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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

InDel Variations of *PRL* and *GHR* Genes Associated with Litter Size in Pirlak sheep breed*

Pırlak Koyun Irkında Bir Batında Doğan Yavru Sayısı ile Ilişkili *PRL* ve *BHR* Genlerindeki inDel Varyasyonlar*

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Abstract

Numerous molecular genotyping methods are available to analyse local livestock populations at molecular level in which traditional Polymerase Chain Reaction (PCR) guided by specific oligonucleotides is a fast and cost-effective method to investigate single genes. Until today, many genes which are of major effects on litter size have been reported in sheep. Genetic variations in these genes shaping the expression profile at DNA level may lead to differences in litter size among the sheep breeds. This is the first attempt to investigate insertion/deletion (inDel) variations in Prolactin (*PRL*) intron 2 and Growth Hormone Receptor (*GHR*) intron 3 and intron 4 genes in Pirlak sheep breed via traditional PCR technique. A total of 100 unrelated animals sampled from representative herds reared in Antalya were genotyped based on absence/presence of 23 base pairs (bp) length inDel in which three genotypes (II, ID, and DD) were detected in all loci. I and D allele frequency were 0.421 and 0.579, respectively in terms of *PRL*-intron 2 locus. I / D allele frequencies were found as 0.599 / 0.401 and 0.372 / 0.628 in *GHR* intron 3 and intron 4, respectively. The lowest II (0.181) and DD (0.177) genotype frequencies were detected in *GHR*-intron 4 and *GHR*-intron 3 loci, respectively. The lowest (0.177 for DD) and highest (0.448 for ID) genotype frequencies were detected in *GHR* intron 3 locus across the population. Significant deviation from Hardy-Weinberg Equilibrium (HWE) was detected only in *PRL*-intron 2 locus. The results of the present study confirm that Pirlak breed conserves sufficient genetic variation in *PRL* and *GHR* gene regions which could be utilized in selection strategies in order to increase litter size in the future.

Keywords: Deletion, Insertion, Litter size, Multiple birth, PCR

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Öz

Çiftlik hayvanı populasyonlarının moleküler seviyede analiz edilmesine olanak sağlayan çok sayıda moleküler genotipleme yöntemleri bulunmakla birlikte özgün oligonükleotidlerle yönlendirilmiş geleneksel Polimeraz Zincir Reaksiyonu (PZR) tekli genlerin incelenmesinde kullanılan hızlı ve uygun maliyetli bir tekniktir. Günümüze kadar koyunlarda bir batında doğan yavru sayısı üzerine major etkileri olan çok sayıda gen bildirilmiştir. DNA seviyesinde ekspresyon profilini şekillendiren bu genlerdeki varyasyonlar koyun ırklarında bir batında doğan yavru sayısında farklılıkların görülmesine neden olabilmektedir. Bu çalışma Pırlak koyun ırkında Prolaktin (*PRL*) intron 2 ve Büyüme Hormonu Reseptörü (*BHR*) intron 3 ve intron 4 gen bölgelerindeki insersiyon/delesyon (inDel) varyasyonlarının geleneksel PZR yöntemiyle incelendiği ilk araştırmadır. Antalya'da yetiştirilen, ırkı temsil eden ve birbiriyle akraba olmayan toplam 100 hayvan 23 baz çifti (bç) uzunluğundaki inDel'in varlığı/yokluğuna göre genotiplendirilmiş ve bütün varyasyonlar bakımından üç farklı genotip (II, ID ve DD) tespit edilmiştir. *PRL*-intron 2 lokusunda I ve D allel frekansı sırasıyla 0.421 ve 0.579 olarak bulunmuştur. *BHR*-intron 3 ve intron 4 lokuslarındaki I / D allel frekansları sırasıyla 0.599 / 0.401 ve 0.372 / 0.628 olarak bulunmuştur. En düşük II (0.181) ve DD (0.177) genotip frekansı sırasıyla *BHR*-intron 4 ve *BHR*-intron 3 lokusunda belirlenmiştir. Populasyon seviyesinde en düşük (DD için 0.177) ve en yüksek (ID için 0.448) genotip frekasnları *BHR* intron 3 lokusunda tespit edilmiştir. Sadece *PRL*-intron 2 lokusunda Hardy-Weinberg (HWD) dengesinden önemli sapma tespit edilmiştir. Bu çalışmanın sonuçları Pırlak ırkının *PRL* ve *BHR* gen bölgeleri bakımından yeterli seviyede genetik varyasyona sahip olduğunu ve bunun gelecekte bir batında doğan yavru sayısını arttırmak için yapılacak seleksiyon stratejilerinde kullanılabileceğini göstermiştir.

