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Analysis of *IGF1* C472T and *TG* C422T polymorphisms in Turkish Grey Steppe and Holstein Crossbred Bulls

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Abstract

The aim of the study was to determine genotypic distribution of bovine insulin-like growth factor 1 and thyroglobulin genes in Turkish Grey Steppe (Boz) and Holstein crossbred bulls. In the study, unrelated 30 Turkish Grey and 26 Holstein crossbred cattle were used. Genomic DNA was isolated from whole blood using standart phenol-chloroform extraction method. The polymerase chain reaction-restriction fragment length polymorphism technique was used for genotyping of C472T and C422T polymorphisms in insulin-like growth factor 1 and thyroglobulin genes, respectively. Hardy-Weinberg equilibrium was calculated by using PopGene software program. In addition the population indexes including heterozygosity, number of effective alleles and polymorphism information content were determined on the basis of allelic distribution. For insulin-like growth factor 1 gene, A allele frequency was higher and BB genotype was not present in both breed groups. For thyroglobulin gene, TT genotype was not present and moreover C allele was almost fixed in both Turkish Grey and Holstein crossbred bulls. In addition, extremely low minor allele frequencies in resulted in low genetic variabilities of population indexes. The present results may be useful for future studies conducted on bovine genome with respect to insulin-like growth factor 1 and thyroglobulin genes.

Keywords: cattle, Turkish Grey, Holstein crossbreds, *IGF1*, *TG*, PCR-RFLP

Introduction

Evaluation of variations at genotypic structure contributes to the genetic characterization of livestock populations and genetic modification in the desired direction may provide novel aspects to achieve permanent and sustainable progress in improving qualitative and quantitative traits that are economically important in cattle breeding. Modern techniques in molecular genetics allow direct genotyping for candidate genes. Variation in the exonic region of a gene may lead to changes in amino acids which induce reorganization in response to expressed protein. Although variations in the non-coding regions of a gene do not change the amino acid sequence of the protein, they may influence gene splicing or binding of regulatory proteins during transcription (Choudhary et al., 2005; Le Hir et al., 2003). Recently in livestock, such variations in DNA may also be associated with, or linked to, economic traits, which are controlled by many genes, and thus, genotypic characteristics associated with quantitative patterns have been identified (defined as quantitative trait loci: QTL) and single nucleotide polymorphisms (SNPs) of many candidate genes have been specifically determined (Hayes et al, 2001; Lande et al, 1990).

Bovine insulin-like growth factor 1 (*IGF1*) gene (GenBank Acc. No: AF210383) is located on chromosome 5, in the centimorgan 73.5 (Grosse et al., 1999). The provisional nucleotide sequence is approximately 72 kb and this gene consists 6 exons responsible for expressing heteroge-

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neous mRNA (De la Rosa Reyna et al., 2010). Although the length and genetic structure between species is variable, the expressed protein of 70 amino acids is highly conserved in vertebrates (Upton et al., 1998). Due to its biological function involving regulation of cell proliferation and animal growth, the *IGF1* gene is considered to be a candidate gene for predicting growth rate and meat production traits in animal genetic improvement programs (Andrade et al., 2008; De la Rosa Reyna et al., 2010; Siadkowska et al., 2006). Thyroglobulin (*TG*) gene (GenBank Acc. No: X05380) is located on the centromeric region of bovine chromosome 14 (Casas et al., 2005). *TG* was suggested as an important molecular regulator which encodes the glycoprotein precursor to the thyroid hormones including T3 and T4 (Pannier et al., 2010; Shin and Chung, 2007). This gene has been shown to be associated with differences in meat quality with respect to its effects on adipocyte growth and differentiation and lipid metabolism (Anton et al., 2008; Casas et al., 2005; Gan et al., 2008).

