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MOLECULAR POLYMORPHISM OF *MYOSTATIN* GENE FOR GROWTH TRAITS IN CROSSBREEDING EXPERIMENT INVOLVING APRI AND MOSHTOHOR RABBIT LINES

Abdelwahab H. Khattab[1,2], Maher H. Khalil[1,*], Mahmoud M. Iraqi[1], Emad M. Amin[2], and Ayman G. EL Nagar[1]

[1]Dep. of Anim. Prod., Fac. of Agric. at Moshtohor, Benha Uni., 13736, Egypt.

[2]Dep. of Anim. and Poult. Breeding, Anim. and Poult. Prod. Divi., Desert Res. Center, Minis. of Agric., El-Matareya 11753, Cairo, Egypt.

[*]**Corresponding author:** Maher H. Khalil[1] E-mail**: maher.khalil@fagr.bu.edu.eg**

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ABSTRACT: Five years crossbreeding experiment was preformed involving two synthetic rabbit lines of APRI (A) and Moshtohor (M) where bucks of APRI line and does of Moshtohor line were used to produce F_1 crossbreds (½A½M), followed by *inter-se* mating to obtain F_2 crossbreds $(½A½M)^2$. PCR-RFLP technique was used to detect the SNP genotyping of *MSTN* gene in APRI and Moshtohor lines and their progenies of F_1 and F_2 crossbreds and to detect the molecular associations between SNP genotypes of *MSTN* candidate gene and growth traits using *FspBI* restriction enzyme. Two genotypes of *MSTN* gene were detected (TT and TA) in each separate genetic group. The variance components estimated by Gibbs Sampling were used to solve the mixed model equations using the PEST software, getting the solutions for genetic groups effects along with the solutions for other fixed effects. Then, the generalized least-square means (GLM) for the two genotypes of the *MSTN* gene were estimated using the PEST software. The GLM obtained for body weights at 4, 6, 8, 10 and 12 weeks of age in both crosses of ½A½M and $(½A½M)^2$ were higher than those of their parental lines of APRI and Moshtohor. But, APRI-line rabbits were nearly similar in body weights to Moshtohor line rabbits at 8, 10 and 12 weeks of age. The effective numbers of alleles (N_e) were 1.26, 1.08, 1.11 and 1.37 in APRI line, Moshtohor line, ½A½M and $(½A½M)^2$ crosses, respectively. High degrees of similarity were detected between the numbers of expected and observed genotypes in all genetic groups and therefore these genetic groups were in Hardy-Weinberg equilibrium *(HWE)* for *MSTN* gene. The values of polymorphic information content (PIC) were low and ranged from 0.073 in Moshtohor line to 0.235 in $(½A½M)^2$ cross. The genotypic frequencies of TT genotype of *MSTN* gene were high in all genetic groups (0.68 to 0.92). The allelic frequency recorded for T allele was much higher than for A allele. The heterozygosis for *MSTN* gene in all genetic groups were low or moderate, ranging from 0.079 to 0.323 for the observed heterozygosity (H_O) and 0.076 to 0.271 for the expected heterozygosity (H_e). The reductions in heterozygosity due to inbreeding (F_{IS}) in all genetic groups were low. In different genetic groups, both TT and TA genotypes of *MSTN* gene were found to be significantly associated with most body weights and gains at different ages. In APRI line and $(½A½M)^2$ cross, TT genotype was positively associated with an increase in most body weights. In Moshtohor and ½A½M cross, TA genotype was positively associated with heavy body weights. High means for TT and TA genotypes of *MSTN* gene in ½A½M and $(½A½M)^2$ crosses were recorded and showing strong positive associations of both TT and TA genotypes of *MSTN* gene with most body weights and gains.

Keywords: Rabbits, crossbreeding, growth, *Myostatin* gene, polymorphism, PCR-RFLP.

INTRODUCTION

In the last three decades, some genetic attempts were conducted to establish new rabbit lines in Egypt through crossing V-line does with Baladi Red bucks to produce APRI line (Youssef et al., 2009) and with Sinai Gabali bucks to produce Moshtohor line (Iraqi et al., 2008&2010a,b). However, post-weaning body weights and gains from weaning to slaughtering age are the criteria commonly used in selection for synthesizing the paternal rabbit lines (Khalil and Al-Saef, 2008; Khalil, 2010).

MSTN as a candidate gene has received much attention in molecular genetic studies of rabbits breeding industry due to the following facts: 1) It has strong association with some production traits like early maturity, high growth rate, efficient feed utilization and carcass traits (Fontanesi et al., 2008; Qiao et al., 2014; Abdel-Kafy et al., 2016; Shevchenko, 2015; El-Sabrout and Aggag, 2018; Yang et al., 2019), 2) It is used in marker assisted selection (MAS) or gene assisted selection (GAS) programs to improve the response of selection productivity (Fontanesi et al., 2011; Peng et al., 2013; Khalil, 2020), 3) It has an important impact on the amount of lean meat and fat deposition (El-Sabrout and Aggag, 2018; Yang et al., 2019), 4) It has the ability to stop the growth of sarcogenic and myogenic cells (Joulia et al., 2003) and 5) It has shown to regulate muscle development and growth (Joulia et al., 2003; El-Sabrout and Aggag, 2018). In practice, few studies reported that the *MSTN* genotypes had relevant impacts and relevant associations with growth traits in rabbits (Peng et al., 2013; Qiao et al., 2014; El-Sabrout and Aggag, 2018).

In reference to the molecular analyses of rabbit populations that are conducted nowadays, Khalil (2020) reported that the technique of Restricted Fragment Length Polymorphism (RFLP) based on Polymerase Chain Reaction (PCR) nominated as PCR-RFLP was used for analyzing the associations between SNP genotypes of candidate genes and economic traits in rabbits (Peiró et al., 2008; Abdel-Kafy et al., 2015; Shevchenko, 2015; Khalil et al., 2021a,b). In this concept, few studies have shown significant associations of some candidate genes like *PGR, FGF, IGF-1, IGF-2, MSTN, MC4R, PGAM2, BFGF, FGF-5, PGAM* and *GH* with body weights and daily weight gains in rabbits (e.g. Fontanesi et al., 2008).

