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Research Article

Analysis of FecB, BMP15 and CAST Gene Mutations in Sakiz Sheep [1]

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Abstract

Fertility traits, such as the ovulation rate and the number of offspring at birth, are genetically regulated by fecundity genes. This study was performed to identify DNA polymorphisms in Booroola *(FecB)*, Galway *(FecX^G)*, Inverdale *(FecX^I)* and Calpastatin *(CAST)* genes in Sakiz sheep. A total of 71 ewes were genotyped for gene or allelic polymorphisms in the genes listed above using the PCR-RFLP method. The results obtained from this study indicated that all of the Sakiz ewes sampled were non-carriers for *FecB, FecX^G* or *FecX^I* mutations. However, genotypic frequencies in the *CAST* gene were 0.59, 0.36 and 0.05 for AA, AB and BB, respectively. A significant deviation from Hardy-Weinberg equilibrium for the *CAST* gene was not observed in the investigated breed (P>0.05). As a result, more extensive screening is required as tests for newly discovered mutations are developed. Additionally, this study is the first to report a genetic polymorphism in the *CAST* gene in Sakiz sheep.

Keywords: *Sakiz sheep, Fertility, FecB, BMP15, CAST*

Sakız Irkı Koyunlarda FecB, BMP15 ve CAST Genlerindeki Mutasyonların Analizi

Özet

Ovulasyon oranı ve bir batındaki yavru sayısı gibi dölverim özellikleri fekondite genleri tarafından regüle edilmektedir. Bu çalışma Sakız ırkı koyunlarda, Booroola *(FecB)*, Galway *(FecX^G)*, Inverdale *(FecX^I)* ve Calpastatin *(CAST)* genlerindeki DNA polimorfizmlerini belirlemek amacıyla yapılmıştır. Araştırılan gen veya aleller açısından toplam 71 koyun PCR-RFLP metoduyla genotiplendirilmiştir. Çalışmadan çıkan sonuçlar, Sakız koyunların *FecB, FecX^G* ya da *FecX^I*'ye ait mutasyonları taşımadığını göstermiştir. Ancak *CAST* geni açısından AA, AB ve BB genotiplerinin sırasıyla 0.59, 0.36 ve 0.05 frekans gösterdiği tespit edilmiştir. *CAST* geni açısından çalışılan sürünün Hardy-Weinberg dengesine uyduğu görülmüştür (P>0.05). Sonuç olarak yeni keşfedilen mutasyonların geliştirilmesi gibi daha yoğun tarama çalışmaları gerekmektedir. Ek olarak Sakız koyunlarında *CAST* genindeki polimorfizmin varlığı ilk defa bu çalışmada bildirilmektedir.

Anahtar sözcükler: *Sakız Koyunu, Fertilite, FecB, BMP15, CAST*

INTRODUCTION

The incorporation of a major gene for prolificacy into a flock using marker-assisted selection (MAS) promotes increased selection pressure on other traits, leading to increased genetic gain [1]. It is hypothesized that MAS using both the bone morphogenetic protein receptor IB *(BMPR-IB)* and the bone morphogenic protein 15 *(BMP15)* genes is warranted to increase litter size in sheep and will be of considerable economic value to sheep breeders.

Sakiz is a dairy sheep breed of considerable economic interest to Turkish farmers, mainly due to its high prolificacy and milk production. These sheep are raised in Cesme, the provinces of Izmir and Aydin, and coastal locations in the Marmara and Aegean regions. The average litter size is 1.7 to 2.3 lambs, and the ovulation rate is 2.9 to 3.3 [2,3].

In recent years, a number of natural genetic mutations have been associated with ovulation rates in sheep breeds, including one mutation in the *BMPR-1B* gene *(FecB)* and ten different mutations in the bone morphogenetic protein 15 gene *(FecX^I, FecX^H, FecX^G, FecX^B, FecX^R, FecX^L, FecX^{Gr}, FecX^{TT}, FecX^O* and *FecX^W)* [4-6]. These mutations are significantly associated with the ovulation rate of different sheep breeds.

