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Original Research

MSTN, *CKM*, and *DMRT3* Gene Variants in Different Lines of Quarter Horses

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ABSTRACT

Single nucleotide polymorphisms (SNPs) in the equine myostatin (*MSTN*) (g.66493737C>T) and creatine kinase muscle (*CKM*) (g.22999655C>A) genes have been associated with optimum racing distance and muscle development and racing performance in Thoroughbred horses, respectively. Considering that, since its formation, the Quarter Horse breed has received important genetic influence from the English breed, the genes cited become important candidates for athletic performance in the racing line of the American breed. An SNP in the equine doublesex and mab-3-related transcription factor 3 (*DMRT3*) gene (g.22999655C>A) has been described, which is responsible for the gait phenotype in homozygous individuals. Using a sample of 296 Quarter Horses of the racing line and 68 animals of the cutting line, the objective of this study was to compare the frequencies of the three SNPs cited above between a random subsample of animals of the cutting line (n = 20) and animals with extreme phenotypes for racing performance (n = 20 per extreme phenotype). The *MSTN* SNP showed practically no variation, with the observation of only one heterozygous animal (CT) in the cutting line, suggesting that this gene has been under great selective pressure within the racing segment. The *CKM* gene variant studied was found to be polymorphic, but no significant associates were observed between its alleles and the different lines or groups. Two animals carrying the CA heterozygous *DMRT3* genotype were identified in the group with poor racing performance and one in the cutting line, indicating that this variant can be a limiting factor for the development of greater speeds.

1. Introduction

The Quarter Horse breed originated in the beginning of the 17th century as a result of crossings between breeds brought by the English settlers to America and wild horses introduced by Spanish colonizers [1]. As a result of different selection objectives, different segments or lines of the breed currently exist, including the cutting line and racing line. Animals of the racing line show a better performance in short-distance races than any other line or breed and are the fastest horses and one of the fastest animals in the world [1]. The cutting line is destined for functional tests, exploring skills such as agility and obedience, traits that are important for cattle management in the field [2].

As observed for humans [3], athletic performance in horses is likely to be influenced by a large number of genes. However, only few genetic variants have so far been related to this trait exclusively in Thoroughbred animals, including single nucleotide polymorphisms (SNPs) in the myostatin

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gene (*MSTN*) [4–8] and the creatine kinase muscle gene (*CKM*) [9]. The *MSTN* gene is expressed in skeletal muscle tissue and acts as a negative regulator of muscle mass growth. Several mutations or polymorphisms have been identified in the *MSTN* gene of cattle [10–12], sheep [13], mice [14], and humans [15], which result in phenotypes of hyperplasia and muscle hypertrophy. The *CKM* gene encodes a muscle isoenzyme of creatine kinase found exclusively in striated muscle, which is involved in important energy processes in the cell, especially the generation of adenosine triphosphate during the first seconds of intense exercise [16]. Recently, an SNP in the equine doublesex and mab-3-related transcription factor 3 (*DMRT3*) gene has shown a highly significant association with the type of gait. This alteration causes significant changes in the motor coordination of trot and gallop (racing) movements [17].

In view of the role of the genes cited in skeletal muscle and nervous system physiology, the influence of Thoroughbreds on the formation of Quarter Horses and the knowledge that the effect of DNA polymorphisms on phenotypes are intrinsic parameters of each line or breed in a certain environment; the objective of the present study was to compare the frequencies of the *MSTN* g.66493737C>T, *CKM* g.15884567A>G, and *DMRT3* g.22999655C>A SNPs between the racing and cutting lines of Quarter Horses and between animals with extreme phenotypes for racing performance. The results of this study are expected to improve the understanding of the importance of the protein products of the *MSTN*, *CKM*, and *DMRT3* genes for performance-related physiology in horses. Furthermore, the results obtained, through the use of molecular markers, may contribute to the selection of superior Quarter Horses for this trait.

2. Material and Methods

2.1. Animals, Blood Collection, and Performance Data

For this study, 364 Quarter Horses of both sexes, born between 1982 and 2011 and registered at the Brazilian Association of Quarter Horse Breeders, were used. Of these, 296 animals were of the racing line and 68 of the cutting line. The racing animals, including 67 males and 229 females born to 95 stallions and 240 mares, were housed at the Sorocaba Jockey Club and on 14 other properties in the state of São Paulo, Brazil. The cutting horses, including 26 males and 42 females born to 44 stallions and 64 mares, were housed on three properties in the state of São Paulo. Blood was collected on the horse farms in the state of São Paulo and at the Sorocaba Jockey Club. The use of full-sibs in the two lines was not practiced.