The main objectives of the present study were: (1) to characterize *MSTN* candidate gene on SNPs molecular basis for post-weaning growth traits in different genetic groups obtained from crossbreeding experiment between bucks of APRI line rabbit and does of Moshtohor line, (2) to detect SNP genotyping of *MSTN* gene in APRI and Moshtohor lines and their crossbred progenies of F_1 and F_2 using PCR-RFLP technique, and (3) to detect the molecular associations between SNP genotypes of *MSTN* candidate gene and post-weaning growth traits using *FspBI* (*BfaI*) restriction enzyme.

MATERIALS AND METHODS

Animals and mating system used, management and feeding regime

Five years crossbreeding experiment was performed between bucks of APRI rabbit line (A) and does of Moshtohor line (M) to produce F_1 crossbred rabbits (½A½M), followed by *inter-se* mating to obtain F_2 crossbreds $(½A½M)^2$. Therefore, four genetic groups were used to assess the crossbreeding effects on post-weaning growth traits in terms of direct additive genetic, maternal effects and direct and maternal heterosis. The total number of sires, dams and kits used in this experiment were presented in Table 1. The experimental work was started from September 2016 to June 2020 in the rabbitry belonging to the Department of Animal Production, Faculty of Agriculture, Benha University, Egypt. APRI line rabbits was synthetized in the

rabbitry of Animal Production Research Institute through crossing Baladi Red bucks with V-line does, followed by two generations of *inter-se* mating with selection for litter weight at weaning (Youssef et al., 2009). Moshtohor line was developed in the rabbitry of Department of Animal Production, Faculty of Agriculture at Moshtohor, Benha University, Egypt through crossing Sinai Gabali bucks with V-line does, followed by four generations of *inter-se* mating along with selection for litter weight at weaning and individual weight at 56 days (Iraqi et al., 2008&2010a,b). The management procedures and feeding regime practiced in this experiment were outlined previously by Khattab et al. (2024).

Growth performance and data collected

In the studied four genetic groups, body weights were recorded individually at weaning at 4 weeks of age (BW4), then at 6 (BW6), 8 (BW8), 10 (BW10) and 12 (BW12) weeks of age, along with daily weight gains (DG) during the age intervals from 4 to 6 (DG4-6), 6 to 8 (DG6-8), 8 to 10 (DG8-10) and 10 to 12 (DG10-12) weeks, respectively.

Blood sampling and DNA extraction for molecular analyses

For genotyping the *MSTN* gene, a total of 105 rabbits at 12 weeks of age were chosen at random from different genetic groups to be genotyped. A 3-5 *ml* sample of venous blood was taken from the rabbit ear vein using a 2-gauge 1.5-injection needle and placed into tubes containing EDTA as an anticoagulant. The blood samples were kept cool in an ice tank until taken to the laboratory and maintained in a freezer at -20 °C. The QIAamp DNA Blood Mini Kit (Cat No. 51104, QIAGEN, Germany) was used to extract genomic DNA from the whole blood samples. A 200 *ml* of blood were placed in a 2 *ml* Eppendorf tube in addition to 20 *ml* of proteinase K solution and mixed by vertexing; after that, 200 *ml* of lysis solution were placed and mixed by pipetting to obtain a uniform suspension. The sample was incubated in a water bath for 10 minutes at 56°C until the cells were lysed. By pipetting, 200 *ml* of ethanol (96–100%) were added. The mixture was taken to the spin column in a 2 *ml* collection tube without wetting the rim. After that, the mixture was centrifuged at 8000 rpm for one minute, discarded the collection tube containing the filtrate, and placed the spin column in a clean 2 *ml* collection tube. Then wash buffer 1 (AW1) in 500 *ml* was added and centrifuged for one minute at 8000 rpm at room temperature. The filtrate was discarded, and the spin column was placed in a new 2 *ml* collection tube. Wash buffer 2 (AW2) in 500 *ml* was added and centrifuged for three minutes at 16000 rpm at room temperature. In a new 2*ml* collection tube, placing the spin column and discarding the filtrate, then centrifuged for one minute at full speed. Finally, the spin column was placed in a new 1.5 *ml* microcentrifuge tube and adding 200 μl of elution buffer (AE) to the eluted DNA. Then, DNA was incubated for one minute at room temperature (15-25^{o}C) and centrifuged at 8000 rpm for one minute. At -20 °C, genomic DNA was kept for further applications pertaining to this study.

Amplification by polymerase chain reaction (PCR)

For PCR amplification of *MSTN* gene, the part of intron 1, exon 2 and part of intron 2 (SNP c.713T>A; on chromosome number 7) with the expected amplicon size of 570 bp was used. According to Fontanesi et al. (2011), the primers used in the amplification were: the forward primer 5′ - TGCATGCATTATCCCAATAGA-3′ and the reverse primer 5′- TCGGTAGTTGTTTCCCACTTT-3′. The PCR was performed in a reaction volume of 25 μl using two μl of template genomic DNA of each sample, one μl of each primer, 12.5 μl of Dream Taq Green PCR

master mix (Thermo Scientific, USA) and nuclease free water up to 25 μl. The thermal cycling protocol was done by initial denaturation at 95°C for five minutes, followed by 35 cycles each at 95°C for 30 seconds, annealing temperature was 57°C for 30 seconds, elongation temperature was 72°C for 30 seconds and final extension at 72°C for 10 minutes. The DNA products were separated on 1.5 % agarose gel in 1 X TBE buffer, stained with ethidium bromide and runed on a standard gel electrophoresis, visualized on a UV Trans-illuminator and photographed using gel documentation system.

