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Effect of selection for eventing on the *MSTN* gene in Brazilian sport horses

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Polymorphisms in MSTN have previously been associated with equine performance. Therefore, the aim of this study was to identify variants in MSTN intron 1 in 16 Brazilian Sport Horses selected for competition in eventing and their possible effects of selection on performance. Among the nine variants identified, eight had already been reported in previous studies or genomic databases, although they showed differences in frequencies when compared with other horse breeds. Moreover, a new mutation was identified in two horses, both in heterozygous form. Considering the absence of molecular studies in this valuable Brazilian breed, these findings represent an important contribution to the characterization of its genetic profile and may possibly aid in further genotype-phenotype association studies.

Key words: *Brazilian horse, myostatin, performance, skeletal muscle*

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Brazilian Sport Horses emerged in 1977, through crossbreeding of the Thoroughbred, Hanoverian, Westfalen, Holsteiner and Trakehner breeds, in order to develop a national breed specifically for show jumping, dressage and eventing [5]. Polymorphisms of the *MSTN* gene were the first to be associated with athletic ability prediction in horses [1, 6, 7]. This gene codifies the Growth Differentiation Factor 8 (GDF8), known as myostatin, which is essential for the regulation of muscle mass growth [10]. Changes in *MSTN* gene expression appear to result in altered oxidative phosphorylation and muscle structure changes in horses [9]. The polymorphism g.66493737C>T (rs.397152648), located in intron 1, is the most powerful athletic performance predictor described to date in Thoroughbreds [7]. Horses with the homozygous genotype CC have been shown to be more efficient in higher-speed, short-distance races; on the other hand, heterozygotes (CT) horses have been shown to perform better in medium-distance races, and homozygotes (TT) have been shown to have higher endurance [6]. Therefore, the aim of this study was to identify the effect of the selection for eventing on the *MSTN* gene, which has been previously associated with equine performance, in Brazilian Sport Horses.

The procedures described in this study were approved by the Ethics Committee for the Use of Animals at the Universidade Federal Fluminense (protocol number 276/2013). The sequence variation analysis was performed according to studies that determined the location of a relevant polymorphisms in this region that were related to the performance of horses [6, 7]. The screening of sequence variations in the intron 1 region of *MSTN* was performed by DNA sequencing in 16 Brazilian Sport Horses that were in training and regularly competing in 3-day events. The two pairs of primers, MSTN_10 and MSTN_11, used for amplification and sequencing of this region were previously described by Hill *et al*. [7] for amplification of the nucleotide sequences at positions chr18:66493261+66493840 and chr18:66493779+66494452, respectively, according to NCBI Genomic Sequence: NC_009161.2, Reference EquCab2.0. The nomenclature used to describe the identified mutations followed the standards established by Den Dunnen *et al*. [4]. Because the Hardy-Weinberg equilibrium (HWE) test demonstrates whether genotypes were randomly sampled from the general population, it was employed as

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Table 1. Allele and genotype frequencies of *MSTN* gene variants identified in the Brazilian Sport Horse (BSH) by the present study and comparisons with the frequencies observed in the Thoroughbred horse (TB) by Hill *et al*. [6]

Study in BSH compared with study in TB horses	Genomic position of variants and nucleotide substitution	Total number of horses studied	Wild-type homozygotes (p^2)	Heterozygotes (2pq)	Mutant homozygotes (q^2)	p	q	F_p	F_q
BSH	18: 66493519 G>A	16	14 (GG)	2 (GA)	0 (AA)	30 (G)	2 (A)	0.937	0.063
BSH	18: 66493525 T>G	16	13 (TT)	3 (TG)	0 (GG)	29 (T)	3 (G)	0.906	0.094
TB		145	138 (TT)	5 (TG)	2 (GG)	281 (T)	9 (G)	0.969	0.031
BSH	18: 66493582 T>G	16	10 (TT)	6 (TG)	0 (GG)	26 (T)	6 (G)	0.813	0.187
TB		138	135 (TT)	3 (TG)	0 (GG)	273 (T)	3 (G)	0.989	0.011
BSH	18: 66493737 C>T	16	1 (CC)	6 (CT)	9 (TT)	8 (C)	24 (T)	0.25	0.75
TB		140	42 (CC)	75 (CT)	23 (TT)	159 (C)	121 (T)	0.568	0.432
BSH	18: 66493745 A>G	16	14 (AA)	2 (AG)	0 (GG)	30 (A)	2 (G)	0.937	0.063
TB		146	139 (AA)	6 (AG)	1 (GG)	284 (A)	8 (G)	0.973	0.027
BSH	18: 66493775 A>G	16	13 (AA)	3 (AG)	0 (GG)	29 (A)	3 (G)	0.906	0.094
TB		145	139 (AA)	5 (AG)	1 (GG)	283 (A)	7 (G)	0.976	0.024
BSH	18: 66494218 A>C	16	9 (AA)	6 (AC)	1 (CC)	24 (A)	8 (C)	0.75	0.25
TB		137	59 (AA)	67 (AC)	11 (CC)	185 (A)	89 (C)	0.675	0.325
BSH	18: 66494302 A>G	16	14 (AA)	2 (AG)	0 (GG)	30 (A)	2 (G)	0.937	0.063
BSH	18: 66494367 G>A	16	9 (GG)	7 (GA)	0 (AA)	25 (G)	7 (A)	0.781	0.219

