VETERINARSKI ARHIV 93 (1), 1-16, 2023 DOI: 10.24099/vet.arhiv.1483

Single nucleotide polymorphisms in *β-Lactoglobulin*, *k-casein* and *DGAT1* genes as candidates for rigorous selection of milk composition and performance traits in Holstein cattle

Ahmed Ateya[1*], Sherif Nasr[2], Hanaa Ghanem[1], Kadry Sadek[3], Mona Al-Sharif[4], and Basma Hendam[1]

[1]Department of Husbandry & Development of Animal Wealth, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt
[2]Department of Animal Husbandry and Animal Wealth Development, Faculty of Veterinary Medicine, Damanhour University, Egypt
[3]Department of Biochemistry, Faculty of Veterinary Medicine, Damanhour University, Egypt
[4]Department of Biology, College of Science, University of Jeddah, Jeddah, Saudi Arabia

ATEYA, A., S. NASR, H. GHANEM, K. SADEK, M. AL-SHARIF, B. HENDAM: Single nucleotide polymorphisms in *β-Lactoglobulin*, *k-casein* and *DGAT1* genes as candidates for rigorous selection of milk composition and performance traits in Holstein cattle. Vet. arhiv 93, 1-16, 2023.

ABSTRACT

The aim of this study was to investigate *β-Lactoglobulin*, *k-casein* and *DGAT1* gene polymorphism and to associate this polymorphism with milk composition and performance traits in Holstein cattle using the PCR-DNA sequencing approach. On the basis of farm records, accurate phenotypic data for milk composition and performance traits were obtained for seventy Holstein dairy cows. Blood samples were collected from each animal into tubes containing disodium EDTA as an anticoagulant for DNA extraction. PCR was carried out for amplification of fragments of exon 4 (301-bp) of *β-Lactoglobulin*, exon 4 (373-bp) of *k-casein*, and exon 7 (321-bp) of *DGAT1* genes. DNA sequencing assessment elaborated single nucleotide polymorphisms (SNPs) in the investigated genes amongst the enrolled dairy cows. On the basis of the dairy cows that harbored identified SNPs in each gene, the animals were allocated into different groups. The least square means of the groups revealed a significant association ($P \leq 0.05$) between SNPs and milk production and performance traits. Logistic regression model confirmed a highly significant effect of the identified SNPs on the studied traits, where a moderate to strong relationship was detected between the predictor (SNPs) and the grouping variable (Milk composition and performance traits). Consequently, the identified SNPs in *β-Lactoglobulin*, *k-casein* and *DGAT1* genes could be used as candidates for developing marker assisted selection (MAS) for milk composition and performance traits in Holstein dairy cattle.

Key words: Holstein cattle; SNPs; genetic variability; milk composition; milk performance

*Corresponding author:
Ahmed Ateya, Department of Husbandry & Development of Animal Wealth, Faculty of Veterinary Medicine, Mansoura University, Gomhoria St., Mansoura, P.O. box 35516, Mansoura, Egypt. Tel: 002-01003541921, FAX: 002-050-2372592, E-mail address: ahmed_ismail888@yahoo.com

ISSN 0372-5480
Printed in Croatia

Introduction

Milk from ruminants is an important component in the human diet as it is the source of a number of valuable nutrients, especially proteins. The association of genetic polymorphism with milk production and composition has stimulated interest in using the genetic polymorphism of candidate genes in marker assisted selection (MAS) to improve milk performance traits in farm animals (MACCIOTTA et al., 2008). Additionally, the increased application of molecular genetic markers associated with various QTL has been elaborated to enhance the effective and rigorous selection and breeding of livestock, particularly for genetic traits including growth rate, body weight, carcass characters, feed intake, milk production and composition (SPELMAN and BOVENHUIS, 1998).

The genetic structure of animals can be described using a variety of proxy molecular indicators according to the point of investigation. One of them, the analysis of single nucleotide polymorphisms (SNP) as a genetic marker has become widely used in this area. The use of SNPs or whole genome scanning may therefore reshape the findings of previous research concerning the assessment of genetic variations and genetic determinants of livestock breeds, and thus could provide more in-depth understanding of molecular basis of genetic diversity (GROENEVELD et al., 2010).

The genetic polymorphism of milk protein have received greater importance in the last decade. This great interest is due to the possible associations between milk protein genotypes and economically important traits in dairy cattle. Previous studies reported the possible association between milk protein gene polymorphism and milk production, milk composition and cheese production (ROBITAILLE et al., 2002). Therefore, milk protein genes could be useful as proxy markers for rigorous selection of milk production performance and composition traits in Holstein cattle. β-Lactoglobulin is considered the principle whey protein in the milk of cows and other ruminants that depends on breed and lactation stage (HEJTMÁNKOVÁ et al., 2012). It was established that β-Lactoglobulin is a polypeptide of a single chain consisting of 162 amino acids, and amino acid sequence variations have been identified (CREAMER et al., 1983). Two variants of β-Lactoglobulin, named A and B, have been identified that differ in two amino acids; however both variants contain five cysteine residues, four of which are involved in forming intra-chain disulphide bridges. The biological functions of this protein are still not known. It could have a role in the metabolism of phosphates in the mammary gland and the transport of retinol and fatty acids in the gut (HILL et al., 1997).

Casein represents 80 % of the total proteins in cow's milk and it is therefore the most abundant protein constituent (KAMIÑSKI et al., 2007). The casein genes are completely linked and inherited as a cluster, so they have potential value and could be candidates for marker assisted selection of milk traits (LIEN and ROGNE, 1993; RISTANIC et al., 2020). It is divided into four fractions: αS1-casein, αS2- casein, β-casein, and κ-casein (EIGEL et al., 1984), where κ-casein accounts for approximately 12% (FIAT and JOLLÈS, 1989). Kappa casein (κ-CN) is determined by the gene positioned on chromosome 6 in cattle (KAMIŃSKI et al., 2007; CAROLI et al., 2009). Two variants of κ-casein gene have been elaborated: A and B variants. Two single nucleotide polymorphisms (C136T and A148C) substitute Thr with Ile and Asp with Ala in the B variant (HRISTOV et al., 2012). The A variant is associated with higher milk yield but lower protein content; while allele B is linked with higher protein (ALIPANAH et al., 2005; OTAVIANO et al., 2005).

Generally, most productivity traits, such as milk production performance and composition, have been shown to be affected by numerous polymorphisms in different loci of genes (BUITKAMP and GÖTZ, 2004). For example, associations have been documented between the contents of milk fat in cattle and the gene encoding acylCoA-diacylglycerol acyltransferase1 (DGAT1) (WINTER et al., 2002). The DGAT1 enzyme has been identified as catalyzing the synthesis of triglycerides, and playing an essential role in the development of fat-rich connective tissue, absorption of fat in the gut, and the synthesis of lipoprotein (CASES et al., 1998). Additionally,

the DGAT1 gene, located on the centromeric end of the BTA14 gene in cattle, has been shown as a core gene affecting the quantity of milk and the percentage of milk fat (GRISART et al., 2002; THALLER et al., 2003).

