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Detection of Growth Hormone (GH) Gene Polymorphism in Norduz Sheep

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ABSTRACT

Background: The Norduz sheep is a local breed of sheep bred for its meat, milk, progeny and fleece and is one of the genetic resources in the rural areas of Norduz, a district of the Turkish province of Van. The PCR-based method involves amplification of a conserved DNA region and digestion of the PCR products with DNA restriction endonucleases throughout the genome. Based on the resulting polymorphisms, statistical methods can be used to identify the desired genotypes among the alleles found. The objective of this study was to identify the polymorphisms of the growth hormone gene and determine their association with six growth traits in Norduz sheep.

Methods: Out of 50 Norduz sheep, three different loci, GHY1, GHY2 and GHY3 (with the length of 599, 690 and 679 bp respectively) of GH gene on chromosome 11 of sheep genome were selected to identify polymorphisms among individuals using the PCR-RFLP technique (GH/MspI).

Result: In this research study, no polymorphisms were detected after digesting three loci of the GH gene with the enzyme MspI. The 50 individuals of Norduz sheep had only AB genotypes for all three loci, indicating that no polymorphisms were present in the regions of the GH genes studied.

Key words: Genetic resources, Growth hormone (GH) gene, Norduz sheep, PCR-RFLP.

INTRODUCTION

The Norduz sheep is a local breed of sheep raised in rural areas in the Norduz region of Grpnar district of Van province in eastern Turkey. The Norduz sheep, a breed with fat tails, is classified as a multipurpose breed that produces meat and milk (Aygün *et al*., 2006 and Yılmaz *et al*., 2012). Growth is the main process of increasing the size of tissues through the proliferation of cells and the increase of intracellular substances.

The growth property is controlled by the growth hormone (GH) gene, which contains the instruction to produce growth hormone. Growth hormone is a single polypeptide hormone composed of 191 amino acids and secreted by somatotropin cells in the anterior pituitary (Afifi *et al*., 2019). GH has a great influence on various physiological traits such as milk production and growth traits. The main effects of GH are related to the stimulation of muscle and bone growth through the activity of insulin-like growth factor (IGF-1) and plays an important role in milk production (Mahrous *et al*., 2018). The GH gene consists of 1800 base pairs and five exons (Moradian *et al*., 2013) and is used as a genetic marker to determine polymorphisms in various livestock species such as pigs, sheep, goats and cattle (Kumari *et al*., 2014).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is one of the simplest and most common methods for analyzing genomic DNA. Recently, the PCR-RFLP technique is widely used by researchers to identify genetic polymorphisms because it is easy to develop and inexpensive to apply (Rasmussen, 2012). In animal production, growth traits are of critical importance to breeders as they affect the financial profitability of the farm. Accordingly, identification of genetic markers for growth traits in livestock is a critical step in establishing and managing a marker-assisted selection system. Although research investigating polymorphisms of the growth hormone gene and its influence on growth traits is very limited in sheep breeds (Seevagan *et al*., 2015). This study was conducted to search for genetic variations in three different loci (GHY1, GHY2 and GHY3) of growth hormone (GH) and reveal their associations with growth traits in Norduz sheep using the PCR-RFLP.

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MATERIALS AND METHODS

Animals and traits

Fifty Norduz lambs (43 females and 7 males) born between November 2017 and February 2018, aged 6 to 9 months,

were randomly selected at the Livestock Research and Application Farm of Van-Yüzüncü Yıl University. All sheep were healthy and in good nutritional condition. The growth measurements of the sheep were measured and recorded five times in 25-day periods, starting in mid-July and ending in mid-November 2018. The recorded quantitative values related to growth performance traits were seven: body length (BL), body weight (BW), body height (BH), breast circumference (CA), breast depth (CD) and breast depth between thighs (BSCD).

DNA isolation, loci determination and primer design

Genomic DNA was purified from animal peripheral blood samples using a Thermo Fisher Scientific mini DNA purification kit. The three loci GHY1, GHY2 and GHY3 of the GH genes were located on sheep chromosome 11 (OAR11). PCR primers were used based on Depison *et al*. (2017) and the length and location of each loci are shown in Table 1.

Recognition site of MspI endonuclease

The PCR amplicons on GHY1, GHY2 and GHY3 were digested with DNA cutting enzyme *MspI* DNA endonuclease (Table 2).