an initial step for checking the possibility of whether genotype frequencies were not well distributed, based on the allelic frequencies observed. Fisher's exact test was used to compare allelic and genotypic distributions between the Brazilian Sport Horses and Thoroughbreds.

Nine sequence variations were identified in *MSTN* intron 1 (Table 1), one of which had not been described in the literature or registered in the GenBank databases [3, 7]. The new mutation, described as g.66493519G>A, was found in two of the 16 Brazilian Sport Horses and was in the heterozygous form (GA) in both animals.

Interestingly, a smaller number of variants was reported by Dall'Olio *et al*. [3] in a screen of the coding, untranslated, intronic, and regulatory regions of the *MSTN* gene by a DNA sequencing method. Specifically, only seven sequence variants, four of which were in intron 1, were observed in 12 horses from ten different breeds. Thus, in contrast to humans and cattle, the presence of mutations in the *MSTN* coding region that alter the protein function does not appear to be common in horses. In fact, the majority of variants described in equines show effects in regulation of gene expression, since they are mostly located in noncoding regions of the *MSTN* gene.

The sequence variant g.66493737C>T, which was described by Hill *et al.* [7] as the most powerful predictor of performance in Thoroughbreds, was identified in Brazilian Sport Horses with a higher frequency of the T allele (Table 1). This allele was identified in 15 of the 16 horses analyzed; 37.5% of the horses were heterozygotes (TC), and 56.2% of the horses were homozygotes (TT). Only one horse (6.3%) showed the sprinting homozygous genotype (CC). Alternatively, a study of genotypes for this variant in Thoroughbred horses showed a higher frequency of the C allele, with approximately 30% of the horses being homozygotes (CC) and approximately 16% of them being homozygotes (TT) [6]. Considering these findings, it is evident that there is a difference in the allele frequencies of the g.66493737C>T polymorphism between Brazilian Sport Horses (25% "C" allele and 75% "T" allele) and Thoroughbred horses (67.5% "C" allele and 32.5% "T" allele). The genotype frequencies identified in this study did not show evidences of the Hardy-Weinberg equilibrium. The comparison of allelic (T/C) and genotypic (CC, CT and TT) distributions between the Brazilian Sport Horses and Thoroughbreds in relation to polymorphism g.66493737C>T did revealed a difference according to Fisher's exact test ($P<0.01$). Furthermore, a study performed in Quarter Horses revealed that almost all equines studied (except one) were homozygotes CC [11].

These results could be explained by the closed stud book of the Thoroughbred in contrast to the great genetic variability with an inbreeding coefficient close to zero of Brazilian Sport Horses. Another factor that should be highlighted is the small sample size of the present study due to selection of a representative group, which was carefully chosen mainly in order to minimize the chance of relationships between individuals.

The higher frequency of the T allele observed in the Brazilian Sport Horses indicates that this breed has a favor-

able genotype for endurance. Working with a larger sample size, Li *et al*. [8] observed a lower frequency of this allele in native Chinese horse breeds when compared with that in Brazilian Sport Horses. This finding is probably associated with the Chinese breed's distinct phenotypes because numerous mutations described in American and European breeds were not found in these horses. Most of the *MSTN* gene sequence variations found in this study have been described in European horse breeds, which could probably be explained by the fact that Brazilian Sport Horses originated mainly from European breeds. The diverse genetic base of this breed may have been reflected in the large number of unique haplotypes.