Associations have been reported between *β-Lactoglobulin*, *k-casein* and *DGAT1* gene polymorphism and milk composition and performance in dairy cattle (KAMINSKI and ZABOLEWICZ, 2000; RACHAGANI et al., 2006; KARIMI et al., 2009; OLEŃSKI et al., 2012; ZAGLOOL et al., 2016; RANGEL et al., 2017; SAFRONOVA et al., 2017; BANKAR et al., 2018; SIGNORELLI et al., 2009; RYCHTÁŘOVÁ et al., 2014). However, the results reported were controversial. Moreover; unlike in our study, previous studies reported relatedness using RFLP (RACHAGANI et al., 2006; BANKAR et al., 2018; RYCHTÁŘOVÁ et al., 2014). Other studies investigated this association using SSCP genetic markers (DINC et al., 2013; BARBOSA et al., 2019).

Consequently, the objective of the present study was to elucidate the efficiency of single nucleotide polymorphism in *β-Lactoglobulin*, *k-casein* and *DGAT1* genes, as a genetic marker for rigorous selection of milk composition and performance traits in Holstein cattle using the PCR-DNA sequencing approach.

Materials and methods

Ethics Statement. The collection of samples and care of the animals used in this study followed the guidelines for experimental animals established by the Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University (code number R/23).

Experimental Animals. Seventy Holstein dairy cows were used in this study. The animals belonged to a private farm located on the Ismailia desert road, Ismailia Governorate, Egypt. The animals were in the third lactation season and were raised in a commercial dairy herd of approximately 450 animals. On the basis of farm records, accurate phenotypic data for milk composition and performance traits (milk yield, fat %, protein %, lactose %, total solids %, milk density, order of lactation, days in milk, dry period, and daily milk yield) were obtained. The cows were 3 years of age on average and 450 kg of average body weight. The animals were housed in a cubicle (free-stall/feedlot) barn with straw-bedded stalls, and a slatted floor that was scraped regularly. They were fed a total mixed ration (TMR), milked twice a day and artificially inseminated.

Sample Collection and DNA Extraction. Under complete aseptic conditions, blood samples were collected from each animal into tubes containing disodium EDTA as an anticoagulant for DNA extraction. Extraction of the genomic DNA was done using a Gene JET whole blood genomic DNA extraction kit, following the manufacturer's procedure (Thermo scientific, Lithuania). NanoDrop was used to assess the quality, purity and concentration of the DNA.

Polymerase Chain Reaction (PCR) for β-Lactoglobulin, k-casein and DGAT gen. PCR was carried out for amplification of fragments of exon 4 (301-bp) of *β-Lactoglobulin*, exon 4 (373-bp) of *k-casein*, and exon 7 (321-bp) of *DGAT1* genes. The primer sequences were designed according to the PubMed published sequences of *Bos taurus* gb|HQ589927.1|, gb|EU348567.1|, and gb|EU348567.1| for *β-Lactoglobulin*, *k-casein* and *DGAT1* genes respectively. The primers used in the amplification are illustrated in Table 1. The polymerase chain reaction mixture was performed in a final volume of 70 µL in a thermal cycler. Each reaction volume contained 4 µL DNA, 29 µL H_2O (d.d water), 35 µL PCR master mix (Jena Bioscience, Germany), and 1 µL of each primer. The reaction mixture was subjected to the following thermal cycler program: an initial denaturation temperature of 95°C for 3 minutes; the cycling proceeded for 35 cycles of 94 °C for 30 sec for denaturation, annealing temperatures (as shown in Table 1) for 45 sec, extension at 72 °C for 45 sec and a final extension at 72 °C for 8 min. Samples were held at 4 °C and representative results of PCR analysis were detected by agarose gel electrophoresis. The fragment patterns were then visualized under UV light using a gel documentation system.

Table 1. Forward and reverse primer sequence, length of PCR product and annealing temperature for *BLG, k-casein,* and *DGAT1* genes

Primer	Forward	Reverse	Annealing Temperature (°C)	Length of PCR Product (bp)	Reference
BLG	5′-ACTCACTTTCCTCCCGTCTTGA-3′	5′-GCTCCCGGTATATGACCACCC-3′	62	301-bp	Current study
k-casein	5′TACCATGGCACGTCACCCACAC-3′	5′-TCGCCTTCTCTGTAACAGATTTA -3′	60	373-bp	Current study
DGAT1	5′-AGGGCTGGGGCCAAGGCCAAG -3′	5-′GGAAGTTGAGCTCGTAGCACA -3′	64	321-bp	Current study

DNA sequencing and Polymorphism Detection. To detect single nucleotide polymorphisms in the three genes between the enrolled Holstein dairy cows, sequencing of PCR products was carried out in forward and reverse directions using an ABI 3730XL DNA sequencer (Applied Biosystem, USA), depending on the enzymatic chain terminator technique developed by SANGER et al., (1977). Chromas and blast 2.0 softwares were used for analysis of DNA sequencing data, and differences were classified as single-nucleotide polymorphisms (SNPs) between PCR products of the selected productive genes, as well as between PCR products for these genes and the reference sequences available in GenBank. On the basis of DNA sequencing data alignment, amino acid sequence variations of the milk production traits between the seventy Holstein dairy cows were shown using the MEGA4 software package (TAMURA et al., 2007).

Statistical Analysis. In this study, the statistical hypothesis was Ho: Single nucleotide polymorphisms in *β-Lactoglobulin*, *k-casein* and *DGAT1* genes cannot be used as candidates for milk composition and performance traits in Holstein cattle

HA: Single nucleotide polymorphisms in *β-Lactoglobulin*, *k-casein* and *DGAT1* genes could be used as candidates for milk composition and performance traits in Holstein cattle.

Associations between the identified SNPs and milk production and performance traits were examined using the least square method of the general linear model (GLM) procedures using SPSS software (SPSS version 18.0, 2009). The following model was used:

$$Y = \mu + b + e$$

Where Y is the value of the studied trait, μ is the overall mean of the population, b is effect of the gene SNP and e is the residual effect.

Results

Single nucleotide polymorphisms in β-Lactoglobulin, k-casein and DGAT1 genes. PCR-DNA sequencing revealed nucleotide sequence variations in the form of SNPs among the Holstein dairy cows. DNA sequencing of the *β-Lactoglobulin* gene (301-bp) revealed one SNP (C129T). DNA sequencing of the *k-casein* gene (373-bp) also

revealed seven SNPs (G61T, G99C, T129C, G137A, C165A, G226A, T234A, and A240G). Regarding the *DGAT1* gene, DNA sequencing for a fragment of 321-bp elicited one SNP (G140A), which seemed to be characteristic for a number of the dairy cows. The nucleotide sequence variations of *β-Lactoglobulin*, *k-casein* and *DGAT1* genes amongst the enrolled dairy cows studied and the reference sequences available in GenBank confirmed all identified SNPs (Figure 1, 2 and 3).