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The *FecB* gene is a dominant autosomal gene responsible for the fecundity of Booroola Merino sheep, and its additive effect on the ovulation rate was first identified in the 1980s [4,7]. The *FecB* gene maps to chromosome 6 in sheep [8]. A point mutation that significantly increases the ovulation rate of ewes is located at base 746 of the coding region (746 A → G) in the highly conserved intracellular kinase signaling domain of the *BMPR-1B*; this mutation causes a glutamine to arginine amino acid substitution [9]. The gene dosage effect of the mutation is additive for the ovulation rate with an increase of 1.5 for each gene copy. These extra ovulations subsequently increased litter size by approximately 1.0 and 1.5, respectively [4,10]. *FecB* gene mutations have been reported in many other sheep breeds, such as Booroola Merino [7], Belclare, Cambridge, China Small Tailed Han, Hu [5], Romney [11], Kendrapada [12], Garole and Javanese sheep [13]. In contrast, *FecB* mutations were not detected in Thoka, Woodlands, Olkuska, Lacaune [13] and Sangsari sheep [14].

The *BMP15* located on the chromosome X [15]. Each *BMP15* gene significantly affects prolificacy [11]. Ewes with two inactive copies of the *BMP15* gene (homozygous animals) are sterile [15,16] and exhibit a similar ovarian phenotype. Ewes with a single inactive *BMP15* gene (heterozygous animals) are fertile and exhibit an increased ovulation rate and an increased incidence of twin or triplet births [4,5,13-18]. In general, heterozygous ewes with mutations in both *FecB* and *FecX*^{G} exhibited increased fertility compared with ewes harboring a mutation in only one of these genes [6]. The Galway mutation has been identified in Cambridge [16], Garole, Kendrapada [12] and Small Tailed Han sheep [19]. Hanrahan et al.[16] estimated the effect of *FecX*^{G} to be 0.77 and 1.18 in Belclare and Cambridge ewes, respectively. The *FecX*^{I} mutation was studied in Inverdale, Belclare, Cambridge, Romney, Hanna and Rasa Aragonese, Cambridge, Small Tailed Han, Sakiz [13,16,19] sheep. Recently, Gursel et al.[20] report that the Inverdale mutation was identified in Sakiz sheep in Turkey.

The *CAST* gene, which is located on sheep chromosome 5, acts on growth characteristics, such as muscle development, birth weight of the lamb, weight at weaning, and postmortem meat qualities, such as tenderness [21-24]. In addition, the *CAST* gene affects cataract development and the reproductive performance of the animals [23-25]. In a study by Garcia et al.[26], calpastatin exerted a significant impact on fertility and longevity on cattle. Based on these results, the use of *CAST* markers together with increased predicted transfer ability (PTA) potential in selection programs for dairy parameters is projected to increase milk yield together with fertility [26].

FecB, FecX^{G} and *FecX*^{I} mutations have been studied in local sheep breeds, such as Akkaraman, Morkaraman, Daglic, Awassi, Tuj, Karakas and Bafra (Sakiz x Karayaka cross) in Turkey as well as in Greek Sakiz ewes; however, these mutations were not identified [10,27,28]. In addition, the *CAST* gene has not been studied in Sakiz or other Turkish domestic sheep breeds.

We tested the hypothesis that a major gene was segregating in the Sakiz breed. Therefore, the aim of this study was to investigate *FecB, FecX*^{G}, *FecX*^{I} and *CAST* gene mutations in the Sakiz sheep breed and the potential introgression of these genes into these breeds to enhance their reproductive rate.

MATERIAL and METHODS

Animals

A total of 71 ewes were used in the study. Ewes were randomly selected from five unrelated herds. These herds were managed within the scope of the Protection of Indigenous Genetic Resources Project under the supervision of the Ministry of Agriculture.

DNA Isolation

Initially, wool samples were prepared by cleaning with EtOH (ethanol). The CTAB (hexa-decyltrimethylammonium bromide) method of DNA isolation was performed using wool samples obtained from selected animals [29]. The amount of DNA obtained and its purity were determined by spectrophotometry (Nanodrop-2000c, Thermo Scientific). The quantified DNA was stored at -80°C until PCR-RFLP (Polymerase chain reaction and Restriction fragment length polymorphism) was performed.

PCR-RFLP Genotyping

DNA was amplified by PCR using the primer sets provided in *Table 1*. The amplification products were cut with restriction enzymes, and the alleles or mutations were determined. The primer sets used for the amplification of *FecB, FecX*^{G}, *FecX*^{I}, and *CAST*; the size of the PCR products; the restriction enzymes used and their product sizes and references for PCR-RFLP are provided in *Table 1*.

