IJAS Iranian Journal of Applied Animal Science

Polymorphisms of Candidate Genes for Muscle Performance and Male Fertility in Brazilian Mangalarga Horses

Short Communication

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Received on: 13 May 2011
Revised on: 19 Aug 2011
Accepted on: 20 Aug 2011
Online Published on: Jun 2012

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Online version is available on: www.ijas.ir

ABSTRACT

The aims of the present study were to propose a polymerase chain reaction-restriction fragment length polymorphism PCR-RFLP genotyping method for the AY_376689:c.773C>T single nucleotide polymorphism SNP of the equine *PRKAG3* gene, related to muscle performance, as well as to characterize this SNP and a second polymorphism, AAWR_02017454:g.121684T>C of the *SPATA1* gene, related to male fertility, in Mangalarga horses, in order to provide a basis for future studies investigating the association between markers and traits of interest in this breed. For this, 151 Mangalarga horses of both sexes, representatives of the population of the Sate of São Paulo, Brazil, were used. Polymerase chain reaction-restriction fragment length polymorphism PCR-RFLP was found to be adequate for the genotyping of the AY_376689:c.773C>T single nucleotide polymorphism SNP of the *PRKAG3* gene. However, this polymorphism probably does not segregate in Mangalarga horses, a fact impairing association studies with muscle performance traits. The estimates of population genetic parameters obtained for the AAWR_02017454:g.121684T>C polymorphism of the equine *SPATA1* gene demonstrated the possibility of association studies between this marker and traits related to male fertility.

KEY WORDS Mangalarga horses, *PRKAG3*, SNP, *SPATA1*.

INTRODUCTION

The varied use of Brazilian Mangalarga horses (working on cattle farms, endurance riding, racing, non-specialized equestrian sports, horse therapy and equestrian tourism) has somehow impaired the clear definition of criteria to be included in selection programs. However, apart of the modality contemplated some features are always important, including the muscularity and fertility. In this respect, the characterization of the variability in genes related to these traits is the first step towards the marker-assisted selection, an approach that will certainly help breeders in their decision-making as to which animals to send for reproduction.

In equines, studies have reported a relation of the protein kinase AMP-activated gamma 3 non-catalytic subunit gene (*PRKAG3*), annotated on equine chromosome 6, and the spermatogenesis-associated 1 gene (*SPATA1*), annotated on equine chromosome 5, with the physiology of muscle performance and male fertility, respectively. With respect to *PRKAG3* gene, the AY_376689:c.773C>T polymorphism, responsible for an amino acid change in polypeptide chain, showed alleles with different distribution in breeds with contrasting phenotypes for development and muscle performance (Park *et al.* 2003).

The AAWR_02017454:g.121684T>C polymorphism (or BIEC2-968854) of the *SPATA1* gene, mapped in intron 6,

showed a significant association with pregnancy rate per oestrus of mares matted by Hanoverian stallions (Giesecke *et al*. 2009).

The objectives of the present study were to propose a polymerase chain reaction-restriction fragment length polymorphism PCR-RFLP genotyping method for the AY_376689:c.773C>T SNP in the equine *PRKAG3* gene, and, in view of the lack of information about the allele distribution of gene polymorphisms in horse breeds, to characterize this single nucleotide polymorphism SNP and a second polymorphism, AAWR_02017454:g.121684T>C of the *SPATA1* gene in Mangalarga, in order to provide a basis for future studies investigating the association between markers and traits of interest in this breed.

MATERIALS AND METHODS

Whole blood samples (5 mL) were obtained from 151 Mangalarga horses of both sexes, representatives of the population of the Sate of São Paulo, Brazil, in agreement with the Brazilian legislation for animal well-being (protocol No. 111/2008 approved by the Ethics Committee on Animal Experimentation CEEA, School of Veterinary Medicine and Animal Science, São Paulo State University, Botucatu/SP, Brazil).

