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Research Article

Associations Between *GH, PRL, STAT5A, OPN, PIT-1, LEP* and *FGF2* Polymorphisms and Fertility in Holstein-Friesian Heifers [1]

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Abstract

In this study, it was aimed to investigate polymorphisms in seven genes (*GH, PRL, STAT5A, OPN, PIT-1, LEP* and *FGF2*) related to reproductive traits in dairy heifers. Frequency distributions of the genotypes between fertile and repeat breeder heifers groups were investigated. Allele effects on fertility were also analyzed. Blood samples were taken from a total of 160 Holstein-Friesian heifers and they were divided into two groups according to their artificial insemination numbers (AI). The heifers becoming pregnant after the first AI were used as the fertile heifers (FH, n=80) and the heifers with 3 or more equal AIs were accepted as the repeat breeder heifers (RBH, n=80). All the animals were genotyped by the PCR-RFLP method for seven genes and the association works were performed for 145 animals (RBH, n=79; FH n=66). For all loci investigated, two alleles and three genotypes were found for overall population with the exception that PRL locus had two alleles and two genotypes. The chi-square test (χ^2) revealed that the whole population and the two groups separately were at Hardy-Weinberg equilibrium. The genotype distributions of *PIT-1* and *STAT5A* conspicuously differed between the FH and the RBH groups; however, these differences were not found significant. Association of *GH*-AB genotype was found significant on AI number for the first pregnancy. Mixed effect logistic regression model was used to investigate the allele effects on fertility. No linkage disequilibrium was detected between the investigated loci.

Keywords: *Polymorphism, Fertility, Infertility, Dairy Heifers*

Holstein-Friesian Düvelerde Fertilite ile *GH, PRL, STAT5A, OPN, PIT-1, LEP* ve *FGF2* Polimorfizimlerinin İlişkileri

Özet

Bu çalışmada sütçü düvelerde reprodüktif özellikler ile ilişkili yedi gendeki (*GH, PRL, STAT5A, OPN, PIT-1, LEP* ve *FGF2*) polimorfizimlerin araştırılması amaçlanmıştır. Genotip frekanslarının fertil ve repeat breeder düve gruplarındaki dağılımı araştırılmıştır. Ayrıca fertilite üzerine allel etkisi de incelenmiştir. Toplam 160 Holstein-Friesian düveden kan alınmış ve bu düveler tohumlama sayılarına (ST) göre iki gruba ayrılmıştır. İlk tohumlamada gebe kalan düveler fertil düve (FH, n=80) olarak kullanılmış ve üç veya daha fazla ST'si olan düveler repeat breeder düve (RBH, n=80) olarak kabul edilmiştir. Tüm hayvanlar yedi gen bakımından PCR-RFLP metodu ile genotiplendirilmiş ve ilişkilendirme çalışmaları toplam 145 hayvanda yapılmıştır (RBH, n=79; FH, n=66). İki allel ve iki genotipin bulunduğu PRL hariç incelenen tüm lokuslarda iki allel ve üç genotip belirlenmiştir. Ki-kare sonuçları (χ2) tüm populasyonun ve ayrı ayrı grupların Hardy-Weinberg dengesinde olduğunu ortaya koymuştur. *PIT-1* ve *STAT5A* lokuslarının genotip frekanslarının dağılımları FH ve RBH grupları arasında belirgin biçimde farklı olmasına rağmen bu farklılık istatistiksel anlamda önemli bulunmamıştır. GH-AB genotipinin fertilite üzerine etkisi önemli bulunmuştur. Fertilite üzerindeki allel etkisini incelemek için karışık etkili lojistik regresyon analizi kullanılmıştır. İncelenen lokuslar arasında bir bağlantı dengesizliği belirlenmemiştir.

Anahtar sözcükler: *Polimorfizm, Fertilite, İnfertilite, Sütçü düve*

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INTRODUCTION

Although female reproduction is essential for the prolificacy of animal production, decreasing reproductive performance is one of the major problems in dairy industry [1,2]. As it is well known, the use of conception rate as an indicator of reproductive performance has decreased in the last decades [1]. Repeat breeder heifers (RBH) cause economic losses due to both the high insemination cost and the increased age of heifers at first calving, which is a source of complex health problems. Identifying dairy cattle with superior genetic potential for improved fertility might increase dairy farm profitability.