Whole blood (5 mL) was collected from each animal by puncture of the left jugular vein in the neck region into vacuum tubes containing 7.5 mg EDTA. Performance data (sped index [SI]) were obtained from the Department of Statistics of the Sorocaba Jockey Club and from the Equibase online database [18]. The SI of several years was available for most of the animals used, and the mean SI was thus calculated. However, only the maximum SI (the best score of the animal obtained during his entire life) was available for other animals. Because the mean SI showed a high correlation with maximum SI ($r = 0.8762$), the latter was used for analysis to prevent the loss of animals. According to Evans [19], the SI was created for Quarter Horse races to permit the comparison of performance between animals under different conditions (distances, racetrack, climate, and country). Details on SI calculation have been described previously by Meira et al [20].

All animal procedures were performed according to Brazilian guidelines of animal well-being (Protocol No. 204/2012-CEUA issued by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science, UNESP, Botucatu, São Paulo, Brazil).

2.2. Determination of Extreme Groups and Genotyping of Animals

For the formation of groups of 20 animals with lower and higher SIs, the phenotypes of 296 animals of the racing line were adjusted for the systematic effects of environment (fixed effects), sex, the interaction between race track (1–14) and distance (228, 275, 301, 320, 365, 402, and 502 m), and the interaction between year of race (1988–2013) and age of animal at race (2, 3, and 4 years). The significance of the effect of sex and of the interactions was tested using the PROC GLM procedure of the SAS v.9.1 program [21].

DNA was extracted from a subsample of 60 animals for subsequent genotyping of the polymorphisms of interest, including 40 animals of the racing line (20 per extreme phenotype) and 20 animals of the cutting line (extracted randomly from the total sample of 68 animals) using the Illustra Blood GenomicPrep Mini Spin Kit (GE Healthcare, USA), according to the manufacturer instructions.

For analysis of the target SNPs of the *MSTN* and *CKM* genes, the polymerase chain reaction (PCR) products, including their flanking regions (463 and 556 bp, respectively), obtained with the primers described in Table 1, were sequenced. The sequences were generated with the ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, USA) and analyzed using the Phred/Phrap/Consed package [22].

The g.22999655C>A SNP of the equine *DMRT3* gene was analyzed by PCR–restriction fragment length polymorphism (RFLP), in which a fragment of 470 bp was amplified and digested with the restriction enzyme *Dde*I

Table 1
Sequences of the primers used for genotyping of the SNPs in the candidate genes analyzed in the sample of animals studied.

Gene/ID	Primer 5′-3′	Genotyping Method
CKM-F	GCATAGGAATATATGCGAATCCT	Sequencing
CKM-R	GAAGAGCATGACGGAGCAG	
DMRT3-F	GGGAACAGAATCACCTCCTG	PCR–RFLP
DMRT3-R	CGACTGGTTTCTTGCCAAAG	
MSTN-F	TATTCTTCTTGGGAGGGAGGACTACT	Sequencing
MSTN-R	GCAAGTAATTAGCACAAAAATTTGAATG	

Abbreviations: *CKM*, creatine kinase muscle; *DMRT3*, doublesex and mab-3-related transcription factor 3; *MSTN*, myostatin; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism.

(New England BioLabs, USA). Each PCR reaction mixture contained 50 ng genomic DNA, 0.25 μM of each primer (Table 1), 0.4 mM of each dNTP, 2.5 mM $MgCl_2$, 1× PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl), and 0.75 U *Taq* DNA polymerase (Fermentas, USA) in a final volume of 25 μL. After the initial denaturation at 95°C for 5 minutes, amplification consisted of 36 cycles at 95°C for 1 minute, 59°C for 30 seconds, and 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. Aliquots (12 μL) of the amplification products were digested with 4 U of the restriction enzyme at 37°C for 12 hours. The DNA fragments obtained by PCR–RFLP were separated on 3% agarose gel in a horizontal electrophoresis system. A 100-bp molecular weight standard was included in each gel to permit calculation of the size of the generated fragments and determination of genotypes.

2.3. Allele and Genotype Frequencies of the SNPs Studied

The allele and genotype frequencies of each genotyped polymorphism were calculated from the genotypes identified on the electropherograms and electrophoresis gels. The allele and genotype frequencies of the *CKM* gene were compared between the cutting and racing lines and between extreme performance phenotypes given by the lowest and highest SI. The associations between alleles and the contrasting groups or lines were evaluated using the odds ratio (OR), 95% confidence interval (95% CI), and *P* value calculated by the χ^2 test. Statistical analysis was performed using the genetics package of the R software [23].

