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SINGLE NUCLEOTIDE POLYMORPHISM OF THE GROWTH HORMONE (GH) ENCODING GENE IN INBRED AND OUTBRED DOMESTIC RABBITS

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Abstract: Taking into consideration that the growth hormone (GH) gene in rabbits is a candidate for meat production, understanding the genetic diversity and variation in this locus is of particular relevance. The present study comprised 86 rabbits (*Oryctolagus cuniculus*) divided into 3 groups: New Zealand White (NZW) outbred rabbits; first-generation inbred rabbits (F_1) and second-generation inbred rabbits (F_2). They were analysed by polymerase chain reaction-based restriction fragment length polymorphism method. A 231 bp fragment of the polymorphic site of the GH gene was digested with Bsh1236 restriction enzyme. Single nucleotide polymorphisms for the studied GH locus corresponding to 3 genotypes were detected in the studied rabbit populations: CC, CT and TT. In the synthetic inbred F_1 and F_2 populations, the frequency of the heterozygous genotype CT was 0.696 and 0.609, respectively, while for the homozygous CC genotype the frequency was lower (0.043 and 0.000), and respective values for the homozygous TT genotype were 0.261 and 0.391. This presumed a preponderance of the T allele (0.609 and 0.696) over the C allele (0.391 and 0.304) in these groups. In outbred rabbits, the allele frequencies were 0.613 (allele C) and 0.387 (allele T); consequently, the frequency of the homozygous CC genotype was higher than that of the homozygous TT genotype (0.300 *vs.* 0.075). Observed heterozygosity for the GH gene was higher than expected, and the result was therefore a negative inbreeding coefficient (Fis=–0.317 for outbred NZW rabbits; –0.460 for inbred F_1 and –0.438 for inbred F_2), indicating a sufficient number of heterozygous forms in all studied groups of rabbits. The application of narrow inbreeding by breeding full sibs in the synthetic population did not cause a rapid increase in homozygosity.

Key Words: *Oryctolagus cuniculus*, growth hormone gene, single nucleotide polymorphism, PCR-RFLP, rabbit.

INTRODUCTION

The common or European rabbit (*Oryctolagus cuniculus*) is one of the domesticated animal species utilised in a wide range of economic and scientific applications and research fields. The NCBI database evaluates the rabbit genome to 2737.46 Mb, comprising 29098 genes and more than 19000 described proteins, so this animal species is an ideal candidate for large-scale *Omics* investigations (Miller *et al.,* 2014). The approach for discovery of candidate genes is successfully used in livestock species for identification of DNA markers associated with production traits, including in rabbits. The principle is based on the circumstance that the variability genes encoding protein products included in key physiological mechanisms and metabolic pathways, and directly or indirectly involved in determination of a given production traits, could at least partly explain the genetic variability for the trait itself (Fontanesi *et al.,* 2008). In this connection, the first step is the identification of variability in genes which could be further analysed in association studies with important livestock traits.

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The development and implementation of new molecular genetic and genomic methods of research allowed for detection of homozygous and heterozygous loci and identification of genes related to inbreeding depression, as well as evaluation of the dynamics of genetic diversity of populations on the basis of specific qualitative and quantitative traits (Tanchev, 2016). Several interesting results and analyses have been presented in this line (Kristensen and Sorensen, 2005; Kristensen *et al.,* 2005; Demontis *et al.,* 2009; Ayroles *et al.,* 2009; Paige, 2010; Vermeulen *et al.,* 2013).