This dramatic reduction in reproductive performance of dairy cows is unlikely that this decline could be reversed only through improved management conditions [3]. In the minimization of these problems, the use of molecular markers may provide rapid genetic gains [4].

As Khatib et al.[5] noted, it might be useful to use the whole pathway rather than a single gene in a selection scheme. This seems logical due to the well-known multi-locus combined effect variations observed in quantitative traits. We selected seven different mutations in different genes involved growth, development and other essential actions for maintaining the pregnancy or other reproductive performance and examined their associations with the conception number for each pregnancy [6-16].

We targeted heifers for not only their economic importance but also avoiding evaluation of endocrinologic and oestrus problems found in repeat-breeder heifers [17]. The number of studies on heifer reproductive performance is limited [18]. The aim of the present study is to investigate the frequency distributions of seven loci, considered to be assocaiated with the reproductive traits, in the fertile and the repeat breeder Holstein heifer groups. We also aimed to examine the associations between these polymorphisms and fertility in dairy heifers.

MATERIAL and METHODS

The study was approved by the Ethics Committee of Uludag University (UUHADYEK), (approval date: 04.06.2013; no: 2013-11/1). This study was carried out in seven different lactating dairy farms located in the Marmara region of Turkey with an average 400-800 milking cows. The reproductive management of the dairy heifers in the farms was based on the artificial insemination following the estrus detection after spontaneous or $PGF_2\alpha$ (one or two doses of $PGF_2\alpha$ apart from 14 days) induced estrus. The first insemination age of the heifers was average 15 months in all the dairy farms. The artificial inseminations (AI) were performed by the farm veterinarians.

The Holstein-Friesian heifers (n=160) between 14-28 months of age were included in the study and the heifers were divided into two groups: fertile and repeat breeder. The heifers that became pregnant after the first artificial insemination (AI) were determined as the fertile group (FH, n=80) and the heifers with 3 or more equal AIs were placed in the repeat breeder group (RBH, n=80). The first and the second pregnancy checks were performed on the 30^{th} and 60^{th} days following the AI in the fertile heifers. If the embryonic loss was detected on the 60th day of the pregnancy check, the heifers were excluded from the fertile group. The blood samples were obtained from the coccygeal vein for DNA isolation.

A total of 160 Holstein Friesian heifers were analyzed for polymorphisms in the seven genes and seven different gene regions by using the PCR-RFLP method. However, the association works were performed for a total of 145 animals, 79 from the RBH group and 66 from the FH group. Fifteen of the 160 animals could not been genotyped for all loci. Due to this limitation these animals were not included to association analysis. The total DNA was extracted by using a genomic DNA purification kit (K0512, Fermentas, Lithuania) according to the instruction manual. The quantity and quality of the DNA were checked with a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). The primers and restriction enzymes used for PCR-RFLP analysis are given in *Table 1*.

The PCR amplifications were performed in reaction mixtures of 25 µl containing 12.5 µl of 2× PCR Master Mix (K0172, Fermentas, Lithuania), 0.5 µM of each primer and 25-75 ng of genomic DNA. The amplification was performed by using a Techgene Thermal Cycler (Techne, Cambridge, UK). The restriction enzyme digestions were performed according to the manufacturer's protocols. The digested restriction fragments were directly analyzed via electrophoresis on 2% and 2.5% agarose gels in 1XTBE buffer, stained with SafeView™ Classic (Applied Biological Materials Inc., Canada) and visualized under UV light.

The allele and genotype frequency calculations as well as the chi-square (χ^2) test were carried out by using the Popgene32 [26] program. The linkage disequilibrium between the investigated loci was analyzed according to Weir [27] by using the Popgene32 [26] program. The differences in the genotype frequency distribution between the FH and the RBH groups were analyzed by the likelihood ratio chi-square (χ^2) [28].

