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IDENTIFICATION OF NUCLEOTIDE VARIATION OF GROWTH HORMONE GENE IN RABBIT POPULATIONS REARED IN BULGARIA

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Abstract: Five rabbit populations of New Zealand White (NZW), Californian (CAL), crossbred NZW×GW and two generations of the synthetic population – SPF_1 and SPF_2 reared in Bulgaria were included in the present study with the aim of detecting the genetic variability of the growth hormone encoding gene (*GH*) via polymerase chain reaction with the restriction fragment length polymorphism analysis and direct sequencing. The targeted region of the rabbit *GH* gene was amplified and a fragment of a total of 231 bp was obtained in all studied populations. Allele identification was determined after enzymatic digestion, where two fragments of 62 and 169 bp correspond to allele C and an undigested fragment of 231 bp corresponds to allele T. Two additional bands of 107 and 124 bp evidenced A/G genetic polymorphism in the rabbit *GH* gene. Thirty-eight percent of the studied rabbits were carriers of the double mutation (C/T+A/G) in the same locus as the studied *GH* gene. The sequence analysis revealed two nucleotide substitutions – g.111C>T and g.156A>G in the non-coding region between the regulatory TATA box and 5' UTR region, and a novel g.255G>A genetic variant in intron 1 of *GH* gene. The A>G transition was most frequent (40.57%), compared to the other ones, G>A (28.57%) and C>T (10.80%), respectively. The most frequent genotype in the NZW population was homozygous TT (0.93), with a prevalence of the T allele (0.97) over allele C (0.03) for g.111C>T SNP site. The distribution of the allele and genotype frequencies at the sites g.156A>G and g.255G>A in this rabbit group was identical, with the highest value of 0.93 for alleles A and G, respectively. The rabbit populations CAL and NZW×GW showed equal frequencies of the prevalent T allele (0.83) and for homozygous TT genotype (0.67) according to g.111C>T SNP. The highest values were obtained for the allele A (0.83) and for homozygous AA genotype (0.67) at c.33A>G SNP in these rabbit groups. The highest values (0.67, 0.60 and 0.80) for the heterozygous genotypes at g.111C>T, g.156A>G and g.255G>A SNPs, respectively, were detected among the SPF_2 rabbit population, compared to the both homozygous genotypes. The results obtained in the present research indicates a significant degree of genetic variability of the studied polymorphic GH locus in the SPF_2 rabbit group.

Key Words: rabbits, New Zealand White, Californian, *GH* gene, single nucleotide polymorphism (SNP), DNA sequencing, PCR-RFLP.

INTRODUCTION

In Bulgaria, rabbit breeding represents a small sector of animal production with big potential, due to the good conditions for producing large quantities of rabbit meat, absence of European limiting quotas and the expansion of market demand for rabbit meat. At the beginning of the transition period in the country (1989-2000), rabbit counts ranged between 335 304 (in 1991) and 667 078 (in 1996). Nowadays, 99.4% of the rabbit population is reared in private farms, while the rest are raised in governmental farms (primarily research institutes). New Zealand White and Californian breed represent the majority of the rabbit population in the country (Dimitrova *et al.,* 2008).

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The candidate gene approach is a powerful process for determination of specific loci responsible for genetic variation related to productive traits of interest in animal species. Recent data shows that some of the growth-related genes have already been investigated (Zhang *et al.,* 2012; Sahwan *et al.,* 2014; Gencheva *et al.,* 2017; Hristova *et al.,* 2017; Migdal *et al.,* 2019). From a research point of view, one of the obvious candidate genes is the growth hormone gene (*GH*), which has a critical role in animals' growth and tissue development (Amalianingsih and Brahmantiiyo, 2014; Abdel-Kafy *et al.,* 2015; Zaghloul *et al.,* 2019). Moreover, single nucleotide polymorphisms (SNPs) constitute a powerful method for detecting nucleotide sequence mutations in the amplified DNA (El-Sabrout and Aggag, 2017; El-Sabrout *et al.,* 2019). Identification of SNPs in the rabbit has recently been started from re-sequencing of gene regions selected for different purposes (Fontanesi *et al.,* 2012).

One of the most investigated rabbit genes, encoding the GH, has already been cloned from New Zealand White genomic library and its sequence determined by Wallis and Wallis (1995), but polymorphisms of this gene had not been identified till now. The *GH* gene consists of five exons split by non-coding regions (introns and 5' and 3' untranslated). The authors reported similarity between the sequences of the rabbit *GH* gene and the one found in most other mammals, which suggested a slow nucleotide evolutionary rate for rabbit *GH* gene (Wallis and Wallis, 1995).