5′ . . . C$^{\vee}$C G G . . . 3′

3′ . . . G G C$_{\wedge}$C . . . 5′

PCR and gel electrophoresis

The total volume of the PCR reaction was 25 µl and contained 23 µl of mastermix and 2 µl of purified DNA. The mastermix solution contained (15.8 µl dH_2O, 2.5 µl 10X buffer, 1.5 µl $MgCl_2$, 0.32 µl DMSO, 0.5 µl dNTPs, 1.5 µl for each primer F and R, 0.14 µl Taq polymerase and 2 µl DNA). Amplification began with initial denaturation at 95°C for 10 minutes followed by 30 cycles; each cycle was programmed for 40 seconds of denaturation at 96°C, annealing at 55-58°C for 40 seconds, extension at 72°C for 45 seconds and extension at 72°C for 10 minutes and finally stored at 4°C. Amplicons were observed by migrating samples through agarose gel electrophoresis at 2% concentration and staining with ethidium bromide.

Hardy-weinberg equilibrium (HWE) analysis

Allele varieties determined on the agarose gel were counted on the corresponding references and calculated online via (http://www.oege.org/software/hwe-mr-calc.shtml).

RESULTS AND DISCUSSION

Three different loci, GHY1, GHY2 and GHY3 of the GH gene on the genome of Norduz sheep were successfully amplified by PCR with a length of 599 bp, 690 bp and 679 bp, respectively (Fig 1). Subsequently, all loci were screened for polymorphisms using the PCR-RFLP technique. Surprisingly, after cleavage of the PCR products GHY1 (599 bp), GHY2 (690 bp) and GHY3 (679 bp) with the enzyme MpsI endonuclease, no polymorphisms were detected in the total number of Norduz sheep examined. The results of this

Table 1: The length and location of the GH genes and primers used for PCR analysis.

Locus name	Chromosome	Forward primer	Reverse primer	TM F/R	PCR product
GHY1	OAR11	5′-TTG CAT AAA TGT ATA GAG CAC ACA G-3′	5′-CCC CAC CTC TAG GAC ACA TC-3′	58.5°C	599 bp
GHY2	OAR11	5′-CTG TTT GCC AAC GCT GTG-3′	5′-AAG CCA CGA CTG GAT AAG GA-3′	57°C	690 bp
GHY3	OAR11	5′-CCG AGG CAG CAG ACA TTG-3′	5′-GAA CAT GCG GCG CTT ACA-3′	55°C	679 bp

study show that GHY1, GHY2 and GHY3 loci of GH gene are monomorphic in the population of Norduz sheep. Only two types of alleles A and B and a specific genotype AB were detected heterozygously (Fig 2, 3 and 4) with frequencies of 1.00 each for all three loci (Table 3). Therefore, no statistical analyses were performed to determine the relationships between alleles and growth traits.

The Norduz sheep breed raised in Grpnar district of Van region (Eastern Anatolia) is highly adapted to altitude, climate and geographical conditions in terms of milk, meat, fertility and wool production and therefore represents one of the most important indigenous genetic resources for animal husbandry or production in Turkey (Aygün and Bingol, 1999). As a source of indigenous genes, the phenotypic and genotypic structure of the breed is gradually decreasing due to unconscious livestock production in the region, so that its originality is lost and the breed traits are becoming more and more extinct every day. As a result, molecular genetic studies and studies on Norduz sheep have gained considerable importance for livestock breeding and selection programs.

This study on growth hormone (GH) gene at different loci is the second study on Norduz sheep genome. The first study was conducted by Mahmood (2018). In this study, PCR-RFLP technique was used to detect genetic variations at two different loci of GH gene. Two loci, 422 bp GH1 and 934 bp GH, from the growth hormone gene family that affect growth traits were first amplified by PCR and cleaved using HaeIII enzyme. The HaeIII-RFLP of the GH1 locus (422 bp) showed two genotypes: AA (366 and 56 bp) and AB (366 and 56 bp). The genotypic frequencies for AA and AB were 0.26 and 0.74 and the allelic frequencies were 0.57 and 0.43, respectively. The genotype BB was not observed in the GH1 locus. However, the GH locus, 934 bp, showed all three genotypes after digestion: AA (277, 202 and 110 bp), AB (277, 256, 202 and 110 bp) and BB (256, 202 and 110 bp). The genotypic frequencies for AA and AB and BB were 0.27, 0.50 and 0.23, respectively and the allelic frequencies were 0.52 and 0.48 for A and B, respectively. Neither loci exhibited Hardy-Weinburg equilibrium (HWE), which was due to the difference in sample size.