After removal of red blood cells from the samples, DNA was extracted from leukocytes by a non-phenol method using digestion with proteinase K and precipitation with NaCl and alcohol (Sambroock *et al*. 1989). Genotyping of the *PRKAG3* and *SPATA1* gene polymorphisms was performed by polymerase chain reaction-restriction fragment length polymorphism PCR-RFLP. A fragment of 182 base pairs (bp) comprising exon 8 of the *PRKAG3* gene was amplified using the primers described by Park *et al*. (2003). The reaction mixture contained 50 ng of genomic DNA, 0.2 µM of each primer, 1 x PCR buffer, 1.2 m*M* $MgCl_2$, 0.24 m*M* dNTPs, and 0.5 U *Taq* DNA polymerase (Fermentas, USA) in a final volume of 25 µL.

The amplification conditions consisted of initial denaturation at 94 ˚C for 5 min, followed by 34 cycles at 94 ˚C for 60 s, 66 ˚C for 30 s and 72 ˚C for 30 s, and a final extension step at 72 ˚C for 5 min. Aliquots of the amplification products (10 µL) were digested with 5.5 U of the restriction enzyme *Alu*I (New England Biolabs, USA), at 37 ˚C for 16 h. For analysis of the *SPATA1* gene polymorphism, a 523-bp fragment of intron 6 was amplified and digested with *Rsa*I (New England Biolabs, USA), according to Giesecke *et al*. (2009).

After digestion of the amplified products, the DNA fragments of the *PRKAG3* and *SPATA1* genes were separated on 3% high-resolution agarose gels and 2% agarose gels, respectively. A 100-bp molecular weight marker was included to permit calculation of the size of the amplified and digested fragments, which were visualized by staining with ethidium bromide under ultraviolet light. The genotypes of the individuals were determined by analysis of the size of the fragments in bp.

On the basis of the genotypes identified, allele and genotype frequencies and Hardy-Weinberg equilibrium were obtained for each polymorphism using the PopGene 1.32 program (Yeh *et al*. 1999).

The selective neutrality was evaluated according to the test of *F* parameter of Ewens-Watterson, with Popgene 1.32 and PyPop programs (Lancaster *et al*. 2007). The Popgene 1.32 established a confidence interval for *F* and PyPop verified the significance of the standard deviation between *F* values, expected and observed, by the exact test of Slatkin (Slatkin, 1996).

RESULTS AND DISCUSSION

With respect to the AY_376689:c.773C>T polymorphism of the *PRKAG3* gene, genotype CC was the only genotype detected in the population studied, which is characterized by the presence of three fragments of 118, 45 and 19 bp. However, the smallest fragment (19 bp) could not be visualized under the electrophoresis conditions used. According to the restriction map, genotype TT would be characterized by the presence of four fragments of 79, 45, 39 and 19 bp, and the heterozygous genotype by the presence of five fragments corresponding to the combination of the two homozygous patterns. Figure 1 shows the band pattern obtained by agarose gel electrophoresis for individuals with genotype CC of the *PRKAG3* gene SNP.

The probable C allele fixation of the SNP in Mangalarga horses is consistent with the fact that polymorphisms that segregate in certain breeds may not segregate or may segregate with a low minor allele frequency in others. According to Van Eenennaam *et al*. (2007), allele distributions are as important as the size of the sample for the detection of the effects of gene polymorphisms on traits of interest. This is due to the fact that in association studies, the number of individuals in the groups to be compared, i.e., the number of different genotypes, is related to the allele frequencies. In addition, the occurrence of a favorable allele of a polymorphism in a population is inversely related to its potential increase in breeding programs. Data analysis of AY_376689:c.773C>T SNP of the *PRKAG3* gene was not possible because of the absence of the polymorphism in the animals studied. PCR-RFLP has been shown to be efficient, inexpensive and appropriate for laboratories possessing a basic infrastructure (equipment and reagents) when compared to the method of direct pyrosequencing of PCR products used by Park *et al*. (2003), a fact permitting to extend

the analysis of this polymorphism to breeds in which it segregate.

