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Associations of *VLDLR* Gene Polymorphism (intron 11, 392C > T) with Egg Production and Weight in Japanese Quails (*Coturnix coturnix japonica*)

ABSTRACT

Objective: This study aimed to assess the association between the *VLDRLR* gene intron 11 (392 C>T) polymorphism and egg production and weight in quails.

Material and Methods: Egg yield and weight were recorded over 90 days for 191 Japanese quails, which were genotyped using the restriction fragment length polymorphism technique for the *VLDLR* gene variation.

Results: The *VLDLR* gene was polymorphic due to conserving all possible genotypes (TT, TC, and CC). The TT genotype was the most common with a frequency of 0.70, while the frequency of the TC and CC genotype were 0.16 and 0.14, respectively. The mean 90-day egg production was 78.31, 76.07, and 73.94 in TT, TC, and CC genotypes, respectively, while the mean egg weight ranged from 915.80 (CC genotype) to 939.19 (TT genotype). Association analysis revealed a significant relationship between the *VLDLR* genotypes and egg production traits (P<0.05), while no significant relationship was detected for egg weight.

Conclusion: This study showed that the TT genotype for *VLDLR* gene intron 11 (392 C>T) polymorphism can be used in marker-assisted selection studies in order to increase egg production in quails.

Keywords: Candidate genes, egg traits, genetic variation, MAS, PCR-RFLP

Japon Bıldırcınlarında (*Coturnix coturnix japonica*) *VLDLR* Gen Polimorfizminin (11 intron, 392C > T) Yumurta Üretimi ve Agırlıgı ile Iliskisi

ÖZ

Amaç: Bu çalışma, Japon bıldırcınlarında *VLDLR* geninin intron 11 (392 C>T) polimorfizmi ile yumurta üretimi ve ağırlığı arasındaki ilişkiyi değerlendirmeyi amaçlamıştır

Materyal ve Metot: *VLDLR g*en varyaysyonu için restriksiyon fragment uzuluğu polimorfizmi tekniğiyle gengenotiplendirilen 191 Japon bıldırcınında 90 günlük yumurta verimi ve ağırlığı verisi kaydedilmiştir.

Bulgular: Muhmetel bütün genotipleri (TT, TC ve CC) içerdiğinden dolayı *VLDLR* geninin polimorfik olduğu belirlenmiştir. En yaygın genotipin 0.70 frekans ile TT olduğu, TC ve CC genotip frekanslarının ise sırasıyla 0.16 ve 0.14 olduğu belirlenmiştir. 90 günlük yumurta üretimi ortalamasının TT, TC ve CC genotipleri için sırasıyla 78.31, 76.07 ve 73.94 olduğu, yumurta ağırlığı ortalamasının ise 915.80 (CC genotipi) ile 939.19 (TT genotipi) aralığında değiştiği gözlemlenmiştir. Yapılan ilişki analizi, *VLDLR* genotipleriyle yumurta üreteimi arasında önemli bir ilişkinin olduğunu (P<0.05), yurmurta ağırlığı açısından ise herhangi bir ilişkinin olmadığını göstermiştir.

Sonuç: Bu çalışma, *VLDLR* geninin intron 11 (392 C>T) polimorfizmini için TT genotipinin japon bldırcınlarında yumurta üretimini arttırmak için uygulanacak olan marker destekli seleksiyon çalışmalarında kullanılabileceğini göstermiştir.

Anahtar Kelime: Aday genler, yumurta özellikleri, genetik çeşitlilik, MDS, PZR-RFLP

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INTRODUCTION

The Japanese quails (*Coturnix coturnix japonica*) are globally recognized laboratory animal species and the smallest avian species bred for egg and meat production. They offer several species-specific advantages such as small body size, ease of breeding, short generation interval, high egg production, being more resistant to diseases than other poultry species and requiring less space for production making the quails a convenient model animal for poultry breeding (Alkan et al. 2010; Alkan et al. 2013). Furthermore, ongoing breeding studies have made quails one of the preferred poultry species for egg and meat traits. Indeed, approximately 250 eggs may be produced per year in quails by traditional breeding studies (Narinç et al. 2013; Akarikiya et al. 2022), which can be further improved by marker-assisted selection (MAS) studies in order to improve egg yield.