As a second genetic investigation of the Norduz sheep genome in relation to growth traits, the GHY1 (599 bp), GHY2 (690 bp) and GHY3 (679 bp) variants of the GH genes in the sheep genome were amplified in this study based on the research of Depison *et al*. (2017). After cleavage with MspI endonuclease, the study by Depison *et al*. (2017) using thin tail sheep (TTS) in the highlands and lowlands in Jambi Province of Indonesia reported that there were two alleles and two genotypes in the GHY1 locus, namely genotype AA with only one fragment of 599 bp and genotype AB with two fragments of 408 bp and 83 bp. Similarly, there were two alleles and two genotypes for the GHY2 locus: genotype AA with only one fragment of 690 bp and genotype AB with three fragments of 291 bp, 206 bp and 173 bp. Finally, two alleles and two genotypes were observed in the GHY3 locus, namely genotype AA with only one fragment of 679 bp and genotype AB with two fragments of 522 bp and 99 bp. Besides, GH related genetic studies in sheep genome have increased especially in recent years, Meena *et al.* (2016) investigated allelic variation in the leptin gene of Malpura sheep in India using the PCR-RFLP method with the digestive enzymes of BcnI, SsiI and OliI. Their results showed that three non-synonymous SNPs were found in Malpura sheep. Loci A271G and A316C were found to be monomorphic, while locus T387G was polymorphic. In Malpura sheep, two genetic variants (G and T) and three genotypes (GG, GT and TT) were found. The allele frequencies of the G and T alleles at locus T387G were 0.82 and 0.18, respectively. In addition, Abdelmoneim *et al*. (2016) investigated the relationship between growth hormone (GH) gene polymorphism and estimated body weight in Harri sheep and discovered three novel SNPs, each of which was found on both alleles, with the homozygous mutation being more frequent (48, 56 and 50%) than the heterozygous (30, 18 and 20%) for G871A, G1383A and A1509G, respectively. The results suggest that these SNPs could be useful markers in selecting lambs for higher growth rate and meat production. Moreover, Afifi *et al*. (2019) conducted a study to investigate the relationship between growth hormone (GH) gene polymorphism and milk production in Najdi sheep. Five novel SNPs were discovered

Table 2: Virtual cutting and expected lengths of locus with DNA cutting enzymes.

Locus	DNA cutting	Cutting outcomes expected enzyme DNA fragment lengths (bp)
GHY1	*MspI*	408+83=599 bp
GHY2	*MspI*	29+206+173=690 bp
GHY3	*MspI*	522+99=679 bp

Table 3: Genotype and allele fequency of GHY1, GHY2, GHY3.

LOCI	Allele number		Allele frequency		Genotype frequency			Heterozygosity	
	n_a	n_e	A	B	AA	AB	BB	H_0	H_e
GHY1	1.00	1.00	1.00	1.00	0.00	1.00	0.00	0.00	0.00
GHY2	1.00	1.00	1.00	1.00	0.00	1.00	0.00	0.00	0.00
GHY3	1.00	1.00	1.00	1.00	0.00	1.00	0.00	0.00	0.00

n_a= Observed number of allele; n_e= Effective number of allele; H_0= Observed heterozygosity; H_e= Expected heterozygosity.

in the GH gene of Najdi sheep (G871A, A1259C, G1383A, A1486G and A1509G). In this study, a significant association was found between the detected SNP genotypes and daily milk yield (DMY). Regression analysis also showed the positive effect of genotype and SNPs on DMY, suggesting that these SNP could be potential markers for selecting ewes for milk production.

In contrast to the studies on sheep genomes mentioned above, only one type of genotype AB was detected at all three loci in the present study. In other words: After cleavage of the PCR products GHY1 (599 bp), GHY2 (690 bp) and GHY3 (679 bp) with the enzyme MspI endonuclease, no polymorphisms were found in the total number of Norduz sheep examined. The three loci of the GH gene were monomorphic, *i.e.* there was only the genotype AB for all three loci. As for the GH gene with different loci, Seevagan *et al.* (2015) in a similar study reported that MspI - PCR-RFLP of three loci (GH2; 422, GH4; 214 and GH5; 365 bp fragments) of GH gene in Vembur and Kilakarsal sheep breeds revealed no variations in the studied regions of GH gene. The GH2, GH4 and GH5 loci were monomorphic. Just like in this study, only genotype AB was found for all three loci.

