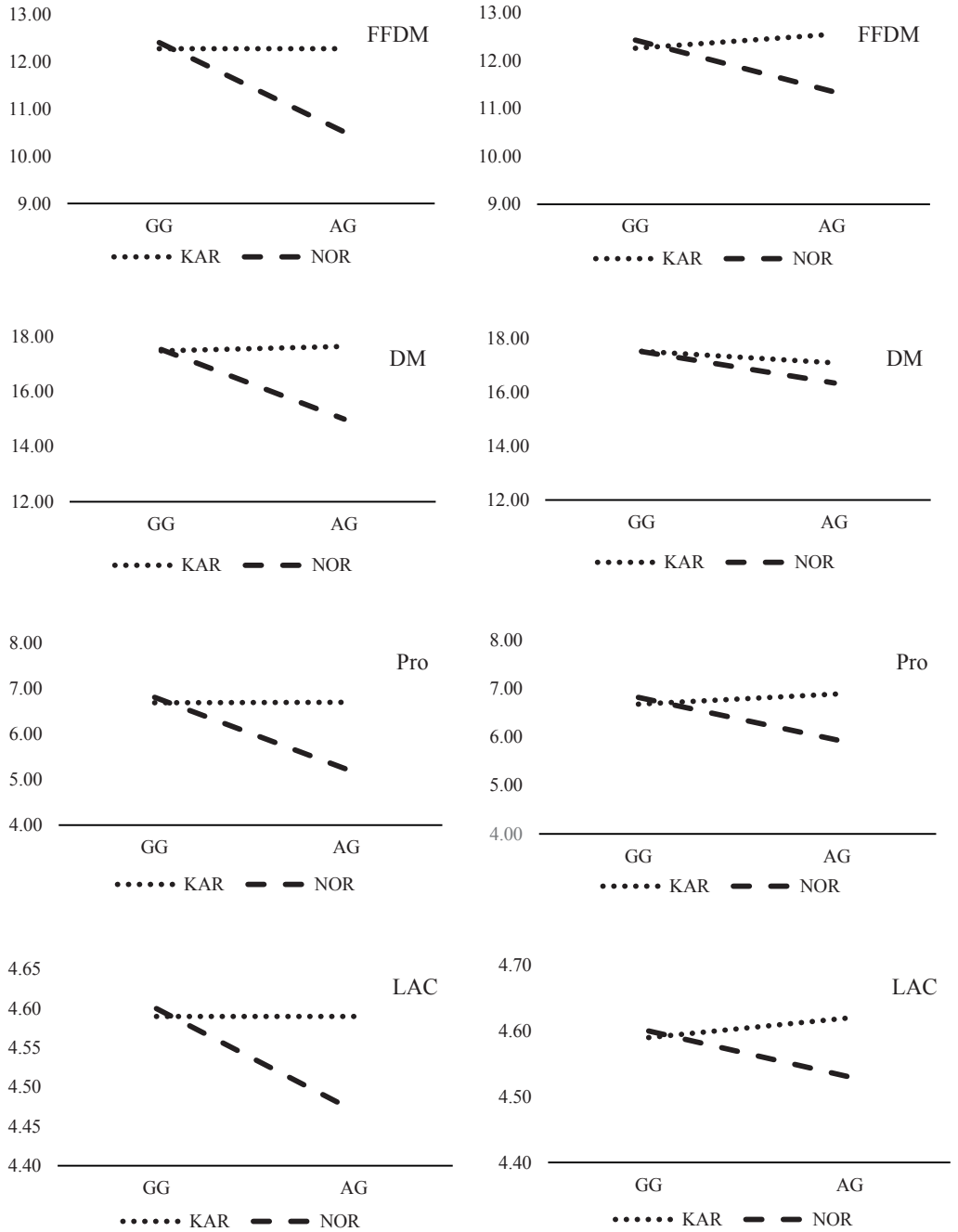

Figure 2. (Continued).

LA. Also, the effect of breed-SNP1 (BXG) interaction on the milk components were estimated as highly significant ($p < 0.01$, Table 2, Figure 2).

On the other hand, taking SNP2 into account, it was observed that SNP2 had statistically significant effects on pH, acidity H (aH) and lactic acid (LA) levels that were influenced with SNP2 genotypes of GG and GA. When compared the GA and GG genotypes of SNP2 in Norduz sheep, the GA genotype increased pH level, but slightly decreased acidity H (aH) and lactic acid (La) levels. In addition, it was found that interaction of breed-SNP2 (BXG) had only significant effects on fat-free dry matter (FFDM), protein (Pro) and lactose (Lac) ($p < 0.01$, Table 2, Figure 2).