Molecular methods are now used in breeding studies to improve the quantity and quality of yields, which have economic importance in farm animals (Demir et al. 2023; Karsli et al. 2020). SNP or InDel mutations may be easily detected across genomes of poultry farm animals (Karsli et al. 2017; Demir et al. 2020a) like quail (Bozkaya et al. 2013; Ahmed and Al-Barzinji, 2020) thanks to the rapid advances in the area of molecular genetics. Associations analyses are available to reveal the relationships between various phenotypic traits (meat, egg production, and quality characteristics) and genomic variations allowing for using candidate genes in MAS (Balcıoglu et al. 2014; Raschia et al. 2018). Previous studies have revealed several candidate genes related to meat yield (Kadlec et al. 2011; Thu et al. 2020; Thu et al. 2021), egg yield, and quality (Karsli et al. 2017; Demir et al. 2020b; Roy et al. 2024) in poultry farm animals. Of these genes, Very Low-Density Lipoprotein Receptor (*VLDLR*) has been studied in several avian species such as chicken (Abdulwahid et al. 2019), duck (Pan et al. 2017) and quail (Wu et al. 2015) due to its regulation functions (lipid, triglycerides and cholesterol metabolism, cell proliferation and differentiation) related to economically important traits (Pan et al. 2017; Abdulwahid et al. 2019; Bello et al. 2022). In poultry, the process of egg production stimulated by estrogen results in notable increases in liver lipid production and leads to alterations in the diameter of assembled *VLDL* (very low-density lipoprotein) from a general *VLDL* (~70 nm in diameter) facilitating the transport of lipids to peripheral tissues, to yolk-targeted VLDL (*VLDLy*; ~30 nm) (Salvante et al. 2007). Yolk lipids are carried to the oocytes by VLDL yolk-targeted (VLDLy), which has a specific receptor *(VLDLR*) located on the surface of the ova (Al-Hassani et al. 2023). VLDLR, a transmembrane lipoprotein receptor within the low-density lipoprotein receptor family, is prevalent in skeletal muscle, adipose tissue, heart, and brain, while being notably lacking in the liver (Nimpf et al. 2000). *VLDLR* is also known as the vitellogenesis receptor or vitellogenin receptor, mediating the absorption of plasma very low-density lipoprotein and vitellogenin (Wang et al. 2011). Additionally, it functions as a part of triglyceride and cholesterol metabolism (Brown and Goldstein, 1986) and plays a role in numerous cellular processes such as cell proliferation, migration, and differentiation (Hussain, 2001). It is reported that *VLDLR* plays a significant function in avian reproduction by influencing oocyte development and yolk lipoprotein deposition (Wang et al. 2011; Abdulwahid et al. 2019). Therefore, this study aimed to i) identify *VLDRLR* gene polymorphism (intron 11, 392C > T), ii) assess relationships between genetic variations and phenotypic traits such as egg production and egg weight and iii) discuss the possibilities of using this gene region in MAS studies to improve egg production in Japanese quails.