Mixed effect logistic regression in framework of generalized linear mixed model was applied to estimate the parameter of linear predictor contains vet and farms as random effects in addition to a set of our fixed explanatory variables (*OPN, STAT5A*, *GH*, *PIT1*, *PRL*, *FGF2*, *LEP*). Parameter estimates of both random and fixed effects were obtained by using the PROC GLIMMIX in SAS [29]. We used a CONTRAST option to test the hypotheses for the comparison of alleles within genotypes.

RESULTS

The distrubutions, numbers of the AIs and the ages of the heifers were shown in *Table 2* and numbers of the AIs and the ages of the heifers were greater (P<0.01) in the repeat breeder heifers (4.5±0.18 and 20.9±0.50) than in the fertile heifers (1.00±0.00 and 15.7±0.52, respectively). All the loci investigated were found to be polymorphic with two alleles and three genotypes for each with the exception that the *PRL* loci had two alleles and two genotypes *(Table 3)*. We found limited variation on the *LEP* locus with a quite low frequency of B allele. There was only one animal carrying BB genotype at this locus.

The observed allele and genotype frequencies and the expected heterozygosity values as well as the χ^2 values and the number of investigated individuals from each group are given for each investigated loci in *Table 3* and *Table 4*. The population was found to be in Hardy-Weinberg equilibrium for the investigated loci. The linkage disequilibrium anaylsis showed that there was no linkage disequilibrium between these loci.

The genotype distributions of some genes were different in the two groups *(Table 3)*. While genotype AB of *GH* was higher in RBH group, genotype AB and GC of *PIT-1* and *STAT5A* loci were higher in the FH group *(Table 3)*. While these differences were not significant for *PIT-1* and *STAT5A* loci, *GH* locus was found to be differ between groups (P=0.05). Heterozygote genotype (AB) at *GH* locus seems to be unfavorable for AI number for the first pregnancy. The heterozygote genotype (AB) at *GH* locus frequency of the FH group was different from that of the RBH group. Odds Ratios with 95% Wald confidence limits graphic and logistic regression graphics are given in *Fig. 1* and *Fig. 2*, respectively. The index plots of the Pearson residuals and the deviance residuals in *Fig. 2* indicate that no cases are poorly accounted for by the model, causing instability in all parameter estimates and goodness of fit. In addition, according to both logistic regression and association analysis, farms and inseminaters effects were found insignificant on fertility.

DISCUSSION

Two alleles and three genotypes were found for SNPs located between in exon 6 and intron 5 of the *PIT1* gene *(Table 3, Table 4)*. Similar to other studies on the B allele, a positive effect on growth and development

Table 1. *Gene locations of loci, size of PCR products, primer sets and restriction enzymes (RE) used for RFLP analysis*

Loci	Primers (5′ → 3′)	R. E	Location within Gene	PCR product size (bp)	References
FGF2	F: CATAGTTCTGTAGACTAGAAG R:CTCTAAAGAAGGATTAAGTCAAAATGGGGCTGGTA	*Csp6*I	Intron 1	207	[19]
OPN	F: GCAAATCAGAAGTGTGATAGAC R: CCAAGCCAAACGTATGAGTT	*Bse*NI	Intron 4	290	[20]
PIT1	F:AAACCATCATCTCCCTTCTT R:AATGTACAATGTGCCTTCTGAG	*Hinf*I	Between Intron 5-Exon 6	447	[21]
STAT5A	F:GAGAAGTTGGCGGAGATTATC R: CCGTGTGTCCTCATCACCTG	*Bst*EII	Exon 8	820	[22]
GH	F:CCCACGGGCAAGAATGAGGC R:TGAGGAACTGCAGGGGCCCA	*Msp*I	Intron 3	329	[23]
PRL	F:CCAAATCCACTGAATTATGCTT R:ACAGAAATCACCTCTCTCATTCA	*Rsa*I	Exon 4	294	[24]
LEP	F: AGTGTCTCTTGGGGCATTTT R: CCTGGGCTCCTATCTTTCTG	*Sau3A*I	Between Intron 2-Exon 3	1147	[25]

Table 2. *The numbers of the AIs and the ages of the heifers according to groups*