The essential functions of the growth hormone (GH) during animal development make it a candidate gene for traits associated with meat production (Fontanesi *et al.,* 2008). The rabbit gene encoding GH has been isolated, cloned from a genomic library and sequenced by Wallis & Wallis (1995). It comprises 4 introns and 5 exons coding for a protein of 216 amino acids with 26 signal and 190-amino acids mature peptides. GH plays a pivotal role in postnatal growth performance and the regulation of multiple biological and metabolic functions related to or involved in muscle mass accretion, lipid metabolism and bone growth, among others. The GH gene alters the metabolism of carbohydrates, proteins and lipids and promotes postnatal growth of mammals exerting direct or indirect effects on numerous tissues (Fontanesi *et al.,* 2012). By means of Southern blotting analysis, the researchers discovered that unlike other animal species, the GH in the rabbit genome was present as a single copy without GH-like genes. GH gene mutations are described in several species, but so far, only few studies have researched its variability in rabbits. Polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) analyses of single nucleotide polymorphisms (SNP) of the rabbit GH gene were recently reported by Fontanesi *et al.* (2012), Amalianingsih *et al.* (2014) and Hussein *et al.* (2015). The GH SNP and its association with body weight and growth performance traits in different domestic rabbit breeds and their strains were successfully determined (Qiao, 2010, Fontanesi *et al.,* 2012).

The effect of inbreeding on the dynamics of genetic population structures has also raised a particular interest. According to most researchers, inbreeding leads to reduced heterozygosity and respectively, lower genetic diversity of populations (Falconer and Mackay, 1996; Keller, 2002; Charlesworth and Willis, 2009; Nietlisbach *et al.,* 2016; Tanchev, 2016). Some studies and analyses however provide evidence that the process of inbreeding-induced reduction of heterozygous genotypes within a population is multifactorial, although the most influential factors are its size, heterogeneity and the rate of achieving a specific inbreeding level (Maiwashe and Blackburn, 2004; Frankham, 2002; Charlesworth and Charlesworth, 1999; Demontis *et al.,* 2009; Liao and Reed, 2009). Hence, the main purpose of the present study was to investigate the genetic C/T variability of the growth hormone (GH) gene in outbred New Zealand White and inbred synthetic populations of rabbits through PCR-RFLP.

MATERIAL AND METHODS

Sample source

A total of 86 rabbits were studied, divided into the following groups:

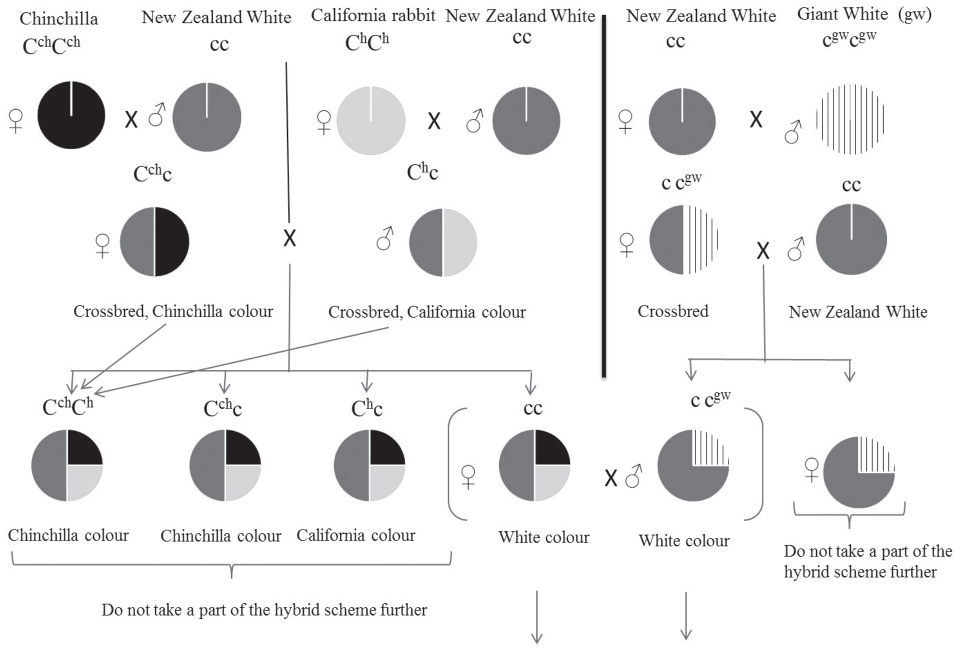
i) Outbred New Zealand White (NZW) rabbits (16 females and 24 males), reared in the Institute of Animal Science – Kostinbrod.

