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Original Article

Genetic Variation of *ACTN3* and *MSTN* Genes in a Cohort of Endurance Arabian Horses

S. Almarzook[1]*, A. Said Ahmed[1], M. Reissmann[1], G. A. Brockmann[1]

[1]Humboldt-University of Berlin, Faculty of Life Sciences, Albrecht Daniel Thaer-Institute for Agricultural and Horticultural Sciences, Department of Animal Breeding Biology and Molecular Genetics, Invalidenstrasse 42, 10115 Berlin, Germany

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Corresponding Author

S. Almarzook

almarzos@hu-berlin.de

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ABSTRACT

Arabian horses are readily distinguishable in form and features, and they are widely known for endurance capability. This study is the first examining of endurance-related genes in Arabian horses born and raised in Syria. The major objective was to identify genetic variation in candidate genes that could potentially affect endurance traits and to associate them with endurance phenotypes. The two genes *Alpha-actinin skeletal muscle isoform 3* (*ACTN3)* and *Myostatin (MSTN)* were sequenced. Performance traits were available for 42 recorded Arabian horses from Syria performed endurance racing over 40, 80, and 120 km distances. Based on the recorded mean speeds, horses were grouped according to their performance index into low and high performers. The comparative sequencing revealed a total of 13 variants in both studied genes, 12 variants in *ACTN3* and one variant in *MSTN*. General linear model analyses showed that none of the analyzed variants has significant effect on any of the studied traits. However, for *ACTN3*, we found a 5' UTR variant (12:26511704G>A) that predicted to cause a gain of an E4BP4 transcription factor binding site, and a variant in the 3´ UTR 12:26524930T>C that predicted to cause the abrogation of two predicted miRNA target sites (eca-miR-1296 and eca-miR-326) and thereby affect gene expression. For *MSTN*, a 5' UTR variant 18:66495696A>G is predicted to cause the substitution of the transcription factor binding sites for HFH-1 and Sox-5 by binding sites for HFH-3 and E4BP4.

Keywords: Endurance, candidate genes, transcription factor binding site, Arabian horse, Syria.

INTRODUCTION

Endurance played a vital role in the evolutionary history of human and other species, because it enabled them to survive and preserve their lives under different conditions (Maffetone, 2010). In horses, endurance is a trait of great economic value. Humans invested in endurance to improve labor capability and athletic performance of horses. Nowadays, endurance performance is very important in the equestrian competitions. According to Bergero *et al.*, (2005), the endurance performance of horses is identified as a low-intensity long-term trial. Most of the international equine endurance distances range between 30 and 500 km that can be run in one to five days. Different parameters were used to measure endurance performance. Endurance horses can achieve an average speed in endurance races exceeds 25 km/h in the last phase of the race (Nagy *et al.*, 2012).

Heritability estimates for mean speed in eight endurance horse breeds (including Arabian horses) vary considerably between 0.16 and 0.40 at distances of 90 km and ≥ 120 km, respectively (Ricard & Touvais 2007). This makes the mean speed as one selection parameter for endurance performance. Studies demonstrated differences in biomarkers related to endurance performance including heart rate, lactate and uric acid (Adamu *et al.*, 2012), physiology and metabolic performance (Bergero *et al.*, 2005; Castejon *et al.*, 2006), morphological factors and gaiting variability (Metayer *et al.*, 2004; Cottin *et al.*, 2010), skeletal muscle fiber types (fast/low twitch fibers), and fiber composition (Rivero *et al.*, 1993; Rivero & Barrey 2001). Pathways involved in endurance as a complex quantitative trait provide a list of candidate genes to be tested for association with endurance trait. There are approximately 230 candidate genes suggested for athletic performance in humans (Bray *et al.*, 2009; Schröder *et al.*, 2011). Recently, studies have tried to identify a DNA profile specific to endurance performance in humans, but it is still under consideration (Rankinen *et al.*, 2016). In contrast to humans, in horses, only a small set of genes related to racing performance has been genotyped until today (Hill *et al.*, 2010a; Silva *et al.*, 2015)

