PL-ISSN 0015-5497 (print), ISSN 1734-9168 (online)
Folia Biologica (Kraków), vol. 62 (2014), No 3
doi:10.3409/fb62_3.219

Identification of a New Haplotype within the Promoter Region of the *MSTN* Gene in Horses from Five of the most Common Breeds in Poland*

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Accepted May 15, 2014

STEFANIUK M., KACZOR U., AUGUSTYN R., GURGUL A., KULISA M., PODSTAWSKI Z. 2014. Identification of a new haplotype within the promoter region of the *MSTN* gene in horses from five of the most common breeds in Poland. Folia Biologica (Kraków) **62**: 219-222.

Myostatin (GDF-8) encoded by the *MSTN* gene is a negative regulator of muscle growth and development and belongs to the TGF-β superfamily of secreted growth and differentiation factors. In Thoroughbred horses, an *MSTN* sequence polymorphism (g.66493737C>T) is associated with optimum race distance. In the present study, a genetic polymorphism of a predicted promoter of the *MSTN* gene was investigated in 451 horses belonging to five different breeds: Arabian, Thoroughbred, Polish Konik, Hucul and Polish Heavy Draft. Two SNPs located at g.66495826T>C and g.66495696T>C (chr;18 EquCab 2.0) showed three haplotypes previously described: [g.66495826:T, g.66495696:T], [g.66495826:T, g.66495696:C], [g.66495826:C, g.66495696:T] with frequencies 0.877; 0.101; 0.005; respectively. Analysis performed on Polish Heavy Draft indicated the occurrence of a new haplotype [g.6649582626:C, g.66495696:C] with frequency 0.016.

Key words: Myostatin, horse, polymorphism, promoter, haplotype.

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Myostatin (encoded by *MSTN*), also called GDF-8, belongs to the TGF-β super family responsible for secreted growth and differentiation factors, and acts as a strong inhibitor of muscle formation (THOMAS *et al*. 2000). *MSTN* appears to be highly conserved across species, but shows a high level of diversity within species (GROBET *et al*. 1998). *MSTN* gene has been characterized in several livestock species and is composed of three exons and two introns. Polymorphisms in the *MSTN locus* have been described so far in humans (SCHUELKE *et al*. 2004), dogs (MOSHER *et al*. 2007), sheep (BOMAN *et al*. 2009), cattle (MCPHERRON & LEE 1997), horses (HILL *et al*. 2010a) and pigs (TU *et al*. 2012).

Advances in genetics have enabled the identification of a region on ECA18 which includes the *MSTN* gene associated with racing phenotypes and influencing racing performance in Thoroughbred horses (HILL *et al*. 2010a; HILL *et al*. 2010b; TOZAKI *et al*. 2011). However, to date only a few studies have analyzed horse *MSTN* gene polymorphisms in different horse breeds and types (DALL'OLIO *et al*. 2010; BARON *et al*. 2012). In this study we analyzed the predicted promoter region of the *MSTN* gene (rev.str.66, 495,813-66,489,608; chr. 18 EquCab2.0) in five breeds; Arabians (OO), Polish Konik horses (PK), Hucul horses (HC), Thoroughbred horses (XX) and Polish Heavy Draft horses (PHD). In Polish horse breeds, sequence variants of the *MSTN* gene have been poorly characterized (MAĆKOWSKI & CHOLEWIŃSKI 2010). The significance of *MSTN* SNPs on economically important traits in horses requires the characterization of *MSTN* gene polymorphisms among Polish horse breeds.

*This work has been approved by the University of Agriculture in Kraków nr.34/2010.

Material and Methods

The promoter region of equine *MSTN* was predicted using MatInspector software (CARTHARIUS *et al*. 2005). In the present study, a total of 451 randomly selected horses belonging to five different breeds were sampled from different stables. DNA was extracted from equine blood using MasterPure Genomic DNA Purification Kit (Epicentre Technologies, USA). PCR reactions were performed in a final volume of 20 μl containing roughly 200 ng of gDNA, 0.25 mM of each dNTP, 10 pmol of each primer and 1.5 U of TaqDNA Polymerase (Thermo Scientific, USA), 2 mM of $MgCl_2$ and 1xPCR buffer containing KCl. The temperature cycles (35) were as follows: denaturation 94ºC for 30 s, annealing at 56ºC for 30 s elongation at 72ºC for 45 s. Primer sequences used for amplification were described by DALL'OLIO *et al*. (2010) and were as follows:
484 bp: F5'TCAGGGAAACAAGTTTCTCAAAT3' and R5'TGCTCCACAATGAATCTCG3': 204 bp: F5'TCAGGGAAACAAGTTTCTCAAAT3' and R5'ACTTCCTCAGAAATTAAGATTTAAT3'.

PCR-RFLP was designed to genotype two SNPs in the predicted promoter region of the *MSTN* gene. PCR products of 484 bp were digested with restriction enzyme *Rsa*I (5'GT↓AC3') (Thermo Scientific, USA). Digestions were performed overnight at 37ºC in a 10 μl volume containing 5 μl of PCR product, 3U *Rsa*I supplied with buffer Tango, resulting in a DNA fragment of 484 bp for allele T and 47 bp for allele C. Digestions of 204 bp PCR product (allele T – 179 and 25bp; allele C – 204 bp) were performed overnight at 37ºC in a 10 μl volume with 5 μl of PCR product and 4 U enzyme *Ssp*I (5'AAT↓ATT 3') supplied with Buffer G.