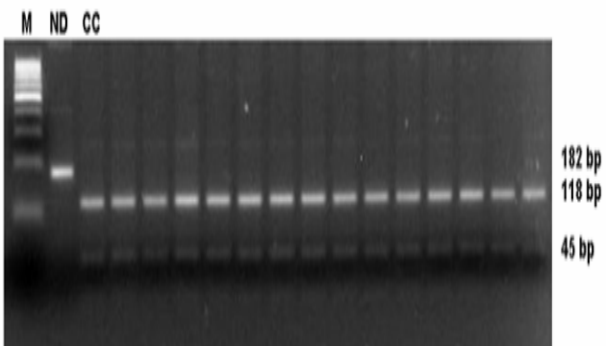

Figure 1 Band pattern obtained for the AY376689:c.773C>T polymorphism of the equine *PRKAG3* gene by PCR-RFLP and electrophoresis. M-100-bp molecular weight marker, ND-amplified DNA not digested with *Alu*I (182 bp), CC-genotype resulting from the digestion of the amplified products with *Alu*I. The numbers on the right indicate the size of the DNA fragments in base pairs

Considering the only report in the literature of study with the AY_376689:c.773C>T SNP of the equine *PRKAG3* gene (Park *et al.* 2003), which found the T allele only in heavy (Belgian) and moderately heavy horse breeds (North Swedish Trotter, Fjord and Swedish Warmblood) but not in lighter breeds selected for racing performance (Standardbred, Thoroughbred and Quarter horse), the lack of detection of this polymorphism in the animals studied was expected. Since the absence of this SNP in Mangalarga horses impairs association studies between this marker and athletic performance in this breed, the use of other polymorphisms described for this gene, the search for new polymorphisms in the DNA sequence of the breed or the search for new candidate genes for the trait is necessary. Although, the AY_376689:c.773C>T polymorphism deserves attention because it is responsible for an amino acid change from proline to leucine at position 258 (Pro258Leu) of the polypeptide chain, a region highly conserved among AMPKγ genes, it was not the only one identified by Park *et al.* (2003). Six other polymorphisms, four of them also causing amino acid substitution, were detected in breeds with contrasting phenotypes for muscle development and performance.

Recently, genome-wide association studies (GWAS) in Thoroughbred breed, have identified new genes related to athletic performance in horses, including the myostatin (*MSTN*) gene (Binns *et al.* 2010; Tozaki *et al.* 2010) and pyruvate dehydrogenase kinase, isozyme 4 (*PDK4*) gene (Hill *et al.* 2010).

With respect to the BIEC2-968854 SNP of the equine *SPATA1* gene, the T and C alleles were detected in the animals studied.

Individuals carrying genotype TT were characterized by the presence of three restriction fragments of 266, 177 and 80 bp, and those carrying genotype CC by the presence of two fragments of 444 and 80 bp. Heterozygous individuals presented fragments of 444, 266, 177 and 80 bp.

The allele distribution of the *SPATA1* gene polymorphism in Mangalarga horses was 0.64 for allele C and 0.36 for allele T. These results are in contrast to those reported by Giesecke *et al.* (2009), the only one in the literature, who found a lower frequency of allele C (0.41) compared to allele T in Hanoverian horses. The genotypic frequencies in present research were 0.40 for genotype CC, 0.48 for CT and 0.12 for TT. The divergent allele frequencies of the *SPATA1* gene polymorphism between Mangalarga and Hanoverian horses may be the result of selection with different intensities for male fertility. In contrast to indirect selection, it may only represents effects of genetic drift. Similar to the study of Giesecke *et al.* (2009) conducted on Hanoverian horses, the calculated chi-square value (0.18) was lower than the tabulated value (3.84), indicating that the locus investigated was in Hardy-Weinberg equilibrium in the population studied. In the Ewens-Watterson test of selective neutrality, the *F* value observed (0.537) was not significant at 5% (p=0.089), being within the range of 0.5-0.99 (interval of *F* with 95% confidence, obtained with one million of simulations), indicating the lack of evidence of preferential matings or of selection favoring one allele over the other. However, this value is very close to the lower limit and is significant at 10%. In this respect, although the Mangalarga population studied was in equilibrium for the *SPATA1* locus, the selective neutrality test did not definitively exclude the potential application of the marker in studies investigating the association with important traits in this breed.