In addition, some similar research studies indicate the absence of polymorphisms in the regions of the various GH gene loci of interest in the sheep genome. Al-Barzinji and Othman (2013) conducted studies to determine the polymorphism in Booroola gene (FecB) in five Iraqi sheep breeds (Hamdani, Karadi, Arabi, Naeimi and Awassi). The amplified Booroola receptor gene (BMPRIB) with a length of 140 bp was digested with the restriction enzyme AvaII. Their results showed that there were monomorphisms for this locus in all races. Similarly, Jamshidi *et al*. (2013)

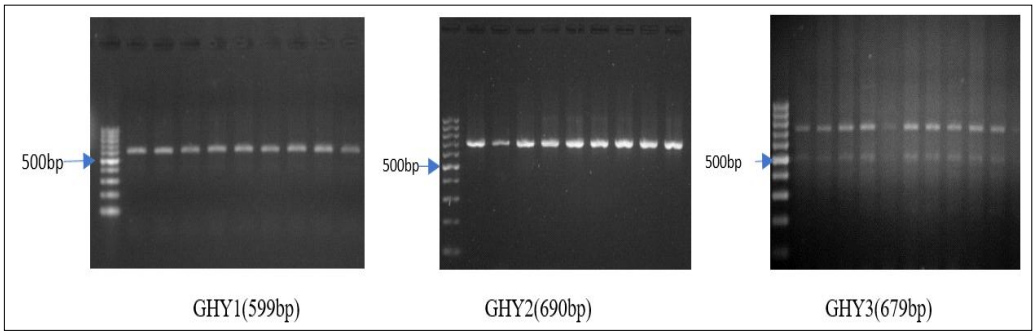

Fig 1: Gel image of PCR amplicons of GHY1, GHY2 and GHY.

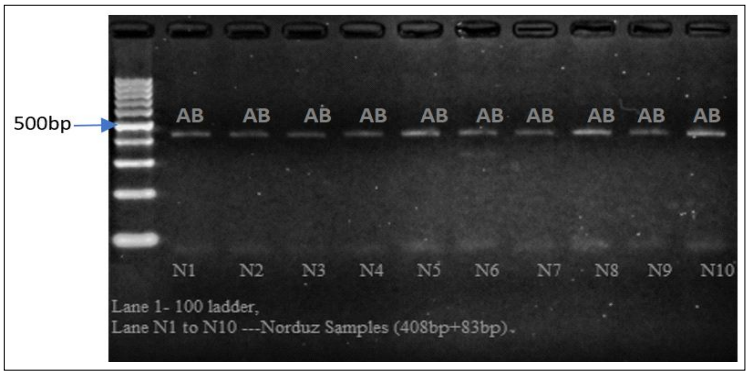

Fig 2: PCR-RFLP results with *MspI* enzyme of GHY1(599 bp) locus.

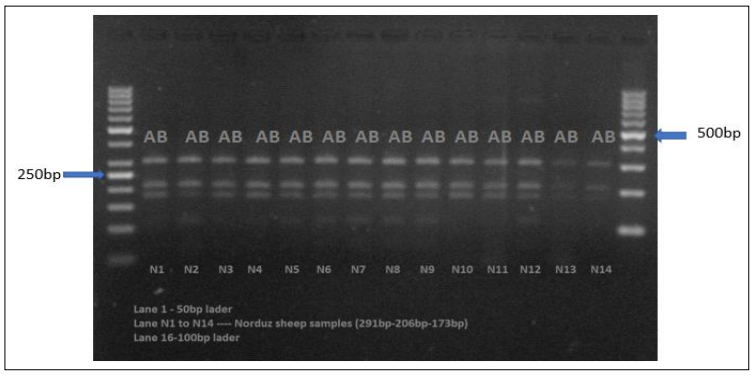

Fig 3: PCR-RFLP results with MspI enzyme of GHY2(690 bp) locus.