Genes of Interest

The *Alpha-actinin skeletal muscle isoform 3* (*ACTN3*) gene (ENSECAG00000018961) encodes the equine α-actinin 3 protein. The gene is located between 26,511,750 and 26,524,992 bp on horse chromosome 12. *ACTN3* is composed of 21 exons. *ACTN3* is expressed mainly in the fast twitch muscle fibers (type 2 muscle fibers) which are responsible for high speed and important for the maintenance of muscle contraction (Yang *et al.*, 2003; MacArthur & North 2004; Sjöblom *et al.*, 2008). The *ACTN3* gene is highly conserved and its mutation rate is lower than average (North 2008; Fattahi & Najmabadi 2012) which reflects the importance of its function. In humans, the homozygosity for a nonsense polymorphism (R577X), which converts Arginine at position 577 of the protein into a stop codon, causes complete deficiency of the fast skeletal muscle fiber protein α-actinin-3 (Mata *et al.*, 2012; Orysiak *et al.*, 2014). In a study of endurance athletes, the XX genotype was over-represented (Yang *et al.*, 2003). This suggest that ACTN3 variants may contribute to enhancing the endurance performance (Yang *et al.*, 2003; Zanoteli *et al.*, 2003). MacArthur *et al.*, (2008) supported this statement by mouse studies. Their analysis of knockout mouse muscle showed a shift in the properties from fast fibers towards slow fibers, increased activity of the metabolic enzymes and better resistance to fatigue. In horses, *ACTN3* is suggested to affect muscle strength and insulin sensitivity which are related to endurance performance in different horse breeds (Gu *et al.*, 2009; Thomas *et al.*, 2014).

The *Myostatin* (*MSTN*) gene (ENSECAG00000021373) is located between 66,490,208 and 66,495,180 bp on horse chromosome 18. *MSTN* comprises three exons. *MSTN* encodes the growth differentiation factor 8 (GDF-8) which belongs to the TGF-β protein family affecting growth, differentiation and regulation of muscle proliferation as well as controlling the muscle fiber's growth (Carnac *et al.,* 2006). Additionally, *MSTN* is involved in performance relevant functions such as regeneration of skeletal muscles, bone formation, glucose metabolisms and adipocyte proliferation. In different species, mutations which result in an inhibition of *MSTN* cause increased muscle mass, for instance in Bully Whippet dogs and Belgian blue cattle (Mosher *et al.,* 2007). In horses, findings implied that *MSTN* variants can be potential predictors of racing performance and morphological traits (Gu *et al.,* 2009; Hill *et al.,* 2010b; Tozaki *et al.,* 2011; Petersen *et al.,* 2014; François *et al.,* 2016).

Horses, in general, vary in their ability to perform endurance due to variability of genetic background and morphological differences, health conditions, and training programs. Arabian and Arabian-cross horses possess morphological and physiological characters making them very suitable for endurance performance and long distance riding under harsh conditions (Wood & Jackson 1989; Metzger *et al.,* 2015; USEF 2016). It is strongly thought that adaptation to extreme endurance exercise is influenced by genetic factors in this breed (Ricard *et al.,* 2017).Therefore, the ultimate purpose of studying the racing genetics was to provide genetic predictors of the horse's potential for high racing ability. In this study, we focus on Syrian Arabian horses which provide a valuable model to investigate genetic variants in candidate genes for endurance in horses.

MATERIALS AND METHODS

Animals and Endurance Data

For this study, we sampled blood or hair from 42 endurance Arabian horses born in Syria between 1994 and 2007. Samples were collected by state veterinarian according to the animal welfare regulations set by the Syrian Ministry of Agriculture and Agrarian Reform and the Syrian Arabian horse official authorities. The studied horses performed endurance in official national events carried out in Syria between 2001 and 2010. Mean speeds for short and medium endurance distances based on international rules (Ricard & Touvais 2007) have been recorded. Based on mean speeds, 24 individuals were indicated as high endurance performers (18-25 km/h) and 18 were indicated as low endurance performers (6-16 km/h). Individuals who were excluded from race due to lameness were included in our study as low performance horses (Table 1).

Weights (jockeys and their kits including lead weights) were optimized to 75 Kg. 78.5% of the horses were born in the south of Syria (Damascus and Dara) which suggests the homogeneity in geographical affiliation of the studied group (supplementary Table S1).