Considerably, SNP1 and 2 had neither increasing nor decreasing effects on the milk yield and milk component traits in Karakaş sheep. Nonetheless, it was figured out that the AG genotype of SNP1 and 2 had statistically significant impacts on reducing milk components in Norduz sheep.

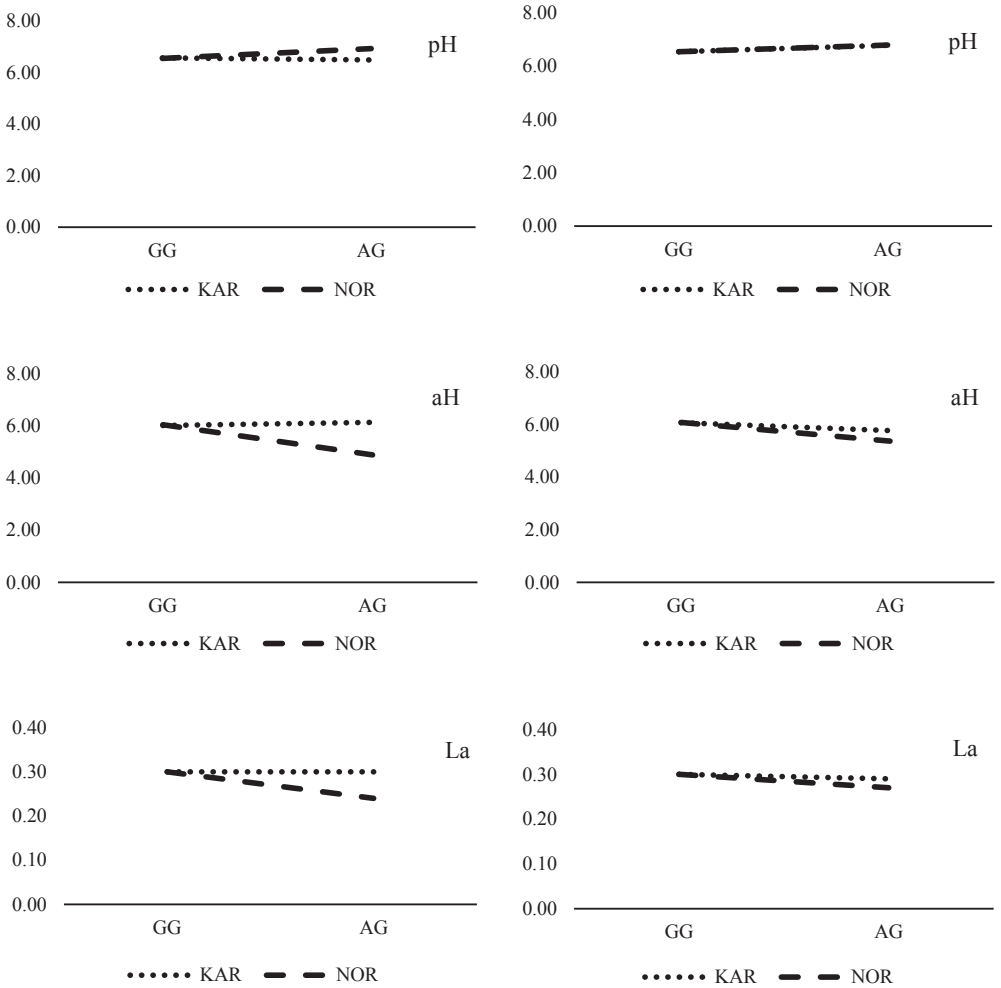

Figure 2. (Continued).

Unlike SNP1 and 2, SNP3 with TT and TC genotypes had no statistically significant evidence for both milk yield and milk component traits.

5. Conclusion

In this study, three novel SNPs have been identified in the exon 2 region that is 815 bp in length of the GDF9 gene. Results indicated that SNP1 and 2 had potential effects on both milk production and milk components while SNP3 had no effects. Karakaş and Norduz sheep carrying the desired SNP1 and SNP2 genotypes should be evaluated for reference flocks. Similar research studies that would be applied to different sheep breeds in and around the Lake Van basin may lead to marker-assisted selection (MAS) or genomic selection attempts in a near future.

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