In order to identify DNA markers useful for association studies with economic traits, Fontanesi *et al.* (2008) used a candidate gene approach and re-sequenced the *GH* gene in four different rabbit breeds (Belgian Hare, Burgundy Fawn, Checkered Giant and Giant Grey). Regardless of the large amount of data, mutations were not detected in the sequenced regions encompassing exons 2, 3 and 4, introns 1 and 3, and parts of introns 2 and 4. Accordingly, it was suggested that there is a lack in information of polymorphisms of *GH* gene, considering that this rabbit gene is located on chromosome 19 (Abdel-Kafy *et al.,* 2016; El-Sabrout and Aggag, 2017). Since the *GH* gene plays an important role in rabbit growth efficiency, we consider the detection of sequence variations within this gene to be critical for rabbit selection programmes and navigation of the breeding process in this species. Therefore, the main objective of the present study was to detect the nucleotide polymorphisms of *GH* gene in five rabbit populations reared in Bulgaria, using the applications of polymerase chain reaction with the restriction fragment length polymorphism (PCR-RFLP) analysis and direct DNA sequencing.

MATERIAL AND METHODS

Animals

The present study was conducted in a frame of 50 rabbits (males and females), divided into five groups: New Zealand White (NZW, n=15), Californian (CAL, n=3), Crossbred (NZW×Giant White [GW], n=6), first *inter se* generation of synthetic population (SPF_1, $[NZW\times GW]^2$, n=11) and second generation of synthetic population (SPF_2, n=15). The second synthetic population was obtained from crossing four rabbit breeds (62.5% NZW, 12.5% CAL, 12.5% Chinchilla (CH) and 12.5% GW; ${}^5/_8NZW{}^1/_8CAL{}^1/_8CH{}^1/_8GW$) as previously described by Hristova *et al.* (2018). All the animals were born and reared in the rabbitry of the Faculty of Agriculture, Trakia University – Stara Zagora, Bulgaria.

Genomic DNA isolation

Blood sample for analysis was collected via puncture of *v. auricularis* and ethylenediamine tetraacetic acid (EDTA) vacuum tubes. Genomic DNA was extracted from whole blood (3 mL) using DNA Purification Kit (Illustra Blood GenomicPrep, GE Healthcare, UK) according to the manufacturer' instructions. The samples were frozen and stored at –20°C until PCR amplification. The quantity (approximately 40-80 ng) and purity of the obtained DNA samples were measured through NanoVue Plus Spectrophotometer (GE Healthcare) at wavelength 260/280 nm and verified by electrophoresis on 1% agarose gel.

Polymerase Chain Reaction (PCR)

The targeted site of the rabbit *GH* gene included part of the 5'-flanking region, 5'-untranslated (UTR) region, exon 1 (CDS) and part of intron 1. The region was amplified via standard PCR, using primers suggested by Fontanesi *et al.* (2012). The PCR conditions were optimised at the annealing temperature in range of 56-62°C and reactions were performed on Doppio (2×48 well) Gradient Thermal Cycler (VWR®, Germany). On chromosome number 19, the amplifications

were carried out using 2×Red Taq DNA Polymerase Master mix (VWR, Belgium), in a final volume of 20 µL, containing 80 ng DNA template, 20 pM of each primer and nuclease-free water (ddH_2O). The reactions were performed under the following cycling conditions: a preliminary denaturation at 94°C/5 min, followed by 30 cycles at 94°C/30 s, primer annealing at 60°C/45 s, extension at 72°C/1 min, final extension at 72°C/10 min, and stored at 4°C/∞.

Restriction Fragment Length Polymorphism (RFLP)

The obtained amplicons were digested using 10 U/µL *Bsh1236I* restriction enzyme (Bioneer, South Korea) in a determined specific site at 5'...CG↓CG...3'. The digestion reactions were carried out in a total volume of 25 µL, containing 10 µL PCR product and incubated at 37°C/15 h. PCR products and restriction fragments were stained with fluorescent nucleic acid dye GelRed® (Biotium, USA), separated on 2.5% agarose gel and visualised using Electrophoresis Gel Imaging Analysis System (Bio-Imaging Systems, Israel). The appropriate endonuclease enzyme for restriction and relevant sites were selected based on Sequence Extractor (Stothard, 2006).