MATERIAL and METHODS

Phenotypic Data Records

This study was designed to cover 250 samples. However, phenotypic records for 198 samples were obtained at the end of the experiment due to mortality and laying problems. Similarly, a total of 7 samples were excluded from the experiment due to unexpected laboratory practices (non-amplification in PCR or non-specific digestion in PRFLP). As a result, this study was carried out with 191 samples, which is still consistent with some previous studies in Japanese quails (Lan et al., 2017; Rifki et al., 2021). Two phenotypic data (egg yield and egg weight) belonging to the studied animals were recorded at Akdeniz University's Poultry Farm, Faculty of Agriculture, Department of Animal Science. Routinely, following the incubation period, chicks are kept in cages for the first 30 days and fed ad libitum with fodder containing 24% raw protein and 2800 kcal/kg metabolic energy for the first 5 weeks. At the end of the growing period, egg yield and egg weight were recorded for 250 female quails taken into individual cages (20x20x29 cm) for 90 days. During the laying period, the quails were fed with a ration consisting of 2800 kcal/kg ME and 21% HP as ad libitum and exposed to 16 h of light and 8 h of darkness. Daily egg collection and weighing procedures were carried out in the morning hours (08:00-10:00).

Collection of Feather Samples and DNA Isolation

The shed feathers inside the individual cages were collected and numbered according to the cages for DNA isolation. DNA was isolated from collected feathers following the protocol described by Bello et al. (2001). Agarose gel and spectrophotometer were used to determine DNA quality and quantity. Before the PCR process, the DNA concentration was adjusted to approximately 20 ng/µl.

Process of PCR-RFLP and Genotyping

VLDLR gene intron 11 (392C > T) variation was identified via PCR-RFLP method utilizing specific oligonucleotide primers (F: CCTCTATTGATACCCGTGAT and R: TTAGGCCATTGGATTTCTGT) reported by Wu et al. (2015). The 493 bp length of VLDLR intron 11 region was amplified by thermal cycler with initial denaturation at 94 ºC for 5 min, followed by 35 cycles consisting of denaturation at 94 ºC for 45 sec, annealing at 55 ºC for 45 sec, extension at 72 ºC for 45 sec, with final extension 72 ºC for 5 minutes. PCR reaction mixture consisted of 20 ng/µl template DNA, 1.2 µL HQ buffer (GeneAll), 2 µL 10X buffer (GeneAll), 2.5 mM dNTPs, 10 pM/µl primer, 2.5 U Taq DNA polymerase, and 11.4 µL H2O. In the RFLP process, the amplified PCR products were digest with *NlaIII* (isoschizomer of *FatI*) (Thermo Scientific, ER1831) restriction enzymes in which 5 µL of PCR product and digestion mixture (2 µL of buffer, 5 µL of H2O and 2.5 U cutting enzyme) were mixed and incubated at the suitable temperature and time recommended by the manufacturer.

Statistical Analysis

Allele and genotype frequency were calculated by the POPGENE 1.31 program (Yeh et al. 1997). Hardy-Weinberg equilibrium was checked by using the chi-square ($\chi 2$) statistic in the population (Hartl and Clark, 1989). The sample size calculation was performed by G*Power 3.1.9.2 (Faul et al. 2007) software with the options of linear multiple regression and a priori, while other input parameters were set to default setting (effect size=0.15, α =0.05, and power=0.95). The phenotypic traits were compared among the genotypes. The genotypes obtained in the relevant gene regions and the model used to determine the 90-day egg yield ad egg weight aregiven below.

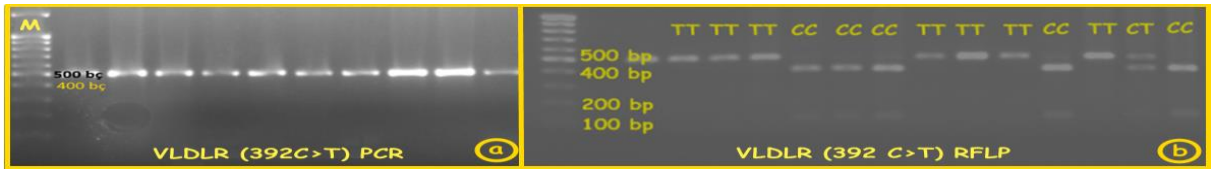
$$Y = \mu + G + e;$$

where Y is the dependent variable (analysed trait), µ is the mean for yields, G is the genotype, and e is the error.

