Characterization Of Polymorphisms In Candidate Genes For Muscle Performance And Male Fertility In Brazilian Mangalarga Horses

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Introduction

The varied use of Brazilian Mangalarga horses (working on cattle farms, endurance riding, racing, non-specialized equestrian sports, horse therapy, equestrian tourism) has somehow impaired the clear definition of parameters to be included in selection programs in view of the number of factors to be considered. In this respect, the characterization of the variability in genes related to traits of interest for all disciplines in which the breed is involved is the first step toward marker-assisted selection, an approach that will certainly help breeders in their decision-making as to which animals to send for reproduction. Studies have reported an association of the protein kinase AMP-activated gamma 3 non-catalytic subunit (*PRKAG3*) gene and the spermatogenesis-associated 1 (*SPATA1*) gene with the physiology of muscle performance (Park, Marklund, Jeon *et al.* (2003)) and male fertility (Giesecke, Hamann and Stock (2009)), respectively. The objectives of the present study were to propose an alternative genotyping method for the AY_376689:c.773C>T SNP in the equine *PRKAG3* gene by PCR-RFLP, as well as to characterize this SNP and a second polymorphism, AAWR_02017454g.121684T>C (or BIEC2-968854) of the *SPATA1* gene in Mangalarga horses, in order to provide a basis for future studies investigating the association between markers and traits of interest in this breed.

Material and methods

Animals and genotyping. 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. 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, Fritsch and Maniatis (1989)). Genotyping of the *PRKAG3* and *SPATA1* gene polymorphisms was performed by PCR-RFLP. A fragment of 182 bp comprising exon 8 of the *PRKAG3* gene was amplified using the primers described by Park, Marklund, Jeon *et al.* (2003). The reaction mixture contained 50 ng of genomic DNA, 0.2 µM of each primer, 1x PCR buffer [10 mM Tris-HCl, pH 9.0, and 50 mM KCl], 1.2 mM $MgCl_2$, 0.24 mM dNTPs, and 0.5 U *Taq* DNA polymerase in a final volume of 25µL. The amplification conditions consisted of initial denaturation at 94 ºC for 5 min, followed by 34

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cycles at 94 ºC for 60 s, 66 ºC for 30 s and 72 ºC for 30 s, and a final extension step at 72 ºC for 5 min. Aliquots of the amplification products (10 µL) were digested with 5.5 U of the restriction enzyme *Alu*I 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, according to Giesecke, Hamann and Stock (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 base pairs.

Statistical analyses. On the basis of the genotypes identified, allele and genotype frequencies, Hardy-Weinberg equilibrium and selective neutrality (Ewens-Watterson test) were obtained for each polymorphism using the PopGene 1.32 program (Yeh, Yang and Boyle (1999)).

Results and discussion

***PRKAG3* gene polymorphism.** 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 smaller 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.

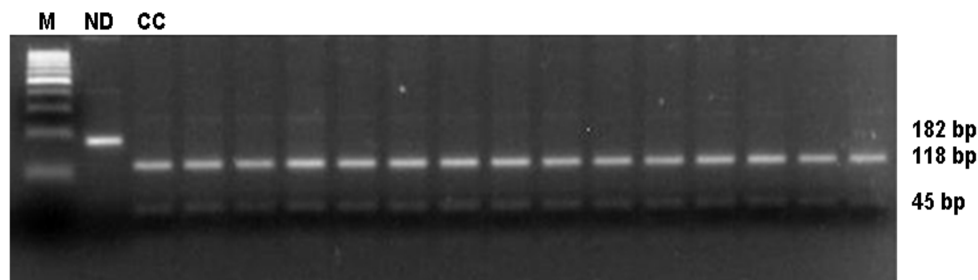

Figure 1: Band pattern obtained for the AY376689:c.773C>T polymorphism of the equine *PRKAG3* gene by PCR-RFLP and electrophoresis on 3% high-resolution agarose gel. M - 100-bp molecular weight marker, ND - amplified DNA not digested with *Alu*I (182 bp), CC - genotype resulting from the digestion of the amplified products with *Alu*I. The numbers on the right indicate the size of the DNA fragments in base pairs.

The probable absence of the SNP in Mangalarga horses is consistent with the fact that polymorphisms that segregate satisfactorily in certain breeds may not segregate or may

segregate inadequately in others. According to Van Eenennaam, Li, Thallman *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. Statistical analysis 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 used by Park, Marklund, Jeon *et al.* (2003), a fact permitting to extend the analysis of this polymorphism to breeds in which it occurs. Considering the results reported by Park, Marklund, Jeon *et al.* (2003) who found the T allele of the AY_376689:c.773C>T SNP of the *PRKAG3* gene 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 not unexpected. Since the absence of this SNP in Mangalarga horses impairs association studies between this marker and important traits in this breed, the use of other polymorphisms described for this gene or the search for new polymorphisms in the DNA sequence of the breed 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, Marklund, Jeon *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.

***SPATA1* gene polymorphism.** 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, Hamann and Stock (2009) who found a lower frequency of allele C (0.41) compared to allele T in Hanoverian horses. The genotype frequency in present research was 0.40 for genotype CC, 0.48 for CT and 0.12 for TT. The discrepant allele frequencies of the *SPATA1* gene polymorphism between Mangalarga and Hanoverian horses might be indicative of the relationship of this polymorphism with phenotype differences between breeds and, in contrast to indirect selection, may only represent effects of genetic drift. Similar to the study of Giesecke, Hamann and Stock (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 (0.54) was within the range (0.5-0.99), 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. 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, Hamann and Stock (2009)). Although 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 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.

Conclusion

PCR-RFLP was found to be adequate for the genotyping of the AY376689:c.773C>T SNP of the *PRKAG3* gene, although this polymorphism probably does not occur in Mangalarga horses, a fact impairing association studies with muscle performance traits in this breed. Taken together, the genetic parameters obtained for the AAWR_02017454g.121684T>C polymorphism of the equine *SPATA1* gene in the population studied demonstrated the possibility of association studies between this marker and traits related to male fertility in the Mangalarga breed.

References

Barendse, W.J. (2005). *Aust. J. Exp. Agric.*, 45:831–836.

Giesecke, K., Hamann, K., and Stock, K.F. (2009). *Anim. Genet.*, 40:359–365.

Park, H.B., Marklund, S., Jeon, J.T. *et al.* (2003). *Cytogenet. Genome Res.*, 102:211–216.

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

Van Eenennaam, A.L., Li, J., Thallman, R.M. *et al.* (2007). *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.