Genetic studies of SNPs in the *IGF1* and *TG* have primarily been performed in various cattle breeds. However, there is a very limited evaluation of these genes in Turkish native cattle breeds. Moreover, to the best of our knowledge, there is no publicly available information about these markers in Turkish Grey Steppe (Boz) breed. Thus, the objective of this study was to assess the genotypic distribution and population genetic indices of *IGF1* C472T and *TG* C422T in Turkish Grey Steppe and Holstein crossbred bulls.

Materials and Methods

Animals and sampling

The present study was conducted on a total of 56 bulls from two different breed groups of cattle, including 30 Turkish Grey Steppe and 26 Holstein crossbreds (Holstein x Turkish native cattle breeds), maintained at private livestock farms located in the South Marmara region of Turkey. From each animal, about 4 mL of venous blood was collected from the jugular vein and placed into a sterile K^3EDTA tube (Vacutest Kima, SRL, Italy). Blood samples were transported in an icebox to the laboratory and stored at -20 °C until needed for DNA extraction. Whole experiment were conducted in accordance with the ethical standards of Uludag University Local Ethical Committee of Animal Experiments (approval number: 2012-10/05).

Genomic DNA isolation

Genomic DNA was isolated by phenol-chloroform extraction method as described by Green et al. (2012). Briefly, 750 µL blood samples was treated with 30 µL Proteinase K with 80 µL sodium dodecyl sulfate and 90 µL, 1 M sodium chloride for 16 h at 56°C, which was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 vol/vol), and afterwards, again with an equal volume of chloroform. DNA precipitated by ethanol (Sigma-Aldrich, St Louis, MO, USA), was dissolved in TE buffer (10 mM Tris–HCl, pH 7.4 and 0.1 mM EDTA). The amount and purity of extracted DNA samples assessed by spectrophotometry (NanoDrop 2000c, Thermo Scientific, Wilmington, DE, USA). The total amount of DNA was approximately 70 – 260 ng. Samples showing an optical density (OD) ratio (260 nm/ 280 nm) of between 1.7 and 1.9 being used for further analysis while samples outside this range were reprocessed.

Genotyping

In this study, genotyping of the SNPs in the *IGF1* and *TG* genes was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). In this context, a 249 bp fragment in the 5'-noncoding region of *IGF1* gene and a 545 bp fragment in the 5'-promoter region of the *TG* gene were amplified from the purified genomic DNA using two different sets of primers as shown in Table 1. The PCR reaction mixture (total of 50 µL) contained 3 µL of genomic DNA, 33.5 µL of ddH2O, 5 µL of 10x buffer, 5 µL of MgSO4, 1 µL of dNTPs (2.5 mM), 2.5 U of Taq DNA polymerase (Biomatik, Cambridge, Canada, A1003-500U, 5 $U\mu L^{-1}$) and 1 µL (0.025 µM) of each primer. The DNA amplification reactions were carried out using two thermal cyclers (Palm Cycler GC1-96, Corbett Research, Sydney, Australia and MyGenie 96 Thermal Block, Bioneer, Daejeon, South Korea) pre-programmed for the following conditions:

(a) T/C polymorphism at position 472 of *IGF1* gene:
Initial denaturation for 5 min at 94 °C followed by 31 cycles (denaturation at 94 °C for 1 min, annealing at 64 °C for 1 min and extension at 72 °C for 1 min) and a final extension at 72 °C for 5 min.

(b) C/T polymorphism at position 422 of the *TG* gene:
Initial denaturation for 5 min at 94 °C followed by 35 cycles (denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min) and a final extension

Table 1. Primers used and corresponding restriction enzymes in the present study.

Gene	PCR amplicon	Forward primer	Reverse primer	Restriction enzyme	Reference
IGF1	249 bp	5'ATTACAAAGCTGCCTGCCCC3'	5'ACCTTACCCGTATGAAAGGAATATACGT3'	*Sna*BI	Siadkowska et al. (2006)
TG	545 bp	5'GGGGATGACTACGAGTATGACTG3'	5'GTGAAAATCTTGTGGAGGCTGTA3'	*Mfl*I	Shin and Chung (2007)

at 72 °C for 7 min.