Digestion and genotyping of *MSTN* gene using PCR-RFLP technique

The amplified DNA fragments of *MSTN* gene were digested with *FspBI* (*BfaI*) restriction enzyme (Thermo Scientific, USA). The RFLP for *MSTN* gene was performed in reaction volume consisted of five μl PCR product, one μl distilled water, 0.7 μl 10 X buffer and 0.3 μl restriction enzyme. A 100 bp ladder was used for gel electrophoresis for affirmation of the length of PCR product. The mixtures were then incubated at 37°C for 15 minutes. Digested restriction fragments were subordinated to electrophoresis in 2 % agarose gel stained with ethidium bromide at 100 volts in 1 X TBE buffer, visualized under UV Trans-illuminator and photographed by gel documentation system.

Molecular characterization of *MSTN* gene in different genetic groups

The genetic diversity of SNP T>A for *MSTN* gene were assessed by calculating the effective number of alleles (*Ne)*, allelic and genotypic frequencies, the observed (*Ho*) and expected (*He*) heterozygosity, Hardy-Weinberg equilibrium (HWE), the reduction in heterozygosity due to inbreeding (i.e. fixation index, F_{IS}) for each genetic group using GENALEX software version 6.5 (Peakall and Smouse, 2006). The following equations were used in estimation of the previous parameters:

$$Ne = \frac{1}{\sum_{i=1}^{n} p_i^2}$$

$$Ho = \frac{No.\ of\ heterozygosity}{n} \quad He = 1 - \sum_{i=1}^{n} p_i^2$$

$$F_{IS} = (H_e - H_o)/H_e$$

where: Pi = the frequency of the i[th] allele, Pj = the frequency of the j[th] allele and n = the number of alleles. The polymorphic information content (PIC) was calculated using CERVUS software, version 3 (Kalinowski et al., 2007):

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2p_i^2\, p_j^2$$

Model for detecting the associations between genotypes of *MSTN* gene and growth traits:

To detect the polymorphic associations, the variances components estimated by Gibbs Sampling Algorithm were used to solve the mixed model equations using PEST software (Groeneveld, 2006), getting the generalized least-square means (GLM) of the two genotypes of *MSTN* gene in each genetic group separately. Two genotypes of *MSTN* gene (TT and TA) were obtained in each of the four genetic groups studied. The molecular associations between the genotypes of *MSTN* gene and growth traits were assessed in each genetic group separately using the following model:

$$y = Xb + Z_a u_a + Z_c u_c + e$$

Where y = vector of the observed growth trait for the weaned rabbit; b = vector of the fixed effects of genetic group of the weaned rabbit (4 levels: A, M, ½A½M and (½A½M)[2]), the fixed effect of T/A SNP genotypes of the *MSTN* gene, kit sex (male and female), year-season of birth (16 levels), parity order (6 levels), and litter size in which the kit was born (10 levels); u_a = vector of random additive genetic effect of the weaned rabbit; u_c = vector of random effects of the non-additive common litter effects; X = incidence matrix of fixed effects; Z_a = incidence matrix of random additive genetic effects; Z_c = incidence matrix of

common litter effects; *e* = vector of the random residual effects. The data were renumbered and recoded using the renumf90 software (Misztal et al., 2018). The pedigree file was checked for the relationship issues using the CFC v.1.0 software (Sargolzaei et al., 2006). The variance components for the random effects of the studied traits and heritabilities were estimated based on a Bayesian Inference of Gibbs Sampling Algorithm using the TM software (Legarra et al., 2008) where the Gibbs sampler algorithm comprised 200,000 iterations and discarding the first 20,000 iteration. Afterwards, one sample in each 200 iteration was saved. The estimated variance components were used to solve the mixed model equations using the PEST software (Groeneveld, 2006), getting the solutions for genetic groups effects along with the solutions for other fixed effects and their error variance-covariance matrix. The generalized least-square means (GLM) for the two genotypes of the *MSTN* gene were estimated using the PEST software (Groeneveld, 2006).

RESULTS AND DISCUSSION

Descriptive statistics of genetic groups for growth traits

The estimates of genetic group comparisons cited in most of the Egyptian studies in rabbits are commonly based on ordinary least-square methodology, but our estimates here depend on generalized least-square methodology. The generalized least square means obtained here for body weights at 4, 6, 8, 10 and 12 weeks of age in both crosses of ½A½M and $(½A½M)^2$ were higher than those of their parental lines of APRI and Moshtohor (Table 2). But, APRI-line rabbits were nearly similar in body weights to Moshtohor line rabbits in 8, 10 and 12 weeks, while the differences were significant for body weights at 4 and 6 weeks of age and not significant for all daily weight gains. Good post-weaning body weights and daily weight gains in crosses of ½A½M and $(½A½M)^2$ thus indicate that incorporating APRI and Moshtohor genes in a crossbreeding programme in a country with a hot climate was linked to improved post-weaning growth performance of the resulting crossbred rabbits. The differences between ½A½M and $(½A½M)^2$ crosses were significantly in favour of ½A½M cross for body weights at 4, 6, 10 and 12 weeks of age and for daily weight gain during the interval from 8 to 10 weeks. The significant positive heterosis on growth traits may be the cause of the crossbred rabbits' superiority over their parent lines. In general, the crossbred rabbits of F_1 and F_2 were somewhat higher than the parental lines and they are within the ranges observed by several Egyptian studies (e.g. Abou Khadiga et al., 2008; Iraqi et al., 2008; Abdel-Hamid, 2015). Iraqi et al. (2008) in a crossbreeding experiment involved Sinai Gabali (G) and V-line rabbits, reported that the F_1 cross (½G½V) was superior by 24, 42 and 106 g over the average of their parents for body weights at 4, 8 and 12 weeks of age, respectively. Youssef et al. (2009) using Baladi Red (BR) and V-line rabbits reported that the F_1 cross (½BR½V) was superior by 90, 128, 163 and 155 g over V-line, and by 105, 153, 230 and 332 g over BR for body weights at 6, 8, 10 and 12 weeks of age. In crossing Saudi Gabali (SG) with V-line rabbits in Saudi Arabia, Khalil and Al-Homidan (2014) stated that the cross $(¾V¼SG)^2$ nominated as Saudi 2 had higher body weights relative to Saudi Gabali rabbits by 39, 65, 98, 132 and 157 g at 4, 6, 8, 10 and 12 weeks, respectively, they added that the cross $(¾SG¼V)^2$ nominated as Saudi 3 had also superiority in body weights and daily weight gains. However, the crossbred rabbit produced by mating bucks of Egyptian rabbits with does of exotic breeds or lines were superior in body weights and gains relative to those produced by the reciprocal mating. This could be attributed

to that Bouscat, New Zealand White, Chinchilla, Californian, V-line, and Hyplus line dams were superior in their mothering and milking abilities than dams of the local Egyptian breeds/lines (Abdel-Ghany et al., 2000; Khalil and Afifi, 2000).