One-way analysis of variance (One-way ANOVA) was performed using the SPSS (version 23) software to determine the relationship between the genotypes obtained in the *VLDLR* (392C>T) gene region and the phenotypic traits. Tukey test for multiple comparisons was performed between groups which were observed to be statistically different.

RESULTS

Initially, records of phenotypic data for a total of 250 animals were collected, but some animals were excluded from the statistical analyses due to various management (survival and laying problems) and laboratory practices (PCR amplification and RFLP digestion problems). Thus, the analyses were conducted based on 191 samples that were recovered during the experiment. It is noteworthy that even the decreased sample size (191 individuals) turned out to be statistically enough for the experiment because the required sample size in power analysis was estimated at 107 individuals. The 493 bp length products obtained by the PCR process were digested with *NlaIII* restriction enzyme, revealing two alleles (C and T) and three genotypes (CC, CT, and TT) (Figure 1). Since the genotypic bands were clearly distinguishable in agarose gel electrophoresis and consistent with the fragment sizes reported by Wu et al. (2015), genotype confirmation via sequencing or other methods was deemed unnecessary.



M: Marker (Thermo 100 bp; Cat.No: SM0241). a) PCR prducts (1 % agarose gel) (493 bp), b) Digestion of VLDLR PCR products by NlaIII (3% agarose gel) (TT genotype: 493 bp; CT genotype: 493 bp, 392 bp, and 101 bp; CC genotype: 392 bp and 101 bp.)

Figure 1 Images of agarose gels from PCR and RFLP processes for *VLDLR* gene intron 11 (392C > T)
Şekil 1. *VLDLR* geni intron 11 (392C > T) için PCR ve RFLP işlemlerinden elde edilen agaroz jellerin görüntüleri

The *VLDLR* (392C>T) gene was found to be polymorphic since studied quail population conserves all possible genotypes. The C and T allele frequencies were 0.22 and 0.78, respectively, while genotype frequencies ranged from 0.14 (CC) to 0.70 (TT) across the population (Table 1).

Table 1. Some descriptive statistics of genotypic and phenotypic data in the studied quail population.
Tablo 1. Çalışılan bıldırcın popülasyonundaki genotipik ve fenotipik verilerin bazı tanımlayıcı istatistikleri.

		Gene Frequencies		Genotype Frequencies			χ2
Gene	**n**	**C**	**T**	**CC**	**CT**	**TT**	
				0.14 (27)	0.16 (31)	0.70 (133)	
	191	0.22	0.78	**Genotypes**			
				CC	**CT**	**TT**	
***VLDLR* (intron 11, 392C>T)**	Egg number of 90 days (Mean±SD)			73.94±4.62[b]	76.07±6.03[ab]	78.31±6.32[a]	53.84**
	Egg weight of 90 days (g) (Mean±SD)			915.80±76.60	933.50±80.20	939.19±81.29	

Different superscript letters (a and b) differ significantly (P < 0.05) in genotypes. χ2 0.01;1:6.63; χ2 0.05;1:3.84;
****: Significant deviation from H-W equilibrium (P ≤ 0.01)**

Significant deviation from Hardy Weinberg equilibrium (P<0.01) was detected in quail population for *VLDLR* gene. The allele and genotype frequencies, as well as the mean number of egg and egg weight per genotype, were summarised in Table 1. The 90-day egg yields were 73.94, 76.07, and 78.31, while 90-day egg weight were 915.80, 933.50, and 939.19 regarding CC, CT, and TT genotypes, respectively. The 90-day egg production of animals with the TT genotype was higher than those with the TC and CC genotypes, according to a one-way analysis of variance across the groups (P<0.05). However, there was no significant difference between the groups in terms of 90-day egg weights.