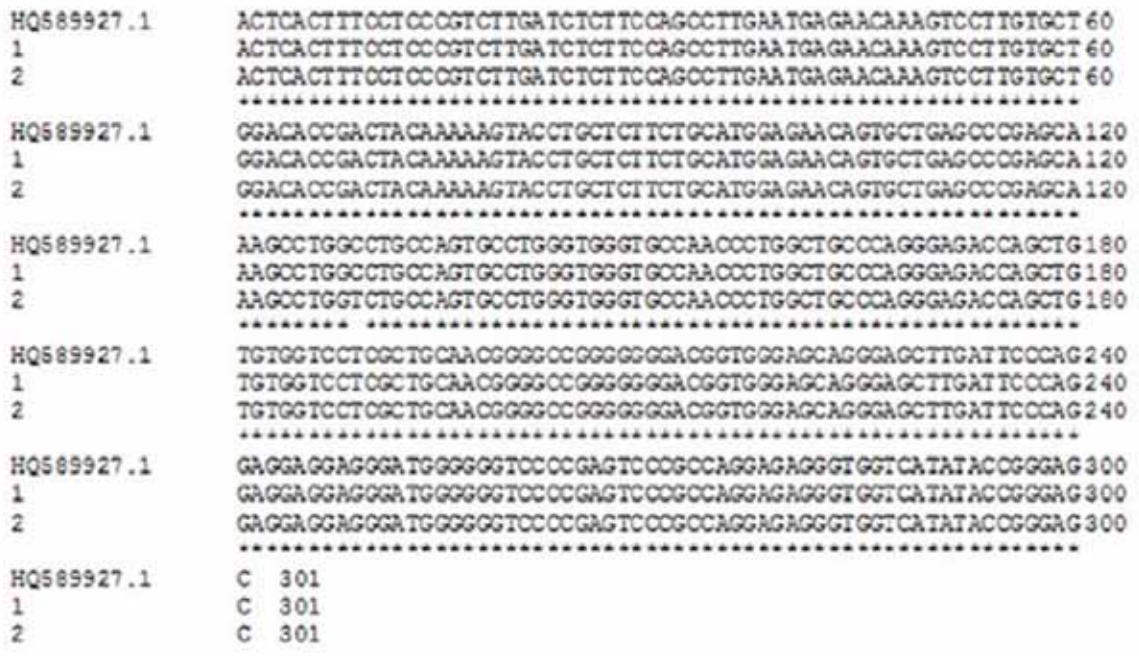

Fig. 1. Representative DNA sequence alignment of BLG gene (301-bp) among Holstein dairy cows and reference sequence available in GenBank gb|HQ589927.1|. Asterisks represent similarity

Association of β-Lactoglobulin, k-casein and DGAT1 Gene Polymorphisms and Milk Performance and Composition Traits. On the basis of the SNPs identified in each gene, the dairy cows were allocated into different groups as follows: dairy cows harboring C129T SNP for *β-Lactoglobulin* gene were represented as G1 *BLG*; while dairy cows that did not exhibit the identified SNP were represented as G2 *BLG*. Regarding the identified SNPs in the *k-casein* gene, dairy cows were distinguished into four groups: dairy cows harboring G61T, G137A, C165A, and A240G SNPs were represented as G1 *k-casein*, dairy cows harboring G99C and T234A SNPs were represented as G2 *k-casein*, dairy cows exhibiting T129C, and G226A SNPs were represented as G3 *k-casein*, and dairy cows that did not harbor either of the identified SNPs were represented as G4 *k-casein*. Along the same line, SNPs identified in the *DGAT* gene led to the dairy cows being divided into two groups: G140A was exhibited by one group of dairy cows and they were represented as G1 *DGAT*; while the other groups that did not exhibit the denoted SNP were represented as G2 *DGAT*.

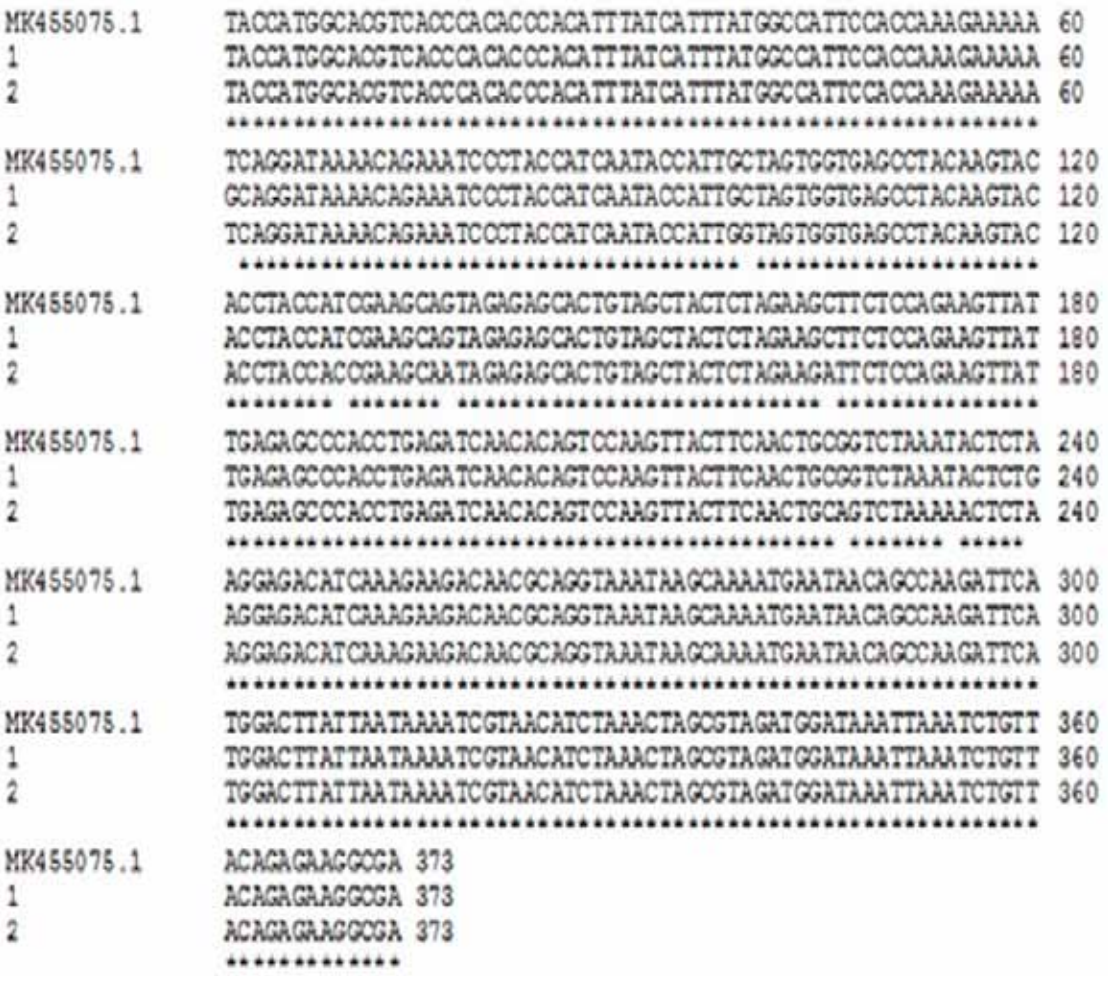

Fig. 2. Representative DNA sequence alignment of K casein gene (373-bp) among Holstein dairy cows and reference sequence available in GenBank gb|EU348567.1|. Asterisks represent similarity.