ii) Synthetic rabbit population obtained from crossing 4 rabbit breeds: NZW (62.5%), Chinchilla (12.5%), California (12.5%) and Giant White (12.5%). When hybridisation schedule (Figure 1) was completed, inbreeding was performed by mating full sibs (a brother mated 5 sisters) to obtain 2 subgroups: First-generation inbred rabbits (F_1) with theoretical inbreeding coefficient Fx=0.250 (14 females and 9 males) and second-generation inbred rabbits (F_2) with Fx=0.375 (8 females and 15 males).

The synthetic rabbit population was reared at the Faculty of Agriculture, Trakia University – Stara Zagora.

DNA extraction and PCR amplification

Blood samples (3 mL) were collected from the auricular vein of rabbits in sterile ethylenediaminetetraacetic acid (EDTA) tubes, mixed thoroughly and stored at –20°C until analysis. Genomic DNA was extracted from whole blood using Illustra Blood GenomicPrep DNA Purification Kit (GE Healthcare, UK). The quality of yielded DNA (about



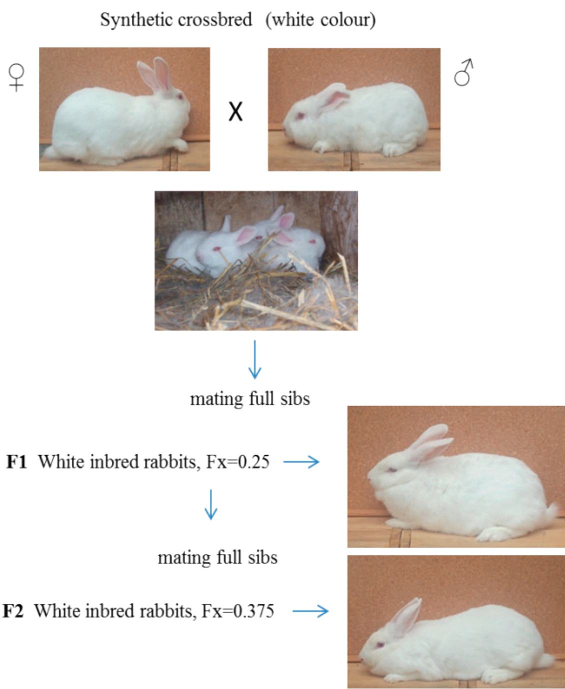


Figure 1: Hybridisation schedule of the synthetic crossbred rabbit population.

Table 1: Allele and genotype frequencies, expected heterozygosity, chi-square test of HWE (χ^2) and coefficient of inbreeding (Fis) for the polymorphic locus in growth hormone gene in the studied rabbit populations.

		Allele frequencies		Genotype frequencies					
Rabbit populations	n	C	T	CC	CT	TT	Nei *	χ^2 **	Fis
Outbred NZW (Fx=0)	40	0.613	0.387	0.300 (12)	0.625 (25)	0.075 (3)	0.475	3.707 (0.050)	–0.317
Inbred F_1 (Fx=0.25)	23	0.391	0.609	0.043 (1)	0.696 (16)	0.261 (6)	0.476	4.437 (0.035)	–0.460
Inbred F_2 (Fx=0.375)	23	0.304	0.696	0.000 (0)	0.609 (14)	0.391 (9)	0.423	4.036 (0.045)	–0.438

NZW: Outbred New Zealand White rabbits; Fis: coefficient of inbreeding; F_1: First-generation inbred rabbits of the Synthetic rabbit population; F_2: Second-generation inbred rabbits of the Synthetic rabbit population.
* Expected heterozygosity calculated as per Nei (1973).
** *P*-value (*P*) and degrees of freedom (df)=1.