Table S1: Origins and sexes of 42 Syrian Arabian horses reported for endurance performance

Region in Syria	Number of individuals	
	Males	Females
Al Hasakah	1	6
Damascus	8	23
Dara	-	2
Hama	1	-
Hims	1	-
Total	11	31

Genotyping of the Candidate Genes

Promoters, exons and 3´ UTRs of two autosomal genes (*ACTN3 and MSTN*) were amplified using primers designed by using Primer3 online tool (Untergasser *et al.,* 2012). If the intron was smaller than 350 bp, two exons were amplified using one primer pair was designed based on the reference equine genome assembly EquCab2. Primers information with PCR product sizes are listed in the supplementary Table S2.

Table 1: Arabian horses reported for endurance performance (high and low) for three distances

distances	40 km		80 km		120 km	
performance	High	Low	High	Low	High	Low
	7	5	7	11	10	2
SUM	12		18		12	

Table S2: Information of gene specific sequences, PCR product sizes and annealing temperature for the analyzed fragments

Primers ID	Sequence	Product (bp)	Annealing T°
ACTN3 prom up	AGG TTG AGC AGC TGG AAG G	633	59
ACTN3 prom low	CTG TTC CAT ATA CTC GCC GC		
ACTN3 Exon1 up	CTT TCC CAA GGT CAC ACA GC	633	59
ACTN3 Exon1 low	TCC CCT TGT CAC CCT AAA CC		
ACTN3 Exon2-3 up	ACT AGA GCT CAG GGA GGG AA	635	59
ACTN3 Exon2-3 low	TGT GAG GCA TGG GTG GTT AT		
ACTN3 Exon4-5 up	GAT CTG AAC CCG TGA AGC TG	802	59
ACTN3 Exon4-5 low	CAT TAC CAG ACT TGC GCC AT		
ACTN3 Exon6-7 up	TGG TAA TGA AGG GCC TCA CA	642	59
ACTN3 Exon6-7 low	GGG ACC AAT ATG CTC CCA GA		
ACTN3 Exon8 up	CAG GGA AGA AGA CAC TGG GT	489	59
ACTN3 Exon8 low	CTC CCT GTG TGA TGC CCT TA		
ACTN3 Exon9 up	CTT TGC ATG GGT CCA GGT TT	363	59
ACTN3 Exon9 low	GAG CTT GGA TGG GCA GAA AG		
ACTN3 Exon10 up	GAG ATG GGT GGA TGA GGT GA	400	59
ACTN3 Exon10 low	CCA TCA CGG TTC ACC CAT TG		
ACTN3 Exon11 up	ATC AAC TTC AAC ACG CTG CA	645	59
ACTN3 Exon11 low	CCT TTG GAC ACC TGC TAT GC		
ACTN3 Exon12 up	TAT CAC ACT AGC GCC TCA GG	482	59
ACTN3 Exon12 low	GGG ACA AGT GAT GAT GGG GA		
ACTN3 Exon13-14 up	GCA GGC AAG GAG GAA ATC TG	462	59
ACTN3 Exon13-14low	AGC TTC CCT GTC ATC CCA TC		
ACTN3 Exon15 up	AAA GCG CCA GTT CTT GAG TG	417	59
ACTN3 Exon15 low	TGA GGT TTC AGG GTG GCT AG		
ACTN3 Exon16-17 up	GTA AAT GGT GCA CTG ACC CC	774	59
ACTN3 Exon16-17low	TTA GAC TGC TCT GTG ACC GG		
ACTN3 Exon18-19 up	AAC CTC CAG ATG CGG ACA G	547	59
ACTN3 Exon18-19low	GCG TGA TGA GGA GGA AGT GA		
ACTN3 Exon20-21 up	TCT GTG TGA CTC CAA AGC CT	1052	59
ACTN3 Exon20-21low	TGT TCC CTT CCA CGG TGT AA		
MSTN prom up	TGC CCT GGT AAT AAC AAT GAA GA	1200	58
MSTN prom low	TGC CTG TAC AGT CTG AGA GA		
MSTN Exon1 up	CTG GTG TGG CAA GTT GTC TC	682	58
MSTN Exon1 low	TGC AGC AGA TTT CAG TCT CA		
MSTN Exon2 up	GTT CCT CCA CGG TGT CTC TT	878	59
MSTN Exon2 low	TTA TTG GGT ACA GGG CTG CC		
MSTN Exon3 up	AAC AAG CGT GAA GAG AGG GA	801	59
MSTN Exon3 low	AAT TGT GAG GGG AAG GCC TT		

