



## Effects of 24-bp Indel Mutation Within the AHR Gene on Litter Size in the Akkaraman Sheep Breed

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### ABSTRACT

The aryl hydrocarbon receptor (Ahr) is a transcription factor that is involved in a variety of biological processes, such as cell proliferation, growth and the immune system. Previous investigations have asserted the importance of Ahr gene in female reproduction. However, there are limited studies on the exact mechanisms of the Ahr gene on sheep litter size. Therefore, this study aimed to investigate the 24-bp indel mutation in region 3 of the Ahr gene in Akkaraman sheep, and its impact on litter size. A total of 100 blood samples were used for the study. Genomic DNA

was obtained from whole blood. Following the polymerase chain reaction (PCR) analysis, all animals were genotyped based on the result of the agarose gel image. In the study, a 24-bp indel mutation was found, and three genotypes (II, ID and DD) were detected in Akkaraman sheep. The ID (Insertion/deletion) genotype (59%) was the most common, followed by DD (deletion/deletion) (36%) and II (Insertion/insertion) (5%). In addition, the association analyses showed that the indel mutation within the Ahr gene had a significant influence on litter size.

Keywords: Sheep, Reproduction, Fecundity, Aryl hydrocarbon receptor, Polymorphism

## 1. Introduction

Akkaraman sheep have been widely bred in the Anatolian region of Türkiye for centuries, and they are known for their long mating season, adaptability, and resistance to disease (Akçapınar 2000). Despite its exquisite features, the twinning rate in this breed ranges from 1.08 to 1.39 per ewe, indicating a low litter size (Esen & Bozkurt 2001; Aksoy et al. 2023; Acı et al. 2024). When it comes to livestock, many factors influence the profitability of both dairy and meat production systems (Soydan et al. 2009; Sen et al. 2020). The main factors affecting profitability, particularly in sheep farming, are litter size, the prolificacy rate, the number of lambs at weaning age, feed conservation, and labor (Cam et al. 2017). Among these, litter size is crucial for increasing breeders' incomes and is commonly used as a selection criterion for reproductive traits in breeding programs (Schmidová et al. 2014). However, due to the low heritability of litter size, it is difficult to achieve the desired results in a short period of time using traditional selection. Therefore, elucidating the genetic basis of reproductive traits will provide genotypic data that will be more useful for selection studies (Acı et al. 2024). Although the Akkaraman breed is a low fertility breed, due to the importance of the breed in the country's animal husbandry, there has recently been an increase in studies examining some genes of fertility traits (Kırıkçı et al. 2021; Kırıkçı 2023; Yiğit et al. 2023; Acı et al. 2024). These limited studies demonstrate that the Akkaraman breed exhibits genetic variation in GDF9 and RORA genes (Kırıkçı 2023; Yiğit et al. 2023).

The Ahr gene, a member of the PER-ARNT-SIM (Pas) family, can be activated by a ligand as a transcription factor. The gene is involved in numerous biological processes, such as the cellular proliferation and differentiation. In its unbound state, the Ahr is restricted to the cytoplasm (Girolami et al. 2013). After activation, the Ahr associates with the core of the Ahr nuclear translocator (Arnt) and forms an active complex with Arnt to alter the expression of target genes (Basham et al. 2015). Bussman et al. (2006) stated that Ahr activation may disseminate the proliferation of granulosa cells, thereby promoting follicular development and enlarging the number of ovulations. One of the most fascinating aspects of the Ahr gene is its ability to promote or inhibit cell proliferation (Larigot et al. 2018). More recently, studies in knockout mice have provided important evidence for the role of Ahr in female reproduction. Specifically, these mice have reproductive defects, such a developmental disorder of the antral follicles and the corpus luteum (Benedict et al. 2000; Benedict et al. 2003), as well as reduced ovulation rates (Barnett et al. 2007). Furthermore, Ahr mRNA levels were shown to increase during follicular maturation in the rat ovary (Chaffin et al. 2000).

In a recent study, a 24-bp indel variant of Ahr gene was shown to increase the litter size of female Australian white sheep (Ren et al. 2022). In addition to these functions, Ahr also plays a role in the immune system (Gutiérrez-Vázquez & Quintana 2018) and in tumorigenesis (Kolluri et al. 2017). There are not enough studies in the literature examining the effects of the Ahr gene on sheep reproduction. Although studies have been conducted on the fertility genes of Turkish sheep breeds, the Ahr gene polymorphism and its role in reproduction have not been investigated. Therefore, this study investigated for the first time a 24-bp indel mutation within the Ahr gene and its effects on litter size in the Turkish sheep breed Akkaraman.