The PCR products were separated by 2% (w/v) agarose gel electrophoresis at 85–90 V for one hour. Afterwards, 15 µL of PCR product was digested with 15 Units of *Sna*BI and *Bst*YI restriction enzymes for *IGF1* and *TG*, respectively by incubating at 37 °C overnight. The digested products were subjected to 3% (w/v) agarose gel electrophoreses and stained with ethidium bromide (Sigma Aldrich, Steinheim, Germany) with the concentration of 1 µg.mL^{-1} as an intercalated reagent. The banding was visualized under UV illumination and documented using a gel documentation system (DNR-Minilumi, DNR Bio-Imaging Systems, Israel). As a reference to estimate the size of fragments we applied DNA ladder (100–1500 bp, Biomatik).

Statistical analysis

The genotype and allele frequencies of each SNP were calculated according to the method of Falconer et al. (1996). A standard χ2 goodness-of-fit was calculated to assess compliance with Hardy–Weinberg Expectations (HWE) using POPGENE software v1.32 (Yeh et al., 2000). On the basis of allele frequencies, the population genetic indexes including gene heterozygosity (He), effective allele numbers (Ne) and polymorphism information content (PIC) were estimated by using the following formulas as described by Nei et al. (1974) and Botstein et al. (1980):

$$He = 1 - \sum_{i=1}^{n} P_i^2$$

$$Ne = 1/\sum_{i=1}^{n} P_i^2$$

$$PIC = 1 - (\sum_{i=1}^{n} P_i^2) - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2$$

Where:

Pi was the frequency of the ith allele,

n was the number of alleles.

Results

In the present study, the 249 bp fragment in the 5'-noncoding region of the *IGF1* gene (Fig 1) has been amplified and the digestion of the PCR product with the *Sna*BI nuclease resulted in two bands (223 bp and 26 bp) for homozygote AA (nt: TT) and three bands (249 bp, 223 bp and 26 bp) for the heterozygote (AB, nt: CT) genotype (Fig 2). The DNA amplified from BB (nt: CC) animals should have been remained undigested with the corresponding restriction enzyme. However, CC genotype was not observed in both breed groups.

The cleavage of a 565 bp PCR product (Fig 3) by *Bst*YI yielded three fragments of 259 bp, 178 bp and 72 bp and was diagnostic for the CC genotype in the *TG* assay. Results revealed that, TG C422T was fixed in Turkish Greys and Holstein crossbreds, and thus, T allele which was characterized by 473 bp and 72 bp, was unseen as shown in Fig 4.

Two alleles and two genotypes in each SNP were found in the present study. In this context, BB genotype in *IGF1* and TT genotype in *TG* were not observed in Turkish Greys and Holstein crossbreds. Moreover, heterozygous genotype frequency of *TG* was rather low in this study. Hence, the A allele seemed to be fixed in both breed groups in evaluating the genotypic distribution *TG* marker. Present results revealed that the most frequent genotype for *IGF1* was AB (0.63 and 0.69) and CC for TG (0.97 and 0.92) C422T marker in Turkish Greys and Holstein crossbred bulls, respectively. The B and A allele frequency was 0.32 and 0.68 for Turkish Grey cattle; 0.35 and 0.65 for crossbreds in *IGF1*, and whereas, C and T allele frequency was 0.98 and 0.02 for Turkish Greys; 0.96 and 0.04 for crossbreds.

The allele and genotype frequencies, population genetic properties including compatibility with the HWE, He, Ne, and PIC are shown in Table 2. Results showed that the

Table 2. Allele and genotype frequencies of polymorphisms in IGF1 and TG genes, population genetic indices (He, Ne, PIC) and compatibility with the Hardy-Weinberg equilibrium (HWE).