Table S3: The customized allele-specific PCR assays and primers for the important identified *ACTN3* and *MSTN* in 32 Arabian horses

SNP	Gene	Primer A1	Primer A2	Primer C	Temp
18:66493737	*MSTN*	TAT TAA GTA ATC AGG TTA TAA TGC ACC AAA	ATT AAG TAA TCA GGT TAT AAT GCA CCA AG	CCA GGA CTA TTT GAT AGC AGA GTC ATA AA	57
18:66495696	*MSTN*	ATT CTT TCT ATT TCA AAT GTT TGC CTA AAT AAT	CTT TCT ATT TCA AAT GTT TGC CTA AAT AAC	GAA ATG TTA CTT CCT CAG AAA TTA AGA TTT	57
12:26511704	*ACTN3*	GGG GCC TCG TTA AGT AGC GT	GGG GCC TCG TTA AGT AGC GC	CCC CAT ATT TAG CGC GAA TCC GAT	57
12:26515885	*ACTN3*	GAC CCC TTG ACC TCT CCT CTT A	GAC CCC TTG ACC TCT CCT CTT T	GAT TTT GTG GAA GCG CAT CTT GCC TT	57
12:26524894	*ACTN3*	GTT CTC CAC GCA AGT AGG AGC	GGT TCT CCA CGC AAG TAG GAG T	TGG GAT CAG CCA GAG GGA GCA A	57

Allele Frequency of the Identified Variants

Genes were initially sequenced in 10 endurance Arabian horses which belong to two sub cohorts of high (n=5) and low performance horses (n=5). PCR products were sequenced using the ABI PRISM 310 sequencer (Applied Biosystems). Sequences were edited using the Sequence Scanner v2.0 (Applied Biosystems 2012, USA) as well as the BioEdit software (Hall 1999). The multiple sequence alignments were done using Clustal Omega package (Sievers & Higgins 2014).

The genomic positions of the identified sequence variants were determined according to the *Equus caballus* genome assembly EquCab2 (GCA_000002305.1) and the protein sequence that are available in Ensembl, Release 90, 2017.

The Variant Effect Predictor toolset (Ensembl) was used to determine functional consequences and novelty of the identified variants. Furthermore, we checked both of the transcription factor binding sites (TFBSs) within promoter regions using ConSite online toolset (http://consite.genereg.net) as well as miRNA target sites in the 5´ and 3´ UTRs using miRBase Database, Release 21, with filtering for *Equus caballus* (Griffiths-Jones *et al.*, 2006; Griffiths-Jones *et al.*, 2007).

For determining alleles and genotypes frequencies after identifying the sequence variants and constructing the haplotypes (manually) based on the common variants, we additionally genotyped 32 Arabian horses for the autosomal genes variants with KASP genotyping method. Reagents were obtained from KBioscience (UK), PCR was performed on a StepOnePlus set, (Applied Biosystems, USA) based on a protocol from Kreuzer *et al.*, (2013) (Table S3).

Genotype and allele frequencies were determined by direct counting. A generalized linear model (GLM) was used to estimate the association of the SNPs with endurance performance traits including the mean speeds and the three distances (40, 80, 120 Km) in both performance indices. The GLM was performed using SAS (version 9.3) with the three genotypes of each SNP as independent variables and the endurance traits as the dependent variable.

RESULTS

The comparative sequence analysis of the candidate genes in the 10 endurance Arabian horses led to identify a total of 13 allelic variants in both *ACTN3* and *MSTN*. The five promising SNPs (three SNPs in *ACTN3* and

two SNPs in *MSTN*) were genotyped across the 42 horses (Table S3).