Polymorphic characterization of *MSTN* gene in different rabbit genetic groups

Molecular weights

The amplified DNA fragment of 570 bp was digested using the *FspBI* restriction enzyme and two genotypes were obtained for *MSTN* gene in the four genetic groups of rabbits. As shown in Figure 1, the banding patterns of *MSTN* gene yielded in PCR product were one band in the TT genotype (570 bp) and two bands in the TA genotype (570 and 445 bp). The AA genotype of *MSTN* gene was not attained in the present study. Similarly, Amalianingsih and Brahmantiyo (2015) found two genotypes of *MSTN* gene, one fragment of 508 bp identified for TT genotype and two fragments of 508 bp and 444 bp for TA genotype. The PCR-SSCP analysis obtained by Makhlouf et al. (2018) showed that the segment of 523 bp of *MSTN* gene was polymorphic in Alexandria and V-line rabbits where two SSCP patterns were detected and identified as AA and AB genotypes in V-line rabbits and only one SSCP pattern was seen in Alexandria line rabbits (AB genotype). On the other hand, Abdel-Kafy et al. (2016) observed three genotypes of *MSTN* gene in Egyptian APRI line rabbits (TT, TA and AA), with AA genotype having the lowest genotypic frequency (20 animals from 284). They added that the digested fragment of 445 and 125 bp indicated to the AA genotype and the fragment of 570, 445 and 125 bp indicated to the TA genotype.

The observed and effective number of alleles, Hardy-Weinberg equilibrium and polymorphic information content in each rabbit genetic group

The observed number of genotypes of *MSTN* gene (N_o), the effective number of alleles (N_e), Hardy-Weinberg equilibrium (*HWE*) and the polymorphic information content (PIC) are presented in Table 3 for each separate genetic group. The differences between the four genetic groups in effective number of alleles were significant where the highest N_e (1.37) was noticed in interse cross $(½A½M)^2$, followed by APRI line (1.26), then ½A½M (1.11) and Moshtohor line (1.08). However, the N_e is an index used to reveal the genetic diversity of the populations studied. Rafayová et al. (2009) reported that the effective number of alleles of *MSTN* gene was 1.79 in M91 and P91 rabbit lines. Peng et al. (2013) found that the effective numbers of alleles of *MSTN* gene were 1.97, 1.96 and 1.57 in Ira, Champagne and Tianfu Black rabbits.

Chi-square values (χ^2) of the *MSTN* gene genotypes were not significant in all genetic groups studied (Table 3), indicating that all genetic groups were in Hardy-Weinberg equilibrium for *MSTN* gene, i.e. there was a high degree of similarity between the numbers of the expected and observed genotypes. Yang et al. (2019) stated that χ^2 value of *MSTN* gene genotypes for *HWE* in six populations of Harbin, Hotot, Tianfu, Belgain, Zika and Californian indicating that the six populations were in *HWE* where χ^2 values were 0.998, 0.963, 0.981, 0.998, 0.995, and 0.986, respectively.

The value of polymorphic information content (PIC) gives an estimate of the marker's discriminating power and, thus, describes the marker's usefulness for identifying polymorphism within a population (Fontanesi et al., 2008; Peng et al.,2013). The current PIC values were low and varied from 0.073 in the Moshtohor line to 0.235 in the $(½A½M)^2$ cross (Table 3). Fontanesi et al. (2008) found that the PIC values for *MSTN* gene were high (0.37) across Belgian Hare, Burgundy Fawn, Checkered Giant, and Giant Grey rabbits. In two rabbit lines, Rafayová et al. (2009) found that the PIC value of an SNP at intron 2 of the *MSTN* gene was 0.34.

Peng et al. (2013) stated that PIC value for *MSTN* gene in the populations of Ira, Champagne and Tianfu Black rabbits were 0.37, 0.37 and 0.30, respectively.

Genotypic and allelic frequencies of *MSTN* gene

The genotypic frequencies of TT genotype of the *MSTN* gene in all genetic groups were high (0.76, 0.92, 0.89 and 0.68 for A, M, ½A½M and $(½A½M)^2$ groups, respectively (Table 4). While the frequencies were low in TA genotype (0.24, 0.08, 0.11 and 0.32 for A, M, ½A½M and $(½A½M)^2$ groups, respectively). The allelic frequency recorded for T allele was much higher than those recorded for A allele (0.88, 0.96, 0.95 and 0.84 vs 0.12, 0.04, 0.05 and 0.16 in A, M, ½A½M and $(½A½M)^2$ groups, respectively (Table 4), i.e. T allele was the most frequent allele compared with A allele in all genetic groups studied. These results were in accordance with those of Abdel-Kafy et al. (2016) who found that the T allele in Egyptian APRI rabbits had a frequency of 0.73, while the A allele had an allelic frequency of 0.27. Fontanesi et al. (2008) in different rabbit breeds stated that the frequencies of C and T alleles were 0.51 and 0.49, respectively. Rafayová et al. (2009) in lines M91 and P91 rabbits showed that the allelic frequency of *MSTN* gene was 0.67 for T allele and 0.33 for C allele. Peng et al. (2013) stated that the T allele frequency in Ira, Champagne and Tianfu Black rabbits was 0.44, 0.42 and 0.22, while it was 0.56, 0.58 and 0.76 for C allele, respectively with genotypic frequencies of 0.12, 0.13 and 0.04 for TT, TC and CC genotypes in Ira rabbits, 0.64, 0.59 and 0.40 in Champagne rabbits and 0.24, 0.28 and 0.56 in Tianfu Black rabbits. El-Sabrout and Aggag (2018) stated that the frequency for A allele was 0.78 in V-line rabbits and 0.54 in Alexandria line, while the genotypic frequencies for AA genotype was 0.72 in V-line and 0.46 in Alexandria rabbit line. Makhlouf et al. (2018) found that the frequency for AA genotype was 0.08 in V-line rabbits with allelic frequency for A allele was 0.54.