30-90 ng) was determined by means of NanoVue Plus Spectrophotometer (GE Healthcare). PCR amplifications were carried out in total volume of 20 µL, containing 80 ng DNA template, 20 pM of each primer and 2× Red Taq DNA Polymerase Master mix (VWR, Belgium). PCR amplifications of the polymorphic site (part of the 5'-flanking region and 5'-untranslated region, exon 1 and part of intron 1) in the GH gene were done with primers designed by Fontanesi *et al.* (2008). PCR reactions were performed in GeneAmp thermocycler (Applied Biosystems, USA) under the following conditions: an initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, primer annealing at 60°C for 45 s, extension at 72°C for 1 min and final extension at 72°C for 10 min.

Genotyping

The genotypes of the studied rabbits with respect to the GH gene were established through RLFP analysis. The digestion reactions were carried out in 25 µL final volume, containing 10 µL PCR product, incubated at 37°C/15h using 10U/µL Bsh1236I enzyme (Bioneer). PCR products were digested with the Bsh1236 I restriction enzyme in a determined specific site at 5'... CG ↓ CG ... 3'. The obtained PCR products and restriction fragments were separated on 2% agarose gel and visualised using Electrophoresis Gel Imaging Analysis System (Bio-Imaging Systems, Israel).

Statistical analysis

Allele and genotype frequencies, expected and observed heterozygosity; the Hardy-Weinberg equilibrium (HWE) test and the coefficient of inbreeding (Fis) were calculated with PopGene32, v. 1.31 software (Yeh and Yong, 1999; Labate, 2000).

RESULTS AND DISCUSSION

A 231 bp fragment of the target polymorphic region of the GH gene in rabbits was successfully amplified in studied rabbit populations. After digestion, 3 different genotypes were identified in the studied rabbit populations —CC, CT and TT regarding to the studied GH locus (Figure 2).

The homozygous CC genotype (2 fragments with size 169 bp and 62 bp) was detected in 12 outbred rabbits, only in one inbred F_1 rabbit and was absent in inbred F_2 rabbits. The heterozygous CT genotype (3 fragments with size 231, 169 and 62 bp) was found out in 16 inbred F_1 rabbits, 14 inbred F_2 rabbits and 25 outbred NZW rabbits.

The frequencies of detected GH gene genotypes and alleles in each of rabbit populations are presented in Table 1.

In the inbred F_1 population, the heterozygous CT genotype frequency was 0.696, whereas the homozygous CC and TT genotypes had frequencies of 0.043 and 0.261, respectively. This implied predominance of the T allele

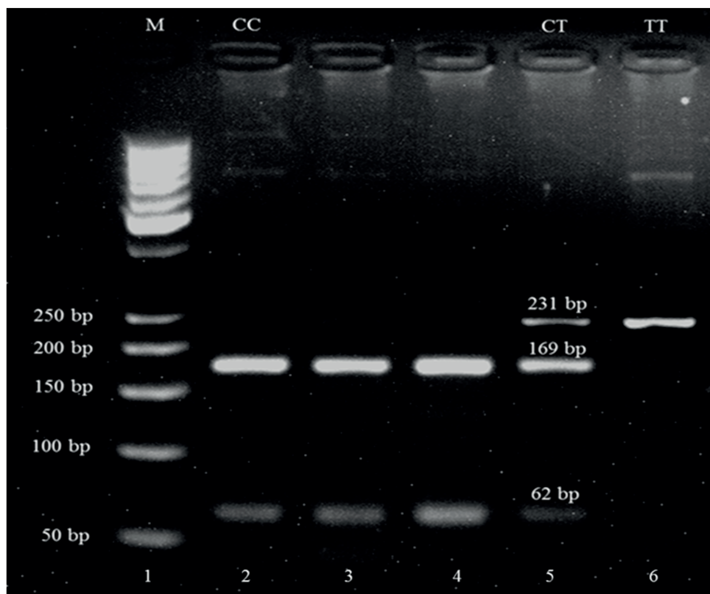


Figure 2: Restriction fragments of amplified polymerase chain reaction products of the polymorphic locus in growth hormone gene with Bsh1236 I in rabbits on 2% agarose gel electrophoresis. Lane 1: M – DNA ladder, 50 bp, lanes 2-6 – samples: CC and TT – homozygous genotype, CT - heterozygous genotype.