ACTN3

By sequencing the promoter and 21 exons with flanking intron regions of *ACTN3* (approximately 8.979 bp), we found 12 variants: one 5´ UTR variant, five intronic variants, four exonic synonymous variants, and two 3´ UTR variants (Table 2). No change in the amino acid was detected. Three *ACTN3* variants were genotyped further in 32 horses which are 12:26511704G>A, 12:26515885A>T, 12:26524894T>C.

In general, the more frequent variants were the intronic variant 12:26515885A>T (splice region variant), and the exonic variant 12:26524717A>G (synonymous variant) but no significant frequency differences have been detected between the high and low performance groups. One variant in the 3´ UTR (12:26524930T>C) was analyzed for its potential effect on miRNA binding (eca-miR-1296 and eca-miR-326). Additionally, the TFBSs analysis of the *ACTN3* 5' UTR 12:26511704G>A was predicted to cause gaining of an E4BP4 binding site (Table 3).

MSTN

By sequencing the promoter and three exons with their flanking intron of *MSTN* in 10 horses, one transition was detected at 18:66495696A>G within the promoter region. The *MSTN* promoter polymorphism 18:66495696A>G was genotyped in further 32 horses. The alternative allele G frequencies of the variant 18:66495696A>G are listed in Table 2.

The analysis of TFBSs of the *MSTN* promoter substitution of A to G at the position 18:66495696 is predicted to cause substitution of two binding sites for HFH-1 and Sox-5 by two binding sites for HFH-3 and E4BP4 (Table 3).

DISCUSSIONS

Although *ACTN3* is an important functional gene previous studies found variants which infer possible functional changes (Thomas *et al.*, 2014). If we underlay one mutation every 644 to 891 bp in horses (Orlando *et al.*, 2013), we would expect 10 to 14 variants in the *ACTN3* gene. In the current study in Syrian Arabian horses, we detected 12 variants, which is consistent with this expectation assuming an average mutation rate. The *ACTN3* 5' UTR variant 12:26511704G>A showed significant frequencies differences between four equine phenotypes including endurance, sprint, pace, and strength. The A-allele is overrepresented in the strength (Clydesdale and Shire breeds, frequency=77%) and pace horses (Standardbred breed, frequency=69%), compared to sprint (Thoroughbreds, frequency=17%) and endurance horses (American Arabian, 38%) (Thomas *et al.*, 2014). The A-allele frequency in our current study of Syrian Arabian horses (frequency=42%) is consistent with their findings. The variant 12:26511704G>A is predicted to cause gain of a binding site for the E4 promoter–binding protein 4 (E4BP4), a basic leucine zipper transcription factor. E4BP4 regulates circadian rhythm by competing for DNA binding with a member of the related PAR family of basic leucine zipper transcription factors. E4BP4, also known as nuclear factor interleukin 3 (NFIL3) is thought to affect exercise in the skeletal muscles (Bottinelli & Reggiani 2007). Based on findings by Thomas *et al.*, (2014), the *ACTN3* exonic variants 12:26515942C>T (Exon3), 12:26519406A>G (Exon10), and 12:26524717A>G (Exon21) are assigned to three conserved domains (Calpomin homology, Spectrin repeats, and the two EF-hands, respectively), which have an important role in calcium ion binding supporting the protein structure (Djinovic-Carugo *et al.*, 2002; Parry & Squire 2005). The 3´ UTR variants 12:26524894T>C and 12:26524930T>C seems to have no direct effect on the gene function.

Table 2. The detected variants in the autosomal genes *ACTN3* and *MSTN*, their locations and the mutated allele's frequencies

