C/T POLYMORPHISM AT SECOND INTRON OF THE MYOSTATIN (MSTN) GENE IN INBRED AND OUTBRED RABBITS

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ABSTRACT

The aim of the present investigation was to establish the genetic structure of inbred and outbred rabbit populations corresponding to myostatin (MSTN) gene. A total of 86 rabbits were analyzed for C/T polymorphism at position 34 in the second intron of the myostatin gene through PCR-RFLP method. Studied rabbits were divided into 3 groups: New Zealand White outbred rabbits (40); first-generation (F1) inbred rabbits (23) and second-generation (F2) inbred rabbits (23). A 80 bp fragment of the polymorphic site of the MSTN gene was digested with the AluI restriction enzyme. In the synthetic inbred F_1 and F_2 populations, the frequency of the homozygous CC genotype was 0.870 and 0.740, respectively, while for the heterozygous genotype CT it was lower (0.130 and 0.260). This presumed a preponderance of the C allele (0.935 and 0.870) over another one – T (0.065 and 0.130) in these groups. In outbred NZW rabbits, the allele frequencies were 0.725 (allele C) and 0.275 (allele T) and the frequency of the homozygous CT genotype was higher than that of the homozygous CC genotype (0.450 vs 0.550). The homozygous genotype TT was not determined in all investigated rabbit populations. The observed heterozygosity was higher than the expected one calculated using Nei's method and consequently, a negative inbreeding coefficient has resulted (Fis<0) in all studied groups of rabbits. The obtained results of MSTN polymorphism demonstrate the possibility to apply C/T SNP as an useful DNA marker for future association studies with meat production traits according to the breeding programs in *Oryctolagus cuniculus*.

Key Words: *Oryctolagus cuniculus*, myostatin (MSTN) gene, single nucleotide polymorphism (SNP), PCR-RFLP

INTRODUCTION

Rabbit meat has become one of the necessary sources of food in some countries. Few research have been performed to improve rabbit genetic potential for high body weight using genotypes based on single nucleotide polymorphisms (SNPs) of candidate genes. Some of the growth genes were previously investigated e.g. myostatin (MSTN) which has an important role in growth and development of animals.

MSTN can be considered as candidate gene for meat production traits in rabbits (**Peng et al. 2013; Qiao et al. 2014**). Moreover, SNP is a potent approach for detecting nucleotide sequence mutation in amplified DNA (**El-Sabrout and Aggag, 2017**). MSTN, also known as GDF8, is a member of the transformic growth factor (TGF)-b superfamily that actively depresses skeletal muscle growth (**Lee, 2004**). The rabbit MSTN gene sequence has been assembled after the initiative of the Broad Institute that shot gun sequenced the rabbit genome at 2X level. It comprises three coding exons and two introns as observed in other species (**Fontanesi et al. 2008; Kurkute et al., 2011**). A single nucleotide polymorphism (C-T transition) has been recently detected at position 34 in intron 2 of myostatin gene in different rabbit breeds and broiler lines (**Fontanesi et al. 2008; Rafayovà et al. 2009; Markowska et**

al. 2010; Bindu et al. 2011).

Understanding the single nucleotide polymorphism in the myostatin encoding (MSTN) gene in rabbits is of particular relevance, taking into consideration that this locus is a candidate gene for meat production. Hence, the aim of the present study was to detect genotypes variation of C/T at position 34 in the second intron of the rabbit myostatin gene and to establish the genetic structure of outbred New Zealand White and inbred synthetic populations of rabbits through PCR-RFLP analysis.

MATERIALS and methods

Sample source and DNA extraction

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

1. Outbred New Zealand White (NZW) rabbits (16♀ and 24♂), reared in the Institute of Animal Science – Kostinbrod.

   Synthetic rabbit population obtained from crossing four rabbit breeds: NZW (62.5%), Chinchilla (12.5%), California (12.5%) and Giant White (12.5%). The synthetic rabbit population was reared at the Faculty of Agriculture, Trakia University – Stara Zagora.
2. When hybridization schedule was completed, inbreeding was performed by mating full sibs (a brother mated 5 sisters) to obtain two subgroups:

2.1. First-generation inbred rabbits (F_1) with theoretical inbreeding coefficient Fx = 0.250 (14♀ and 9♂).

2.2. Second-generation inbred rabbits (F_2) with Fx = 0.375 (18♀ and 15♂).

Blood samples (3 ml) were collected from the auricular vein of rabbits in sterile 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 30-90 ng) was determined by means of NanoVue Plus Spectrophotometer (GE Healthcare).