Heterozygosis and inbreeding in *MSTN* gene genotypes

The values of heterozygosity for *MSTN* gene were low to moderate (Table 5) and ranged from 0.079 to 0.323 for the observed heterozygosity (H_O) and 0.076 to 0.271 for the expected heterozygosity (H_e). Fontanesi et al. (2008) found that the level of heterozygosity was high (0.50) for *MSTN* gene across Belgian Hare, Burgundy Fawn, Checkered Giant, and Giant Grey rabbits. Rafayová et al. (2009) stated that the observed and expected heterozygosities for *MSTN* gene in M91 and P91 lines were 0.47 and 0.44, respectively. Peng et al. (2013) reported that heterozygosity for *MSTN* gene in three populations of Ira, Champagne and Tianfu Black rabbits were 0.49, 0.49 and 0.36, respectively.

The reductions in heterozygosity due to inbreeding in different genetic groups were low as shown in Table 5. The highest F_{IS} was observed in Moshtohor line (-0.041), and the lowest value was -0.192 in $(½A½M)^2$ cross. The negative value of F_{IS} indicating an excess in heterozygosity and the low F_{IS} value (very close to zero) indicating low level of inbreeding within the populations, while the high positive value indicating high levels of inbreeding. Rafayová et al. (2009) stated that the inbreeding coefficient for *MSTN* gene in M91 and P91 lines was low (0.051).

Polymorphic associations between *MSTN* gene genotypes and post-weaning growth traits in each genetic group separately

The molecular association analyses in each separate genetic group revealed that two genotypes of the *MSTN* gene of TT and TA were detected (Table 6). However, there were abundant reports evidencing that the *MSTN* gene is associated with growth traits in different breeds/lines of rabbits (Fontanesi et al., 2011; Peng et al., 2013; Qiao et al., 2014; Abdel-Kafy et al., 2016; El-Sabrout and Aggag, 2018; Yang

et al., 2019). Moreover, the *MSTN* gene in rabbits has shown an increase in body weight and relevant increase in the weight ratios of muscles in the whole body (Fontanesi et al., 2008&2011; Qiao et al., 2014).

The generalized least square means (GLM) estimated by PEST software for SNP genotypes of the *MSTN* gene in APRI, Moshtohor, ½A½M and $(½A½M)^2$ showed molecular associations of these genotypes with most body weights and daily weight gains studied (Table 6). The differences in GLM for body weights between TT and TA genotypes of *MSTN* gene in APRI line at different ages were mostly significantly in favour of TT genotype ($p<0.01$; Table 6). But, the differences in GLM in body weights in ½A½M genetic group were mostly significantly in favour of TA genotype of *MSTN* gene ($p<0.01$) since high means were recorded for TA genotype to be 688 *vs* 652 g at 4 weeks, 1170 *vs* 1079 g at 6 weeks, 1480 *vs* 1304 g at 8 weeks, 1965 *vs* 1688 g at 10 weeks and 2200 *vs* 1908 g at 12 weeks of age, while the respective means for $(½A½M)^2$ cross were slightly different between TT and TA genotypes where the estimates were nearly similar to be 659 and 660 g at 4 weeks, 1018 and 1009 g at 6 weeks, 1331 and 1347 g at 8 weeks, 1750 and 1742 g at 10 weeks and 2080 and 2028 g at 12 weeks of age. For the parental genetic groups, the GLM for body weights of TT and TA genotypes in APRI line were in favour of TT genotype to be 575 *vs* 543 g at 4 weeks, 795 *vs* 790 g at 6 weeks, 1037 *vs* 928 g at 8 weeks, 1211 *vs* 1160 g at 10 weeks and 1525 *vs* 1488 g at 12 weeks (mostly $p<0.01$), while the respective means for body weights in Moshtohor line were in favour of TA genotype to be 500 *vs* 472 g at 4 weeks, 773 *vs* 756 g at 6 weeks, 995 *vs* 998 g at 8 weeks, 1217 *vs* 1243 g at 10 weeks and 1462 *vs* 1437 g at 12 weeks of age (mostly $p<0.01$).

The GLM for most body weights in all genetic groups indicate that TT genotype of *MSTN* gene was positively associated with an increase in most body weights and gains in APRI line and $(½A½M)^2$ cross, while TA genotype was positively associated with most body weights in Moshtohor and ½A½M cross (mostly $p<0.01$, Table 6). These strong molecular associations of both TT and TA genotypes with growth traits lead us to conclude that both TT and TA genotypes could be used in selection to improve rabbits' growth of synthesized lines in Egypt. Peng et al. (2013) reported that *MSTN* gene genotypes had relevant impacts on body weights at 84 days of age in Ira, Champagne and Tianfu Black rabbits where the genotype TC exhibited significant associations with greater body weights relative to the genotype CC. Qiao et al. (2014) revealed that *MSTN* gene affected significantly body weights at 70 and 84 days of age in specialized Chinese rabbit strains and their crosses. El-Sabrout and Aggag (2018) found that the genotypes of *MSTN* gene in V-line and Alexandria line rabbits were significantly associated with higher body weights. Contrarily, Fontanesi et al. (2011) and Abdel-Kafy et al. (2016) found no significant association between the c.713T>A SNP of *MSTN* gene in commercial lines and APRI line rabbits, respectively. Yang et al. (2019) reported that the *MSTN* gene was significantly associated with growth and carcass traits in six populations of rabbits (Harbin, Hotot, Tianfu, Belgain, Zika, and Californian); the CC genotype had the highest weights at 135 days relative to CT and TT genotypes.