Gene	SNP position	Reference> mutated allele	Amino acid change and positions	Variant effect	SNP ID	Frequency of the mutated allele in Arabian horses			
						Sequenced individuals N=10	Total N=42	High performers N=24	Low performers N=18
ACTN3	12:26511704	G>A	-	5´ UTR	I1 (Thomas *et al.*, 2014)	0.50	0.42	0.33	0.53
	12:26515793	C>G	-	Intronic (I12)	rs68947239	0.45	0.43	0.46	0.39
	12:26515795	C>T	-	Intronic (I12)	rs68947240	0.45	0.43	0.46	0.39
	12:26515807	T>C	-	Intronic (I12)	I4 (Thomas *et al.*, 2014)	0.45	0.43	0.46	0.39
	12:26515885	A>T	-	splice region (I12)	rs68947241	0.45	0.43	0.46	0.39
	12:26515942	C>T	Ile105Ile	Synonymous (E3)	rs68947242	0.45	0.43	0.46	0.39
	12:26516020	G>C	-	splice region(I3)	rs68947243	0.45	0.43	0.46	0.39
	12:26519406	A>G	Pro366Pro	synonymous (E10)	E3 (Thomas *et al.*, 2014)	0.60	-	-	-
	12:26524504	T>C	Ala814Ala	synonymous (E20)	rs394353570	0.95	-	-	-
	12:26524717	A>G	Leu858Leu	synonymous (E21)	E6 (Thomas *et al.*, 2014)	0.30	0.61	0.58	0.64
	12:26524894	T>C	917	3´ UTR	rs396350893	0.70	0.39	0.42	0.36
	12:26524930	T>C	929	3´ UTR	rs396948497	0.30	0.61	0.58	0.64
MSTN	18:66495696	A>G	-	Upstream	(Stefaniuk *et al.*, 2016)	0.15	0.09	0.10	0.08

E: Exon, I: Intron

Frequencies were calculated after aligning the variants, and constructing the haplotypes of *ACTN3* common variants.

Table 3: Potential effects of variants within the untranslated and promoter regions of *ACTN3* and *MSTN* genes on miRNA and transcription factor binding sites (TFBSs)

Gene	Variant position	Location of variant	Effect of the allelic change	
	miRNA gain/loss of binding	**TFBSs*** gain/loss of binding		
ACTN3	12:26511704G>A	5 ´ UTR	-	E4BP4 [+]
ACTN3	12:26524930T>C	3 ´ UTR	eca-miR-1296 [-] eca-miR-326 [-]	-
MSTN	18:66495696A>G	Promoter	-	HFH-1 [-] Sox-5 [-] HFH-3[+] E4BP4[+]

*TFBSs: transcription factor binding sites, [+]=gain, [-]=loss

No clear *ACTN3* allele frequency differences were detected in our study between high and low endurance performance horses. However, all variants in the 5´ and 3´ UTR were analyzed for their potential effects on miRNA binding. Interestingly, one variant in the 3´ UTR of *ACTN3* (12:26524930T>C) is located within two predicted miRNA target sites (eca-miR-1296 and eca-miR-326). The variant C is responsible for abrogation of these miRNA sites, which might affect their post-transcriptional regulation and consequently the gene expression.

In the *MSTN* gene, the promoter variant 18:66495696A>G has been previously reported in different horse breeds including Arabian, Thoroughbred, Polish Konik and Hucul horses (Binns *et al.*, 2010; Dall'Olio *et al.*, 2010; Hill *et al.*, 2010c; Tozaki *et al.*, 2012; Stefaniuk *et al.*, 2014). The G-allele has a frequency of 0.23 in Arabian horses in Poland (Stefaniuk *et al.*, 2014). Furthermore, 18:66495696A>G is suggested to be associated with height at withers in Arabian horses and Uruguayan Creolo horses (Dall'Olio *et al.*, 2012; Stefaniuk *et al.*, 2016). In the current study, the frequency of the alternative allele G was 0.09, and it did not differ considerably between high and low performing groups.