The least square means in the two groups (G1 *BLG* and G2 *BLG*) of the *β-Lactoglobulin* gene for milk composition and performance traits are presented in Table 2. There was a significant association ($P \leq 0.05$) between the identified SNPs and all studied traits, except for fat %, lactose %, and milk density. Cows harboring C129T SNP (G2 *BLG*) had higher milk yield, protein %, total solids %, order of lactation, days in milk, dry period, and daily milk yield and dry period compared to cows that did not exhibit the identified SNP (G1 *BLG*). However, G2 *BLG* and G2 *BLG* had the same trend for fat %, lactose %, and milk density.

The least square means for the effect of *k-casein* SNPs on milk composition and performance traits are presented in Table 3. The *k-casein* SNPs had a significant effect ($P \leq 0.05$) on the latter traits. The results revealed that cows harboring T129C, and G226A SNPs G3 *k-casein* were superior in terms of the studied traits. In the same way, *DGAT1* SNPs had a significant effect ($P \leq 0.05$) on milk composition and performance traits (Table 4). Cows harboring G140A SNP (G1 *DGAT*) had a higher trend for the studied traits than the cows that did not exhibit the identified SNP (G2 *DGAT*).

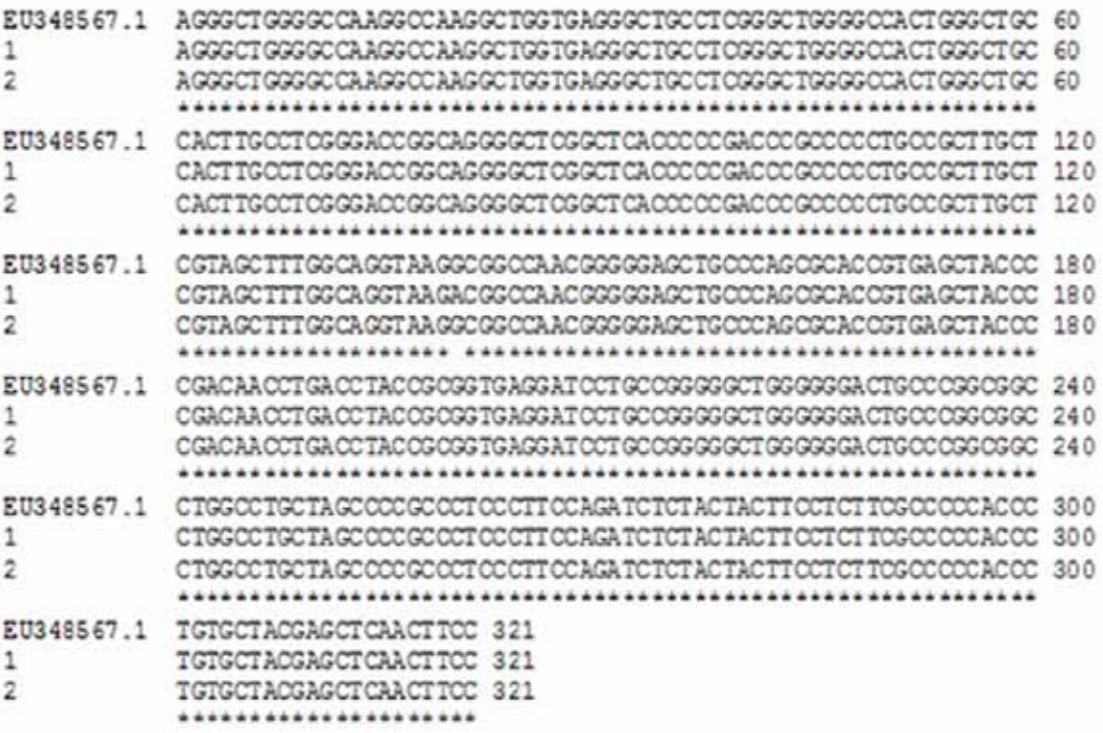

Fig. 3. Representative DNA sequence alignment of DGAT1 gene (339-bp) among Holstein dairy cows and reference sequence available in GenBank gb|EU348567.1|. Asterisks represent similarity.

Table 2. Association of *BLG* SNPs with milk production and performance traits

SNP group (Means ± SE)		
Trait	G1 *BLG*	G2 *BLG*
Milk yield	11231 ± 424[a]	8475 ± 675[b]
Fat %	1.8 ± 0.154[b]	2.9 ± 0.287[b]
Protein %	3.59 ± 0.241[a]	2.56 ± 0.114[b]
Lactose %	4.64 ± 0.038[a]	4.32 ± 0.047[a]
Total solids %	36.8± 0.097[a]	33.4± 0.19[b]
Milk density	28.4± 0.42[a]	27.8± 0.74[a]
Order of lactation	3.98± 0.23[a]	3.31± 0.11[b]
Days in milk	275.52± 11.46[a]	242.41 ± 9.84[b]
Dry period	64.52 ± 1.31[a]	60.17 ± 1.14[b]
Daily milk yield (kg)	34.47± 0.37[a]	21.56± 0.28[b]

G1 *BLG* is cows harboring C129T SNP (n= 37) and G2 *BLG* is cows did not exhibit the identified SNP (n= 33).
Means of different levels within the same row having different superscript are significantly different (P<0.05).

Table 3. Association of k-casein SNPs with milk production and performance traits

SNP group (Means ± SE)				
Trait	G1 *k-casein*	G2 *k-casein*	G3 *k-casein*	G4 *k-casein*
Milk yield	9654 ± 235^{b}	6524 ± 354^{c}	12124 ± 541^{a}	6195 ± 451^{c}
Fat %	1.8 ± 0.321^{c}	2.1 ± 0.121^{b}	1.6 ± 0.114^{c}	2.7 ± 0.324^{a}
Protein %	2.61 ± 0.214^{b}	2.43 ± 0.107^{b}	3.61 ± 0.338^{a}	2.08 ± 0.154^{c}
Lactose %	4.15 ± 0.056^{b}	4.09 ± 0.086^{b}	4.84 ± 0.054^{a}	3.97 ± 0.099^{b}
Total solids %	32.6± 0.116^{b}	31.8± 0.321^{b}	37.4± 0.214^{a}	31.6± 0.320^{b}
Milk density	27.6± 0.65^{a}	21.6± 0.22^{b}	29.4± 0.11^{a}	20.32± 0.83^{b}
Order of lactation	3.41± 0.11^{b}	3.15± 0.05^{c}	3.99± 0.49^{a}	3.16± 0.34^{c}
Days in milk	251.41 ± 11.94^{b}	213.88 ± 9.64^{c}	284.65± 10.52^{a}	206.54 ± 12.62^{c}
Dry period	61.17 ± 2.14^{ab}	60.17 ± 1.04^{c}	63.44 ± 1.31^{a}	60.13 ± 1.08^{c}
Daily milk yield (kg)	29.42± 0.35^{b}	22.85± 0.48^{c}	36.57± 0.51^{a}	18.62± 0.67^{d}

G1 *k-casein* is cows harboring G61T, G137A, C165A, and A240G SNPs SNP (n= 19) and G2 *k-casein* is cows harboring G99C and T234A SNPs (n= 21), G3 *k-casein* is cows harboring T129C, and G226A SNPs (n= 15), and G4 *k-casein* is cows did not exhibit the identified SNPs (n= 15).
Means of different levels within the same row having different superscript are significantly different (P<0.05).