Direct sequencing

To identify SNPs, the PCR products representing different alleles of the targeted region of *GH* gene were obtained from a total of 50 rabbits of the studied populations, then purified and subjected to direct sequencing in both directions (forward and reverse) by biotech company Bioneer, South Korea. Bioneer Sequencing Service is a fast and highly reliable service performed on the ABI 3730XL DNA Analyzer, which provides high-quality sequence analysis data (Phred Score (QV): ≥20, guaranteed read lengths: ≥700 bp) within 24 h from the arrival of sample. We also used Bioneer's oligo synthesis supports for *GH* custom primer.

Statistical analyses

Data analysis was conducted via the MEGA7 software version 7.0 for bigger data sets (Kumar *et al.,* 2016). The nucleotide sequences were edited, assembled and aligned by CLUSTAL-W (Thompson *et al.,* 1994) and compared with those available in the GenBank database accession number Z38127 for the *GH* gene (Wallis and Wallis, 1995). Comparison of the obtained sequences was performed using the BLAST program from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). Identified differences between individual sequences of each breed were classified as single nucleotide polymorphisms (SNPs). The sequence files were converted in FASTA input format through DNA Baser v.4.36.0 (Heracle BioSoft, 2013) to analyse the genetic information. Additionally, maximum likelihood of different substitutions was estimated according to Tamura and Nei (1993), through MEGA v.7 software, based on analysis of 50 nucleotide forward sequences (Kumar *et al.*, 2016). In each rabbit population separately, allele and genotype frequencies, expected heterozygosity (He) and Chi-square (χ^2) for the Hardy-Weinberg equilibrium (HWE) test were calculated by POPGENE 32 program, v.1.31 (Yeh and Yong, 1999).

RESULTS AND DISCUSSION

PCR-RFLP analysis of the polymorphic GH locus

The results of the molecular genetic analysis in the present study revealed the presence of single nucleotide polymorphisms (SNPs) at the *GH* gene in NZW and CAL rabbit populations and their crosses, reared in Bulgaria. The targeted locus of rabbit *GH* gene including 5'-flanking region, 5'-untranslated region (UTR), exon 1 (CDS) and part of intron 1 was amplified and a total of 231 bp (primer regions included), between nucleotides 51 and 262 bp was obtained in the studied populations (Figure 1). The digestion with restriction enzyme *Bsh1236I*, at positon 113, revealed RFLP patterns, as follows: one undigested fragment of 231 bp corresponds to allele T, two fragments of 62 and 169 bp correspond to allele C. The presence of three bands (62, 169 and 231 bp) resulted in heterozygous (CT) genotype and after the restriction, two bands of 107 and 124 bp were identified in the *GH* locus under the study, which evidenced for new genetic polymorphism in the rabbit *GH* gene, named further as A>G (Figure 2). Thirty-eigh percent of the studied rabbits (n=19) were carriers of the double mutation (C>T and A>G) in the same locus of studied *GH* gene. Sequencing analysis was applied for detection and confirmation of this A>G variant in the targeted region of *GH* gene.

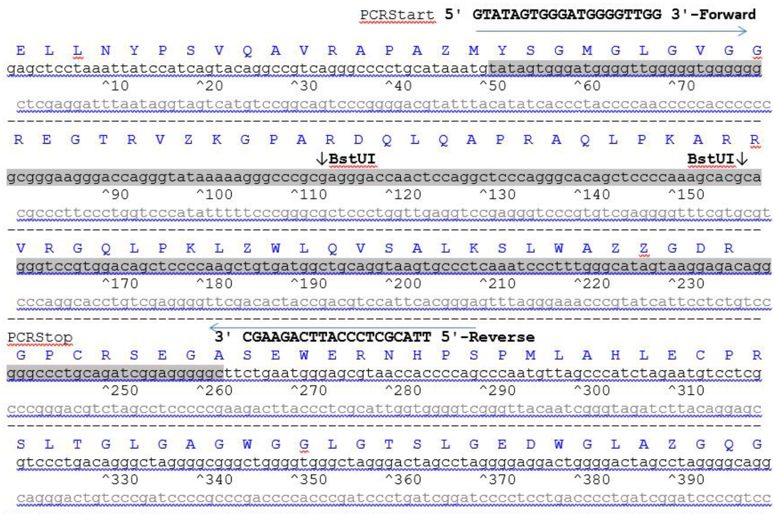

Figure 1: Sequence extracted for PCR product; the range of PCR amplification (from 51 to 262 bp) is shaded and restriction sites are labelled with an arrow.