Locus	Breed	n	Allelic frequencies		Genotypic frequencies			χ2(HWE)	He	Ne	PIC
			B	A	BB	AB	AA				
IGF1 C472T	Turkish Grey	30	0.32	0.68	0 (n=0)	0.63 (n=19)	0.37 (n=11)	6.442*	0.4352	1.7705	0.3405
	Holstein Crossbred	26	0.35	0.65	0 (n=0)	0.69 (n=18)	0.31 (n=8)	7.297*	0.4551	1.8348	0.3515
			C	T	CC	CT	TT				
TG C422T	Turkish Grey	30	0.98	0.02	0.97 (n=29)	0.03(n=1)	0 (n=0)	0.008	0.0392	1.0407	0.0384
	Holstein Crossbred	26	0.96	0.04	0.92 (n=24)	0.08 (n=2)	0 (n=0)	0.041	0.0768	1.0831	0.0739

Figure 1. The electrophoresis pattern of PCR amplification for C472T polymorphism within the bovine IGF1 gene.

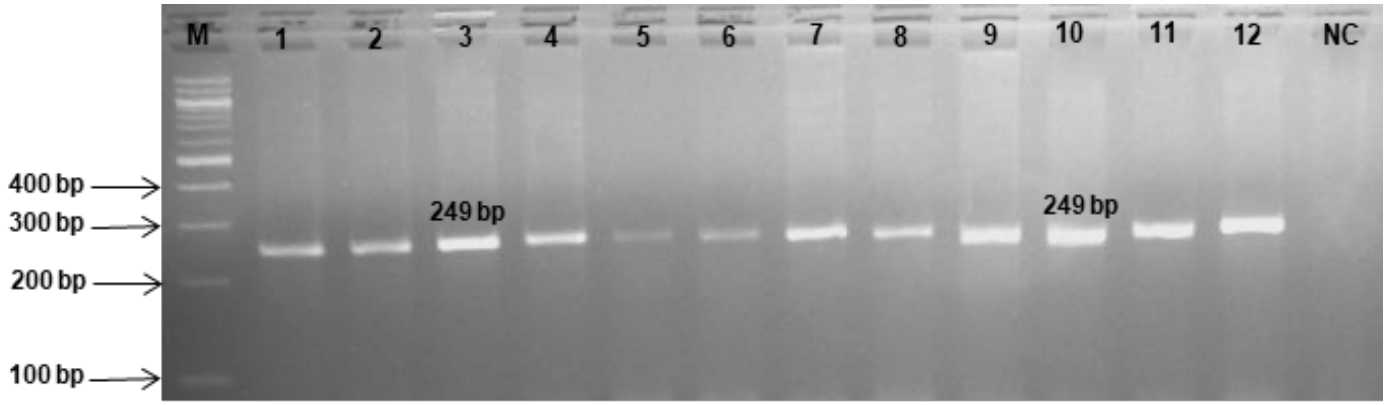


Figure 2. The electrophoresis pattern of restriction enzyme digestion of PCR product with SnaBI for C472T polymorphism within the bovine IGF1 gene.