For the PCR amplification of *FecB, FecX*^{G}, *FecX*^{I} and *CAST*, 33.5 µl of dH_2O, 5 µl of 10X buffer, 5 µl of $MgSO_4$, 1 µl of dNTPs (2.5 mM), 2.5 U Taq DNA polymerase (Biomatik Corp., Cambridge,Canada) and 1 µl of (0.025 µM) forward primer and 1 µl of (0.025 µM) reverse primer from each primer sets presented in *Table 1* were used. The final volume was 50 µl after the addition of 3 µl of 100 ng/mL DNA sample.

The PCR conditions for *FecB* [5,13], *FecX*^{G} [16,27], *FecX*^{I} [5,15,27] and *CAST* [21,30] were as follows: initial denaturation at 94 to 95°C for 0.30 s to 5 min followed by 33 to 35 cycles of denaturation at 94 to 95°C for 0.30 s to 3 min, annealing for 40 s to 1 min at 60 to 61°C, elongation at

Table 1. *Primer sets and enzymes for PCR-RFLP of FecB, BMP15 (FecX^G,FecX^I) and CAST genes*
Tablo 1. *PCR-RFLP için FecB, BMP15 (FecX^G, FecX^I) ve CAST genlerinin primer set ve enzimleri*

Gene-Allele	Primer Sets*	PP (bp)	RE	RP (bp)		References
FecB	**F:**5′-CCAGAGGACAATAGCAAAGCAAA-3′ **R:**5′-CAAGATGTTTTCATGCCTCATCAACAGGTC -3′	190	*AvaII*	AA	190	[5,13]
				BB	159, 31	
				AB	190, 159, 31	
FecX^G	**F:**5′CACTGTCTTCTTGTTACTGTATTTCAATGAGAC-3′ **R:**5′-GATGCAATACTGCCTGCTTG-3′	141	*Hinf I*	AA	141	[16,27]
				BB	112, 29	
				AB	141, 112, 29	
FecX^I	**F:**5′-GAAGTAACCAGTGTTCCCTCCACCCTTTTCT–3′ **R:**5′-CATGATTGGGAGAATTGAGACC-3′	154	*XbaI*	AA	154	[5,15,27]
				BB	124, 30	
				AB	154, 124, 30	
CAST	**F:**5′-TGGGGCCCAATGACGCCATCGATG - 3′ **R:**5′- GGTGGAGCAGCACTTCTGATCACC - 3′	622	*MspI*	AA	336, 268	[21,30]
				BB	622	
				AB	622, 336, 268	

** **GenBank accession no:** BMP15(FecX^G, FecX^I)(AH009593), FecB (AF312016), CAST (AF016006-8); (**PP:** PCR Product, **RE:** Restriction Enzyme, **RP:** Restriction Product)*

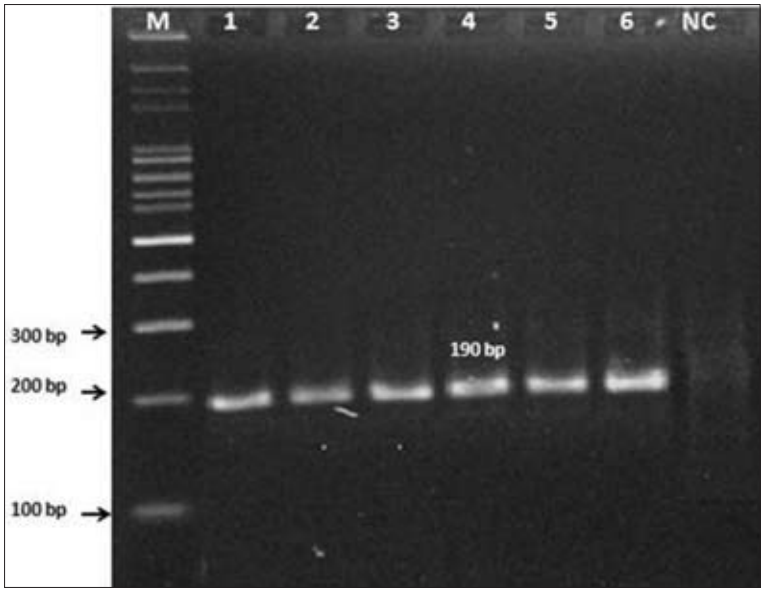
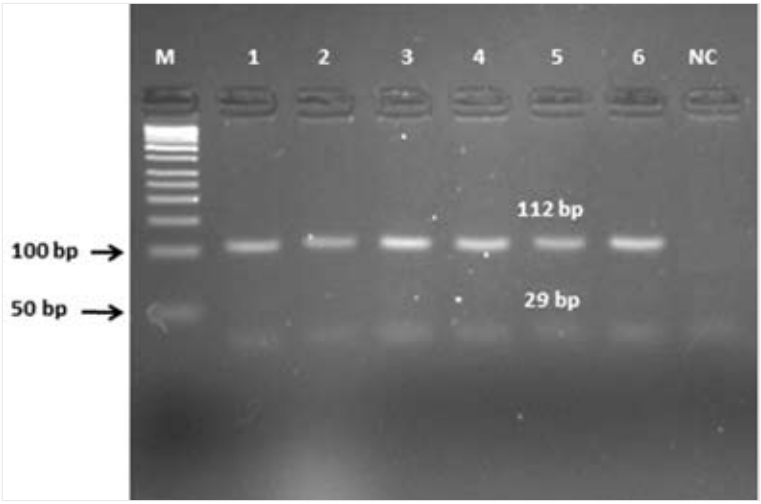