## 2. Material and Methods

### 2.1. Material

A total of 100 blood samples previously collected from female Akkaraman sheep for another project, were used as research material in this study (Kırıkçı 2023). The animals ranged in age from 2 to 6 years and were all bred under similar conditions in the Yozgat province of Türkiye. Based on interviews with farmers, the ewes were divided into two groups: single and twin lambs.

### 2.2. Method

The research was carried out in the Laboratory, Department of Zootechnics, Kırşehir Ahi Evran University, Kırşehir, Türkiye.

### 2.3. DNA isolation

Genomic DNA was extracted from whole blood using the protocol provided by the manufacturer's guidelines using a GeneJET Genomic DNA Purification Kit (Thermo Scientific, Lithuania). The following DNA isolation, samples were stored at -20 °C.

### 2.4. Amplification of the Ahr gene and genotyping of animals

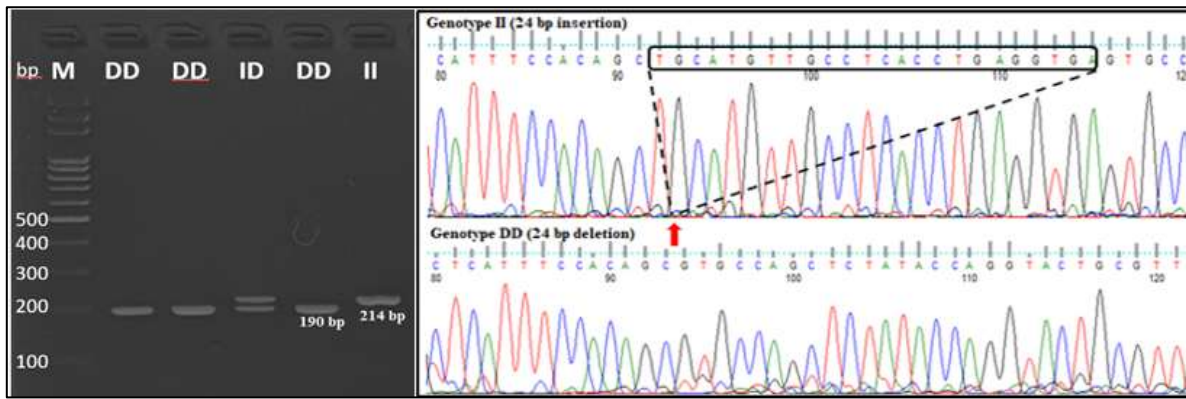
To amplify the intron region 3 of the Ahr gene, PCR analysis was performed using the primers F: GCGTCCTGTCTGGTGTGTAA and R: ACAGGCTCTTTATCGGGTGG designed by Ren et al. (2022). In the PCR reaction, 4 µL of PCR master mix was used for the amplification of the Ahr gene fragment, including 1.6 µL of DNA template, 0.7 µL of primer (each primer), and added ultrapure water was added to a final volume of 25 µL. The samples were denatured for 5 min, followed by 35 cycles of 94 °C for 30 s each, annealing at 61 °C for 30 s, extension at 72 °C for 1 min, and finally extended at 72 °C for 7 min. Indel variants were determined by electrophoresizing 4.0 mL of the PCR products on 3.5% agarose gels. Afterwards, the gel images were visualized using a uv transilluminator. To confirm the existence of the indel mutation, a total of three samples of genotypes DD, ID, and II were subjected to sequence analysis using an ABI 3100 capillary electrophoresis device. The obtained sequences were aligned with Mega X (Molecular Evolutionary Genetics Analysis) software and compared with each other (Kumar et al. 2018).

### 2.5. Statistical analysis

The population parameters of the Ahr gene, such as allele and genotype frequencies, were accurately calculated using the website [www.msrcall.com](http://www.msrcall.com). The Hardy-Weinberg equilibrium (HWE) and heterozygosity values were calculated using the same program. The association between the indel variant and litter size was analyzed using the independent chi-square test and Fisher's exact test (Ren et al. 2022) in SPSS 22 software (IBM SPSS Inc., Chicago, IL, USA).

## 3. Results and Discussion

The intronic mutations may regulate transcriptional activity by preventing or increasing the binding of transcription factors, thus affecting gene expression, resulting in phenotypic differences (Shaul et al. 2017; Cui et al. 2018). For example, an insertion/deletion mutation (indel) within the ATBF1 gene strongly influences litter size in goats (Wang et al. 2020). Similarly, a 23-bp indel mutation within the RORA gene significantly affects sheep litter size (Yang et al. 2022). In the present study, we investigated a 24-bp indel mutation in the intron 3 region of the Ahr gene for the first time in the Akkaraman sheep breed. The Ahr gene was successfully amplified by PCR, and all individuals were genotyped using the agarose gel electrophoresis technique. The gel results showed two band patterns with lengths of 190 and 214 bp. The presence of the 24-bp mutation identified in the Ahr gene from the agarose gel analysis images was also confirmed by DNA sequence analysis, as shown in Figure 1.