The GLM for daily weight gains in APRI parental line were in favour of TA genotype relative to TT genotype, being 20.7 *vs* 19.7 g at 4-6 weeks, 22.1 *vs* 13.9 g at 6-8 weeks, 19.5 *vs* 15.3 g at 8-10 weeks and 23.1 *vs* 20.8 g at 10-12 weeks of age (mostly $p<0.01$), 29.7 *vs* 19.5 g at 4-6 weeks, 25.1 *vs* 16.7 g at 6-8 weeks, 15.7 *vs* 15.6 g at 8-10 weeks and 17.6 *vs* 17.7 g at 10-12 weeks in Moshtohor line (mostly

p<0.01). The respective means for daily body gains in ½A½M cross were significantly in favour of TA genotype of *MSTN* gene, being 33.0 *vs* 30.2 g at 4-6 weeks, 29.9 *vs* 18.4 g at 6-8 weeks, 42.9 *vs* 25.7 g at 8-10 weeks, and 26.5 *vs* 24.1 g at 10-12 weeks of age. The respective means for interse $(½A½M)^2$ group were mostly in favour of TA genotype, being 31.2 vs 25.9 g at 4-6 weeks, 26.0 *vs* 23.6 g at 6-8 weeks, 27.3 *vs* 30.5 g at 8-10 weeks, and 26.0 *vs* 22.6 g at 10-12 weeks (p<0.01). However, the GLM for TT and TA genotypes of *MSTN* gene in ½A½M and $(½A½M)^2$ crosses were higher in body gains than the parental APRI and Moshtohor lines (Table 6), *i.e*. high means for TT and TA genotypes of *MSTN* gene in ½A½M and $(½A½M)^2$ crosses showing strong positive associations of both TT and TA genotypes of *MSTN* gene with most body weights and gains studied. Synonymous single base mutations can lead to variations in the way that mRNA is folded or modified, which can lead to changes in biological processes (Yang et al., 2019). Consequently, growth traits may be influenced by *MSTN* mutations as a potential genetic marker to enhance meat production through the understanding precisely the effects of *MSTN* gene on growth traits.

CONCLUSIONS

1) A candidate gene to be used in marker-assisted selection programmes aimed at enhancing post-weaning performance in rabbits, the *MSTN* gene has been found to be significantly associated with post-weaning growth traits. To enhance the growth of synthesized line rabbits in Egypt, selection might also be conducted using the TT and TA genotypes of the *MSTN* gene.

2) The significant molecular associations between post-weaning growth traits and *MSTN* gene genotypes are not fully indicative to that it is unclear if the present DNA polymorphisms in *MSTN* gene represent an actual mutation, or they are just molecular markers associated with growth traits. Accordingly, more research is required to validate the existing molecular association between growth traits and *MSTN* gene, including linkage analysis and functional genomics, based on larger data sets.

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DECLARATION OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

All experimental procedures involving animals handling and treatment were approved by the Research Ethics Committee of the Faculty of Agriculture, Benha University, Egypt (REC-FOABU).

SOFTWARE AND DATA REPOSITORY RESOURCES

Data used is available from the corresponding author upon reasonable request.

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Table (1): Genetic groups and numbers of sires, dams and kits categorized according to their genetic groups

Sire genetic group (N)	Dam genetic group (N)	Kit genetic group	No of kits weaned
APRI line (95)	APRI line (141)	APRI line	520
Moshtohor line (92)	Moshtohor line (164)	Moshtohor line	604
APRI line (64)	Moshtohor line (104)	½A½M	374
½A½M (12)	½A½M (36)	(½A½M)²	500
Total No. = 263	Total No. = 445		Total No. = 1998

N = Numbers given in brackets.

Table (2): Descriptive statistics for body weights and daily weight gains in different genetic groups in terms of generalized least square means (GLM) ± standard errors (SE) obtained by PEST software

Trait[+]	Genetic group											
	APRI line (A)			Moshtohor line (M)			½A½M			(½A½M)^2		
	N	GLM	SE	N	GLM	SE	N	GLM	SE	N	GLM	SE
Body weight (g):												
BW4	520	490^{d}	3.4	604	505^{c}	3.1	374	585^{a}	4.0	500	565^{b}	3.4
BW6	471	775^{d}	4.0	542	790^{c}	3.7	358	920^{a}	4.5	491	880^{b}	3.8
BW8	436	1060^{b}	5.4	491	1055^{b}	5.0	350	1205^{a}	5.3	468	1195^{a}	5.2
BW10	423	1325^{c}	7.1	478	1330^{c}	6.7	340	1580^{a}	7.2	459	1475^{b}	6.7
BW12	347	1635^{c}	9.2	367	1630^{c}	8.3	301	1925^{a}	14.2	444	1850^{b}	7.7
Daily weight gain (g):												
DG4-6	415	21.9^{a}	0.47	488	23.4^{a}	0.43	317	23.9^{a}	0.56	440	22.9^{a}	0.43
DG6-8	390	19.3^{b}	0.40	449	19.2^{b}	0.37	301	21.3^{ab}	0.45	409	22.4^{a}	0.35
DG8-10	369	18.9^{b}	0.39	426	19.7^{b}	0.37	294	26.3^{a}	0.42	390	20.2^{b}	0.35
DG10-12	336	22.5^{b}	0.45	365	22.3^{b}	0.42	280	25.7^{a}	0.72	374	26.9^{a}	0.36

[+]BW4, BW6, BW8, BW10 and BW12= Body weight at 4, 6, 8, 10 and 12 weeks of age, respectively; DG4-6, DG6-8, DG8-10, and DG10-12= Daily weight gain during the intervals from 4 to 6, 6 to 8, 8 to 10 and 10 to 12 weeks of age, respectively; ½A½M= F_1 cross resulted from crossing APRI bucks and Moshtohor does and $(½A½M)^2$ = F_2 cross resulted from interse mating between bucks and does of the F_1 crossbred.