Table 4. Association of *DGAT1* SNPs with milk production and performance traits

SNP group (Means ± SE)		
Trait	G1 *DGAT*	G2 *DGAT*
Milk yield	10952 ± 561^{a}	6954 ± 425^{b}
Fat %	2.2 ± 0.353^{b}	2.8 ± 0.117^{b}
Protein %	2.85 ± 0.116^{a}	2.04 ± 0.312^{b}
Lactose %	3.95 ± 0.112^{a}	3.76 ± 0.214^{a}
Total solids %	32.8± 0.128^{a}	31.6± 0.22^{a}
Milk density	26.4± 0.53^{a}	22.3± 0.21^{b}
Order of lactation	3.45± 0.21^{a}	3.21± 0.28^{a}
Days in milk	257.14± 12.18^{a}	221.22 ± 7.65^{b}
Dry period	62.33 ± 1.65^{a}	61.53 ± 0.85^{a}
Daily milk yield (kg)	30.61± 0.24^{a}	19.95± 0.88^{b}

G1 *DGAT* is cows harboring G140A SNP (n= 28) and G2 *DGAT* is cows did not exhibit the identified SNP (n= 42).
Means of different levels within the same row having different superscript are significantly different (P<0.05).

The logistic regression model assessed the degree to which the studied traits were affected by *BLG*, *k-casein*, and *DGAT1* SNPs. The results showed the fit of the overall model to the data using -2Log Likelihood with a p- value 0.000**; where a highly significant effect of *BLG*, *k-casein*, and *DGAT1* genes was reported on the milk composition and performance traits. Also, values of Cox, Snell and Nagelkerke (R^2) indicated a moderately strong relationship between the predictor (SNP) and the grouping variables (Milk composition and performance traits), as presented in Tables 5, 6, and 7.

Table 5. Logistic regression model for studying effect of *BLG* SNPs on milk production and performance traits

Trait	-2 Log Likelihood	Cox & Snell R Square	Nagelkerke Square
Milk yield	483.840	0.642	0.845
Fat %	512.380	0.624	0.792
Protein %	486.420	0.521	0.684
Lactose %	574.542	0.443	0.641
Total solids %	344.582	0.547	0.705
Milk density	498.830	0.448	0.654
Order of lactation	416.273	0.432	0.642
Days in milk	540.210	0.597	0.727
Dry period	479.321	0.472	0.695
Daily milk yield	396.870	0.423	0.621

Table 6. Logistic regression model for studying effect of *k-casein* SNPs on milk production and performance traits

Trait	-2 Log Likelihood	Cox & Snell R Square	Nagelkerke Square
Milk yield	417.882	0.585	0.743
Fat %	574.380	0.597	0.782
Protein %	486.420	0.511	0.692
Lactose %	574.542	0.427	0.638
Total solids %	314.582	0.431	0.641
Milk density	541.631	0.531	0.724
Order of lactation	395.241	0.473	0.681
Days in milk	479.310	0.438	0.652
Dry period	517.248	0.621	0.798
Daily milk yield	473.521	0.521	0.713

Table 7. Logistic regression model for studying effect of *DGAT1* SNPs on milk production and performance traits

Trait	-2 Log Likelihood	Cox & Snell R Square	Nagelkerke Square
Milk yield	576.885	0.424	0.638
Fat %	412.413	0.451	0.675
Protein %	410.534	0.412	0.618
Lactose %	399.521	0.402	0.611
Total solids %	450.521	0.482	0.693
Milk density	574.054	0.531	0.724
Order of lactation	574.054	0.621	0.754
Days in milk	453.410	0.458	0.681
Dry period	513.214	0.564	0.751
Daily milk yield	418.425	0.532	0.721

Discussion

Analysis of the candidate genes controlling quantitative traits is becoming more advantageous than traditional selection methods. This form of selection directly relies on analysis of genotype, and does not consider the variety of useful properties stipulated by the environment (NG-KWAI-HANG et al., 2002; TSIARAS et al., 2005. Another criterion for the candidate gene approach is its role in discovering whether particular genes are associated with economically important traits in farm animals. Therefore, accurate selection of young animals has been possible irrespective of their sex, which increases selection efficiency. Selection of marker genes is based on their plausible role in biochemical and physiological processes and their polymorphisms, stipulated by point mutation. Mutations may be located in coding regions and lead to variations in the amino acid composition of proteins, and in regulatory elements, thus influencing gene transcription (MATEJICEK et al., 2008; ZAGLOOL et al., 2016)

Milk components, being quantitative traits, are influenced by environmental and genetic factors. Molecular technologies have been developed to detect alleles and frequencies within protein milk genes, including specific PCR sequences, restriction enzymes, and also single nucleotide polymorphism (MEDRANO and AGUILAR-CORDOVA, 1990; MITRA et al., 1998; REN et al., 2011). The development of distinguishing SNPs for each breed is necessary for genotyping and association mapping to milk traits. Polymorphism of genes associated with the parameters of milk yield makes it possible to carry out selection of cattle with consideration for valuable genotypes related to useful properties. Their polymorphisms mostly clarify the genetic variance and enhance the estimation of breeding value. Bovine milk proteins are divided into two main groups: caseins (alphas1-casein, alphas2-casein, beta-casein and kappa-casein) and whey proteins, composed of several different proteins, of which *beta-lactoglobulin* is one (EIGEL et al., 1984). The genetic variants of milk proteins differ from each other by one or more amino acid residues in the polypeptide chains, which is due to various types of mutations in the genes encoding them (HRISTOV et al., 2012; KAMIŃSKI et al., 2007).

In this context, PCR-DNA sequencing of the *β-Lactoglobulin* gene (301-bp), *k-casein* gene (373-bp), and *DGAT1* (321-bp) genes revealed single nucleotide polymorphisms which seemed to be characteristic for a number of dairy cows. Nucleotide sequence variations of *β-Lactoglobulin*,

k-casein and *DGAT1* genes between the studied dairy cows and the reference sequences available in GenBank confirmed all the identified SNPs (Figures 1, 2 and 3). Interestingly, our results indicated that the polymorphisms identified are reported here for the first time. The identified SNPs distinguished dairy cows into different groups. The least square means of the discriminated groups in each gene elucidated a significant association ($P \leq 0.05$) between the identified SNPs and milk composition and performance traits. A novelty of this study is the use of a logistic regression model to reveal how the studied traits were highly significantly affected by *BLG*, *k-casein*, and *DGAT1* SNPs, where a moderate to strong relationship was detected between the predictor (SNPs) and grouping variables (milk production and performance traits). Our results confirmed the alternative Hypothesis H_A regarding genetic variations between the enrolled animals. Consequently, *β-Lactoglobulin*, *k-casein* and *DGAT1* genes could be used as proxy biomarkers for milk composition and performance traits in Holstein cattle

Several studies have reported the possible association of *β-Lactoglobulin*, *k-casein*, and *DGAT1* genes with milk composition and performance traits in Holstein dairy cows. However, some studies have shown contradictory results and confirmed no significant association. For instance, BANKAR et al., (2018) reported that the genotype had no significant effect ($P>0.05$) on milk components (Fat %, protein%, Lactose% and SNF %) and Total Milk Yield (kg). However, ALIM et al., (2015) indicated that β-lactoglobulin and κ–casein genes possibly contributed to association analysis, and can be recognized as genetic markers in programs of gene-assisted selection for the genetic improvement of milk production traits in dairy cattle. The opposing results may be attributed to the genetic background differences between the studied animals. They may also be attributed to the investigation of genetic polymorphisms on different amplified fragments of β-Lactoglobulin, k-casein, and DGAT1 genes.