Given the importance of the variant g.66493737C>T due to its strong association with equine performance [7] and the different genotypic frequencies found in Thoroughbreds, the results found in Brazilian Sport Horses suggest that there is likely a real difference in the genotypic profile and, consequently, in the performance phenotype between these two breeds of horses. Corroborating this hypothesis, a recent study by Velie *et al*. [13] performed in Icelandic horses suggested that MSTN polymorphisms reflect not only performance but also the general phenotype of breeds selected for specific purposes. Considering the fact that the variant g.66493737C>T is located in an intronic region, its relevant effects on the muscularity phenotype would possibly be explained by a linkage disequilibrium between this and another variant with an effect. Interestingly, Hill *et al.* [7] observed a 227 bp insertion in the *MSTN* promoter region, located only 1605 bp away from the variant g.66493737C>T, that was in concordance with the C allele. Further, Santagostino *et al*. [12] demonstrated that this insertion causes a 5- to 6-fold decrease in gene transcription, suggesting a direct influence of the insertion on myostatin expression.

According to Bower *et al.* [2] there is evidence indicating that introduction of the C-allele occurred at a foundation stage of the Thoroughbred (introduced probably by a local British horse). Thereafter, the frequency of the C allele increased among Thoroughbreds, resulting in a phenotype selected for sprint racing. In contrast, the frequency of the T allele remained high in Brazilian Sport Horses, as observed in thoroughbred ancestors before the introduction of the C allele, resulting in a phenotype selected for long-distance races (endurance).

Another important difference between genotype frequencies identified by Hill *et al.* [7] and those found in this study was related to variant g.66493582T>G. In the analysis of 138 horses, Hill *et al.* [7] identified only three heterozygotes (showing a G allele frequency of 0.01). In this study, six of the 16 horses studied presented the heterozygous genotype for this variant (a G allele frequency of 0.19), suggesting a possible association between this variant allele and the genetic profile of the Brazilian Sport Horse breed.

The present study also revealed that some variants appear to be in linkage disequilibrium, as shown in Fig. 1.

In conclusion, the results of the present study suggest that Brazilian Sport Horses training and competing for eventing show *MSTN* gene variants related to their exercise performance, resulting in animals that are selected for

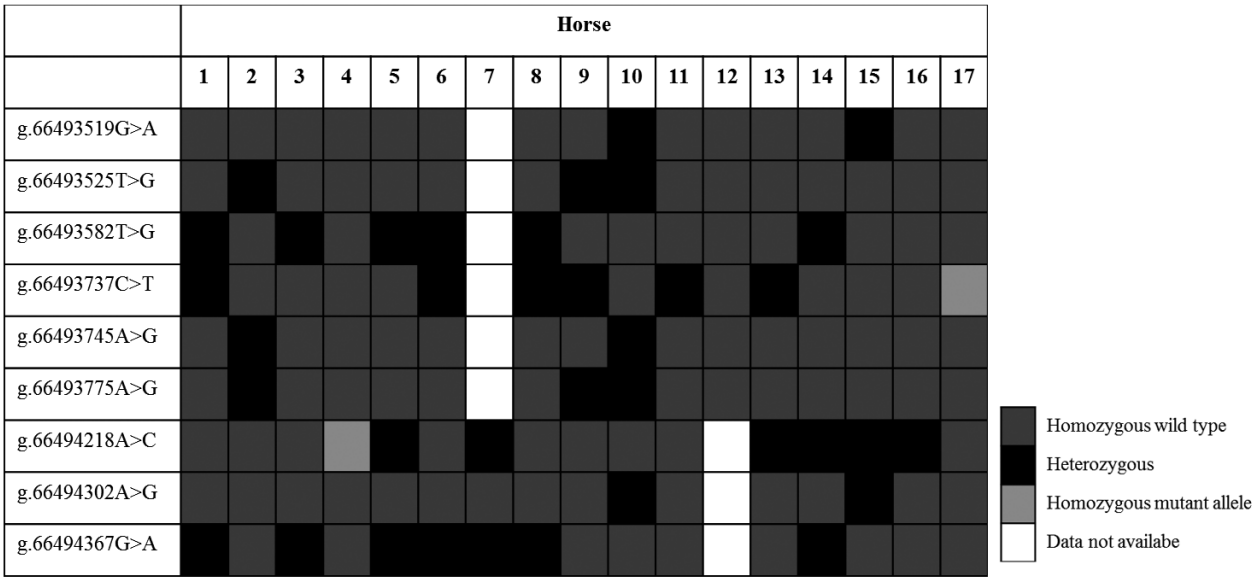

Fig. 1. Genotypes of *MSTN* gene variants identified in Brazilian Sport Horses (BSH).

better competition performance. These findings represent an important contribution to the characterization of the *MSTN* genetic profile of Brazilian Sport Horses and may possibly add to further genotype-phenotype association studies in this valuable Brazilian breed.

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