**Figure 1- Agarose gel and DNA chromatogram results**

The Ahr gene, a ligand-activating transcription factor, is involved in a variety of biological processes, such as cell proliferation, growth and the immune system (Barouki et al. 2007; Roman et al. 2018). Zheng et al. (2020) and Bosse et al. (2014) found that copy number variations and non-synonymous mutations in the eleventh exon within the Ahr gene have a positive impact on reproductive traits of Chinese and European pigs, respectively. More recently, Ren et al. (2022), who first discovered a 24-bp indel mutation in the sheep Ahr gene, showed that this mutation has a significant impact on litter size in the Australian white sheep breed. Depending on previous evidence of the Ahr gene, we hypothesized that this gene may be a candidate gene for selection studies in litter size. The allele and genotype frequencies and population parameters are summarized in Table 1.

**Table 1- Genotype and allele frequency values and population parameters**

Genotypic frequencies			Allelic frequency		Heterozygosity values			
DD	ID	II	D	I	Ho	He	Ne	PIC
0.36	0.59	0.05	0.66	0.34	0.548	0.452	1.825	0.350

In this study, genotype ID was the most common genotype, with a frequency of 59%, followed by genotypes DD and II, with a frequency of 36% and 5%, respectively. In contrast, the DD genotype was most common in Australian white sheep (Ren et al. 2022). The genetic frequencies calculated for the Ahr gene deviated from Hardy-Weinberg equilibrium (HWE) ( $P < 0.05$ ); it is unlikely to be due to migration, genetic drift (Wang et al. 2020), inbreeding, or sample size in the study. In this study, the PIC value was 0.350, indicating moderate polymorphism according to the polymorphism criteria. The value calculated for Akkaraman sheep was higher than those reported for the Australian white sheep (AuW) (Ren et al. 2022). According to Fisher's exact test results, a significant association was found between the indel mutation and litter size in Akkaraman sheep ( $P < 0.05$ ). Similar results were found in the study by Ren et al. (2022) reported that the 24-bp indel mutation within the Ahr gene has a significant impact on the litter size in AuW sheep. As shown in Table 2, the number of individuals with DD genotype was higher in the twin group. However, this situation was opposite in the AuW sheep (Ren et al. 2022). Considering the role of intronic mutations in gene activity, they could affect such phenotypes differently. For example, the intronic 11-bp indel mutation of the DNMT3B gene in goats modulated ovarian gene expression and increased litter size, which was higher in DD genotypes than in other genotypes (Hui et al. 2020). In another study, Australian white sheep with the Cry2 II genotype exhibited a larger litter size than other breeds (Huang et al. 2022). Litter size, a quantitative trait, has an extremely complex mechanism involving many genes or loci as well as gene interactions (Davis 2005).

**Table 2- Association between indel genotypes and litter size in Akkaraman ewes**

Litter size	Genotypes			Total
	DD	ID	II	
Singleton	12 (18)	36 (29.5)	2 (2.5)	50
Twin	24 (18)	23 (29.5)	3 (2.5)	50
Total	36 (36)	59 (59)	5 (5)	100

Fisher's p-value = 0.022

To the best of our knowledge, there was only one study on the intron 3 region of the sheep Ahr gene (Ren et al. 2022). The current study is the first research investigating the Turkish sheep breed. Here we found that the 24-bp indel variant significantly influenced litter size in Akkaraman sheep. Because the results of this study are preliminary, there is clearly a need for further

studies to investigate the effects of Ahr genes and their mechanisms of action on reproductive traits in sheep species with large sample sizes and phenotypic records.

#### 4. Conclusions

Results of this study suggest that a novel 24-bp indel variant within the Ahr gene significantly influences litter size in Akkaraman sheep, implying its role in sheep reproduction. However, this conclusion should be confirmed in a large scale. Further studies may provide insights into the effects of a 24-bp indel variant within the Ahr gene on reproductive traits.

#### Conflict of interest

The authors declare that they have no conflicts of interest.

#### Ethical statement

The blood samples used in this study were collected with ethical approval (03/31/2021, 68429034/04) from the Ethics Committee of Ahi Evran University (AEUHAYDEK), Türkiye.

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