over allele C (0.609 *vs.* 0.391) in this group of rabbits. Similar results were established in inbred F_2 rabbits where the heterozygous and the homozygous TT genotypes were presented with respective frequencies of 0.609 and 0.391. On the contrary, the distribution of allele frequencies in outbred NZW rabbits was 0.613 (allele C) and 0.387 (allele T) and the homozygous CC genotype was therefore more frequently encountered than the homozygous TT genotype (0.300 *vs.* 0.075). The observed preponderance of allele C compared to allele T was also reported by Amalianingsih *et al.* (2014) in New Zealand White and California rabbit breeds (0.625 *vs.* 0.375). Our results for the NZW breed disagreed with data reported by Fontanesi *et al.* (2012) for higher prevalence of allele T (frequency 0.594) over allele C (frequency 0.406) in Checkered Giant rabbits. In a recent study by Hussein *et al.* (2015) comprising 202 rabbits from the APRI strain, observed frequencies were 0.540 for allele T and 0.460 for allele C.

The value of the inbreeding coefficients is a measure of heterozygosity deficiency or excess in a given population (Wright, 1978). The values of observed heterozygosity (ranging from 0.609 to 0.696) were higher than theoretically expected ones (from 0.423 to 0.476) and generated negative coefficients of inbreeding (Fis<0), evidencing excess of heterozygotes in studied rabbit populations. It could be then inferred that the outbred NZW rabbit population whose theoretical coefficient of inbreeding is Fx=0 (according to Wright, 1921), maintained an expected relatively high degree of heterozygosity, as seen from the calculated Fis= –0.317 (Table 1). In the other group of rabbits from the synthetic population with 2 inbred progenies, individual coefficients of inbreeding were Fx_1=0.250 and Fx_2=0.375 respectively. Although it was anticipated that the mating of full sibs would enhance the homozygosity, the calculated coefficients of inbreeding (Fis), characterising the relationship of the observed *vs.* expected heterozygosity (Berg and Hamrick, 1997) in both inbred groups were Fis_1= –0.460 and Fis_2= –0.438. This relatively very high degree of heterozygosity in the 2 successive inbred generations was probably due to the strong heterogeneity of the synthetic population created with the participation of 4 rabbit breeds. Nevertheless, the 2nd inbred generation tended to exhibit reduced number of heterozygous and increase in homozygous subjects with the TT genotype as proved by the calculated coefficient of inbreeding Fis= –0.438. The maintenance of high heterozygosity in heterogeneous populations, despite the presence of narrow inbreeding, allowed expecting a weaker negative effect of inbreeding depression on members of such populations (Zainulin *at al.*, 2006; Tanchev, 2006; Tanchev, 2015).

Chi-square values with regard to the analysed polymorphic locus of the GH gene in studied rabbit populations are presented in Table 1. The χ^2 values showed deviation from the Hardy-Weinberg equilibrium in all 3 investigated populations at probability level $P\leq0.05$ and degree of freedom df=1. This tendency could be attributed to the fact that observed heterozygosity values were substantially higher that theoretically expected ones in all studied rabbit groups.

CONCLUSIONS

The results from the present PCR-RFLP study confirmed the presence of SNP in the relevant polymorphic region of the GH gene in studied outbred and inbred rabbit populations. The application of narrow inbreeding by breeding full sibs in the synthetic highly heterogeneous population did not cause a rapid increase in homozygosity. The distribution of allele frequencies in the GH gene suggested that the observed genetic polymorphism could be a useful marker in future research on association analysis for growth performance traits in rabbits. Therefore, additional investigations are planned to estimate the favourable GH genotypes that would allow accurate rabbit selection.

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