PCR amplification and genotyping

PCR amplifications with respect to the MSTN gene 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 in the GH gene were done with primers designed by **Fontanesi et al.** (**2008, 2011**). PCR reactions were performed in GeneAmp thermocycler (Applied Biosystems, USA) under the following conditions: an initial denaturation at 94ºC for 5 min, followed by 30 cycles at 94ºC for 30 sec., primer annealing at 59ºC for 45 sec, extension at 72ºC for 1 min and final extension at 72ºC for 10 min. The genotypes of the analyzed rabbits were established through RLFP analysis. The digestion reactions were carried out in 25 µl final volume, containing 10 µl PCR product, incubated at 37ºC/10 min using 10 U/µl AluI enzyme (Bioneer). The obtained PCR products and restriction fragments were separated on 2% agarose gel and visualized using Electrophoresis Gel Imaging Analysis System (Bio-Imaging Systems, Israel).

Statistical analysis

PopGene32, v. 1.31 software (**Yeh and Yong, 1999; Labate, 2000**) was applied to

calculate allele and genotype frequencies, expected and observed heterozygosity; the Hardy-Weinberg equilibrium (HWE) test and the fixation index or coefficient of inbreeding (Fis).

RESULTS AND DISCUSSION

As expected, a 231 bp fragment of the target polymorphic region of the MSNT gene in rabbits – intron 2 was successfully amplified in studied rabbit populations. PCR products were digested with the Alu I restriction enzyme in a determined specific site at 5'...AG↓CT...3′. As a result, in all studied rabbit populations, two different genotypes were identified in this locus - CC and CT (figure 1). Homozygous TT genotype was absent in all assessed rabbit groups.

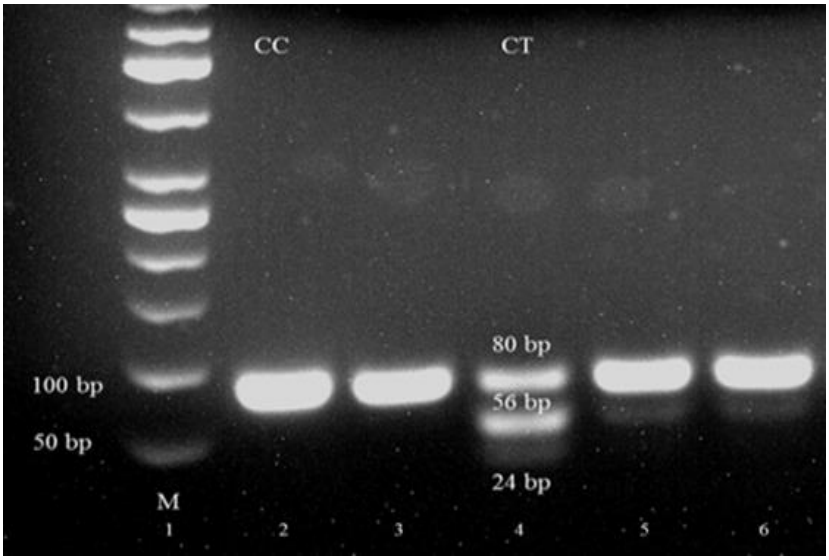

Figure 1. Restriction fragments of amplified PCR products of the MSTN gene with Alu I in rabbits on 2% agarose gel electrophoresis.
Lane 1: M – DNA ladder, 50 bp; lanes 2, 3, 5 and 6 – CC; lanes 4 – CT heterozygous genotype; homozygous genotype TT – absent

The homozygous CC genotype, presented as an undigested fragment with length 80 bp was detected in 18 outbred NZW rabbits, in 20 inbred F_1 rabbits and in 17 inbred F_2 rabbits, respectively. The heterozygous CT genotype (three fragments with size 80, 56 and 24 bp) was found out in 22 outbred NZW rabbits, in 6 inbred F_2 rabbits and only 3 inbred F_1 rabbits. The homozygous genotype TT (two fragments with length 56 bp and 24 bp) was not determined in all investigated rabbit populations.

The allele frequencies and detected genotypes in assessed rabbit populations, corresponding to the MSTN gene are summarized and presented in table 1.

Table 1. Distribution of the allele and genotype frequencies, expected heterozygosity, chi-square test of HWE (χ^2) and coefficient of inbreeding (Fis) for the MSTN gene in the studied rabbit populations.

Rabbit populations	n	Allele frequency		Genotype frequency			Nei*	χ^2 (**)	**Fis****
		C	**T**	**CC**	**CT**	**TT**			
Outbred (Fx=0)	40	0.725	0.275	0.450 (18)	0.550 (22)	0.000 (0)	0.399	5.450 (0.019)	-0.379
Inbred F_1 (Fx=0.250)	23	0.935	0.065	0.870 (20)	0.130 (3)	0.000 (0)	0.122	0.073 (0.7870)	-0.070
Inbred F_2 (Fx=0.375)	23	0.870	0.130	0.740 (17)	0.260 (6)	0.000 (0)	0.227	0.423 (0.515)	-0.150

*Expected heterozygosity computed using Nei's method (1973)
**P-value (P) and degree of freedom (df) = 1
***Wrights coefficient of inbreeding (1978)

