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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Determination of polymorphisms in the *HSP90AA1* gene region in some Turkish sheep populations by AS-PCR

Bazı Türkiye yerli koyun populasyonlarında *HSP90AA1* gen bölgesindeki polimorfizlerin AS-PZR ile belirlenmesi

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ABSTRACT

Heat shock proteins (HSPs) are molecular chaperones protecting living cells from the negative effects of extreme ambient temperatures. In this study, genetic polymorphisms of the *HSP90AA1* gene were monitored via Allele-Specific Polymerase Chain Reaction (AS-PCR) in three native Turkish sheep populations namely İvesi (IVS), Güney Karaman (GKR), and Karakaş (KRK). The *HSP90AA1* was polymorphic in all populations yielding two alleles (C and G) and three genotypes (CC, CG, and GG). The G allele frequency was higher than the C allele frequency in all populations. The lowest (0.100) and highest (0.246) CC frequency was observed in KRK and IVS, respectively, while the GG genotype frequency varied between 0.250 (GKR) and 0.600 (KRK). Significant deviation ($p<0.001$) from Hardy-Weinberg Equilibrium (HWE) was detected in the IVS breed in terms of the *HSP90AA1* gene. The genetic distance-based phylogenetic tree indicated that GKR was genetically different from IVS and KRK populations in terms of the *HSP90AA1* polymorphism. These variations regarding the *HSP90AA1* gene should be conserved, since the negative effects of global warming and climate change are expected to be more hazardous in the future. Besides, these genetic variations may be utilized by the farmers to design comprehensive selection strategies against heat stress in native Turkish sheep populations.

ÖZET

Isı şok proteinleri (HSPs) canlı hücreleri aşırı ortam sıcaklıklarının olumsuz etkilerinden koruyan moleküler şaperonlardır. Bu çalışmada, İvesi (IVS), Güney Karaman (GKR) ve Karakaş (KRK) olarak bilinen üç farklı Türkiye yerli koyun populasyonunda *HSP90AA1* genindeki genetik polimorfizmler Allel-Spesifik Polimeraz Zincir Reaksiyonu (AS-PZR) yöntemiyle incelenmiştir. İki allel (C ve G) ve üç genotipin (CC, CG ve GG) belirlendiği çalışmada, *HSP90AA1* geni bütün populasyonlarda polimorfizm göstermiştir. Bütün populasyonlarda G allel frekansı C allel frekansından yüksek bulunmuştur. En düşük (0.100) ve en yüksek (0.246) CC frekansı sırasıyla KRK ve IVS populasyonlarında belirlenirken, GG genotip frekansı 0.250 (GKR) ile 0.600 (KRK) aralığında değişmiştir. IVS ırkında *HSP90AA1* geni bakımından Hardy-Weinberg dengesinden (HWE) olan sapma önemli ($p<0.001$) bulunmuştur. Genetik mesafe temelli filogenetik ağaç, *HSP90AA1* polimorfizmi bakımından GKR populasyonunun IVS ve KRK populasyonlarından genetik olarak farklı olduğunu ortaya koymuştur. Gelecekte küresel ısınma ve iklim değişikliğinin olumsuz etkileri daha da tehlikeli olacağından *HSP90AA1* genindeki bu varyasyonların korunması gerekmektedir. Ayrıca, bu genetik varyasyonlar çiftçilerin Türkiye yerli koyun populasyonlarında ısı stresine karşı yapılacakları kapsamlı seleksiyon çalışmalarında kullanılabilecektir.

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INTRODUCTION

Türkiye significantly contributes to world animal genetic resources, since 24 of 45 sheep breeds reared in the country are native genetic resources (Karsli et al., 2020; Taşkın & Kandemir, 2022). Based on their tail phenotype, native Turkish sheep are divided into two categories namely fat-tailed and thin-tailed populations (Koyun et al., 2021). IVS, GKR, and KRK are clustered into fat-tailed populations. Of these, IVS is recognized as a unique breed and is preferred by farmers due to its higher milk yield compared to other native Turkish sheep. As reported by Biçer et al. (2019), the milk yield of IVS reared by smallholder farmers is nearly 80 - 100 kg lactation^{-1}, while it may reach up to approximately 300 kg lactation^{-1} via suitable management practices. On the other hand, GKR and KRK are considered the varieties of the Akkaraman breed (Özmen et al., 2020). However, a recent study conducted via 21 microsatellite markers indicated that GKR has become genetically different from the Akkaraman breed (Karsli et al., 2020). Moreover, the authors highlighted that more comprehensive studies based on array technologies and next-generation sequencing instruments are needed to detect the genetic distinctiveness of the GKR population (Karsli et al., 2020).