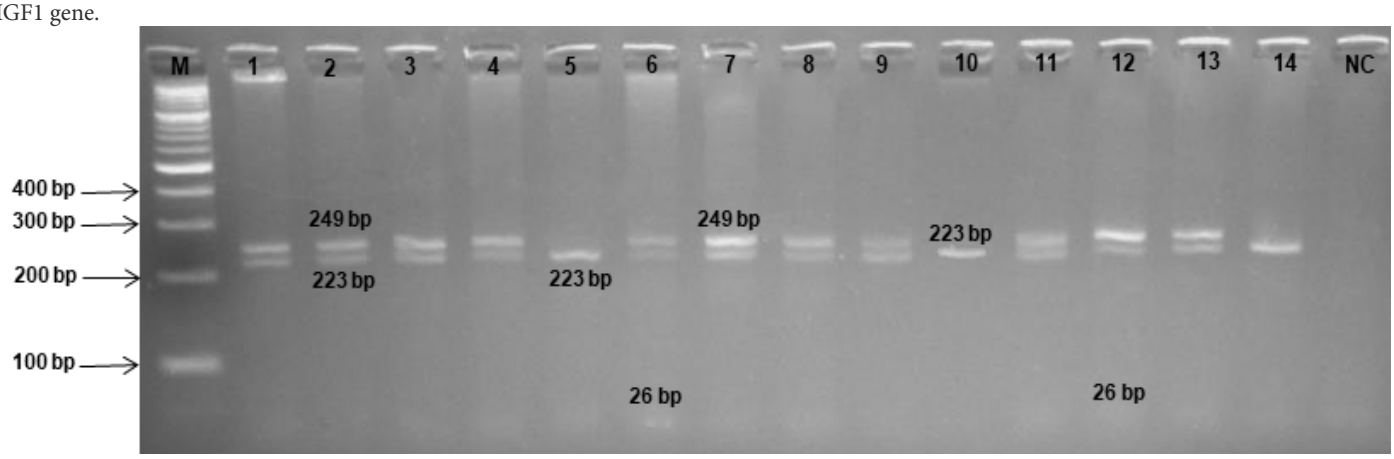
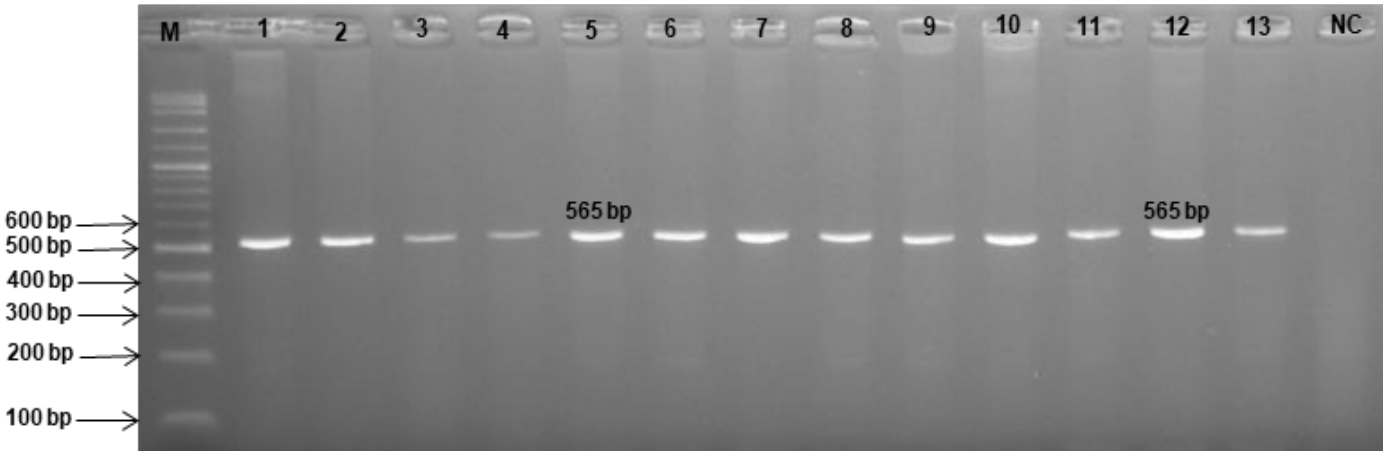
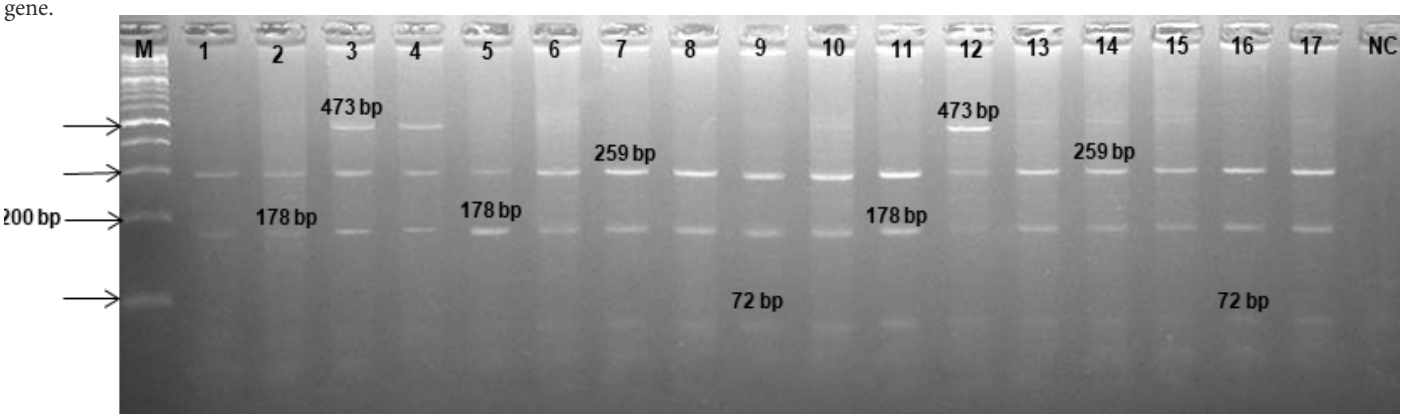