3. Results

There was practically no variation in the g.66493737C>T SNP of the equine *MSTN* gene in the subsample of genotyped animals. Allele C was present in the homozygous condition in all genotyped animals of the Quarter Horse racing line (upper SI, n = 20 and lower SI, n = 20). Only one heterozygous animal (CT) was found in the cutting line (n = 20; f(CT) = 0.05 and f(T) = 0.025).

The g.15884567A>G SNP of the *CKM* gene was found to be polymorphic in Quarter Horses. Analysis of the probability of occurrence of the alternative allele of this polymorphism based on OR and significance assessment by the χ^2 test indicated that this variant is not associated with the different lines (OR, 0.7235; 95% CI, 0.1123–3.3761; *P* value = .7434) or with the contrasting groups of the racing line (OR, 1.1149; 95% CI, 0.2197–5.7905; *P* value = .8764) (Table 2).

With respect to the g.22999655C>A SNP of the equine *DMRT3* gene, individuals carrying genotype CC were characterized by the presence of fragments of 441 and 29 bp. Genotype AA, which was not observed in the subsample analyzed, would present fragments of 221, 220, and 29 bp according to the restriction map. Heterozygous individuals (AC) exhibited the expected combination of fragments of the two homozygous genotypes, that is, fragments of 441, 221, 220, and 29 bp. The fragments of 221 and 220 bp could not be separated, and 28-bp fragment could not be visualized under the electrophoresis conditions of the present study. One of the two alleles of the *DMRT3* polymorphism was practically fixed in the genotyped subsample of Quarter Horse animals. Allele A, which favors the gait phenotype, was not detected in animals with the upper SI (n = 20). However, two heterozygous (AC) animals were observed in the group with lower SI (n = 20; f(AC) = 0.1 and f(A) = 0.05) and one heterozygous animal in the cutting line (n = 20; f(AC) = 0.05 and f(A) = 0.025).

Table 2
Odds ratios for the comparison of allele frequencies between the cutting and racing lines of Quarter Horses and between groups with extreme performance phenotypes in the racing line.

Gene	Allele	Groups (n)[a]		OR (95% CI)	*P* Value
		Cutting (20)	Racing (40)		
CKM	A	0.83	0.78	0.7235 (0.1123–3.3761)	.7434
	G	0.17	0.22		
		Upper SI (20)	Lower SI (20)		
CKM	A	0.77	0.79	1.1149 (0.2197–5.7905)	.8764
	G	0.23	0.21		

Abbreviations: 95% CI, 95% confidence interval; *CKM*, creatine kinase muscle; OR, odds ratio; *P* value, significance of χ^2 test.
[a] Allele frequencies between lines and between groups of contrasting phenotypes in the racing line.

4. Discussion

The results of sequencing of the *MSTN* g.66493737C>T SNP, which is located on equine chromosome 18 (ECA 18), showed that allele C is fixed in the racing line of Quarter Horses. Association tests to verify its relationship with racing performance was therefore not possible. Hill et al [6] genotyped 35 Quarter Horses and also found a high frequency of allele C (0.9) and genotype CC (CC 0.83; CT 0.14; TT 0.03). These results suggest that this *MSTN* polymorphism has been under selective pressure in Quarter Horses. Indeed, a recent study involving 33 breeds (n = 744) detected a selection signature in the region of the *MSTN* gene in Paint Horses and Quarter Horses [24]. Sequencing and histologic analysis showed that the SNP studied (g.66493737C>T), located in intron 1, and a highly correlated (95%) variation in the promoter region (227-bp SINE insertion) were significantly associated with the volume of type IIx (fast glycolytic) muscle fibers [24].

Hill et al [6] investigated the g.66493737C>T SNP near the equine *MSTN* gene in a sample of 148 Thoroughbred horses. The authors identified a strong association of the polymorphism with optimum racing distance, with animals carrying genotype CC being adequate for short-distance (sprint) races, animals with genotype CT for medium-distance races, and animals with genotype TT for long-distance (endurance) races. However, no association between the *MSTN* g.66493737C>T polymorphism and racing performance was found in evaluations of Thoroughbreds from different countries in Europe, North America, and Oceania [25], except for Thoroughbreds from Japan in which a significant association of this SNP with performance ranks and lifetime earnings was observed [8].