Bioinformatics sequencing and pairwise sequence of GH gene

In the present study, sequencing of an amplified fragment (231 bp) in a total of 50 rabbits, in both nucleotide directions (forward and reverse) was performed to identify any SNPs of *GH* gene in NZW and CAL and their crosses. In each sample, we analysed a fragment of the *GH* gene that contained a part of the 5'-flanking region, 5'-untranslated region

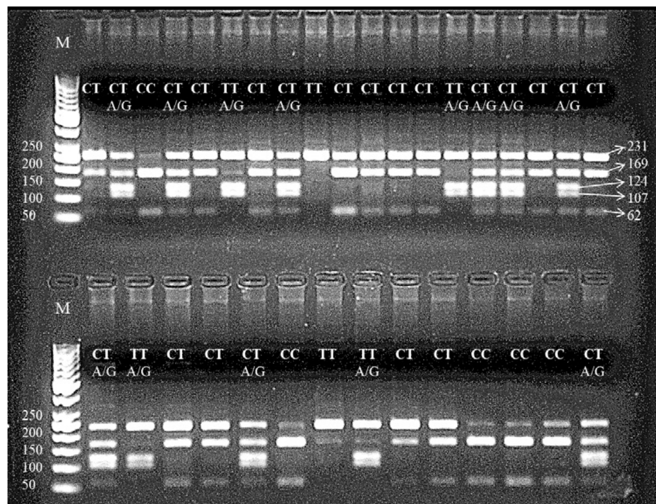

Figure 2: PCR-RFLP analysis of polymorphic *GH* gene; Electrophoregram with restriction fragments of the amplified PCR products of the targeted region of *GH* gene in different rabbit populations, obtained after digestion with restriction enzyme Bsh12361. M – DNA marker (Ladder, 50 bp); different genotypes: homozygous TT and homozygous CC (not presented in this research); heterozygous (CT); 107 and 124 bp – two additional bands correspond to newly discovered single nucleotide polymorphism A/G.

(UTR), exon 1 (CDS) and part of intron 1. Fontanesi *et al*. (2012) re-sequenced a total of 1337 bp of the *GH* gene in 14 rabbbits of different breeds (Belgian Hare, Burgundy Fawn, CAL, Checkered Giant, Giant Grey, Vienna Blue) and commercial lines, and two SNPs (c.78C>T and c.33A>G) have been identified in the 5'-flanking region. In this study, the positions containing gaps and missing data in the sequencing panel were eliminated in advance. An alignment of direct sequencing data of rabbits is shown in Figure 3. Analysis of partial sequencing of target locus showed the presence of three nucleotide substitutions in the studied rabbits, as follows: C/T, A/G and G/A. The pairwise sequence alignment of *GH* gene of the parents compared with F_1 generation is illustrated in Table 1, Figures 3a and 3b, and Supplement 1.

Figure 3: Alignment of direct sequencing data of studied rabbits. Discovery of the single nucleotide polymorphisms (SNPs) C/T, A/G and G/A of the *GH* gene in studied rabbit populations. Figure 3a: Substitutions C→T and A→G; Figure 3b: Substitution G→A. * marks similar sequence in alignment.

Table 1: Pairwise sequence alignment of *GH* gene of the parents compared to NZW×Giant White and F_1 generation.

Pairwise genetic groups	Rabbit samples	No. of SNPs	No. of gaps	Identity ratio (%)
NZW with NZW×GW	2E	0	0	100
	3E	1	2	99
	4E	1	1	99
	5E	2	2	99
	6E	2	1	98
	7E	3	0	98
NZW with $^{5}/_{8}$NZW$^{1}/_{8}$CAL$^{1}/_{8}$CH$^{1}/_{8}$GW	6B	2	0	99
	7B	1	0	99
	8B	3	1	98
	9B	0	0	100
	10B	0	0	100
	1C	2	0	99
	2C	2	0	99
	3C	2	2	98
	4C	3	1	98
	5C	2	2	98
	6C	3	2	98

SNPs: single nucleotide polymorphisms; NZW: New Zealand White; CAL: Californian; CH: Chinchilla; CW: Giant White.

