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Short Communication

A Genome-Wide Association Study Reveals Differences in the Genetic Mechanism of Control of the Two Gait Patterns of the Brazilian Mangalarga Marchador Breed

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ABSTRACT

The *DMRT3* gene is described as the main gene involved in the determination of gait phenotypes in horses, and the allele A of the 22999655C>A single nucleotide polymorphisms (SNP) has been reported as a causal variant of this trait. In the Mangalarga Marchador breed, which exhibits two gait patterns with well-defined characteristics, genotypes AA and CA are associated with marcha picada and genotype CC with marcha batida. In this breed, allele A of the *DMRT3* gene is only related to the marcha picada gait. The objective of this study was to identify the type of control of the marcha batida gait and to investigate SNPs and genomic regions responsible for this phenotype in Mangalarga Marchador horses. Forty-eight horses belonging to the two gait groups, marcha picada with AA and CA genotypes of the 22999655C>A SNP (n = 20) and marcha batida with CC genotype (n = 28), were analyzed using the Equine SNP70 BeadChip. The genome-wide association study result shows for the first time that, in contrast to the marcha picada gait phenotype that is apparently determined by a single gene (*DMRT3*) in which allele A of variant g.22999655C>A controls the trait, the marcha batida gait is controlled by a larger number of genes. Because of the small number of animals used in the two groups compared, the genomic regions associated with smaller effects on the marcha batida gait could not be identified.

1. Introduction

The Mangalarga Marchador (MM) breed was officially declared as the Brazilian National Horse and is the most numerous equine breed in Brazil. The breed emerged about 200 years ago in southeastern Brazil from the crossing of Alter horses from the "Coudelaria de Alter do Chão" situated in Portugal with other native horses selected by local breeders. The Alter breed has roots in the Spanish Andalusian, whose ethnic origin comes from native horses of the Berber, Germanic, and Iberian Peninsula [1]. In December 2015, the Brazilian Mangalarga Marchador Breeders Association featured 11,400 associated members, over 600,000 registered horses, 67 regional breeders organizations in Brazil, and 4 abroad (Germany, Italy, Argentina, and USA). Mangalarga Marchador horses have been exported to Belgium, Holland, Portugal, Israel, Canada, Uruguay, Peru, USA, Germany, Congo, Argentina, France, Austria, and Italy.

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The *DMRT3* gene is described as the main gene involved in the determination of gait phenotypes in different horse breeds. The 22999655C>A single nucleotide polymorphisms (SNP) of this gene, which is responsible for alterations in the locomotion pattern of horses, was described by Anderson et al [2] in studies involving gaited and nongaited breeds. The recessive A allele of this variant has been described to be responsible for gait in the Icelandic horse. A subsequent study including different breeds from various countries confirmed the predominant action of this gene on gait phenotypes. However, some gaited breeds showed high frequencies of allele C, including the Brazilian Mangalarga Marchador breed in which the frequencies of allele C and genotypes AC and CC were high [3].

The relationship of allele A of the *DMRT3* gene with gait types in the MM breed was described by Patterson et al [4]. Genotypes CC and AA were associated with marcha batida and marcha picada, respectively. The heterozygous genotype (AC) was identified in individuals with both marcha batida and marcha picada gait. However, the proportion of heterozygous individuals was much higher among animals with marcha picada (43.3% vs. 6%). Thus, the results found by Patterson et al [4] suggest that the *DMRT3* gene is only related to marcha picada and it may not be the only gene responsible for gait phenotype in the Brazilian Mangalarga Marchador breed.

In view of the above considerations, the objectives of this study were to identify the type of control of gait phenotype (monogenic or polygenic) by genome-wide association study (GWAS) and to perform prospecting for SNPs and genomic regions that contain possible genes responsible for the marcha batida phenotype in the Brazilian Mangalarga Marchador breed.

2. Material and Methods

Blood samples were collected from 55 horses (34 marcha batida and 21 marcha picada) participating in the 34th National Mangalarga Marchador Show. The study was conducted according to ethical principles in animal experimentation (UNESP, Jaboticabal, Brazil; Approval number: 12,807/15).

The typical gait of the MM breed is the stepping four-beat marcha, with alternate lateral and diagonal support interspersed by moments with triple support [5]. The lack of suspension favors the stability of the animal's torso, provides more comfort to the rider, and is favorable to activities like cattle work, cavalcade, working equitation, therapeutic riding, and Marcha Competitions. Marcha batida and marcha picada are the only intermediate-speed natural gaits allowed in the MM breed, and the horses are classified according to the gait by visual evaluation of the Brazilian Mangalarga Marchador Breeders Association official technicians at the moment of breed registration [5]. Marcha picada has a higher proportion of lateral and triple support and a lower proportion of diagonal support compared to marcha batida [6]. Marcha picada is visually compared to a "broken pace," and marcha batida is compared to a "broken trot" although the two marcha gaits have no suspension moment and excess of lateral or diagonal supports with triple support suppression is undesirable.