Table (3): The observed number of genotypes (N_O) and effective number of alleles (N_e), Chi-square values (χ^2) for Hardy-Weinberg equilibrium (*HWE*) and polymorphic information content (PIC) for the *MSTN* gene in each genetic group studied

Genetic group	No of rabbits	Number of animals in each *MSTN* genotypes (N_O)			Effective number of alleles (N_e)	χ^2 value for *HWE*	PIC
		TT	TA	AA			
APRI line (A)	17	13	4	--	1.26^{a}	0.302^{NS}	0.186
Moshtohor line (M)	38	35	3	--	1.08^{b}	0.064^{NS}	0.073
½A½M	19	17	2	--	1.11^{b}	0.059^{NS}	0.095
$(½A½M)^2$	31	21	10	--	1.37^{a}	1.146^{NS}	0.235
Total	105	86	19	--	--	χ^2= 1.571^{NS}	--

[a,b] The estimate superscripted with different letters in each column are significantly different (P≤0.05); NS= Non-significant (P>0.05).

Table (4): The genotypic and allelic frequencies for *MSTN* gene genotypes in each genetic group studied

Genetic group	No of rabbits	Genotypic frequency			Gene frequency	
		TT	TA	AA	T	A
APRI line (A)	17	0.76	0.24	--	0.88	0.12
Moshtohor line (M)	38	0.92	0.08	--	0.96	0.04
½A½M	19	0.89	0.11	--	0.95	0.05
$(½A½M)^2$	31	0.68	0.32	--	0.84	0.16

Table (5): The observed (H_O) and expected (H_e) heterozygosities and fixation index (F_{IS}) for *MSTN* gene genotypes in each genetic group studied

Genetic group	No of rabbits	H_o	H_e	F_{IS}
APRI line (A)	17	0.235	0.208	-0.133
Moshtohor line (M)	38	0.079	0.076	-0.041
½A½M	19	0.105	0.100	-0.056
$(½A½M)^2$	31	0.323	0.271	-0.192

Table (6): The polymorphic associations of the *MSTN* gene genotypes with body weights (BW) and daily weight gains (DG) estimated by PEST software and expressed as generalized least square means and their standard errors (GLM±SE) for each genetic group separately

Genetic group	*MSTN* gene genotypes					*MSTN* gene genotypes				
	Trait[+]	TT (N= 86)		TA (N= 19)		Trait[+]	TT (N= 86)		TA (N= 19)	
		GLM	SE	GLM	SE		GLM	SE	GLM	SE
A line	BW4	575[a]	29.2	543[b]	41.3	DG4-6	19.7[b]	0.36	20.7[a]	0.50
M line		472[b]	21.6	500[a]	46.7		19.5[b]	1.33	29.7[a]	2.03
½A½M		652[b]	21.7	688[a]	53.0		30.2[b]	2.24	33.0[a]	6.33
$(½A½M)^2$		659[a]	23.2	660[a]	29.0		25.9[b]	1.96	31.2[a]	2.26
A line	BW6	795[a]	19.8	790[a]	56.1	DG6-8	13.9[b]	1.85	22.1[a]	4.89
M line		756[b]	16.3	773[a]	37.6		16.7[b]	1.6	25.1[a]	3.03
½A½M		1079[b]	24.1	1170[a]	76.1		18.4[b]	4.89	29.9[a]	12.96
$(½A½M)^2$		1018[a]	25.6	1009[b]	30.6		23.6[b]	3.62	26.0[a]	4.18
A line	BW8	1037[a]	25.7	928[b]	44.5	DG8-10	15.3[b]	2.75	19.5[a]	4.76
M line		998[a]	25.7	995[a]	66.3		15.6[a]	1.32	15.7[a]	2.63
½A½M		1304[b]	39.6	1480[a]	142.8		25.7[b]	2.74	42.9[a]	9.88
$(½A½M)^2$		1331[b]	31.7	1347[a]	43.3		30.5[a]	4.00	27.3[b]	5.37
A line	BW10	1211[a]	40.6	1160[b]	76	DG10-12	20.8[b]	3.30	23.1[a]	5.72
M line		1243[a]	35.3	1217[b]	88.7		17.7[a]	2.86	17.6[a]	5.22
½A½M		1688[b]	49.0	1965[a]	138.7		24.1[b]	1.67	26.5[a]	2.89
$(½A½M)^2$		1750[a]	66.5	1742[a]	85.9		22.6[b]	2.71	26.0[a]	3.58
A line	BW12	1525[a]	55.3	1488[b]	110.6					
M line		1437[b]	47.3	1462[a]	105.7					
½A½M		1908[b]	66.7	2200[a]	163.4					
$(½A½M)^2$		2080[a]	78.8	2028[b]	93.3					

[+] Traits as defined in Table (2).

[a,b] Means within each classification, not followed by the same letter in the row differed significantly (P<0.01)

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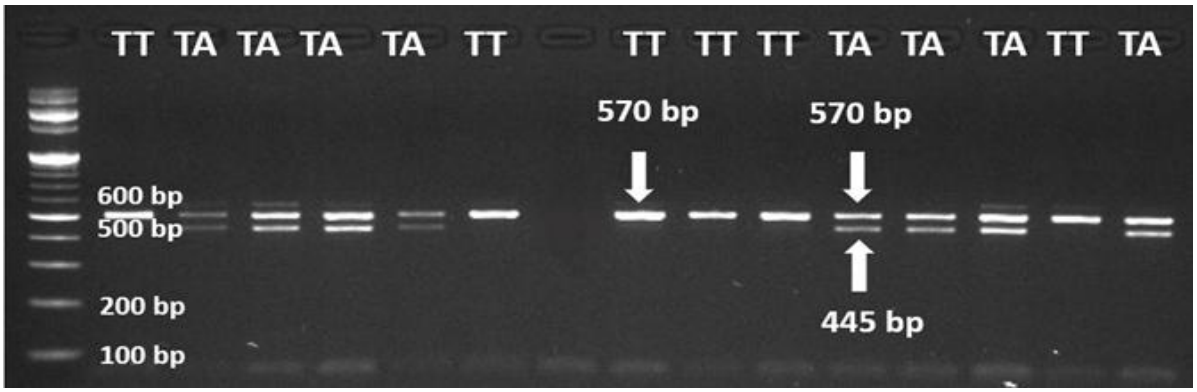

Figure (1): Gel electrophoresis showing the PCR-RFLP products of the c.713T>A SNP identified in the rabbit *Myostatin* gene (*MSTN*). The genotypes are indicated at the top of each lane. M is 100 bp ladder DNA molecular marker.