Fig 1. The *FecB* gene cut with the *AvaII* restriction enzyme. The products were electrophoresed on a 2.5% agarose gel. No mutations were detected in any of the individuals (M: Marker, Columns 1 to 6: 190 bp band, NC: Negative Control)

Şekil 1. *FecB* geninin *AvaII* restriksiyon enzimi ile kesimi (%2.5'lik agaroz jel- tüm bireylerde mutasyon yok) (M: Markör, Sütun1-6: 190 bç'lik bant, NK: Negatif Kontrol)

Fig 2. The *FecX^G* allele cut with the *HinfI* restriction enzyme. The products were electrophoresed on a 4% agarose gel. No mutations were detected in any of the individuals (M: Marker, Columns 1 to 6: 29 and 112 bp bands, NC: Negative Control)

Şekil 2. *FecX^G* allelinin *Hinf I* restriksiyon enzimi ile kesimi (%4'lük agaroz jel- tüm bireylerde mutasyon yok) (M: Markör, Sütun1-6: 29 ve 112 bç'lik bant, NK: Negatif Kontrol)



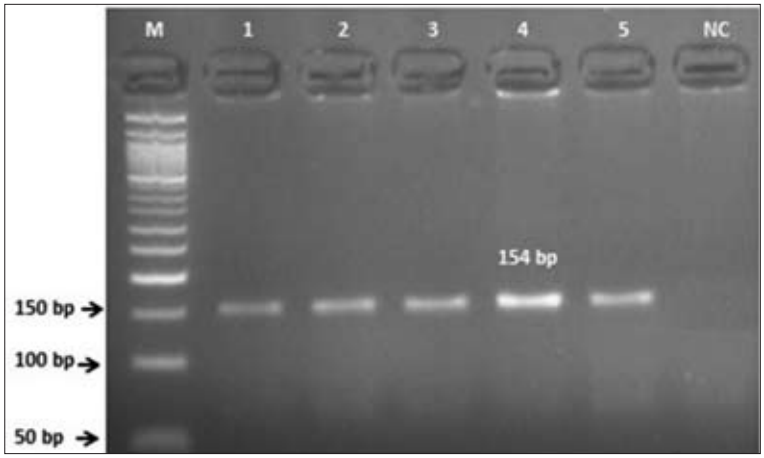


Fig 3. The *FecX*^{I} allele cut with the *XbaI* restriction enzyme. The products were electrophoresed on a 3.5% agarose gel. No mutations were detected in any of the individuals (M: Marker, Columns 1-5: 154 bp band, NC: Negative Control)

Şekil 3. *FecX*^{I} allelinin *XbaI* restriksiyon enzimi ile kesimi (%3.5'luk agaroz jel- tüm bireylerde mutasyon yok.) (M: Markör, Sütun1-5: 154 bç'lik bant, NK: Negatif Kontrol)