GenePop version 4.2 was used for statistical analysis (ROUSSET 2008). Population structure was analyzed by evaluation of expected and observed heterozygosity. Significance of differences in genotype frequencies between each population pair was evaluated by using the exact G test implemented in GenePop software. The haplotype frequencies were calculated using the expectation maximization algorithm (EM) implemented in Arlequin software (EXCOFFIER & LISCHER 2010).

Results

The obtained results are shown in Table 1.

Two transitions were detected within the predicted promoter region: the first was located at g.66495826T>C, and the second at g.66495696T>C. Both polymorphisms have been previously described and g.66495696T>C is located within a functional motif of a TATA box (DALL'OLIO *et al.* 2010). The test for genotypic linkage disequilibrium gave P-value of 0.878 indicating that the examined *loci* are not linked. At g.66495826T>C *locus* the observed heterozygosity was 0.333 and the expected heterozygosity was 0.041. At *locus* g.66495696T>C these parameters equaled 0.179 and 0.185, respectively. Significant deviation from Hardy-Weinberg equilibrium ($p<0.05$) was observed for *locus* 66495826T>C. At both *loci* the EM algorithm identified three haplotypes previously described: [g.66495826:T, g.66495696:T], [g.66495826:T, g.66495696:C], [g.66495826:C, g.66495696:T] (DALL'OLIO *et al*. 2010) and one new haplotype [g.6649582626:C, g.66495696:C] with frequencies of 0.877; 0.101; 0.005 and 0.016, respectively. Haplotypes TT and TC were observed in all breeds, the CT haplotype was identified in Polish Heavy Draft, Thoroughbred and Hucul horses and the CC haplotype only in Polish Heavy Draft (Table 1). The g.66495696 T>C polymorphism was associated ($P<0.05$) with height at withers in Uruguayan Creolo breed horses (DALL'OLIO *et al*. 2012), but not in Italian Heavy Draft Horses (IHDH). Furthermore, a detailed analysis of association between morphological traits in IHDH and polymorphisms of these two transitions showed that g.66495826 T>C was associated with circumference of cannon bone, rear leg side view and

Table 1

Minor allele frequencies and haplotype frequencies in five common horse breeds in Poland

Horse breeds	n	MAF g.66495826C	MAF g.66495696C	Hap[T:T]	Hap[T:C]	Hap[C:T]	Hap[C:C]
PHD	63	0.086	0.218	0.711	0.201	0.066	0.021
XX	86	0.005	0.014	0.983	0.012	0.005	–
OO	99	–	0.230	0.768	0.231	–	–
PK	113	–	0.043	0.956	0.043	–	–
HC	90	0.033	0.039	0.921	0.039	0.039	–

fleshiness. Heterozygous g.66495826 T>C compared to homozygous TT obtained a greater score for fleshiness, sickle rear legs and greater cannon bone circumference (DALL'OLIO *et al*. 2014).

Discussion

It was previously shown that none of the polymorphisms studied in this paper displayed a minor allele frequency (MAF) greater than 0.05 in Thoroughbred (HILL *et al*. 2013) in our results, MAF in Thoroughbred were 0.005 for g.66495826C and 0.014 for g.66495696C. In Arabians, the second light breed included in our study, MAF greater than 0.05 was shown for g.66495696C; 0.230. In our study MAFs greater than 0.05 were observed for both SNPs (g.66495826C – 0.086 and g.66495696C – 0.218) in Polish Heavy Draft horses and these results correspond to MAF obtained for other heavy breeds: IHDH and Noriker horse (DALL'OLIO *et al*. 2010, 2014). MAF values for g.66495696T>C in Polish Konik and Hucul horses were lower than 0.05 and differed from the values obtained for breeds classified in the same type by previous authors (DALL'OLIO *et al*. 2010). Between pairs of breeds highly significant differences (P<0.001) in frequencies of genotypes of the studied polymorphisms were observed. Polish Heavy Draft horses showed a highly significant difference compared to Thoroughbred, Polish Konik horses and Hucul horses. Also Arabians reveal highly significant differences compared to Thoroughbred, Polish Konik and Hucul horses ($p \leq 0.001$, Fisher's method) According to the obtained results, Polish Heavy Draft showed higher levels of differentiation at the investigated *loci.* Furthermore previous reports indicated that the *MSTN* promoter polymorphisms may have a potential effect on morphological traits and body measurements in heavy horse breeds.

On the other hand *MSTN*, referred to as a speed gene, has been identified and associated with race performance in Thoroughbred horses. Moreover, a genome-wide association study (GWAS) confirmed a strong association between a fragment of chromosome 18 within the myostatin gene *locus* with the best race distance in Thoroughbreds (HILL *et al*. 2010b). Thus further studies are required to find potential genetic markers which may have an influence on production traits in horses.

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