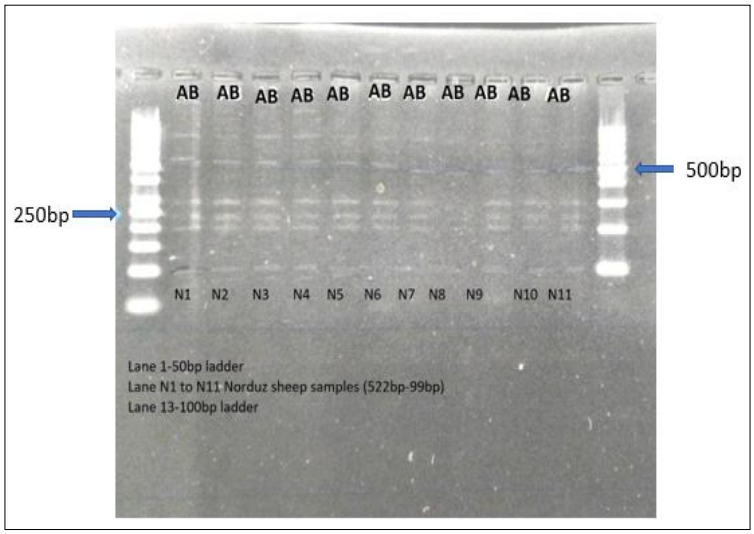

Fig 4: PCR-RFLP results with *MspI* enzyme of GHY3 (679 bp) locus.

attempted to detect polymorphisms in the Sangsari sheep breed by using a different FecB locus with a length of 190 bp and applying PCR-RFLP with the restriction enzyme AvaII. Their results showed that there is a wild monomorphic genotype (++) of the Booroola gene in the studied flock. Moreover, Nanekarani *et al*. (2014) studied the polymorphisms in the 426 bp long callipyge locus (CLPG) digested with FaqI enzyme in Lori sheep. According to PCR-RFLP, their results showed that the overall population of sheep was monomorphic for the CLPG gene. In a similar study, Tolee *et al* (2021) investigated the polymorphism of the CLPG gene using the PCR-RFLP method in Iraqi and Belarusian sheep breeds and found that the entire flock of sheep was monomorphic for the CLPG gene.

Additionally, Bozhilova-Sakova and Dimitrova (2016) conducted a study to identify allelic variations of the calpastanin gene (CAST) associated with meat traits in the native Bulgarian sheep breed Karakachan. A PCR-RFLP technique was performed and the restriction enzyme MspI was used to digest the PCR products. The results of this study showed that no polymorphism was found in all tested animals. Only the genotype MM was observed, so the CAST gene is monomorphic in the whole population of the sheep breed.

Furthermore, Koyun *et al*. (2019) applied the PCR-RFLP technique to study polymorphisms in milk protein genes in Norduz and Karakaş sheep breeds. In this study, four different gene loci affecting milk yield and milk content were selected: Casein (CSN3), Lactalbumin (LALBA1), Lactalbumin (LALBA2) and Lactoglobulin (LGB). The PCR products were digested with EcoRI and Hind III endonuclease enzymes. Their results showed that there were no polymorphisms between individuals in the relevant gene regions in Norduz and Karakaş sheep breeds.

Finally, Pakoohan *et al*. (2021) attempted to uncover allelic variants of FecG gene in sheep from Arunachal Pradesh. Their results showed that the locus of FecG gene is monomorphic. Based on the technique of PCR-RFLP using restriction enzyme DdeI.

The results of the current study showed that the GHY1, GHY2 and GHY3 loci of the GH gene are monomorphic in the population of Norduz sheep. It was also found that not all animals were carriers of the mutation at the GH loci studied. Only two alleles (A and B) and the genotype AB were detected in all animals with a frequency of 1.00 for each of the three loci. Consequently, none of the statistical analyses were applied to detect associations between alleles and growth traits. The absence of polymorphism at three loci suggested that GH gene is highly conserved and could be of use in evolutionary studies.

CONCLUSION

To obtain and maintain more accurate results in similar GH gene polymorphisms, it could be suggested to include more animals of the same and different sheep breeds and other related loci of GH gene in further studies. It is also recommended to use single nucleotide polymorphism (SNP) method as it is one of the most reliable methods to identify polymorphisms in genomes.

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Conflict of interest: None.

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