Figure 3. The electrophoresis pattern of PCR amplification for C422T polymorphism within the bovine TG gene.



M: Marker; NC: Negative control

Figure 4. The electrophoresis pattern of restriction enzyme digestion of PCR product with BstYI for C422T polymorphism within the bovine TG gene.



M: Marker; Lanes 3, 4, 12: CC genotype; Lanes 1, 2, 5-11, 13-17: CT genotype; NC: Negative control Note: TT genotype was not present.

genotypic distribution was determined to be incompatible with either of the breed groups in the HWE for *IGF1* C472T marker (P<0.05). He values ranged from 0.0392 to 0.4551; Ne values ranged from 1.0407 to 1.8348 and PIC values ranged from 0.0384 to 0.3515 were observed in the current study. Extremely low minor allele frequencies resulted in low genetic variabilities of He, Ne and PIC for *TG* C422T marker.

Discussion and Conclusion

IGF-1 protein, which belongs to somatomedins, plays a key role in regulation of metabolism and is encoded by *IGF1* gene (Andrade et al., 2008). In cattle, this gene was localized in chromosome 5, which harbors a high number of quantitative trait loci (QTL) for multiple traits associated with growth rate and meat production (De la Rosa Reyna et al., 2010; Siadkowska et al., 2006). The SNP *IGF1* C472T has been studied in various cattle breeds, including dairy and beef breeds. There are contrasting results obtained from genotypic distribution evaluation in the literature. Siadkowska et al. (2006) reported the genotype frequencies of 0.29, 0.47 and 0.24 for AA, AB and BB genotypes, respectively in Polish Holstein-Friesians. In the study performed by Hines et al. (1998), the frequencies of A and B alleles were 0.55 and 0.45, respectively for a population of Holstein cattle. Similarly, Li et al. (2004) reported the frequencies of 0.56 and 0.44 for the mentioned alleles in the commercial lines of dairy cattle. In Angus cattle the frequencies of both alleles, which was estimated 0.64 for A and 0.36 for B, were different than dairy-purpose breeds, as suggested by Ge et al. (2001). However, as reported by De la Rosa Reyna et al. (2010), the frequency of allele B in the two Charolais groups evaluated was 0.54 and 0.74, and moreover, these researchers found a rather high frequency of B allele (0.97) in Beefmaster cattle. In a study performed by Akis et al. (2010), the frequency of allele B was found to be much higher (0.77 and 0.62, respectively) in South Anatolian Red and East Anatolian Red cattle. Curi et al. (2005) found that B allele was fixed in a Nellore population and these researchers proposed that allele B is characteristic of indicus populations. The fixation of B allele in indicine cattle might be the result of genetic drift, selection or mutation as suggested by Curi et al. (2005) and Akis et al. (2013). In the present study, conversely, allele A is dominant over allele B in *IGF1*, and moreover, BB genotype was not present in both breed groups including Turkish Grey and Holstein crossbred bulls. In the literature there is limited information about the *IGF1* C472T (IGF1-SnaBI) polymorphism in Turkish native cattle breeds. In addition, to our knowledge, there is no information about this marker in Turkish Grey breed. Hence, present results may be useful for future genetic studies conducted on Turkish native cattle.