Following SNP detection at position 34 of the second intron of the myostatin gene, two different genotypes – homozygous CC and heterozygous CT were established in assessed rabbit populations. In the inbred F_1 rabbit group, the homozygous CC genotype frequency was 0.870, whereas the heterozygous CT genotype had low frequency (0.130), suggesting a predominance of the C allele over allele T (0.935 vs 0.065) in this group of rabbits. Similar results were established in inbred F_2 rabbits, where the homozygous CC and the heterozygous CT genotypes were presented with respective frequencies of 0.740 and 0.260. The distribution of the allele frequency in this rabbit groups showed higher prevalence of the allele C over allele T (0,870 vs 0.130). The observed preponderance of allele C (0.510) compared to allele T (0.490) was also reported by **Fontanesi et al. (2008)** in eight rabbit breeds (Checkered Giant, Giant Grey, Dwarf, Burgundy Fawn, Giant White, Lop, Belgian Hare and New Zealand White). These frequencies are similar to those reported in the study of **Bindu et al. (2011)** in a pooled population of New Zealand White and Soviet Chinchilla and their crosses, where the frequencies across the populations were 0.570 and 0.430, respectively. On the contrary, relatively higher prevalence of allel T over allele C were reported by **Rafayovà et al. (2009)**, with frequency 0,669 and 0.331 in rabbit lines M91 and P91 and by **Abdel-Kafy et al. (2016)**, with frequency 0.730 and 0.270 in a total of 286 rabbits of the APRI line. Similar results were also established by **Sternstein et al. (2014)** in an comparatively study of myostatin C/T SNP associated with carcass composition traits where the allele frequencies within two rabbit breeds (Giant Grey and New Zealand White) were the same, with a value of 0.330 for the minor allele C.

In the present research, although the distribution of allele frequencies in outbred NZW

rabbits was 0.725 (allele C) and 0.275 (allele T), the heterozygous CT genotype was more frequently encountered than the homozygous CC genotype (0.550 vs 0.450). Similar results were established by **Markowska et al. (2010)** in three rabbit breeds (White Flemish Giant, Badana and Hermelin) where the heterozygous CT and homozygotic CC genotypes were presented with frequencies of 0.783, 0.692 and 0.592 for CT and 0.167, 0.272 and 0.389 for CC, respectively. Very low frequency of the homozygous genotype TT (0.050, 0.035 and 0.018) was also reported by authors in the examined rabbit populations. **Rafayovà et al. (2009)** noted slight predominance of genotype CT (0.457) over genotype TT (0.441) and low frequency of genotype CC (0.102) in 127 rabbits from M91 and P91 broiler lines.

The values of observed heterozygosity (ranging from 0.550 to 0.260) were higher than theoretically expected ones (from 0.399 to 0.227) and generated negative coefficients of inbreeding (Fis<0) in studied rabbit populations (table 1). It could be then inferred that the outbred NZW rabbit population whose theoretical individual coefficient of inbreeding is Fx=0, maintained an expected relatively high degree of heterozygosity as seen from the calculated Fis= –0.379. Sufficient number of heterozygous form of genotypes also were obtained by **Rafayovà et al. (2009)** in 127 broiler rabbits of M91 and P91 lines, with coefficient of inbreeding Fis=0.051. In the other group of rabbits from the synthetic population with two inbred progenies, individual coefficients of inbreeding were Fx_1=0.250 and Fx_2=0.375 respectively. The calculated coefficients of inbreeding in both inbred groups (Fis_1= –0.070 and Fis_2= –0.150) evidenced that the mating of full sibs would enhance the homozygosity, as it was expected. The obtained results in terms of the fixation index in the present investigation agreed with the explanations by **Berg and Hamrick (1997)** and **Nagylaki (1998)** who considered the fixation index as proportional deviation of observed heterozygosity from HWE expected heterozygosity. When there are fewer heterozygotes than expected, as with inbreeding, F is positive. In addition, it is possible that some pairs of alleles may exhibit a deficit of heterozygotes (F > 0), while others exhibit an excess of heterozygotes (F < 0).

Chi-square values with regard to the MSTN gene in studied rabbit populations are presented in table 1. The χ^2 values showed deviation from the Hardy-Weinberg equilibrium in investigated outbred NZW rabbit population, at probability level P=0.019 and degree of freedom df = 1. This tendency could be attributed to the fact that observed heterozygosity value (0.550) was substantially higher that theoretically expected one (0.399) in this studied rabbit group. Synthetic rabbit population with two inbred progenies was in with HWE (P=0.787 for F_1 and P=0.515 for F_2).

CONCLUSIONS

In conclusion, the obtained experimental results based on PCR-RFLP analysis confirmed the presence of C/T SNP in the second intron of the MSTN gene in studied rabbit populations. The allele distribution in the analyzed populations confirmed the relevance of the MSTN gene as a marker candidate for association analysis of meat production traits in rabbits. Additional investigations are planned to estimate the favourable MSTN genotypes that would allow accurate meat selection in rabbits.

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