Previous studies reported gene polymorphisms for *Lactoglobulin*, *k-casein*, and *DGAT1* genes using RFLP and SSCP genetic markers, however the current study investigated gene polymorphisms by SNP markers. SNP genetic markers may revolutionize previous achievements in conservation decisions, biodiversity assessment and genetic characterization of breeds, providing more understanding of the molecular basis of functional diversity (GROENEVELD et al., 2010). SNP analysis could explain the history of European cattle more accurately than other markers (GAUTIER et al., 2010; SVENSSON et al., 2007; MCKAY et al., 2008; SOCOL et al., 2015). Particular importance is also attributed to SNPs in the search for links between a marker with a specific location in the genome and an unknown gene locus. The search for such associations is important because they allow a phenotypic effect to be assessed by identifying its genetic basis (SVENSSON et al., 2007; MCKAY et al., 2008). An association between *β-Lactoglobulin*, *K-casein* and *DGAT1* genes with milk yield and milk composition traits has been reported via a PCR-RFLP approach (KAMINSKI and ZABOLEWICZ, 2000; RACHAGANI et al., 2006; KARIMI et al., 2009; OLEŃSKI et al., 2012; ZAGLOOL et al., 2016; RANGEL et al., 2017; BANKAR et al., 2018; RISTANIC et al., 2020).

In the same respect, it has been established that the *DGAT1* gene is considered a candidate for milk production traits and composition. It has also been reported that variations in this gene could affect levels of milk yield, protein and fat, as well as milk energy content (GRISART et al., 2002; SANDERS et al., 2006; HARDECKA et al., 2008; SIGNORELLI et al., 2009; RYCHTÁŘOVÁ et al., 2014). Along the same lines, previous studies pointed out *DGAT1* gene polymorphisms and their association with milk production traits and composition by means of RFLP genetic markers.

Conclusion

PCR-DNA sequencing of *BLG*, *k-casein*, and *DGAT1* genes revealed nucleotide sequence variations in the form of SNPs amongst Holstein dairy cows. These findings suggest that variability in productive genes could be used as biomarkers for quick and rigorous selection within dairy cattle. The variability at these markers also makes it

possible to assess the predisposition of animals to a specific type of production. Further studies should be carried out to characterize polymorphisms located in different regions of *BLG*, *k-casein*, and *DGAT1* genes. Other breeds of cattle should also be considered, and functional analysis, such as RNA interference (RNAi) and overexpression studies, may also be needed for these markers to be considered as a guide reference.

Acknowledgements
We gratefully acknowledge all members of the Husbandry and Development of Animal Wealth Department, Faculty of Veterinary Medicine, Mansoura University, for their help and support.

Authors' contributions
Ahmed Ateya conceived and designed the experiment and collected blood samples. Ahmed Ateya, Sherif Nasr, Basma Hendam and Mona Al-Sharif performed PCR-DNA sequencing. Hanaa Ghanem and Kadry Sadek analysed data. All authors contributed to writing the manuscript.

Funding
The author(s) received no financial support for the research, authorship, and/or publication of this article.

Data availability
Data that support the findings of this study are available from the corresponding author upon reasonable request.

Compliance with ethical standards and Conflicts of interest
The authors declare no conflicts of interest.

Ethical approval
The authors confirm that the ethical policies of the journal were followed, as noted on the journal's author guidelines page. The protocol of the study was approved by the Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University, Egypt (code number R/23).

References

ALIM, M. A., D. SUN, Y. ZHANG, Y. ZHANG, Q. ZHANG, L. LIU (2015): DNA Polymorphisms in the β-lactoglobulin and κ–casein Genes Associated with Milk Production Traits in Dairy Cattle. Biores Comm. 1, 82-86.

ALIPANAH, M., L. KALASHNIKOVA, G. RODIONOV (2005): Kappa-casein genotypic frequencies in Russian breeds Black and Red Pied cattle. Iranian J. Biotech. 3, 191-194, http://ijb.nrcgeb.ac.ir/index.php/ijb/article/view/108/91

BANKAR, P. S., S. V. KURALKAR, R. N. DHORE, S. J. MANWAR, M. V. INGAWALE, S. S. ALI (2018): Impact of kappa casein gene polymorphism on milk components of gaolao cattle. Int. J. Sci. Environ. Technol. 7, 1520-1527.

BARBOSA, S. B. P., I. I. M. DE ARAÚJO, M. F. MARTINS, E. C. DA SILVA1, L. A. JACOPINI1 , A. M. V. BATISTA1, M. V. B. DA SILVA (2019): Genetic association of variations in the kappa-casein and β-lactoglobulin genes with milk traits in girolando cattle. Rev. Bras. Saúde Prod. Anim., Salvador. 20, 1-12.
DOI: 10.1590/s1519-9940200312019

BUITKAMP, J., K. U. GÖTZ (2004): Use of milk samples from an evaluation program for the genotyping of cows. Archiv. Fur. Tierzucht. 47, 15-26.
DOI: 10.5194/aab-47-15-2004

CAROLI, A. M., S. CHESSA, G. J. ERHARDT (2009): Invited review: Milk protein polymorphisms in cattle: Effect on animal breeding and human nutrition. J. Dairy Sci. 92, 5335-5352.

CASES, S., S. J. SMITH, Y. W. ZHENG, M. H. MYERS, R. S. LEAR, E. SANDE, S. NOVAK, C. COLLINS, B. C. WELCH, J. A. LUSIS, K. S. ERICKSON, V. R. FARESE (1998): Identification of a gene encoding an acyl CoA: diacylglycerol acyltransferase, a key enzyme in triglycerol synthesis. Proc. Natl. Acad. Sci. 95, 13018–13023.
DOI: 10.1073/pnas.95.22.13018

CREAMER, L., D. PARRY, G. MALCOLM (1983): Secondary structure of β-lactoglobulin. J. Dairy Sci. 227, 98-105.

DINC, V., O. EMEL, K. EVREN, T. INCI (2013). Beta-casein A1/A2, kappa-casein and beta-lactoglobulin polymorphisms in Turkish cattle breeds. Archiv Tierzucht 65, 650-657.
DOI: 10.7482/0003-9438-56-065

EIGEL, W. N., J. E. BUTLER, C. A. ERNSTROM, JR. H. M. FARRELL, V. R. HARWALKAR, R. JENNESS, R. M. L. WHITNEY (1984): Nomenclature of Proteins of Cow's Milk: Fifth Revision1. J. Dairy Sci. 67, 1599-1631.
DOI: 10.3168/jds.S0022-0302(84)81485-X

FIAT, A. M., P. JOLLÈS (1989): Caseins of various origins and biologically active casein peptides and oligosaccharides: structural and physiological aspects. Mol. Cell. Biochem. 87, 5-30.
DOI: 10.1007/BF00421079.