Farms	N		Age of Heifers*		Numbers AI of Heifers	
	FH	RBH	FH	RBH	FH	RBH
Farm 1	7	11	16.55±1.46	23.10±3.00	1.00±0.00	4.18±1.40
Farm 2	20	15	15.58±1.16	22.87±5.05	1.00±0.00	5.20±1.93
Farm 3	9	11	14.64±1.17	17.91±2.97	1.00±0.00	4.54±0.93
Farm 4	14	12	14.38±0.95	19.01±1.66	1.00±0.00	5.00±2.33
Farm 5	10	12	15.35±1.49	18.87±0.63	1.00±0.00	4.50±0.90
Farm 6	10	12	14.70±0.26	18.80±1.39	1.00±0.00	4.16±1.93
Farm 7	10	7	15.47±0.70	18.88±1.89	1.00±0.00	3.86±0.80

** Month,* ***FH:*** *Fertile Heifers;* ***RBH:*** *Repeat Breeder Heifers,* ***AI:*** *Artificial Insemination*

***Table 3**. Allele and genotype frequencies observed (Ho) and expected heterozygosity (He) as well as chi-square test values for all loci investigated for overall population*

Locus	N	Allele Frequency (%)					Genotype Frequency (%)									Ho	He	χ^2
		A	B	G	C	T	TC	TT	CC	GC	GG	AG	AA	AB	BB			
OPN	160				52.81	47.19	48.13	23.13	28.75							0.481	0.500	0.226^{ns}
STAT5A	160			50.94	49.06				23.13	51.88	25.00					0.519	0.501	0.193^{ns}
GH	160	83.75	16.25										70.00	27.50	2.50	0.275	0.273	0.0084^{ns}
PIT1	146	25.34	74.66										5.48	39.73	54.79	0.373	0.380	0.316^{ns}
PRL	159	11.01		88.99							77.99	22.01				0.220	0.196	2.356^{ns}
FGF2	146	34.25		65.75							43.84	43.84	12.33			0.438	0.452	0.132^{ns}
LEP	160	89.69	10.31										80.00	19.38	0.63	0.194	0.185	0.320^{ns}

***Table 4.** Allele and genotype frequencies, observed (Ho) and expected heterozygosity (He) and chi-square test values for all loci investigated according to groups*

Groups	Locus	N	Allele Frequency (%)					Genotype Frequency (%)									Ho	He	χ^2
			A	B	G	C	T	TC	TT	CC	GC	GG	AG	AA	AB	BB			
RBH	*OPN*	80				50.63	49.38	43.75	27.5	28.75							0.438	0.500	1.247^{ns}
	STAT5A	80			52.50	47.50				23.75	47.50	28.75					0.475	0.499	0.181^{ns}
	GH	80	80.63	19.38										62.5	36.25	1.25	0.363	0.312	2.055^{ns}
	PIT1	79	26.58	73.42										3.80	45.57	50.63	0.456	0.390	2.216^{ns}
	PRL	80	10.00		90.00							80.00	20.00				0.200	0.180	0.988^{ns}
	FGF2	79	37.98		62.03							39.24	45.57	15.19			0.456	0.471	0.084^{ns}
	LEP	80	88.75	11.25										78.75	20	1.25	0.200	0.200	0.000^{ns}
FH	*OPN*	80				55.00	45.00	52.5	18.75	28.75							0.525	0.495	0.294^{ns}
	STAT5A	80			49.38	50.63				22.50	56.25	21.25					0.563	0.500	1.254^{ns}
	GH	80	86.88	13.13%										77.5	18.75	3.75	0.188	0.228	2.529^{ns}
	PIT1	67	23.88	76.12%										7.46	32.84	59.70	0.328	0.364	0.628^{ns}
	PRL	79	12.03		87.98							75.95	24.05				0.241	0.212	1.476^{ns}
	FGF2	67	29.85		70.15							49.25	41.79	8.96			0.418	0.419	0.000^{ns}
	LEP	80	90.63	9.38										81.25	18.75		0.188	0.170	0.856^{ns}

as well as on growth hormone expression was reported and found to be predominant [30]. The allelic frequency of this locus is in line with previous studies [30-32]. Although the allelic frequencies of this locus in the FH and the RBH groups were similar, the genotype distribution was different *(Table 4)*. The number of AB genotype animals was higher than the number of those in the RBH group for *PIT1* loci such as the heterozygote genotype of *GH (Table 4)*.