Anahtar Kelimeler: Delesyon, Insersiyon, Bir batında doğan yavru sayısı, Çoklu doğum, PZR

1. Introduction

Small ruminant breeding is of significant potential to effectively utilise the lands which are not suitable for crop production. Since the meadows and pastures of Türkiye are more suitable for small ruminants rather than cattle (Şişman et al., 2009), sheep rearing is commonly practiced by smallholder farmers (Ertuğrul et al., 2009). Official data reported in 2023 indicates that approximately 45 million sheep most of which are native breeds and their crossbreeds are reared in Türkiye (Anonymous, 2023). Sheep rearing plays an important role in meeting demands for beef in Türkiye (Kocaman and Günal, 2007). Therefore, commercial companies prefer to rear sheep breeds famous for multiple birth traits as well as conducting selection practices in order to increase litter size. In this regard, Pirlak is increasingly preferred in Türkiye due to its advantages characteristics such as high adaptation ability, milk yield, as well as litter size. As highlighted by the General Directorate of Agricultural Research and Policies of Türkiye (GDARP) in 2009, possessing thin-tailed phenotype, Pirlak is reared for dual purposes mainly in Burdur, Isparta and Antalya provinces (Özçelik and Bayram, 2012; Çelikeloğlu et al., 2018). There are several studies in the literature indicating that Pirlak is derived from crossbreeding studies between Daglic and Kivircik breeds (Koyuncu et al., 2005; Özçelik and Bayram 2012; Çelikeloğlu et al., 2018).

Being one of the most important reproductive traits, litter size is of the importance for farm animals such as sheep to survive as well as making contribution to incomes of farmers (Karsli et al., 2012; Tao et al., 2021). Advances in feeding techniques, biotechnology, and molecular genetics have created several alternatives for farmers to improve litter size trait in local sheep breeds. For example, Koyuncu and Canbolat (2009), revealed that energy supplementation at pre-mating (21-day) could increase litter size in Kivircik sheep breed. Similarly, Cam and Kuran (2004), studied the effects of single injection of hCG and GnRH at post-mating (12-day) on litter size in Karayaka and Karayayaka x Sakiz F2 crossbreds in which more twins birth was reported in test group than control group. Although feeding and biotechnological applications could increase litter size in a given sheep population, it may be neither practical nor affordable for smallholder farmers. Multiple births supported by feeding and biotechnological applications are not inherited to the next generations and should be repeated for each mating season due to the nature of quantitative traits including litter size. Indeed, as highlighted by Karsli and Balcioglu (2010), litter size is a complex trait which is influenced by numerous environmental factors and controlled by polygenes. This complexity has been forcing scientists to detect genomic regions having minor and major effects on litter size in sheep. Indeed, numerous genomic regions have been reported to influence litter size in sheep (Karsli and Balcioglu et al., 2010; Karsli et al., 2012). Bone Morphogenetic Protein Receptor IB (*BMPR-IB*) region containing Fecundity Booroola (*FecB*) mutation was reported to be associated with litter size in sheep for the first time (Davis et al., 1982). Since then, scientific efforts making use of developing molecular genotyping methods have yielded numerous gene regions such as Growth Differentiation Factor 9 (*GDF9*) (Hanrahan et al., 2004), *BMP15* (Bodin et al., 2007), Histone Cell Cycle Regulator (*HIRA*) (Zhou et al., 2018), Thyroid Stimulating Hormone Receptor (*TSHR*) (Tao et al., 2021), Neurotrophic Receptor Tyrosine Kinase 2 (*NTRK2*) (Esmaeili-Fard et al., 2021), etc., which are of minor and/or major effects on litter size in sheep. Among litter size-associated genes, *PRL* which is located on chromosome 20 with five exons and four introns in the sheep genome, is known to encode prolactin hormone (Al-Thuwaini, 2021). Being an anterior pituitary peptide hormone, *PRL* plays a key role in many endocrine activities to maintain reproduction in mammals (Ran et al., 2011). The *GHR* gene, on the other hand, encodes a protein called growth hormone receptor which affects follicular growth by stimulating Insulin-like Growth Factor 1 (*IGF-1*) gene (Ghiasi and Abdollahi-Arpanahi, 2021). Genetic variations in reproduction-related genes may lead to different amino acid syntheses as well as affect expression profiles at DNA level. Of these genetic variations, insertion and deletion define the acquisition and loss of different numbers of nucleotides in a given sequence, respectively. InDels are of potential to cause different variations across the genome by causing small frameshift mutations, radically altering genes, changing the binding and splicing sites of the genes as well as disrupting other genomic regions (Narzisi and Schatz, 2015).