GAUTIER, M., D. LALOË, K. GOUDARZI (2010): Insights into the Genetic History of French Cattle from Dense SNP Data on 47 Worldwide Breeds. PLoS ONE. 5(9), e13038.
DOI: 10.1371/journal.pone.0013038

GRISART, B., W. COPPIETERS, F. FARNIR, L. KARIM, C. FORD, P. BERZI, N. CAMBISANO, M. MNI, S. REID, P. SIMON, R. SPELMAN, M. GEORGES, R. SNELL (2002): Positional candidate cloning of a QTL in dairy cattle: identification of a missense mutation in the

bovine DGAT1 gene with major effect on milk yield and composition. Genome Res. 12, 222-31.

DOI: 10.1101/gr.224202

GROENEVELD, L. F., A. J. LENSTRA, H. EDING, A. M. TORO, B. SCHERF, D. PILLING, R. NEGRINI, K. E. FINLAY, H. JIANLIN, E. GROENEVELD, S. WEIGEND, G. CONSORTIUM (2010): Genetic diversity in farm animals a review. Anim. Genet. 41, 6-31.

DOI: 10.1111/j.1365-2052.2010.02038

HEJTMÁNKOVÁ, A., V. PIVEC, E. TRNKOVÁ, H. DRAGOUNOVÁ (2012): Differences in the composition of total and whey proteins in goat and ewe milk and their changes throughout the lactation period. Czech J. Anim. Sci. 57, 323-331.

HILL, J. P., W. C. THRESHER, M. J. BOLAND, L. K. CREAMER, S. G. ANEMA, G. MANDERSON, D. E. OTTER, G. R. PATERSON, R. HOWE, R. G. BURR, R. L. MOTION, A. WINDELMAN, B. WICKHAM (1997): The polymorphism of the milk protein β-lactoglobulin. In Milk composition, production and biotechnology. CAB International, Wallingford, UK. 173-213.

HRADECKA, E., J. ČITEK, L. PANICKE, T. V. ŘEHOU, L. HANUSOVA (2008): The relation of *GH1, GHR* and *DGAT1* polymorphisms with estimated breeding values for milk production traits of German Holstein sires. Czech J. Anim. Sci. 53, 238–45.

DOI: 10.17221/362-CJAS

HRISTOV, P., T. DENITSA, M. IVAN, Z. LYUBEN, R. GEORGI (2012): Application of Milk Proteins Genetic Polymorphism for Selection and Breeding of Dairy Cows in Bulgaria.

DOI: 10.5772/50758

KAMIŃSKI, S., A. CIESLIŃSKA, E. KOSTYRA (2007): Polymorphism of bovine beta-casein and its potential effect on human health. Appl. Genet. 48, 189-198. DOI: 10.1007/BF03195213

KAMINSKI, S., T. ZABOLEWICZ (2000): Associations between bovine beta-lactoglobulin polymorphism within coding and regulatory sequences and milk performance traits. J. Appl. Genet. 41, 91-99.

KARIMI, K., M. T. B. NASSIRI, K. MIRZADEH, A. ASHAYERIZADEH, H. ROUSHANFEKR, J. FAYYAZI (2009): Polymorphism of the beta-lactoglobulin gene and its association with milk production traits in Iranian Najdi cattle. Iranian J. Biotech. 7, 82-85.

LIEN, S., S. ROGNE (1993): Bovine casein haplotypes: number, frequencies and applicability as genetic markers. Anim. Genet. 24, 373-6.

DOI: 10.1111/j.1365-2052.1993.tb00343.x.

MACCIOTTA, N. P., M. MELE, G. CONTE, A. SERRA, M. CASSANDRO, R. DAL ZOTTO, A. C. BORLINO, G. PAGNACCO, P. SECCHIARI (2008): Association between a polymorphism at the stearoyl CoA desaturase locus and milk production traits in Italian Holsteins. J. Dairy Sci. 95, 7346-7354.

DOI: 10.3168/jds.2007-0947

MATEJICEK, A., J. MATEJICKOVA, M. STIPKOVA (2008): Joint effects of *CSN3* and *LGB* genes on milk quality and coagulation properties in Czech Fleckvieh. Czech J. Anim. Sci. 53, 246-252.

DOI: 10.17221/363-CJAS

MCKAY, D. S., D. R. SCHNABEL, M. B. MURDOCH, K. L. MATUKUMALLI, J. AERTS, W. COPPIETERS, D. CREWS, D. E. NETO, A. C. GIL, C. GAO, H. MANNEN, Z. WANG, V. P. C. TASSELL, L. J. WILLIAMS, F. J. TAYLOR, S. S. MOORE (2008): An assessment of population structure in eight breeds of cattle using a whole genome SNP panel. BMC Genet. 9, 1-9.

DOI: 10.1186/1471-2156-9-37

MEDRANO, J. F., E. AGUILAR-CORDOVA (1990): Polymerase chain reaction amplification of bovine β-lactoglobulin genomic sequences and identification of genetic variants by RFLP analysis. Anim. Biotech. 1,73-74.

DOI: 10.1080/10495399009525730

MITRA, A., P. SCHLEE, I. KRAUSE, J. BLUSCH, T. WERNER, C. R. BALAKRISHNAN, F. PIRCHNER (1998): Kappa-casein polymorphisms in Indian dairy cattle and Buffalo: A new genetic variant in Buffalo. Anim. Biotech. 9, 81-87.

DOI: 10.1080/10495399809525896

NG-KWAI-HANG, K. F., D. E. OTTER, E. LOWE, M. J. BOLAND, M. J. AULDIST (2002): Influence of genetic variants of beta-lactoglobulin on milk composition and size of casein micelles. *Milchwissenschaft*. 57, 303-306.

OLEŃSKI, K., A. CIEŚLIŃSKA, T. SUCHOCKI, J. SZYDA, S. KAMIŃSKI (2012): Polymorphism in coding and regulatory sequences of beta casein gene is associated with milk production traits in Holstein-Friesian cattle. Anim. Sci. Pap. Rep. 30, 5-12.

OTAVIANO, A. R., H. TONHATI, J. A. D. SENA, M. F.C. MUÑOZ (2005): Kappa-casein gene study with molecular markers in female buffaloes *(Bubalus bubalis)*. Genet. Mol. Biol. 28, 237-241.

DOI: 10.1590/S1415-47572005000200010

RACHAGANI, S, D. G. ISHWAR, G. NEELAM, S. C. GUPTA (2006): Genotyping of β-Lactoglobulin gene by PCR-RFLP in Sahiwal and Tharparkar cattle breeds. BMC Genet. 7, 31.

DOI:10.1186/1471-2156-7-31

RANGEL, N. A. H., L. G. ZAROS, T. C. LIMA, F. L. H. BORBA, L. P. NOVAES, M. L. F. MOTA, M. S. SILVA (2017): Polymorphism in the Beta Casein Gene and analysis of milk characteristicsin Gir and Guzerá dairy cattle. Genet. Mol. Res. 16, 1-9.

DOI: 10.4238/gmr16029592

REN, D. X., S. Y. MIAO, Y. L. CHEN, C. X. ZOU, X. W. LIANG, J. X. LIU (2011): Genotyping of the k-casein and β-lactoglobulin genes in Chinese Holstein, Jersey and water Buffalo by PCR-RFLP. Genet. 90, 1-5, https://doi.org/10.1007/s12041-011-0048-z

RISTANIC, M, U. GLAVINIC, B. VEJNOVIC, M. MALETIC, D. KIROVSKI, V. TEODOROVIC, Z. STANIMIROVIC (2020): Beta-casein gene polymorphism in Serbian Holstein-Friesian cows and its relationship with milk production traits. Acta Vet. Beograd. 70, 497-510.