In Türkiye as well as other countries, sheep rearing plays a crucial role in human life and the sustainable use of pastures. Because grassland-based rearing allows for the effective use of green areas in the long term (Papadopoulou et al., 2021), while animal-derived products such as meat and milk are essential for human nutrition (Pinotti et al., 2021). Therefore, farmers adopt suitable management practices to maintain the production level of economically important traits such as milk and meat. On the other hand, numerous environmental stresses caused by internal and external factors have negative effects on the welfare, growth, development, and productivity in domestic animals (Demir et al., 2021). Of these environmental stressors, global warming and climate change are one of the most threatening for the agricultural sector, since they are ongoing phenomena and are expected to be more devastating in the future (Rovelli et al., 2020).

In farm animals, a balance between heat gain and heat loss is observed during non-stress conditions (Rovelli et al., 2020). Naturally, metabolic heat occurs due to feed intake, while it is tolerated via water intake as well as respiration. However, during extreme changes in ambient temperatures, animals show abnormal behaviors such as consuming less feed, drinking more water, and seeking shades in order to decrease metabolic activities (Perini et al., 2021). These kinds of behavioral adaptation mechanisms may prevent animals from the negative effects of heat stress, while economically important yields significantly decrease mainly due to less feed intake (Demir et al., 2022). The fact that animal-derived products such as milk and meat are essential for human nutrition has encouraged scientists to detect thermo-tolerant animals via molecular genotyping methods. It is of vital importance in the agriculture sector, since thermo-tolerant animals may maintain their productivity in higher ambient temperatures compared to those thermo-susceptible animals (Seijan et al., 2018).

During heat stress, HSPs are activated to protect living cells from the adverse effects of heat and other related stress factors (Demir et al., 2022). Among HSPs, *HSP90* is one of the most abundant proteins in eukaryotic cells. It is reported to compromise 1-2% of the cellular proteins under non-stress conditions, whereas its amount may reach up to 4-6% in heat-stressed cells (Kumar et al., 2016). The *HSP90AA1* gene is particularly interesting among scientists because its variations in different genomic regions are related to various phenotypes. For instance, Marcos-Carcavilla et al. (2010) reported that two polymorphisms located in the *HSP90AA1* 5' flanking region (660 and 528) were associated with the scrapie incubation period in Romanov sheep populations. Another study assessing correlations among several climatic variables and allele frequencies in 31 sheep breeds confirmed that an insertion (C allele) between 667-668 nucleotides of the *HSP90AA1* promoter region was associated with tolerance to heat stress (Salces-Ortiz et al., 2015). By sequencing a 300 bp length of the 5' flanking region of the *HSP90AA1* gene, Oner et al., (2012) reported that a new indel located at the 704 position was present in ten native Turkish sheep breeds (Akkaraman, Çine Çaparı, Dağlıç, Gökçeada, Hemşin, Karayaka, Kıvırcık, Morkaraman, Sakız, and IVS).

Authors highlighted that this indel may partially or completely inhibit the expression level of the gene by creating a glucocorticoid receptor transcription site, whereas no clear-cut relationship was reported between this variation and climatic factors (Oner et al. 2012).

Thanks to molecular genotyping techniques, native sheep populations may be screened for previously reported polymorphisms regarding heat stress-related genomic regions. Moreover, the frequency of thermo-tolerant animals may be increased via suitable mating programs. Hence, this study aimed to reveal *HSP90AA1* polymorphisms in three native Turkish sheep populations (IVS, GKR, and KRK) by AS-PCR.

MATERIALS and METHODS

Sample collection and DNA isolation

In this study, a total of 105 animals from both sexes were randomly sampled from IVS (n=61), GKR (n=24), and KRK (n=20). IVS and GKR were sampled from five representative flocks raised in Antalya province, while KRK samples were obtained from three representative flocks reared in Van province. Blood samples taken from the jugular vein into vacutainer tubes containing anticoagulant were stored at -20 ºC until DNA isolation was carried out. A salting-out method was used to isolate DNA from whole blood samples (Miller et al., 1988). The quality and quantity of isolated DNA were checked via 1% agarose gel electrophoresis and spectrophotometer (NanoDrop-DS 1000). DNA concentration was optimized at 50 ng/µL before PCR amplification.