Variations in the genomic regions related to litter size allow farmers to improve selection strategies in which animals with desired genotype are used for mating programs (Demir et al., 2022). Also known as Marker Assisted Selection (MAS), this kind of selection is of possibility to increase the frequency of the desired genotype by which litter size are increased from one generation to another. Indeed, a recent study showed that the variations caused by inDel in *PRL* and *GHR* genes are responsible for litter size in Australian White sheep breed (Akhatayeva et al., 2020). Moreover, the authors stressed that these variations could be utilized to improve reproductive traits in sheep

breeds via MAS. Hence, this study aims to detect inDel variations in *PRL* and *GHR* genes in Pirlak sheep breed for the first time. In this regard, the objective of the current study is to assess for the first time genetic variations of Pirlak sheep in terms of *PRL* and *GHR* genes which were previously reported to be associated with litter size in different sheep breeds. Additionally, in case of the detection of advantages genotypes, it is aimed to evaluate the usefulness of these genes in further MAS studies to increase litter size in Pirlak sheep.

2. Materials and Methods

2.1. Sample Collection and DNA Extraction

A total of 100 female animals belonging to Pirlak breed were randomly chosen from five representative herds reared in Antalya province. Blood samples were collected from jugular vein into vacutainer tubes containing EDTA solution as an anticoagulant and stored at -20 °C till DNA extraction was performed. DNA was extracted from whole blood samples via a salting-out method reported by Miller et al. (1988). DNA quality and quantity were checked by both 1% agarose gel electrophoresis and spectrophotometer (NanoDrop-SD 1000). DNA concentration was optimised at 50 ng μL^{-1} for PCR amplification.

2.1. PCR Amplification and Genotyping

In this study, a total of three primer sets (*Table 1*) were used to amplify *PRL* intron 2 *GHR* intron 3, and intron 4 regions.

Table 1. An overview of primer sequence of PRL and GHR gene polymorphisms

Gene	Locus	InDel Type	Primer Sequence (5'-3')	Tm (°C)	Reference
PRL	Intron 2	Insertion	F: GGGAAGGGAAGAGAAACAGAGG R: GCTTGTAGGGTGGAACACTGA	60-59.9	Akhatayeva et al., 2020
GHR	Intron 3	Deletion	F: TGCTGTATGGCCCCTCTAGTA R: CTAAAGAGTTTCCCCAGTCCCC	59.8-60	
GHR	Intron 4	Deletion	F: GCTTCTTGCCCAACCCAATG R: CTGGGCAGTGGAGGAGAAAG	60	

PRL: Prolactin; ***GHR:*** Growth hormone receptor; **F:** Forward primer; **R:** Reverse primer; **Tm:** Annealing temperature.