DOI: 10.2478/acve-2020-0037

ROBITAILLE, G., M. BRITTEN, J. MORISSET, D. PETITCLERC (2002): Quantitative analysis of β-Lactoglobulin A and B genetic variants in milk of cows β-Lactoglobulin AB throughout lactation. J. Dairy Res. 69, 651-654.

DOI: 10.1017/s0022029902005733

RYCHTÁŘOVÁ, J., Z. SZTANKÓOVÁ, J. KYSELOVÁ, V. ZINK, M. ŠTÍPKOVÁ, M. VACEK, L. ŠTOLC (2014): Effect of *DGAT1, BTN1A1, OLR1*, and *STAT1* genes on milk production and reproduction traits in the Czech Fleckvieh breed. Czech J. Anim. Sci. 59, 45-53.

DOI: 10.17221/7228-CJAS

SANDERS, K., J. BENNEWITZ, N. REINSCH, G. THALLER, E. M. PRINZENBERG, C. KÜHN, E. KALM (2006): Characterization of the DGAT1 mutations and the CSN1S1 promoter in the German Angeln dairy cattle population. J. Dairy Sci. 89, 3164–3174.

DOI: 10.3168/jds.S0022-0302(06)72590-5

SANGER, F., S. NICKLEN, A.R. COULSON (1977): DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. 74, 5463-5467,

DOI: 10.1073/pnas.74.12.5463

SAFRONOVA, O. S., E. A. BABICH, A.A. OVCHINNIKOV (2017): Polymorphism of Kappa-Casein, Somatotropin, BetaLactoglobulin, Prolactin, and Thyreoglobulin Genes of Black and White Cattle of North Kazakhstan. J. Pharm. Sci. Res. 9, 568-573

SIGNORELLI, F., L. ORRÙ, F. NAPOLITANO, G. DE MATTEIS, M. C. SCATA, G. CATILLO, C. MARCHITELLI, B. MOIOLI (2009): Exploring polymorphisms and effects on milk traits of the DGAT1, SCD1 and GHR genes in four cattle breeds. Livest. Sci. 125, 74-79.

DOI: 10.1016/j.livsci.2009.02.014

SOCOL, C. T., L. IACOB, I. MIHALCA, F. L. CRISTE (2015): Molecular and population genetics tools for farm animal genetic resources conservation: brief overview. Anim. Sci. Biotech. 48 95-102.

SPELMAN, J. R., H. BOVENHUIS (1998): Moving from QTL experimental results to the utilization of QTL in breeding programmes. Anim. Genet. 29, 77-84.

DOI: 10.1046/j.1365-2052.1998.00238.x

SVENSSON, E., C. ANDERUNG, J. BAUBLIENE (2007): Tracing genetic change over time using nuclear SNPs in ancient and modern cattle. Anim. Genet. 38, 378-383.

DOI: 10.1111/j.1365-2052.2007.01620.x

TAMURA, K., J. DUDLEY, M. NEI, S. KUMAR (2007): MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Edu. 24, 1596-1599.

DOI: 10.1093/molbev/msm092

THALLER, G., C. KÜHN, A. WINTER, G. EWALD, O. BELLMANN, J. WEGNER, H. ZÜHLKE, R. FRIES (2003): DGAT1, a new positional and functional candidate gene for intramuscular fat deposition in cattle. Anim. Genet. 34, 354-361.

DOI: 10.1046/j.1365-2052.2003.01011.x

TSIARAS, A. M., G. G. BARGOULI, G. BANOS, C. M. BOSCOS (2005): Effect of kappa-casein and beta-lactoglobulin loci on milk production traits and reproductive performance of Holstein cows. J. Dairy Sci. 88, 327- 334.

DOI: 10.3168/jds.S0022-0302(05)72692-8

WINTER, A., W. KRÄMER, F. A. O. WERNER, S. KOLLERS, S. KATA, G. DURSTEWITZ, J. BUITKAMP, E. L. WOMACK, G. THALLER, R. FRIES (2002): Association of a lysine-232 alanine polymorphism in a bovine gene encoding acyl-CoA:diacylglycerol acyltransferase (DGAT1) with variation at a quantitative trait locus for milk fat content. Proc. Natl. Acad. Sci. 99, 9300-9305.

DOI: 10.1073/pnas.142293799

ZAGLOOL, A., A. AWAD, I. ELBAYOMI (2016): Association of beta Lactoglobulin Gene Polymorphism with Milk Yield, Fat and Protein in Holstein-Friesian Cattle. World's Vet. J. 6, 117-122.

Received: 17 February 2021
Accepted: 7 June 2021

ATEYA, A., S. NASR, H. GHANEM, K. SADEK, M. AL-SHARIF, B. HENDAM: Jednonukleotidni polimorfizmi gena za β-laktoglobulin, k-kazein i DGAT1 kao kandidati za stroge selekcijske kriterije holštajnskih krava s obzirom na sastav i proizvodnost mlijeka. Vet. arhiv 93, 1-16, 2023.

SAŽETAK

Cilj rada bio je, primjenom PCR-DNA metode i analize sljedova, istražiti polimorfizme gena za β-Lactoglobulin, k-kazein i DGAT1 te procijeniti njihovu povezati sa sastavom mlijeka i svojstvima proizvodnosti goveda holštajnske pasmine. Na temelju evidencija s farmi dobiveni su točni fenotipski podaci o sastavu mlijeka i proizvodnosti 70 muznih krava. Za ekstrakciju DNK prikupljeni su uzorci krvi pojedinačnih krava u epruvete koje su sadržavale dinatrijev EDTA kao antikoagulans. PCR je proveden za amplifikaciju fragmenata egzona 4 (301-bp) β-laktoglobulina, egzona 4 (373-bp) k-kazeina i egzona 7 (321-bp) gena DGAT1. Analiza sljedova DNK prikazala je jednonukleotidne polimorfizme (SNPs) u istraženim genima. Uzevši u obzir krave kod kojih su utvrđeni SNP-ove u svakom genu, životinje su raspoređene u različite skupine. Srednje vrijednosti (LSM) skupina pokazale su znakovitu povezanost ($P<0,05$) između SNP-ova i svojstava proizvodnosti mlijeka. Model logističke regresije potvrdio je visoko znakovit učinak identificiranih SNP-ova na istraživana svojstva, pri čemu je ustanovljena umjerena do jaka povezanost između prediktora (SNP-ovi) i varijabli grupiranja (sastav mlijeka i proizvodnost mlijeka). Posljedično, identificirani SNP-ovi u genima β-Lactoglobulina, k-kazeina i DGAT1 mogli bi se koristiti kao kandidatni pri razvoju postupaka selekcije uz pomoć markera (MAS) za sastav mlijeka i svojstva proizvodnosti u mliječnih goveda pasmine holštajn.

Ključne riječi: goveda holštajn; SNP; genetska varijabilnost; sastav mlijeka; proizvodnost mlijeka