The associations between the A→G SNP in the *FGF2* gene and fertilization and embryonic survival were reported by Khatib et al.[19]. They found a higher embryonic survival rate among the embryos produced by the GG genotype compared to the dams with the AG and AA genotype. In another study [18], no relationship was found between SNP 11646 and the reproductive, productive and health traits in cows. In the present study, we found the favorable G allele of *FGF2* gene to be predominant in both the overall population and two separate investigated groups *(Table 3, Table 4)*.

As it is seen in *Table 3* and *Table 4*, the frequency of the C allele was slightly higher than the others [20,33]. Furthermore, genotype and allele frequency distrubitions were not differed between groups for *OPN* locus in our study. The allelic frequency of this locus was found to be similar in the studies where the frequency of the T allele was higher, with the exception of Jersey cows having a much higher C allele frequency [34].

Two alleles and three genotypes were also detected in exon 4 of the *PRL* locus resulting in the A→G nucleotide substitution in the synthesized protein *(Table 3, Table 4)*. The G allele and the GG genotype were found to be predominant. The frequency of the G allele was found to vary between 0.61-0.914 in previous studies [35,36]. The reverse was observed for only the Shimal and Jersey breeds with frequencies of 0.49 and 0.294, respectively [35,36].

Polymorphisms in *STAT5A* and their associations with the reproductive and other economically important traits were investigated [5,15]. Of them, the C→G transversion in