The polymorphisms identified and their *GH* gene sites in the studied rabbit populations, obtained by direct sequencing of PCR products at the polymorphic locus, are presented in Figure 4. The shaded sequence is exon 1 (starting codon), non-shaded is 5'-flanking region. Regulatory class="TATA box" and 5'-untranslated region (UTR) are shown in bold. Single sequence underlined indicates intron 1. The 3 SNPs sites discovered are designed according to the available sequence from NCBI database and labelled in boxes: g.C>T at position 111, g.A>G at position 156 and g.G>A at position 255. However, the exact positions are numbered according to the reference sequence (GenBank accession no. Z38127) of rabbit GH, published by Wallis and Wallis (1995). Two SNPs were identified in the non-coding region between the regulatory TATA box and 5'-untranslated region (UTR) – a substitution C→T at position 111 and named as g.111C>T and another substitution A→G at position 156 and named as g.156A>G. A novel G→A variant was found in intron 1 at position 255 and designated as g.255G>A and, probably, g.255G>A SNP could represent a

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    5'-flanking region
  1 GAGCTCCTAA ATTATCCATC AGTACAGGCC GTCAGGGCCC CTGCATAAAT GTATAGTGGG
                                           TATA box     g.111C>T
 61 ATGGGGTTGG GGGTGGGGGG GCGGGAAGGG ACCAGGGTAT AAAAAGGGCC CGCGAGGGAC
                                       g.156A>G
121 CAACTCCAGG CTCCCAGGGC ACAGCTCCCC AAAGCACGCA GGGTCCGTGG ACAGCTCCCC
          5'UTR  Exon 1(CDS)    Intron 1
181 AAGCTGTGAT GGCTGCAGGT AAGTGCCCTC AAATCCCTTT GGGCATAGTA AGGAGACAGG
                g.255G>A
241 GGGCCCTGCA GATCGGAGGG GGCTTCTGAA TGGGAGCGTA ACCACCCCAG CCCAATGTTA

301 GCCCATCTAG AATGTCCTCG GTCCCTGACA GGGCTAGGGG CGGGCTGGGG TGGGCTAGGG

361 ACTAGCCTAG GGGAGGACTG GGGACTAGCC TAGGGGCAGG GCAAGAGCTG ATCGCCTGCT

421 CCCAGGCCCT GCCCTGACCT CCTGTCTCTC TCCCTCTAG
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Figure 4: Identified polymorphisms obtained by direct sequencing of PCR products of the polymorphic locus of the rabbit growth hormone gene (*GH*).

Table 2: Maximum likelihood estimates of substitution matrix based on analysis of 50 nucleotide forward sequences in studied rabbit populations (the rates of different transitional substitutions are labelled in bold).

	A	T	C	G
A	-	0.00	0.00	40.57
T	0.00	-	20.04	0.00
C	0.00	10.50	-	0.00
G	28.57	0.00	0.00	-

The analysis was computed in MEGA7 (Kumar *et al.,* 2016), using Tamura and Nei (1993).
A, T, C, G: nucleotides.

population-specific polymorphism. The G/A site in the Californian rabbit population was monomorphic. Within the coding region (exon 1) of the rabbit GH locus, none of the 100 tested samples (forward and reverse) contained any SNPs.

Likelihood estimate of GH substitutions

The number of nucleotide substitutions corresponding to *GH* locus in studied rabbit populations is presented in Table 2. The analysis involved 50 nucleotide forward sequences and estimations were performed according to Tamura and Nei (1993). The obtained results in this study revealed that transition A>G was the most frequent (40.57%), compared to G>A (28.57%) and C>T (10.80%). As a whole, the nucleotide frequencies were as follow: A=23.03%, T=15.49%, C=28.76% and G=32.71% in the populations studied.

Allele and genotype frequencies in each rabbit population studied

The values of allele and genotype frequencies of SNPs at the examined polymorphic *GH* locus in each rabbit population are presented in Table 3. In NZW rabbits, the most frequent genotype was homozygous TT (0.93), which implied prevalence of T allele (0.97) over C allele (0.03), with respect to g.111C>T SNP. Based on PCR-RFLP analysis of the same C>T site, the observed preponderance of allele T compared to allele C was also reported by Hussein *et al.* (2015) in a total of 202 rabbits from APRI line (0.54 *vs.* 0.46). However, the results for NZW rabbits disagreed with frequencies of C allele over allele T cited by Fontanesi *et al.* (2012) in 16 rabbits of the Checkered Giant breed (0.59 *vs.* 0.41) and by Amalianingsih *et al.* (2014) in 12 NZW rabbits (0.63 *vs.* 0.37) and in 11 rabbits of Indonesian Rex breed (0.86 *vs.* 0.14), respectively.