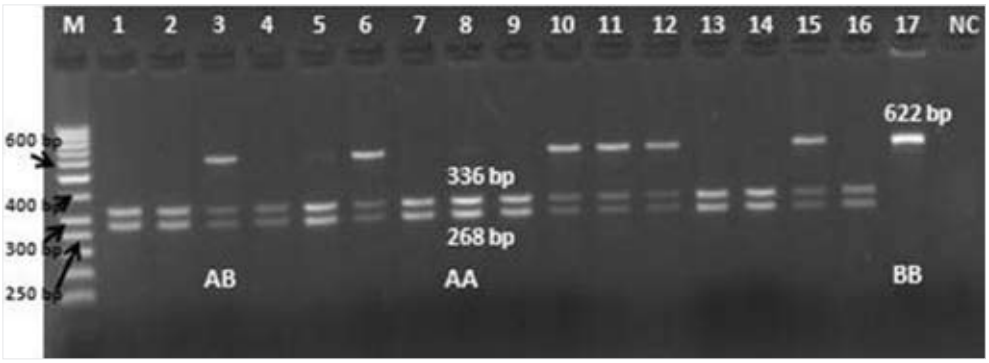


Fig 4. The 622-bp PCR product from the *CAST* gene cut by the *MspI* enzyme. The products were electrophoresed on a 2.5% agarose gel. AA, AB and BB genotypes were detected (M: Marker, Columns 1, 2, 4, 5, 7, 8, 9, 13, 14, 16: 268 and 336 bp bands, Columns 3, 6, 10, 11, 12, 15: 268, 336 and 622 bp bands, Column 17: 662 bp band, NC: Negative Control)

Şekil 4. *CAST* geninin 622 bç'lik PCR ürününün *MspI* enzimi ile kesimi (%2.5'lik agaroz jel görüntüsü - AA, AB, BB genotipi) (M: Markör, Sütun 1,2,4,5,7,8,9,13,14,16: 268 ve 336 bç'lik bant, Sütun3,6,10,11,12,15: 268-336-622 bç'lik bant, Sütun17: 662 bç'lik bant, NK: Negatif Kontrol)

70 to 72°C for 30 s to 2 min, and a final extension at 72°C for 4 to 8 min.

The PCR products for the amplification of the *FecB* gene (product size 190 bp), *FecX*^{G} allele (product size 141 bp), *FecX*^{I} allele (product size 154 bp) and *CAST* gene (product size 622 bp) were subjected to electrophoresis using 2%, 3%, 2.5% and 1.5% agarose gels, respectively.

For RFLP, 15 µl of the PCR products were incubated with 4 µl buffer, 4 µl dH_2O and 15 U of restriction enzymes at 37°C for 16 h. The restriction digestion products *(FecB, FecX*^{G}*, FecX*^{I} and *CAST)* were visualized in agarose gels stained with 2.5%, 4%, 3.5% and 2.5% ethidium bromide, respectively. The PCR and RLFP product bands were by visualized using the DNr Minilumi imaging system *(Fig. 1, 2, 3, 4)*.

Statistical Analysis

Data were processed by POPGENE V1.32 software to calculate genotypic frequencies and Hardy-Weinberg equilibrium [31].

RESULTS

In the present study, *FecB*, *FecX*^{G}, *FecX*^{I} and *CAST* mutations were investigated in Sakiz sheep breeds. The agarose gel images of PCR-RFLP products are presented in *Fig. 1, 2, 3, 4* respectively. Mutations in the *FecB gene* and at the *FecX*^{G} *and FecX*^{I} loci in the *BMP15* gene were assessed in the samples. Based on the PCR-RFLP analysis, no digested fragments were observed for *FecB*, *FecX*^{G} *and FecX*^{I} at 190, 141 and 154 bp, respectively, if the herd did not carry the mutations [5]. All of the 71 individuals were homozygous for *FecB* and *BMP15*. None of the samples harbored the *FecB* mutation, and similarly no *FecX*^{G} *and FecX*^{I} mutations were noted in the *BMP15* gene. Hardy-Weinberg equilibrium and χ^2-values were not estimated due to the genotypic frequency.