PCR amplification and genotyping

AS-PCR protocol recommended by Singh et al. (2017) was used to amplify C and G alleles in the *HSP90AA1* promoter region in the ovine genome. As reported by Singh et al. (2017), two primer sets were utilized to amplify 254 base pairs (bp) length of C and G alleles in the PCR stage. PCR was performed in 50 µL reaction volume with 50 ng template DNA, 5 µL 10X reaction buffer, 0.6 mM dNTPs, 2.5 mM $MgCl_2$, 10 pM of each primer, 1 U of Taq DNA polymerase (GeNet Bio, Korea) and 31.25 µL nuclease-free water. PCR amplification was carried out in initial denaturation at 94 °C for 10 min, followed by 31 cycles at 94 °C for 40 s, at 60 °C for 40 s, and at 72 °C for 40 s. The final extension was applied at 72 °C for 10 min. Amplified C and G alleles were visualized by agarose gel electrophoresis in order to genotype animals. Individuals with both amplifications were considered heterozygous (CG), while single amplifications allowed to genotype individuals as homozygous (CC or GG) based on the type of amplified nucleotide.

Statistical analysis

Allele and genotype frequencies were calculated via GenAlEx 6.5 software (Peakall & Smouse, 2012). The same software was also used to test the HWE by the chi-square approach as well as the calculation of observed and expected heterozygosity values. Nei's standard genetic identity and distance values (Nei, 1972) among populations were calculated by Popgene32 (Yeh et al., 2000). Additionally, the genetic distance matrix was processed via MEGA 11 software (Kumar et al., 2008) in order to draw the phylogenetic tree per population.

RESULTS

In this study, C and G alleles regarding the *HSP90AA1* gene were amplified by AS-PCR after DNA isolation results of which were visualized on agarose gel electrophoresis (Figure 1).

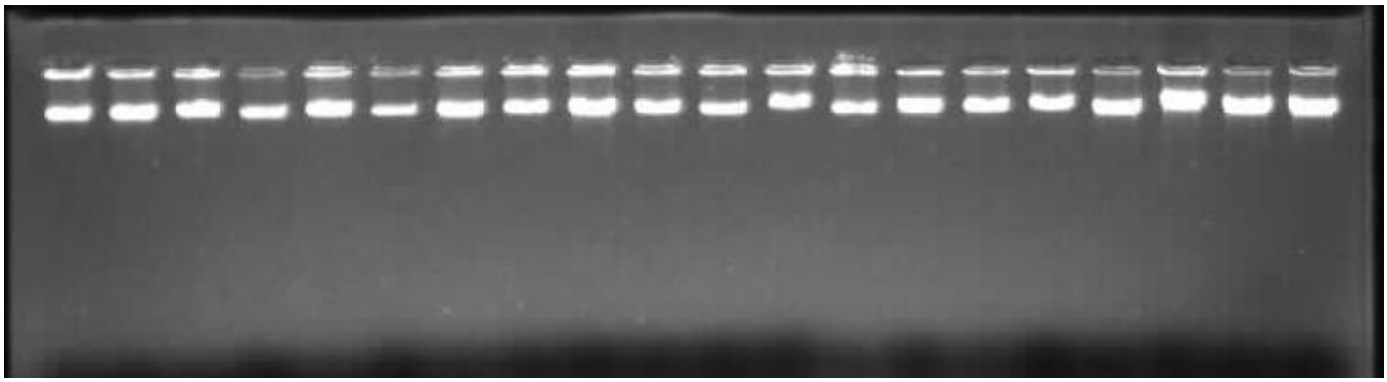

Figure 1. Agarose gel image of some isolated DNA samples
Şekil 1. İzole edilen bazı DNA örneklerine ait agaroze jel görüntüsü

Based on the presence/absence of C (Figure 2a) and G (Figure 2b) alleles, animals were genotyped as CC, CG, and GG.