After DNA extraction, genotyping of the g.22999655C>A SNP of the equine *DMRT3* gene was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) according to Pereira et al [7], in which a 470-bp fragment was amplified and digested with endonuclease *Dde*I (New England BioLabs, USA). From the total of 55 individuals sampled, only 48 were considered to the GWAS analysis and were distributed into two groups of 28 and 20 samples each. The first group consisted of marcha batida horses with genotype CC and the second of marcha picada horses with genotypes AA (n = 11) and CA (n = 9). The DNA samples of each individual were genotyped using the Equine SNP70 BeadChip (Illumina, Inc, USA).

Quality control of the genotyping data for the individuals and SNPs was performed using the Genome Studio 2011.1 (Illumina, Inc, USA) and PLINK v1.07 [8] programs. Animals with a call rate <0.95 or a gender-labeling error were excluded from the sample. Additionally, SNPs located on the X chromosome and SNPs with a low genotyping quality (cluster separation < 0.3), call frequency < 0.9, minor allele frequency < 0.1 (including fixed SNPs), and *P* value for Hardy–Weinberg equilibrium $<1 \times 10^{-4}$ were excluded.

To adjust for inflation due to population stratification (genomic inflation factor = 1.12488), structured association by genetically matching cases and controls using identity-by-state (IBS) similarity was implemented in PLINK v1.07 [8]. Stratified case-control association analyses were performed using Cochran–Mantel–Haenszel tests ($2 \times 2 \times K$, $K = 4$), adjusted for the population substructure by inclusion of the IBS cluster information [8]. Multiple tests were corrected by the FDR-BH (false discovery rate–Benjamini Hochberg) [9]. Metric multidimensional scaling was performed by the $N \times N$ matrix of genome-wide IBS pairwise distances [8].

3. Results

The following genotype frequencies were observed for the 55 animals analyzed by PCR-RFLP of the g.22999655C>A SNP of the *DMRT3* gene: f(CC) = 0.94/0.05, f(CA) = 0.06/0.43, and f(AA) 0/0.52 in the marcha batida (n = 34) and marcha picada (n = 21) groups, respectively.

Because none of the individuals had a call rate < 0.95 and gender-labeling error, no animal was excluded from the sample. After application of the quality control filters to the markers, 25,252 of the 65,157 SNPs present on the Equine SNP70 BeadChip were excluded. Thus, 48 individuals and 39,905 SNPs were considered for the subsequent analyses.

Substructures were observed in the sample, but these clusters are not associated with individuals with different gait types, showing that marcha picada and marcha batida groups are part of the same population (Fig. 1A). Despite the small sample size used in this study, Q–Q plot (Fig. 1B) showed a satisfactory fitness of frequency distribution of data to a normal distribution.

The GWAS results are illustrated in the Manhattan plot shown in Fig. 1C, in which significant SNPs were