As reported by Akhatayeva et al. (2020), the genetic variations in these genomic regions occurred due to a 23 bp length of inDel variations. Therefore, traditional PCR was preferred to genotype the individuals. 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. All PCR products were separated on 3.5% agarose gel in order to genotype individuals as follows II, ID and DD based on the presence or absence of the PCR fragments.

2.2. Statistical Analysis

GenAlEx software (Peakall and Smouse, 2012) was used to calculate allele and genotype frequencies and to test HWE via chi-square ($\chi 2$) approach.

3. Results and Discussion

While some samples (5 samples in *PRL*-intron 2 locus, 4 samples in *GHR*-intron 3 locus, and 6 samples in *GHR*-intron 4 locus) were removed from the analyses due to failures that occurred in DNA isolation and PCR amplification stages, three genomic regions belonging to *PRL* (intron 2) and *GHR* (intron 3 and 4) turned out to be polymorphic yielding three genotypes such as II, ID and DD in Pirlak breed (*Figure 1*). I allele frequency varied between 0.372 (*GHR*-intron 4) and 0.599 (*GHR*-intron 3), whereas D allele frequency ranged from 0.401 (*GHR*-intron 3) to 0.628 (*GHR*-intron 4) (*Table 2*). Comparatively II genotype frequency was low in terms of *PRL*-intron 2 (0.232) and *GHR*-intron 4 (0.181), DD genotype was detected at lowest frequency in *GHR*-intron 3 polymorphisms. *GHR* gene region was at the HWE, whereas a significant deviation from HWE was detected in *PRL* polymorphism (*Table 2*). Selection

practices on multiple birth traits over generations could significantly change allele frequencies leading to deviation from HWE in Pirlak population.

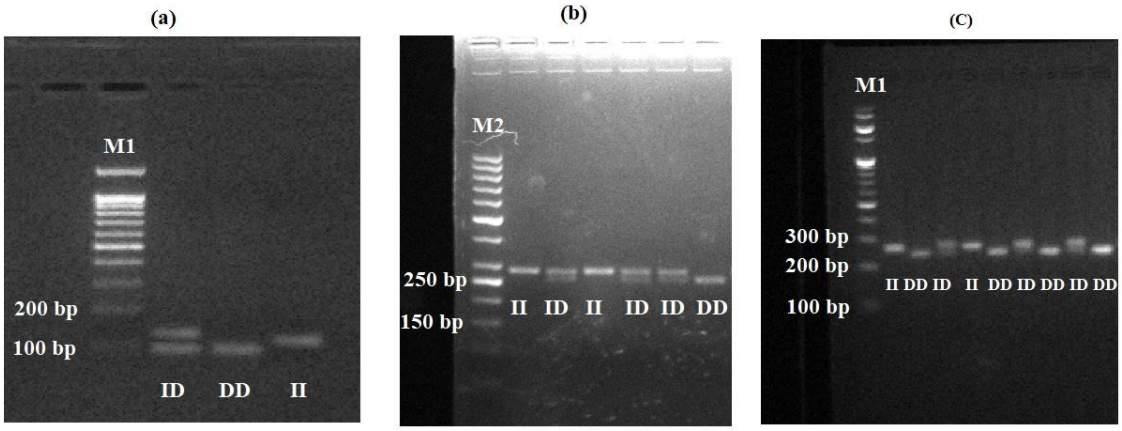

Figure 1. Agarose gel image of (a) PRL-intron 2 (M1: 100 bp ladder, ID: 87 and 110 bp, DD: 87 bp and II: 110 bp), (b) GHR-intron 3 (M2: 50 bp ladder, ID: 260 and 283 bp, DD: 260 bp and II: 283 bp) and (c) GHR-intron 4 ((M1: 100 bp ladder, ID: 238 and 261 bp, DD: 238 bp and II: 261 bp) polymorphisms.