DISCUSSION and CONCLUSIONS

Various studies have shown that *VLDLR* gene is polymorphic and that variations in this gene were associated with phenotypic traits particularly egg yield and egg weight in several avian species (Wang et al. 2011; Cao et al. 2012; Wu et al. 2015; Zhao et al. 2015; Abdulwahid et al. 2019, Zhou et al. 2020). For example, Wu et al. (2015) focused on two polymorphisms (363T>C and 392C>T) in yellow-feather quail and chestnut feather quail populations and reported that variations in this gene were associated with several phenotypic traits such as first egg, the age of first egg, and egg number of 20-week-old birds. Abdulwahid et al. (2019) showed that variations in the *VLDLR* gene were also associated with egg production and egg weight in Iraqi local Brown Chickens. Moreover, Zhao et al. (2015) detected five SNPs and three InDel variations between exon 14 and exon 16 of *VLDLR* gene in Gaoyou duck breed and reported that of the 11 haplotypes, 4 haplotypes were reported to be associated with body weight at 10 weeks (P<0.05) and abdominal fat percentage (P<0.01).

The results of this study, combined with findings from the literature, support the idea that *VLDLR* polymorphisms could be used in MAS to improve performance traits with higher genetic gain in avian species, including quails. The study showed that animals with TT genotypes are advantageous in terms of the egg number of 90 days trait (p<0.05). While no statistically significant evidence was observed, animals with the TT genotype were also of the highest value in terms of egg weight at 90 days. Similarly, Wu et al. (2015) reported that animals with the TT genotype were of the highest value for egg number of 20-week in chestnut feather quails. However, in this study, the highest allele frequency was observed in TT (0.70), while the CC genotype was of the highest frequency in yellow-feather (0.62) and chestnut-feather quail (0.60) populations (Wu et al., 2015). Differences between genotype distribution between quail populations raised in Turkiye and China could be due to different raising practices. While selection occurs in quail reared in China, no systematic selection process was conducted in the quail population we studied. Besides, quail populations sampled for this study have been closely raised for several generations. This kind of close breeding may cause significant variations in allele and genotype distribution, resulting in deviation from HWE in poultry populations (Karsli et al. 2019, Karsli and Fidan, 2019).

VLDLR gene, directly associated with egg yield in avian species, functions especially in the formation process of yolk. Variations in this gene may also interact with other genes affecting egg yield. For example, the *VLDLR* gene has been reported to affect egg yield via interaction with several genes playing a key role in yolk

protein synthesis (vitellogenin, *VTG*), fat metabolism in ovary (lipoprotein lipase, *LPL)*, hormonal signaling pathway during laying period (estrogen receptor, *ESR*), and folicul development (follicle-stimulating hormone receptor*, FSHR*) (Li et al. 2003; Huang et al. 2016; Ma et al. 2020; Liu et al. 2021). Therefore, the combination of genetic variations in *VLDLR* and related genes in MAS studies could be efficient to improve egg yield in Japanese quails.

Wu et al. (2015) reported that the change in intron 11 of the *VLDLR* gene may be linked to another change in *the VLDLR* gene leading to an amino acid change. Although introns do not encode proteins, they play an important role in gene regulation through mechanisms such as splicing mechanisms, affecting regulatory elements, creating new sites where miRNAs can bind or destroying existing binding sites (Le Hir et al. 2003; Lin et al. 2006; Riethoven 2010).

In conclusion, the effect of a point mutation on the *VLDLR* gene intron 11 (392C>T) on 90-day egg production and egg weight was studied in Japanese quails. A significant association was observed between the *VLDLR* gene (392C>T) variation and 90-day egg production. Quails with the TT genotype for the *VLDLR* gene (392C>T) have better egg production than those with the CC and TT genotypes. Since quail is a model organism for avian species, this study confirms that *VLDLR* gene intron 11 (392C>T) polymorphisms could be utilised for other poultry species in MAS to improve phenotypic traits (egg production). In addition, supporting MAS with a higher number the candidate genes will directly increase the success rate and genetic gain due to the nature of polygenic inheritance.

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Author contributions: All authors contributed equally to the preparation of the article.

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