Although, located in an intron region, *in silico* analysis demonstrated that the BIEC2-968854 SNP presents a potential to affect gene regulation since the substitution of nucleotide T with C (T>C) creates a binding site for transcription factor SP1 (Giesecke *et al.* 2009). Even though biological assays confirm the *in vivo* regulatory effect of the mutation in the *SPATA1* gene, different epistatic interactions between candidate genes and the genetic basis of populations and distinct breeds do not permit the association results obtained for Hanoverian animals (Giesecke *et al.* 2009) to be immediately extrapolated to other breeds. Thus, before the transition of markers originally identified in other populations, it is fundamental to confirm their effects on traits of interest in different breeds and environments (Barendse, 2005), a process known as marker validation. This need opens the possibility for future studies involving the Mangalarga breed, among others. In conclusion, PCR-RFLP was found to be adequate for the genotyp-

ing of the AY376689:c.773C>T SNP of the *PRKAG3* gene, although this polymorphism probably does not segregate in Mangalarga horses, a fact impairing association studies with muscle performance traits in this breed. Taken together, the estimates of population genetic parameters obtained for the AAWR_02017454:g.121684T>C polymorphism of the equine *SPATA1* gene in the population studied demonstrated the possibility of association studies between this marker and traits of interest in the Mangalarga breed.

ACKNOWLEDGEMENTS

To CNPq and Fundunesp for financial support provided. To farmers, represented by Raul Sampaio Almeida Prado, for the availability of animals for research.

REFERENCES

Barendse W.J. (2005). The transition from quantitative trait loci to diagnostic test in cattle and other livestock. *Australian J. Exp. Agr*. **45**, 831-836.

Binns M.M., Boehler D.A. and Lambert D.H. (2010). Identification of the myostatin locus (*MSTN*) as having a major effect on optimum racing distance in the Thoroughbred horse in the USA. *Anim. Genet.* **41(2),** 154-158.

Giesecke K., Hamann K., Stock K.F., Woehlke A., Sieme H. and Distl O. (2009). Evaluation of *SPATA1*- associated markers for stallion fertility. *Anim. Genet.* **40**, 359-365.

Hill E.W., Gu J., McGivney B.A. and MacHugh D.E. (2010). Targets of selection in the Thoroughbred genome contain exercise-relevant gene SNPs associated with elite racecourse performance. *Anim. Genet.* **41(2),** 56-63.

Lancaster A.K., Single R.M., Solberg O.D., Nelson M.P. and Thomson G. (2007). PyPop update-a software pipeline for large-scale multilocus population genomics. *Tissue Antig.* **69,** 192-197.

Park H.B., Marklund S., Jeon J.T., Mickelson J.R., Valberg S.J., Sandberg K. and Andersson L. (2003). Molecular characterization and mutational screening of the *PRKAG3* gene in the horse. *Cytogenet. Genome Res*. **102,** 211-216.

Sambroock J., Fritsch E.F. and Maniatis T. (1989). Molecular Cloning: A Laboratory Manual. 2nd Ed. Cold Spring Harbor Laboratory Press, New York, USA.

Slatkin M. (1996). A correction to the exact test based on the Ewens sampling distribution. *Genet. Res*. **68,** 259-260.

Tozaki T., Miyake T., Kakoi H., Gawahara H., Sugita S., Hasegawa T., Ishida N., Hirota K. and Nakano Y. (2010). A genome-wide association study for racing performances in Thoroughbreds clarifies a candidate region near the *MSTN* gene. *Anim. Genet.* **41(2),** 28-35.

Van Eenennaam A.L., Li J., Thallman R.M., Quaas R.L., Dikeman M.E., Gill C.A., Franke D.E. and Thomas M.G. (2007). Validation of commercial DNA tests for quantitative beef quality traits. *J. Anim. Sci*. **85,** 891-900.

Yeh F.C., Yang R.C. and Boyle T. (1999). Popgene version 1.32. Microsoft Windows-Based Freeware for Population Genetic Analysis, University of Alberta, Canada.