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الملخص العربي

التنوع الجزيئي لجين الميوستاتين لصفات النمو في تجربة لخلط أرانب خط أبري مع خط مشتهر

عبد الوهاب حسن خطاب[1،2] ، ماهر حسب النبي خليل[1]، محمود مغربي عراقي[1]، عماد محمد أمين[2]، أيمن النجار[1]
1- قسم الإنتاج الحيواني - كلية الزراعة بمشتهر - جامعة بنها - مصر
2- قسم تربية الحيوان والدواجن – شعبة الإنتاج الحيواني والدواجن – مركز بحوث الصحراء – وزارة الزراعة – مصر

أجريت تجربة خلط لمدة خمس سنوات بين خطين مستنبطين من الأرانب (خط أبري وخط مشتهر)، حيث تم الخلط بين ذكور أرانب أبري مع إناث أرانب مشتهر لإنتاج الجيل الأول الخليط (½أبري½مشتهر)، متبوعاً بالتزاوج البيني بين أفراد الجيل الأول لإنتاج الجيل الثاني الخليط (½أبري½مشتهر)^2. تم تطبيق تقنية التنوع الجزيئي لأطوال القطع المحدد والمعتمد على تفاعل إنزيم البلمرة المتسلسل لمعرفة نمط التنوع الجزيئي للنيوكليوتيدة المفردة لجين الميوستاتين في أرانب أبري، مشتهر، وخلطان الجيل الأول والثاني، وكذلك الكشف عن الإرتباطات الجزيئية بين الأنماط المختلفة لجين الميوستاتين وصفات النمو بعد الفطام بإستخدام إنزيم القطع *FspBI*. تم الكشف عن تركيبين وراثيين لجين الميوستاتين (TT, TA) في كل مجموعة وراثية على حده، أستخدمت مكونات التباين المحسوبة بواسطة برنامج Gibbs sampling في حل معادلات النموذج المختلط MME مستخدما برنامج PEST وذلك للحصول على حلول للمربعات الدنيا المعممة Generalized Least Square solutions للمجموعات الوراثية تحت الدراسة مصحوبة بحلول للتأثيرات الثابتة Fixed effects. وعند ذلك تم تقدير متوسطات المربعات الدنيا المعممة Generalized Least Square Means (GLM) للتراكيب الوراثية لجين الميوستاتين مستخدما برنامج PEST . أظهرت النتائج تفوقاً ملحوظاً في متوسطات المربعات الدنيا المعممة في كل من خلطان الجيل الأول والثاني (½أبري½مشتهر) ، (½أبري½مشتهر)^2 في وزن الجسم عند عمر 4، 6، 8، 10 ،12 أسبوع مقارنة بمتوسطات الأباء من خط أبري وخط مشتهر. وكانت متوسطات خط أبري متقاربة في وزن الجسم لخط مشتهر عند عمر 8، 10 ،12 أسبوع. كانت الأعداد الفعلية للأليلات هى 1.26، 1.08، 1.11، 1.37 في خط أبري، خط مشتهر، خليط الجيل الأول، خليط الجيل الثاني، على الترتيب. دلت الدرجة العالية من التشابه بين الأعداد الفعلية والمتوقعة للتراكيب الوراثية في المجاميع الوراثية المختلفة أنها في حالة إتزان هاردي-فينبرج بالنسبة لجين الميوستاتين. كانت قيم محتوى معلومات التنوع الجزيئي منخفضة وكانت 0.073 في خط مشتهر، 0.235 في خليط الجيل الثاني. كانت قيم تكرارات التركيب الوراثي TT لجين الميوستاتين عالية في المجاميع الوراثية المختلفة وتراوحت بين 0.68 إلى 0.92. سجلت قيم تكرارات الأليل T قيم أعلى مقارنة بالأليل الأخر A. كانت قيم نسب التراكيب الوراثية الخليطة heterozygosis لجين الميوستاتين في كل المجاميع الوراثية منخفضة إلى متوسطة وتراوحت بين 0.079 إلى 0.323 للقيم المشاهدة، وبين 0.076 إلى 0.271 للقيم المتوقعة. وكانت قيم الإنخفاض في هذه التراكيب نتيجة إتباع التربية الداخلية في كل المجاميع الوراثية منخفضة. وجد أن التركيبين الوراثيين لجين الميوستاتين في المجاميع الوراثية المختلفة مرتبطان معنوياً مع معظم صفات وزن الجسم ومعدل الزيادة اليومية في الوزن في الأعمار المختلفة. كان التركيب الوراثي TT في خط أبري، وخليط الجيل الثاني مرتبط إيجابياً بالزيادة في معظم صفات وزن الجسم. كان التركيب الوراثي TA في خط مشتهر، وخليط الجيل الأول مرتبط إيجابياً مع أوزان الجسم الثقيلة. تم تسجيل متوسطات عالية للتركيبين الوراثيين TT وTA لجين الميوستاتين في خلطان الجيل الأول والثاني، وأظهرت هذه التراكيب إرتباطات وراثية موجبة وقوية مع معظم صفات وزن الجسم ومعدل الزيادة اليومية في الوزن.