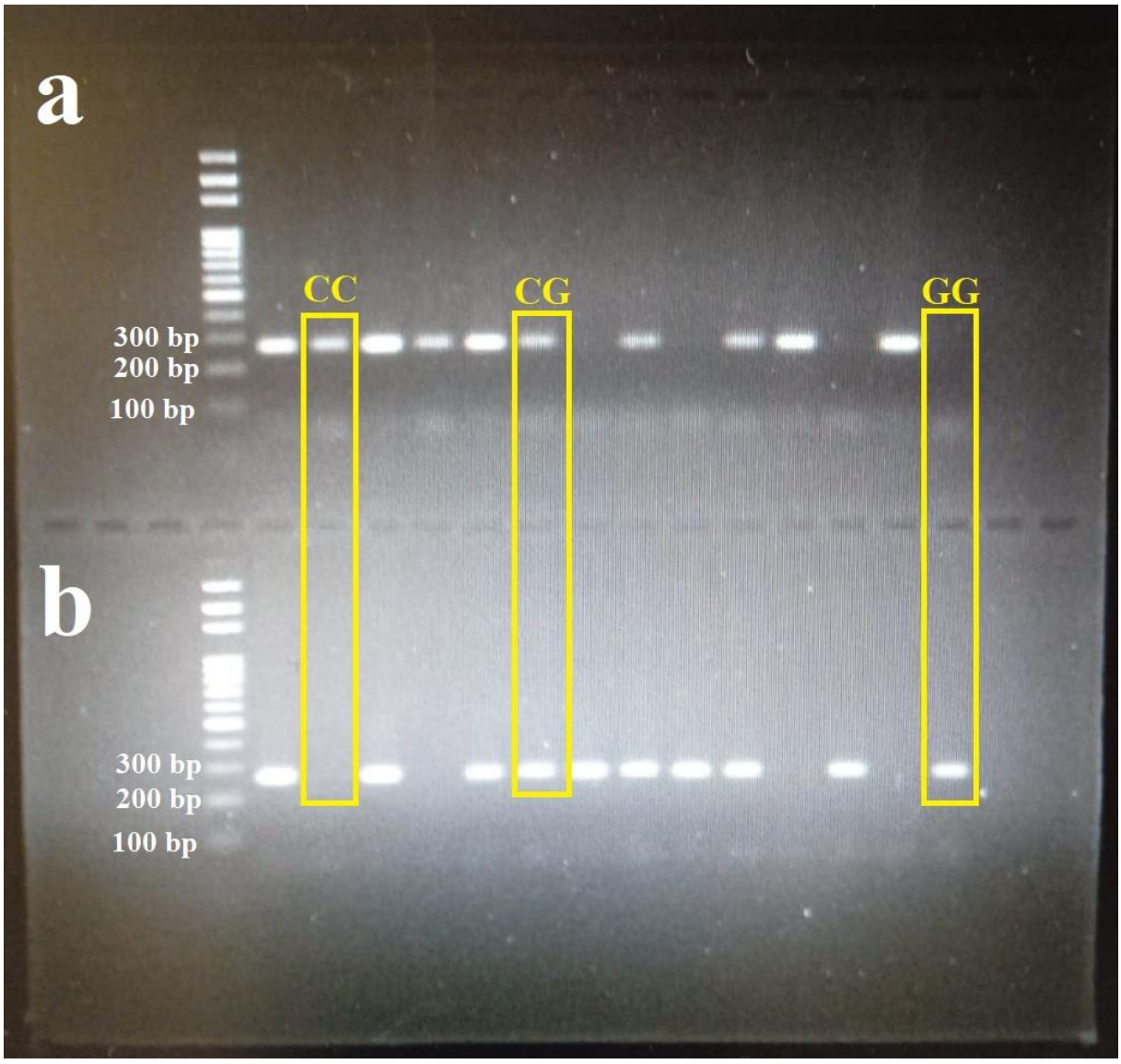

Figure 2. Agarose gel image of (a) C and (b) G alleles of the *HSP90AA1* gene
Şekil 2. HSP90AA1 geninde (a) C ve (b) G allelerine ait agaroz jel görüntüsü

C allele frequency ranged from 0.250 (KRK) to 0.458 (GKR), whereas G allele frequency was higher than C allele frequency in all populations (Table 1). The lowest CC frequencies were detected in KRK (0.100) and GKR (0.166), respectively, while it (0.246) was higher than the CG genotype frequency (0.180) in the IVS breed. The highest CG (0.584) and GG (0.600) genotype frequencies were observed in GKR and KRK, respectively. The highest observed heterozygosity (0.497) was detected in GKR, while it was lower than the expected value (0.583). On the contrary, observed heterozygosity was higher than the expected value in IVS and KRK populations. Significant deviation from HWE in terms of the *HSP90AA1* gene region was observed only in the IVS breed (Table 1).