Different studies reported promoter variants of the equine *MSTN* gene. Among those promoter variants, 18:66495826A>G was also found in Hucul, Polish Heavy Draft, and Thoroughbred horses (Stefaniuk *et al.*, (2014). Studies in Thoroughbred horses showed an association between racing performance phenotypes and promoter insertion polymorphisms (227 bp SINE insertion located at -373/-147 bp upstream of the translation start codon ATG). Animals homozygous for the SINE insertion allele were most frequent short distance racing, heterozygous allele carriers were more frequent in horses performing at middle-distance, while homozygous carriers for the wild type allele were most often found in long-distance endurance races (Hill *et al.*, 2010c; Dall'Olio *et al.*, 2014). The SINE insertion is suggested to affect the *MSTN* gene expression (Santagostino *et al.*, 2015). None of the above promoter variants are observed in the Syrian Arabian horses. Interestingly, the intronic variant 18:66493737T>C has been widely known for its strong positive association with short racing distance, speed and body composition in different racehorse breeds (e.g. Thoroughbred) (Binns *et al.*, 2010; Hill *et al.*, 2010a; Hill *et al.*, 2010c; Tozaki *et al.*, 2011; Hill *et al.*, 2012; Tozaki *et al.*, 2012). In all examined Syrian Arabian horses of the current study, this locus was homozygous for the reference genotype (TT). Homozygosity for the T allele was also found in Arabian horses sampled in Poland (Stefaniuk *et al.*, 2016). In another study, a an unique haplotype was found in the second exon (EU241341) present in 12 of 19 Arabian horses (Baron *et al.*, 2012). In our Syrian Arabian horses, the three *MSTN* exons were conserved and identical to the reference. As shown in our study, *MSTN* exons were conserved in 96 Arabian horses from Poland (Stefaniuk *et al.*, 2016).

The TFBSs analysis of the *MSTN* promoter substitution of A to G at the position 18:66495696 predicted to cause a loss of the Helix factor hepatocyte nuclear factor-3 homologue 1 (HFH-1) and the sex-determining region SRY-box 5 (Sox-5) transcription factor binding sites (UniProt), as well as, gaining of both: Hepatocyte Nuclear Factor 3 Forkhead Homolog 3 (HFH-3) and E4 promoter–binding protein 4 (E4BP4). HFH-1 is one of the nuclear factors that share a conserved DNA-binding domain (winged helix domain). It is thought to have an impact on the nerve and skeletal systems in mammals, particularly during the early stages of the embryo development (Altaba *et al.*, 1993; Hong *et al.*, 1999; Hoggatt *et al.*, 2000). HFH-1 can act as inhibitor for the abundant protein found in smooth muscle-specific promoters (Hoggatt *et al.*, 2000). Smooth muscles are not directly involved in the athletic performance, but their

contractions are regulating the internal organs (e.g. blood vessels).

The Sox family of transcription factors is involved in regulating cell development and tissue regeneration (Sarkar & Hochedlinger 2013). The transcription factor Sox-5 (sex-determining region SRY-box 5) is considered to be an enhancer of chondrogenesis; as such it controls the correct development of the skeleton (Smits *et al.*, 2001). Hepatocyte Nuclear Factor 3 Forkhead Homolog 3 (HFH-3 or FoxO1) is a member of the forkhead domain transcription factors family, which are all expressed in skeletal muscle (Sanchez *et al.*, 2014) and involved in a wide range of cellular functions including energy metabolism (Ogg *et al.*, 1997). HFH-3 has a vital role in development the sense of balance, mediating the formation of fatty acids and glycerol to be consumed by muscle cells under exercise, regulating of glucose metabolism in skeletal muscle, as well as, muscle energy homeostasis (Furuyama *et al.*, 2003; Sanchez *et al.*, 2014).

The correlation between endurance traits and *ACTN3* and *MSTN* genotypes was tested by GLM analysis, and we found that none of the analyzed SNPs has significant effect on the endurance traits. Only SNP 12:26511704G>A (located in the 5´UTR of *ACTN3*) has marginally significant effect (0.04) on the mean speed traits in both performance indices.

CONCLUSION

In the current study, we could not detect any significant difference in allele frequencies of the 13 variants in both genes *ACTN3* and *MSTN*, therefore no differentiation was detected between the high and low performance groups. But these variants could influence the transcription factor binding sites (TFBSs) and the miRNAs. Results could not show to which extent the possible effects of the changed transcription binding factor sites have a direct influence on the endurance performance of the Arabian horses.

However, this study contributes to the knowledge of candidate genes that are related to endurance performance in Syrian Arabian horses. Detection of the genetic background of the endurance-related genes in Arabian horses is still a question to be answered. This can be achieved if we test the association between polymorphisms of a multi-gene panel in a larger group of endurance Arabian horses using more endurance data. The information of the candidate genes variants could be beneficial in improving selecting criteria and breeding programs in the future.

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