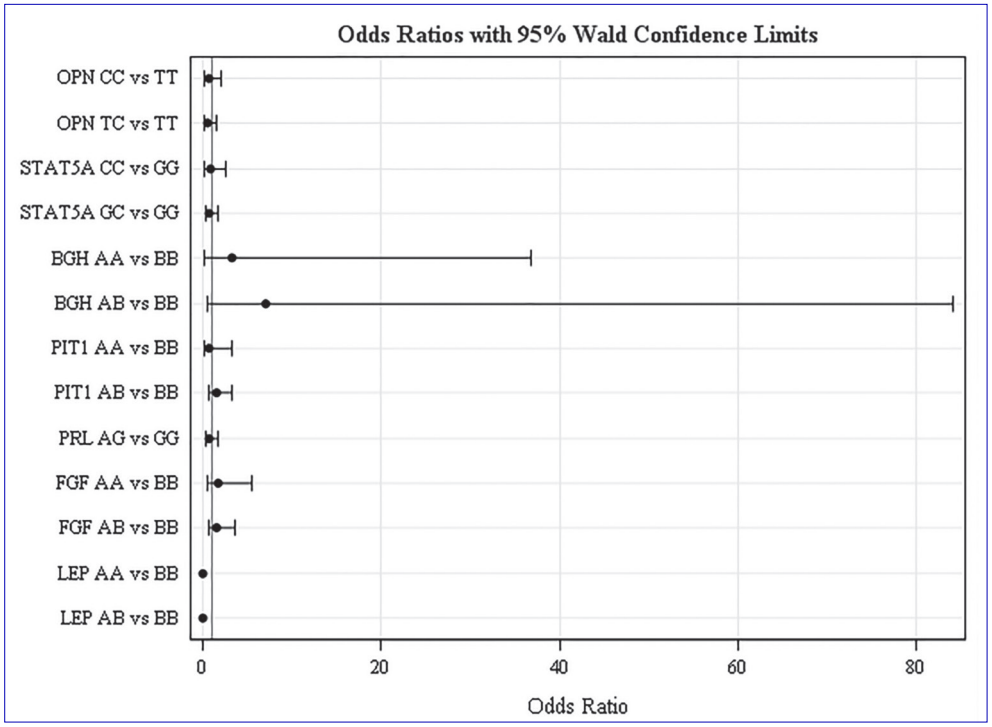

Fig 1. Odds ratios with %95 wald confidence limits

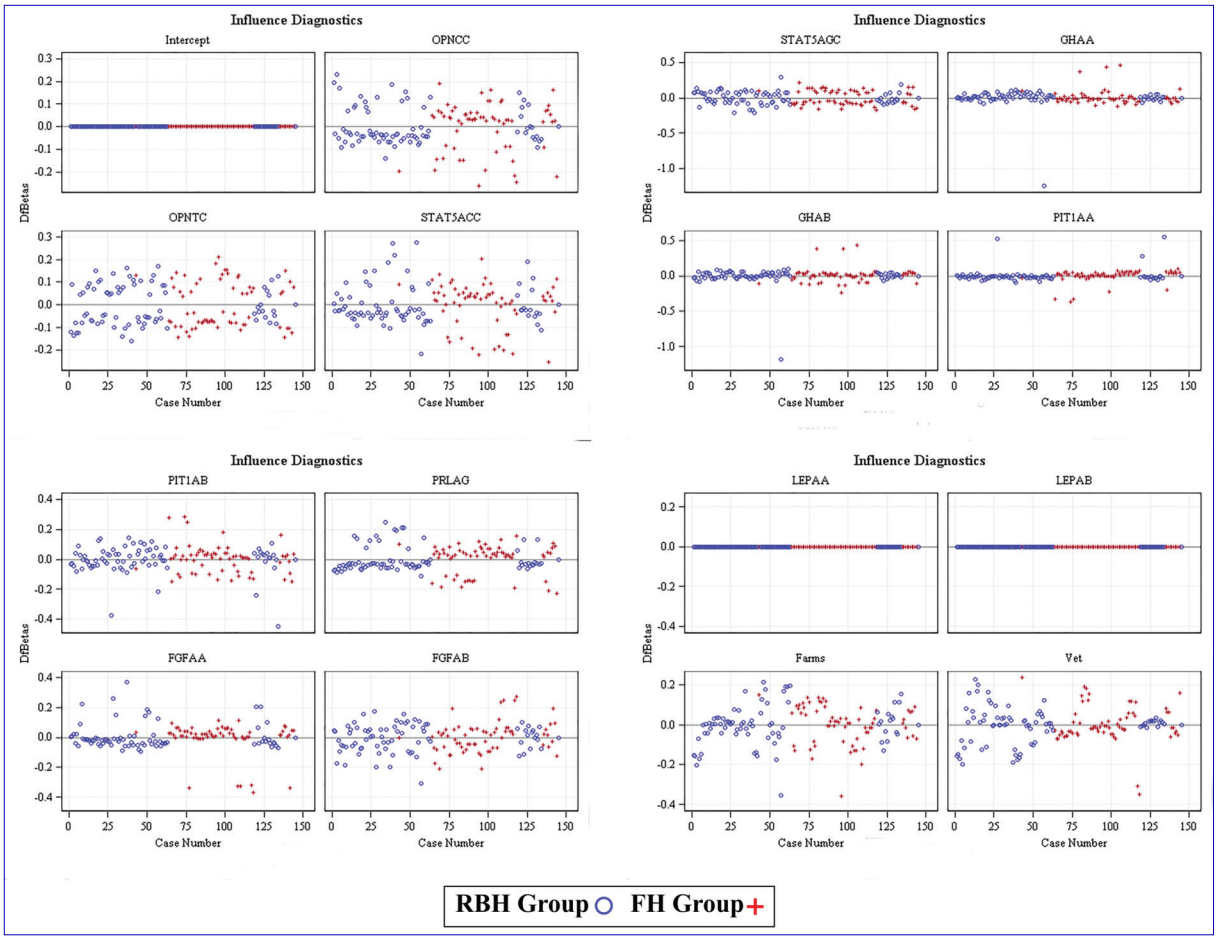

Fig 2. Influence diagnostics in logistic regression

Exon 8 of the *STAT5A* gene was found to be associated with the influence on embryonic survival in cattle [5,37] and previous reports on the expression of this gene support this finding [38]. In our study, the frequencies of the C and G alleles were the same for the two groups *(Table 4)*. On the other hand, while the C allele was present in the FH group, the unfavourable G allele frequency was higher in the RBH group. The genotypic frequencies for GC and GG also seemed different, but this difference was not significant *(Table 4)*.