Table 3: Frequencies of alleles and genotypes of the SNPs at the polymorphic loci of *GH* gene in the rabbit populations examined.

	C>T				A>G				G>A			
	Allele frequency		Genotype frequency		Allele frequency		Genotype frequency		Allele frequency		Genotype frequency	
Genetic group	C	T	CT	TT	A	G	AA	AG	G	A	GG	GA
NZW	0.03	0.97	0.07	0.93	0.93	0.07	0.87	0.13	0.93	0.07	0.87	0.13
n=15			(1)	(14)			(13)	(2)			(13)	(2)
CAL	0.17	0.83	0.33	0.67	0.83	0.17	0.67	0.33	1.00	0.00	1.00	0.00
n=3			(1)	(2)			(2)	(1)			(3)	(0)
NZW×GW	0.17	0.83	0.33	0.67	0.83	0.17	0.67	0.33	0.92	0.08	0.83	0.17
n=6			(2)	(4)			(4)	(2)			(5)	(1)
SPF_1	0.27	0.73	0.55	0.45	0.77	0.23	0.55	0.45	0.77	0.23	0.55	0.45
n=11			(6)	(5)			(6)	(5)			(6)	(5)
SPF_2	0.33	0.67	0.67	0.33	0.70	0.30	0.40	0.60	0.60	0.40	0.20	0.80
n=15			(10)	(5)			(6)	(9)			(3)	(12)

NZW: New Zealand White rabbits; CAL: Californian rabbits; CW: Giant White; NZW×GW: crossbred rabbits. SPF_1 and SPF_2: first- and second- generation *inter se* rabbits from synthetic population (Hristova *et al.,* 2018); SNPs: single nucleotide polymorphisms. A, T, C, G: nucleotides.

Table 4: Nei's expected heterozygosity (He), Chi-square values (χ^2) and *P*-value of the SNPs at the polymorphic loci of *GH* gene in the examined rabbit populations.

	C>T		A>G		G>A	
Genetic group	He	χ^2 (P)	He	χ^2 (P)	He	χ^2 (P)
NZW, n=15	0.06	0.00 (1.00)	0.12	0.04 (0.85)	0.12	0.04 (0.85)
CAL, n=3	0.28	0.00 (1.00)	0.28	0.00 (1.00)	-	-
NZW×GW, n=6	0.28	0.11 (0.74)	0.28	0.11 (0.74)	0.15	0.00 (1.00)
SPF_1, n=11	0.40	1.25 (0.26)	0.35	0.73 (0.39)	0.35	0.73 (0.39)
SPF_2, n=15	0.44	3.31 (0.07)	0.42	2.4 (0.12)	0.48	6.04 (0.01)

NZW: New Zealand White rabbits; CAL: Californian rabbits; GW: Giant White; NZW×GW: Crossbred rabbits; SPF_1 and SPF_2: first- and second-generation *inter se* rabbits from synthetic population (Hristova *et al.,* 2018); H_e=Expected heterozygosity computed according to Nei (1973); SNPs: single nucleotide polymorphisms; A, T, C, G: nucleotides.

For g.156A>G and g.255G>A, the distribution of the allelic and genotypic frequencies in NZW rabbit population corresponding to the other SNPs was equal, with the highest value (0.93) for allele A (in the former site) and allele G (in the second site), i.e. both frequencies were 0.87 for relevant homozygous genotypes AA and GG. We should clarify that Fontanesi *et al.* (2012) have identified A>G SNP in the GH gene and only one rabbit was in heterozygote state (c.33AG), whereas the other animals were homozygous (c.33AA). Therefore, the authors considered this c.33A>G SNP as a very rare polymorphic site and did not perform further investigation.