Table 2. Allele and genotype frequency of PRL and GHR indels in Pirlak

Gene	Region	n	Allele Frequency		Genotype Frequency			χ^2
			I	D	II	ID	DD	
PRL	Intron 2	95	0.421	0.579	0.232	0.379	0.389	*
GHR	Intron 3	96	0.599	0.401	0.375	0.448	0.177	ns
GHR	Intron 4	94	0.372	0.628	0.181	0.383	0.436	ns

PRL: Prolactin; ***GHR:*** Growth hormone receptor; **n:** Number of samples; *: Significant deviation from HWE ($p<0.05$), **ns:** Non-significant deviation from HWE.

The same InDel variation of *PRL* intron 2 locus was also studied in Australian White and Luxi Blackhead sheep breeds (Akhatayeva et al., 2020; Mao et al., 2021). Akhatayeva et al. (2020), highlighted that animals with the DD genotype showed superior values in terms of the first parity litter size. In this study, the DD genotype frequency in Pirlak sheep (0.389) was higher than the values reported in Australian White (0.107) and Luxi Blackhead (0.326) sheep breeds (Akhatayeva et al., 2020; Mao et al., 2021). On the contrary, Akhatayeva et al. (2020), confirmed that animals with the II genotype showed superior values of first parity litter size in terms of the *GHR* intron 3 and intron 4 loci. The II genotype frequencies for *GHR* intron 3 and intron 4 were lower in Pirlak sheep (0.375-0.181) compared to Australian White breed (0.378-0.287). Unfortunately, no previous studies focusing on revealing genetic variations of *PRL* and *GHR* genes in Pirlak breed are available in the literature. Moreover, while few similar studies in other native Turkish sheep breeds were available in terms of the *PRL* gene, no studies were detected for the *GHR* gene. For example, Ozmen and Kul (2016) investigated Single Nucleotide Polymorphisms (SNPs) in *PRL* intron 2 across three native sheep breeds namely Sakiz, Akkaraman, and Awassi. The authors detected two alleles (A and B) and three genotypes (AA, AB, and BB) in which A was the most frequent allele (0.77) in Sakiz, whereas the frequency of B allele was higher in Akkaraman (0.85) and Awassi sheep (0.77). Ozmen et al., (2011) sequenced *PRL* receptor intron 1 and exon 10 loci in three native Turkish sheep breeds (Chios, White Karaman, and Awassi) in which 6 and 7 different haplotypes were reported for intron 1 and exon 10, respectively. The authors highlighted that White Karaman and Awassi were similar to each other in terms of *PRL* receptor intron 1 and exon 10, whereas Chios had unique variations.

Except for *PRL* and *GHR*, numerous genes (*AA-NAT*, *GDF9*, *PRLR*, *BMP15*, *BMPR-1B*, *PRL*) were previously studied in other native Turkish sheep breeds rather than Pirlak sheep (Karsli and Balcioglu, 2010; Karsli et al., 2011; Ozmen et al., 2011; Öner et al., 2014; Ozmen and Kul, 2016; Al-Anbari et al., 2018; Kirikci et al., 2021). Both the present research and previous studies evidenced that native Turkish sheep breeds conserve a considerable genetic variation in terms of litter size-related genes. On the other hand, the number of studies is still scarce, and more scientific

studies focusing on different genes associated with reproduction traits are required in Pirlak as well as other native Turkish cattle breeds in order to obtain deeper results which could be used to improve selection strategies.

4. Conclusions

Genetic variations, which were previously reported to be associated with litter size in sheep and caused by 23 bp length of inDel in *PRL* and *GHR* genes, were assessed in Pirlak breed for the first time. These gene regions were highly polymorphic conserving all the possible genotypes including II, ID and DD. Priority should be given to conserve of these variations not only for animals to survive but also for farmers to improve selection strategies in terms of reproductive traits such as litter size. However, Pirlak sheep breed has been neglected so far, since molecular studies are very scarce in the literature. We highly recommend further studies focusing on the other genetic variations in the related gene regions in Pirlak breed.

Ethical Statement

This study was approved by the Akdeniz University Animal Experiments Local Ethics Committee (Protocol No. 1391/2022.01.003).

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