A total of 71 Sakiz individuals were genotyped. Of these sheep, 42 were AA, 25 were AB and 4 were BB for the *CAST* gene. Genotypic frequencies were estimated as 0.59, 0.36 and 0.05 for AA, AB and BB, respectively, for the *CAST* gene *(Table 2)*. Significant deviation from Hardy-Weinberg

Table 2. *Genotypic frequencies of FecB, BMP15 (FecX^G,FecX^I) and CAST genes*

Tablo 2. *FecB, BMP15 (FecX^G, FecX^I) ve CAST genlerinin genotipik frekansları*

Regions	Allele	n	Frequency	Chi²	P
FecB	AA	71	1.00	NE	NE
	AB	0	0.00		
	BB	0	0.00		
FecX^G	AA	0	0.00	NE	NE
	AB	0	0.00		
	BB	71	1.00		
FecX^I	AA	71	1.00	NE	NE
	AB	0	0.00		
	BB	0	0.00		
CAST	AA	42	0.59	0.01	0.91
	AB	25	0.36		
	BB	4	0.05		

NE: *Not Estimated*

equilibrium was not observed for the *CAST* gene in the investigated breed (P>0.05).

DISCUSSION

The results of our study indicate that *FecB, FecX^G* and *FecX^I* mutations, which have a major effect on litter size, are not present in Sakiz sheep.

These results are consistent with reports in Romanov, Finn, Thoka, Woodlands, Olkuska, Lacaune [13], Sangsari [14], Akkaraman, Morkaraman, Daglıc, Tuj, Karakas [10], Awassi, Imroz, Kivircik [20] and Bafra [28] sheep breeds wherein *FecB* mutant alleles do not segregate in these breeds. In contrast, *FecB* mutations are reported in several sheep breeds, such as Booroola Merino [7], Belclare, Cambridge, China Small Tailed Han, Hu [5], Garole, Javanese [13], Kendrapada [12] and Romney sheep [17].

The *FecX^G* mutation was analyzed in Garole, Kendrapada [12], Malpura, Deccani, Baluchi [32] and Sangsari sheep [14]; however, none of these sheep breeds carried the *FecX^G* mutation in the *BMP15* gene. Additionally, the *FecX^G* mutation was identified in Small Tailed Han, Belclare and Cambridge sheep [1,33]. In contrast with results from Gursel et al.[20], our results indicate that the point mutation in *FecX^G* might not serve as a major gene that influences prolificacy in Sakiz sheep. Galway and Inverdale gene polymorphisms were analyzed as likely candidate genes influencing high prolificacy in Sakiz breeds of Greece, but no polymorphism has been identified [27]. The *FecX^I* mutation was first identified in the Romney sheep breed, which exhibits high fertility traits [1]. Studies indicate that the Small Tailed Han, Hu [33], Olkuska, Garole, Javanese, Woodland, Lacaune [13], Egyptian [34], East Friesien, Finn, Romanov [5] and Greek Sakiz [27] sheep do not carry the *FecX^I* alleles. Our results demonstrate that the *FecX^I* mutation we examined was not present in Sakiz sheep. In addition our results are consistent with reports by Gursel et al.[20].

In this study, we analyzed a *CAST* gene polymorphism. The genotypic frequencies of the AA, AB and BB alleles for the *CAST* gene were calculated as 0.59, 0.35 and 0.56, respectively. These frequencies are similar to those reported in Arabic [21], Iranian Zel [23] and Makoi sheep [24]. The *CAST* gene, which encodes calpastatin, regulates the activity of calpain as a protease inhibitor [35]. In addition to its role in determining the quality of meat, the *CAST* gene has also effects on reproductive activity [23]. However, studies on reproductive activities are very limited. Garcia et al.[26] reported that the *CAST* gene was strongly correlated with fertility and longevity. However, Byun et al.[35] reported that the *CAST* gene did not affect fertility or longevity in Romney, Corriedale, Merino, Polwarth, Kelso and Coopworth ewes. Proof of the relationship between the *CAST* genes and reproductive activity may explain the variety of litter sizes in Sakiz ewes.

These results suggest that the high prolificacy of the Sakiz breed does not result from *FecB*, *FecX^G* and *FecX^I* mutations. Further investigation should be directed at other loci of the *BMP15* gene or other genes and involve larger sample sizes. Furthermore, the effect of *CAST* gene alleles on reproductive parameters should be investigated. Studies on the genomic aspects of fertility, while improving the accuracy of selection, will allow for more economical and efficient use of resources.

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