A similar distribution of the allele and genotype frequencies was established for both rabbit populations of CAL and crossbred NZW×GW, according to the g.111C>T SNP (Table 3), where the frequencies obtained for allele T and homozygous TT genotype (0.83 and 0.67) were lower than those in NZW rabbits (0.97 and 0.93). On the contrary, Fontanesi *et al.* (2012) reported higher prevalence of C allele over allele T of *GH* gene in CAL rabbits (0.63 *vs.* 0.37) and in GW rabbits (1.00 *vs.* 0.00), where the frequencies for *GH* c.78C>T polymorphism were based on PCR-RFLP analysis. For c.33A>G SNP, the highest frequencies in the studied rabbit population CAL and NZW×GW were 0.83 for the allele A and 0.67 for homozygous AA genotype, compared to 0.17 for the allele G and 0.33 for heterozygous AG genotype (Table 3). According to the polymorphism g.255G>A in CAL rabbits, we established a fixation of allele G, resulted in a frequency of 1.00 for homozygous GG genotype, considering this SNP site as a monomorphic in Californian rabbits.

In regard to the rabbits of the synthetic populations, the obtained frequencies for the heterozygous genotypes at SNPs g.111C>T, g.156A>G and g.255G>A were 0.67, 0.60 and 0.80 in SPF_2, and these frequencies are unexpectedly showing relatively high values, compared to both homozygous genotypes in the population. The frequencies of heterozygous and homozygous genotypes in SPF_1 were 0.55 for CT genotype and 0.45 for TT genotype at g.111C>T SNP site; 0.45 for AG and 0.55 for AA at g.156A>G SNP site; 0.45 for GA and 0.55 for GG at g.255G>A SNP site (Table 3).

Heterozygosity and Hardy-Weinberg Equilibrium in each population

The Nei's expected heterozygosity (H_e), Chi-square (χ^2) and *P*-values for testing probability deviation from the Hardy-Weinberg equilibrium (HWE) of SNPs at the examined polymorphic *GH* loci in each rabbit population are presented in Table 4. In regard to g.111C>T SNP, the value of H_e varied from 0.06 in NZW population to 0.44 in SPF_2. The highest value of *He* (0.42 and 0.48) was established in SPF_2 according to two SNPs, g.156A>G and g.255G>A, respectively. Recently, Hristova *et al.* (2018) reported a relatively high degree of heterozygosity (0.61 and 0.70) for F_1 and F_2 generations of the synthetic rabbit populations. The authors pointed out that this high degree of heterozygosity was probably due to strong heterogeneity of the population, created by four-way crossing of four rabbit breeds. Hence, the maintenance of high heterozygosity allows an expected weaker negative effect of inbreeding depression on the individuals in the population.

The Chi-square test for Hardy-Weinberg equilibrium showed values of χ^2 at the level of probability P>0.05 (Table 4), confirming the validity of HWE in all studied rabbit populations.

CONCLUSIONS

The results obtained convincingly revealed the presence of single nucleotide polymorphisms (SNPs) at the *GH* gene in NZW and CAL rabbit populations and their crosses reared in Bulgaria. Alignment of sequence data obtained with the reference sequence from the GenBank identified three SNPs – two nucleotide substitutions g.111C>T and g.156A>G, and a novel g.255G>A in the non-coding regions of the rabbit *GH*. This indicates a significant degree of genetic variability in the examined *GH* locus and a relatively high level of genetic diversity in the rabbit population studied. The results obtained in this paper could be useful particularly for rabbit breeders in Bulgaria, as research in the rabbit breeding sector is newly developed.

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Supplement 1: Pairwise sequence alignment of *GH* gene of the parents compared to NZW×Giant White. 2E-7E – rabbit samples of genetic group NZW×Giant White.

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2E

Sequence ID: Query_25954 Length: 204 Number of Matches: 1

Range 1: 20 to 203 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
340 bits(184)	5e-98	184/184(100%)	0/184(0%)	Plus/Plus

```
Query  18   TATAAAAGGGCCTGCGAGGGACCAACTCCAGGCTCCCAGGGCACAGCTCCCCAAAGCACG  77
Sbjct  20   ............................................................  79

Query  78   CAGGGTCCGTGGACAGCTCCCCAAGCTGTGATGGCTGCAGGTAAGTGCCCTCAAATCCCT  137
Sbjct  80   ............................................................  139

Query  138  TTGGGCATAGTAAGGAGACAGGGGGCCCTGCAGATCGGAGGGGGCTTCTGAATGGGAGCG  197
Sbjct  140  ............................................................  199

Query  198  TAAA  201
Sbjct  200  ....  203
```