Table 1. Allelic and genotypic distribution of the *HSP90AA1* gene in three sheep populations
Çizelge 1. Üç koyun populasyonunda HSP90AA1 genine ait allelik ve genotipik dağılımlar

Population	N	Heterozygosity		Allele frequency		Genotype frequency			X^2
		Expected	Observed	C	G	CC	CG	GG	
IVS	61	0.180	0.446	0.336	0.664	0.246	0.180	0.574	***
GKR	24	0.583	0.497	0.458	0.542	0.166	0.584	0.250	ns
KRK	20	0.300	0.375	0.250	0.750	0.100	0.300	0.600	ns

IVS = İvesi; **GKR** = Güney Karaman; **KRK** = Karakaş; **n** = Number of genotyped animals, **ns** = Deviation from HWE is non-significant; ******* = Deviation from HWE is significant ($p<0.001$).

Nei's standard genetic identity and distance values among studied sheep populations are summarised in Table 2.

Table 2. Genetic identity (above diagonal) and distance (below diagonal) values among three native Turkish sheep populations (Nei, 1972)
Çizelge 2. Üç yerli Türkiye koyun populasyonu arasındaki genetik benzerlik (üst köşegen) ve mesafe (alt köşegen) değerleri (Nei, 1972)

	IVS	GKR	KRK
IVS	-	0.9793	0.9844
GKR	0.0209	-	0.9285
KRK	0.0157	0.0742	-

IVS = İvesi; **GKR** = Güney Karaman; **KRK** = Karakaş.

The lowest (0.0157) and highest (0.0742) genetic distance values were detected between IVS-KRK and KRK-GKR, while it was 0.0209 between IVS and GKR (Table 2). The genetic distance-based phylogenetic tree demonstrated that GKR is genetically different from IVS and KRK populations in terms of the polymorphism of the *HSP90AA1* gene region (Figure 2).