The *CKM* gene (ECA 10) was found to be polymorphic in the sample of animals studied, but there were no significant associations of its variants with the racing or cutting line of Quarter Horses or with the groups of contrasting performance phenotypes in the racing line (Table 2). On the basis of their importance for muscle energy metabolism during intense and short exercises [16,26–28], it was expected that *CKM* gene variants would play a major role in the racing performance of Quarter Horses or even that they were associated with different lines of the breed. However, some studies on humans and horses have associated the *CKM* gene with the efficiency of the aerobic muscle energy metabolism. In the study of Echegaray and Rivera [29], polymorphisms in the human *CKM* gene were associated with an increase in cardiorespiratory endurance and maximal oxygen uptake after 20 weeks of training. Gu et al [9] investigated the effect of the g.15884567A>G polymorphism of the equine *CKM* gene on the retrospective racing performance of 148 Thoroughbreds competing in races that were substantially longer than those disputed by Quarter Horses. The authors found allele A to be favorable for the trait, with animals carrying genotypes AA and AG being superior to GG animals.

The relationship of the *CKM* gene with aerobic metabolism may be explained by its interaction with interferon regulatory factor 1 (IRF-1), an oxygen-activated transcription factor involved in mitochondrial biogenesis and metabolism. In humans, this transcription factor has been shown to be significantly more expressed after a period of persistent exercise [30]. Gu et al [9] proposed that the g.15884567A>G SNP of the equine *CKM* gene, which is located in intron 4, disrupts a putative IRF-1 binding site. It is possible that the relationship of the *CKM* gene with aerobic metabolism through its interaction with IRF-1 may therefore explain the no significant association of this SNP with racing performance in Quarter Horses because this type of metabolism is practically absent in races of this breed.

The present study demonstrated the presence of allele A of the *DMRT3* g.22999655C>A polymorphism in Quarter Horses (frequency of 2.5%). In the study of Andersson et al [17], the frequency of allele A ranged from 0.95 to 1.0 in different gaited breeds and was 0 in nongaited breeds. In a subsequent study conducted on 4,396 animals of 141 breeds, including Quarter Horses, the presence of allele A in nongaited breeds was demonstrated for the first time, with frequencies ranging from 2.4% (Quarter Horse) to 14.3% (Colombian Creole) [31]. According to these authors, the presence of allele A of *DMRT3* in breeds that are not generally classified as gaited might be explained by a preference for ambling gaits in the past, by historical crossing with gaited breeds, or by segregation of the mutation in the ancestral population before breed formation. In addition to Thoroughbreds, Arab and Turkish horse breeds brought to North America by English settlers extensively participated in the formation of the Quarter Horse breed, especially the racing line. Observation of the gait allele in Quarter Horses would therefore not be expected. However, the participation of wild horses of Iberian origin in the formation of Quarter Horses should be considered, a means whereby this allele was probably introduced. According to Promerova et al [31], a possible explanation for the current low frequency of the gait mutation in modern Spanish horses is that ambling is considered a negative trait in most Spanish breeds because the use of horses has changed since the time Spanish conquistadors arrived in North and South America. However, some of the current American breeds originating from Iberian breeds are gaited horses, including Mangalarga, Campolina, and Paso Fino, among others.

The *DMRT3* gene, mapped to ECA 23, encodes a transcription factor that plays an important role in the configuration of spinal cord circuits, controlling movement in vertebrates. The g.22999655C>A SNP creates a premature stop codon that results in a truncated protein, which therefore lacks physiological activity [17]. According to these authors, the mutant allele (A) of the *DMRT3* gene may be harmful to speedy breeds, reducing the performance of horses in races. Nevertheless, Promerova et al [31] stated that it will be of considerable interest to study genotype–phenotype relationships between *DMRT3* mutant and wild-type horses of nongaited breeds. The occurrence of the gait allele in the group with poor racing performance and in the cutting line suggests this allele to be a limiting factor for greater racing speeds, although the effect of heterozygosity in speedy breeds has not been demonstrated. Therefore, despite the low frequency of allele A, genotyping for the g.22999655C>A SNP of the *DMRT3* gene in Quarter Horses may be useful to eliminate heterozygotes and to prevent the formation of homozygote mutant horses.

5. Conclusions

Allele C of the g.66493737C>T SNP of the *MSTN* gene, which is related to short-distance racing in Thoroughbreds, was found to be almost fixed in Quarter Horses. This result confirms the relationship of allele C with short-distance racing and possibly with higher body weight of horses.

No significant associations were observed between alleles of the g.15884567A>G SNP of the equine *CKM* gene and the racing or cutting line of Quarter Horses or between these alleles and the groups of contrasting performance phenotypes in the racing line, suggesting that the product of this gene may be more important in predominately aerobic races, such as the Thoroughbreds.

Allele A (gait allele) of the *DMRT3* g.22999655C>A SNP was detected in a nongaited breed selected for speed, in this case Quarter Horses. Although at low frequency, this variant was only observed in animals with poor racing results and cutting, suggesting that even when present in the heterozygous condition, the effect of this variant can be harmful to racing performance.

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