TG was suggested as an important molecular store for the thyroid hormones which affect fat cell growth and differentiation (Shin et al., 2007). In cattle, this gene was mapped to BTA 14 where the positional and functional candidate genes associated with lipid metabolism located, and hence, *TG* has been widely used in marker assisted selection (MAS) programs not only to improve meat quality traits such as marbling level and tenderness in beef cattle (Thaller et al., 2003), but also to evaluate fat yield and percentage in milk of dairy cattle (Anton et al., 2012). Recently, the DNA tests have been used by breeders in the United States, Canada, Argentina, Japan, and Australia. To give an example, GeneSTAR Marbling is a DNA diagnostic test for the *TG* gene and commercially available marker for carcass quality traits (Shin et al., 2007). Population genetic proper-

ties widely vary among cattle breeds, and thus, knowledge about the genotypic distribution in selected markers may play an important role to perform an adequate evaluation. Previous studies have reported TT genotype frequencies of *TG* C422T marker, ranged from 6.7 to 18.9 % in Bos taurus (Moore et al., 2003; Rincker et al., 2006; Thaller et al., 2003), whereas its frequency was rather low (1.5 %) in Bos indicus (Casas et al., 2005). Similarly, Shin et al. (2007) reported that allelic frequencies including C and T were 0.641 and 0.359, respectively, and genotype frequencies for CC, CT and TT were 0.41, 0.46 and 0.13, respectively. In this study, TT genotype was not present, and moreover, T allele frequencies were extremely low (0.02 and 0.04 in Turkish Greys and Holstein crossbreds, respectively) in both genotyped breed groups.

In the present study, minor allele frequencies widely ranged from 0.02 to 0.98 among two breed groups. Menezes et al. (2006) suggested that a locus can be considered as polymorphic when the frequency of the most common allele was lower than 0.95, accordingly, *TG* C422T may not be polymorphic in respect to genotypic distribution obtained from cattle breed groups analysed. In addition, HWE was tested for each locus and was not compatible for *IGF1*. Such incompatibilities in HWE analysis may result from population substructure in relation to selection, inbreeding and indirect selection (Lacorte et al., 2006). The effectiveness of loci allele impact in populations has been expressed by Ne with respect to He (Trakovicka et al., 2013). Moreover, the PIC values indicate the quality of markers in genetic studies. Hence, evaluating the population genetic indices may be helpful to understand the properties of population substructure. According to the classification reported by Botstein et al. (1980), a marker with a PIC value higher than 0.5 is considered to be very informative, whereas values between 0.25 and 0.5 are mildly informative, and values lower than 0.25 are not informative. The analyses showed median and low levels of PIC for *IGF1* C472T and *TG* C422T, respectively in this study. Moreover, extremely high major allele frequencies revealed low levels of He and Ne for *TG* marker. Population genetic structure and distribution of alleles may vary among breeds and even among different populations of the same breed (Lacorte et al., 2006). Hence, the differences between cattle breed groups in the mentioned status can be evaluated as a common circumstance.

Consequently, this study focused on genotypic distribution of *IGF1* and *TG* genes in Turkish Grey and Holstein crossbred bulls. These genes have been previously identified as genotypic indices contributing to genetic control of lipid metabolism in both beef and dairy cattle. To the best of our knowledge, there is limited publicly available data of mentioned genes in Turkish native cattle breeds. The BB genotype in *IGF1* C472T and the TT genotype in *TG* C422T were not present. Moreover, A allele in *TG* was almost fixed. Accordingly, current results may present not only the genotypic distribution of the markers, but also the difference between culture crosses and native breeds with respect to *IGF1* and *TG* genes. Further genetic studies conducted on native breeds should be performed to achieve an adequate characterization among breeds and to conserve animal genetic resources in Turkey.

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