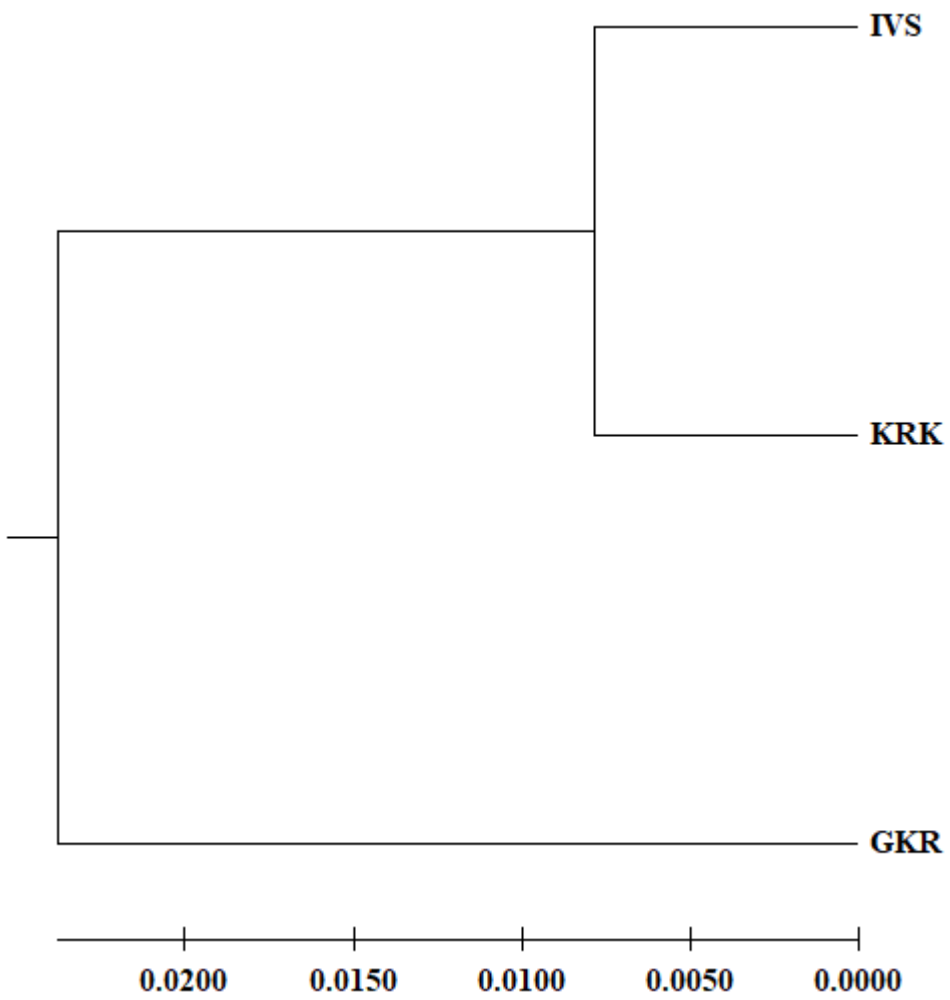

Figure 3. Image of the genetic distance-based phylogenetic tree in three studied sheep populations
Şekil 3. Çalışılan üç koyun populasyonunda genetik mesafe temelli filogenetik ağaç görüntüsü

DISCUSSION

Several studies revealed that polymorphisms in the *HSP90AA1* gene are associated with heat tolerance in different livestock species (Marcos-Carcavilla et al., 2010; Kumar et al., 2016). For example, by monitoring some Indian sheep breeds, Singh et al. (2017) reported that a variation called SNP4 in the *HSP90AA1* gene is directly related to thermo-tolerance in which animals with the C allele and CC genotype were superior in terms of heat stress parameters (rectal temperature, pulse rate, and neutrophil/lymphocyte ratio). The authors reported that no animals with CC genotype were detected in Chokla, Marwari, and Magra breeds, while it was present in Madras Red breed with a low frequency (0.16) (Singh et al., 2017). On the contrary, the CC genotype was detected in three native Turkish sheep populations the frequency of which ranged from 0.100 in KRK to 0.246 in IVS. In Türkiye, grassland-based rearing is preferred by the farmers, since it decreases feeding costs. This rearing also allows animals to be exposed to higher ambient temperatures thereby heat stress-associated genotypes may be observed in some animals. Assessing the same polymorphism, Yurdagül et al., (2023) confirmed that the C allele and CC genotype frequencies were 0.390 and 0.183, respectively in Pırlak sheep which is another native breed derived from crossbreeding of Dağlıç and Kıvırcık (Çelikeloğlu et al., 2018). It is detected that CC genotype frequency was lower in GKR (0.166) and KRK (0.100) populations compared to the Pırlak breed (0.183) (Yurdagül et al., 2023). However, a higher CC genotype frequency was observed in IVS (0.246) than in Pırlak sheep (Yurdagül et al., 2023). Moreover, IVS was

found to be deviated from HWE in terms of the *HSP90AA1* gene which may be due to non-intensive selection practices done by the farmers.

In this study, the *HSP90AA1* polymorphism showed that IVS and KRK were close to each other in tree-based phylogenetic analysis, while GKR was genetically different from both populations. However, it is noteworthy that this result may be misleading due to the fact that the current study benefits from only one gene region. Therefore, previous studies conducted with denser genetic data such as 9-21 microsatellite markers give better information on the genetic structure of native Turkish sheep breeds (Yilmaz et al., 2014; Ameur et al., 2020; Karsli et al., 2020; Kirikci et al., 2020). Surprisingly, similar results were reported by some of these studies. For example, using a total of 18 microsatellite loci, Yilmaz et al., (2014) showed that IVS and KRK were genetically close to each other in factorial correspondence analysis. On the other hand, Karsli et al. (2020) indicated that GKR was genetically different not only from KRK but also from Norduz and Kangal populations according to structure and factorial correspondence analyses via 21 microsatellite markers.

In conclusion, heat will be one of the most devastating environmental stressors in the future due to ongoing climate change and increasing global warming. Therefore, a genetic-based adaptation mechanism will be essential to facilitate the negative effects of heat stress in sheep populations. In this regard, genetic variations in the heat stress-related genomic regions such as the *HSP90AA1* will be required to take measures against environmental stressors. This study confirmed that the *HSP90AA1* gene conserves genetic variations in three native Turkish sheep populations. These variations will allow farmers to design various selection strategies against heat stress in the future. Hence, these variations should be conserved and more studies focusing on different genomic regions related to heat stress should be conducted.

STATEMENT OF CONFLICT OF INTEREST

The author declares no conflict of interest for this study.

STATEMENT OF ETHICS CONSENT

Ethical approval and permission for this study were obtained from the Akdeniz University Animal Experiments Local Ethics Committee. The approval letter's number is 1575/ 2023.04.002 and its date is 04.04.2023.

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