Due to its key role in energy metabolism, the *LEP* gene was also investigated a lot. In the promoter and protein coding regions, several SNPs and microsatellite were reported. Polymorphisms in promoter region of the gene were found associated with the food intake leptin concentations, energy metabolisms and the reproductive parameters [39,40]. One of the most frequently investigated polymorphism was *Sau3AI* RFLP in the region located between intron II and exon II of the *LEP* gene. In the majority of previous studies, this polymorphism was not found influential on the investigated traits such as the age of puberty, BCS, milk production and properties [25,41,42]. We did not find any relationships between AI numbers for the first pregnancy, either. The results for allele and genotype frequencies were also in line with those studies. Allele A was predominant while the frequency of allele B was quite low. According to these results, it can be stated that the allele and genotype distributions were not suitable for the association analysis and this polymorphism was not suitable for being a marker in the selection scheme.

A strong relationship was suggested between the growth hormone circulation and the calving interval [43]. Therefore, polymorphisms on this gene seem to be potential selection criteria for improving reproductive performance. Mullen et al.[44] found six SNPs in 5'UTR region of *GH* in Irish Holstein Frisian cows and reported that some of these polymorphisms were associated with the reproductive traits. Some restriction fragment length polymorphims were also reported on Bovine *GH* locus [45-47]. One of these ploymorphisms in intron III of the gene creates *Msp*I recognizing site and was intensively studied due to its location near a transcription-binding site [23]. Various investigations revealed associations between *GH-Msp*I polymorphism and both famele and male reproductive traits [48-50]. At the same time, it was observed that the distribution of this polymorphism obviously differed between geographic regions [51,52]. The A allele was predominant among breeds from Europe while the frequency of B allele was higher among Bos indicus cattle [51,52]. The B allele was found related to meat quality and the lower frequencies of the allele among Holstein-Frisian cows were explained with this finding [23]. In line with this, the A allele was also found to be predominant in the Holstein Friesian heifer population investigated in our study *(Table 3, Table 4)*. The allelic frequency of the A allele was similar for the heifer groups *(Table 4)*. On the other hand, the genotypic distribution of *GH-Msp*I polymorphism differed between groups. While the frequency of the AB genotype in the RBH group was much higher than the FH group in our study, the BB genotype was observed in the RBH group at very low frequency *(Table 4)*. According to the statistical analyses, the difference between the frequency distributions of the groups was significant. Significant associations were found between the testis quality and the *GH-Msp*I polymorphism in previuos studies carried out on male fertility [48,49]. Arango et al.[50] reported a strong relationship between *GH-Msp*I genotypes and weight in the first estrus and first calving. Associations between the AB genotype and the milk components and the somatic cell counts were also reported [45,47]. In the literature, we've encountered no studies on associations between reproductive performance and *GH-MspI* polymorphism in heifers. Our results were in line with those of some previous studies revealing the opportunity of *GH-Msp*I polymorphism in the selection scheme.

No relationships were found between the *PRL, STAT5A, OPN, PIT-1*, *LEP* and *FGF2* polymorphisms and the AI numbers for the first pregnancy except for the *GH-Msp*I locus. On the other hand, the genotype distributions of *PIT-1* and *STAT5A* loci were also quite different between these two heifer groups. These differences were not statistically significant.

It can be suggested from these results that studies covering more individuals with an extended dataset should be performed to determine more accurate relationships. These polymorphisms may be important for the improvement of reproductive traits. Gene regions effect reproductive and productive traits should be also investigated in native breeds to reveal genetic composition of these breeds as some investigation groups have already done [53,54].

To enhance animal reproductive performance management of environmental conditions may be more expensive or unsustainable. It would be better to produce genetically valuable herds for a more profitable dairy industry. The loci investigated in this study were located on the strong candidate genes for reproductive performance. We observed differences in *PIT-1* and *STAT5A;* nevertheless, we could not prove these differences statistically. Studies with more animals from each heifer group will reveal the accuracy of these loci. On the other hand, we found a possible effect of polymorphism in *GH-Msp*I locus on the fertility in Holstein heifers, which is in line with previous studies finding associations between the *GH-Msp*I locus polymorphism and the reproductive traits. We suggest that *GH-Msp*I locus may be used as the selection criterion in breeding programs and phenotypic effects on herds should be monitored. There is also a need for studies to be made by using more animals, phenotypic data and epigenetic tools to prove this possible relationship.

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