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3E

Sequence ID: Query_25955 Length: 201 Number of Matches: 1

Range 1: 10 to 201 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
344 bits(186)	4e-99	191/193(99%)	2/193(1%)	Plus/Plus

```
Query  10   GTAGAAGGTATA-AAAGGGCCTGCGAGGGACCAACTCCAGGCTCCCAGGGCACAGCTCCC  68
Sbjct  10   ........-...T..............................................  68

Query  69   CAAAGCACGCAGGGTCCGTGGACAGCTCCCCAAGCTGTGATGGCTGCAGGTAAGTGCCCT  128
Sbjct  69   ............................................................  128

Query  129  CAAATCCCTTTGGGCATAGTAAGGAGACAGGGGGCCCTGCAGATCGGAGGGGGCTTCTGA  188
Sbjct  129  ............................................................  188

Query  189  ATGGGAGCGTAAA  201
Sbjct  189  .............  201
```

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4E

Sequence ID: Query_25956 Length: 201 Number of Matches: 1

Range 1: 10 to 200 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
342 bits(185)	1e-98	190/192(99%)	1/192(0%)	Plus/Plus

```
Query  10   GTAGAAGGTATAAAAGGGCCTGCGAGGGACCAACTCCAGGCTCCCAGGGCACAGCTCCCC  69
Sbjct  10   ......C....-................................................  68

Query  70   AAAGCACGCAGGGTCCGTGGACAGCTCCCCAAGCTGTGATGGCTGCAGGTAAGTGCCCTC  129
Sbjct  69   ............................................................  128

Query  130  AAATCCCTTTGGGCATAGTAAGGAGACAGGGGGCCCTGCAGATCGGAGGGGGCTTCTGAA  189
Sbjct  129  ............................................................  188

Query  190  TGGGAGCGTAAA  201
Sbjct  189  ............  200
```

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5E

Sequence ID: Query_25957 Length: 204 Number of Matches: 1

Range 1: 12 to 204 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
344 bits(186)	4e-99	191/193(99%)	2/193(1%)	Plus/Plus

```
Query  11   TAGA-AGG-TATAAAAGGGCCTGCGAGGGACCAACTCCAGGCTCCCAGGGCACAGCTCCC  68
Sbjct  12   ....C...T...................................................  71

Query  69   CAAAGCACGCAGGGTCCGTGGACAGCTCCCCAAGCTGTGATGGCTGCAGGTAAGTGCCCT  128
Sbjct  72   ............................................................  131

Query  129  CAAATCCCTTTGGGCATAGTAAGGAGACAGGGGGCCCTGCAGATCGGAGGGGGCTTCTGA  188
Sbjct  132  ............................................................  191

Query  189  ATGGGAGCGTAAA  201
Sbjct  192  .............  204
```

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6E

Sequence ID: Query_25958 Length: 203 Number of Matches: 1

Range 1: 11 to 203 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
339 bits(183)	2e-97	190/193(98%)	1/193(0%)	Plus/Plus

```
Query  10   GTA-GAAGGTATAAAAGGGCCTGCGAGGGACCAACTCCAGGCTCCCAGGGCACAGCTCCC  68
Sbjct  11   ...G...T....................................................  70

Query  69   CAAAGCACGCAGGGTCCGTGGACAGCTCCCCAAGCTGTGATGGCTGCAGGTAAGTGCCCT  128
Sbjct  71   ............................................................  130

Query  129  CAAATCCCTTTGGGCATAGTAAGGAGACAGGGGGCCCTGCAGATCGGAGGGGGCTTCTGA  188
Sbjct  131  .............................................A..............  190

Query  189  ATGGGAGCGTAAA  201
Sbjct  191  .............  203
```

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7E

Sequence ID: Query_25959 Length: 202 Number of Matches: 1

Range 1: 11 to 202 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
339 bits(183)	2e-97	189/192(98%)	0/192(0%)	Plus/Plus

```
Query  10   GTAGAAGGTATAAAAGGGCCTGCGAGGGACCAACTCCAGGCTCCCAGGGCACAGCTCCCC  69
Sbjct  11   ...T..C.............C.......................................  70

Query  70   AAAGCACGCAGGGTCCGTGGACAGCTCCCCAAGCTGTGATGGCTGCAGGTAAGTGCCCTC  129
Sbjct  71   ............................................................  130

Query  130  AAATCCCTTTGGGCATAGTAAGGAGACAGGGGGCCCTGCAGATCGGAGGGGGCTTCTGAA  189
Sbjct  131  ............................................................  190

Query  190  TGGGAGCGTAAA  201
Sbjct  191  ............  202
```