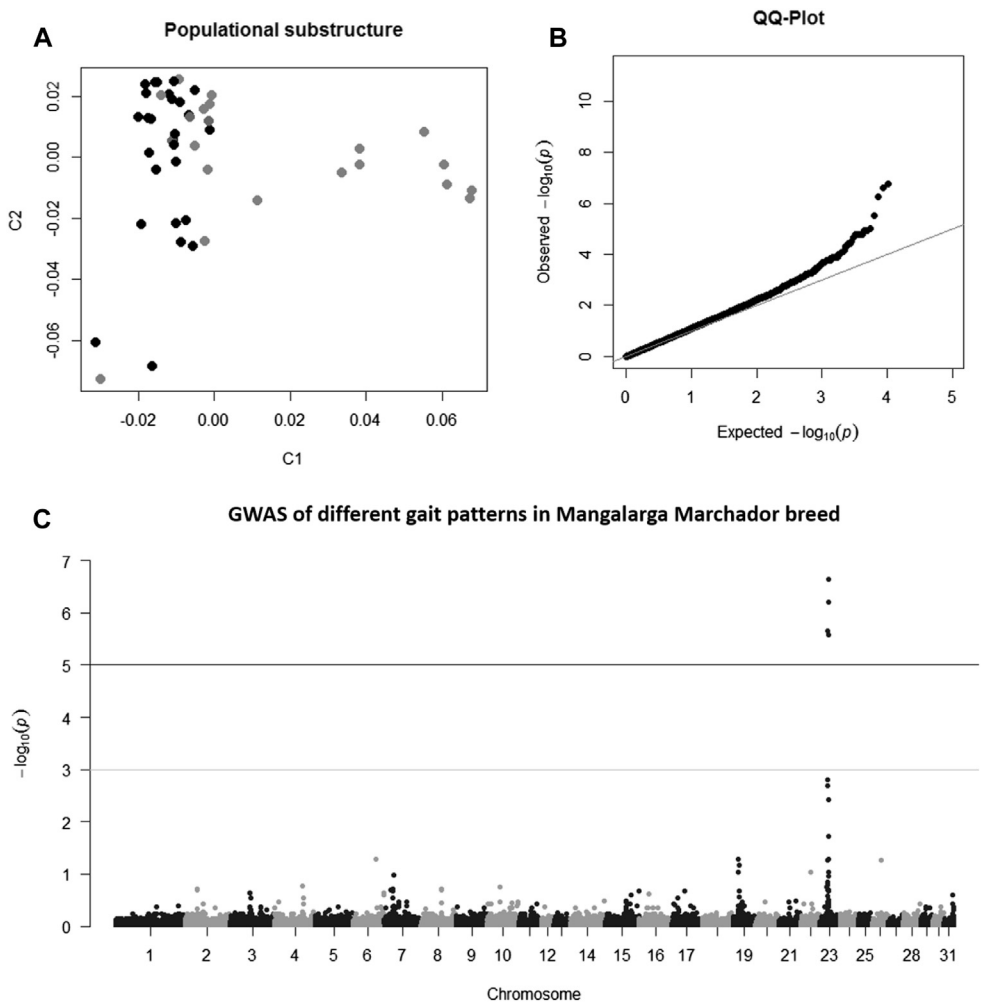

Fig. 1. (A) MDS plot of all samples (marcha batida and marcha picada groups). Each point in the MDS plot corresponds to an individual, and different grayscale shades indicate to which cluster (k = 4) the individuals belong. The position in the Cartesian plane shows the genetic distance among the horses. (B) Q–Q plot indicates the fitness of frequency distribution of data to a probability distribution. (C) Manhattan plot of all single-nucleotide polymorphisms for gait type in the Brazilian Mangalarga Marchador breed. The inverse log *P* values estimated for each polymorphism are plotted on the y-axis. The chromosome number is plotted on the x-axis. The black line indicates the significant threshold ($P < 10^{e-5}$). MDS, metric multidimensional scaling.

situated above the black line. Four markers were significantly associated ($P < 10^{e-5}$) with marcha batida and marcha picada, all of them located on *Equus caballus* autosomal chromosome (ECA) 23 (Table 1). All markers located on ECA 23 are mapped in the region of the *DMRT3* gene. It is noteworthy that the BIEC2_620109 SNP of significant association ($P = 2.3^{e-7}$) was linked to SNP g.22999655C>A.

Table 1
Allele (A_1/A_2) and genotype ($A_1A_1/A_1A_2/A_2A_2$) frequencies of SNPs with significant association in the marcha batida and marcha picada groups of the Mangalarga Marchador breed.

Chr.	SNP_ID	Position (bp)	A_1	A_2	Allele/Genotype	Marcha Picada	Marcha Batida	*P*
ECA 23	BIEC2_620109	22,967,656	A	G	Allele	0.78/0.22	1/0	2.31×10^{-7}
					Geno.	0.55/0.45/0	0/0/1	
ECA 23	BIEC2_651731	22,866,017	A	G	Allele	0.80/0.20	0.96/0.04	6.29×10^{-7}
					Geno.	0.60/0.40/0	0/0.07/0.93	
ECA 23	BIEC2_619750	22,464,604	G	A	Allele	0.85/0.15	0.91/0.09	2.24×10^{-6}
					Geno.	0.70/0.30/0	0/0.18/0.82	
ECA 23	BIEC2_651671	22,756,820	A	G	Allele	0.83/0.18	0.91/0.09	2.62×10^{-6}
					Geno.	0.65/0.35/0	0/0.18/0.82	

Abbreviations: Chr., equine chromosome on which the marker was mapped; ECA, *Equus caballus* autosomal chromosome; *P*, corrected *P* value; SNP_ID, SNP identification.

4. Discussion

The genotyping results obtained by PCR-RFLP for the g.22999655C>A SNP of the *DMRT3* gene confirmed that the marcha batida gait is associated with genotype CC and the marcha picada gait with genotypes AA and AC. However, this study identified for the first time an animal with marcha picada carrying genotype CC. This finding can be explained by the possible influence of training on gait pattern and the consequent change in gait type. In a pilot study [10], lowering the neck exercise produced different effects on marcha batida and marcha picada gaits in the MM breed. In marcha picada horses, the exercise increased the laterality of walking, approaching its characteristics to that of pace gait, which is undesired in the breed [10]. Thus, horses that do not exhibit pronounced gait phenotypes, either marcha batida or marcha picada, may be able to alter their natural gait type through conditioning. This fact has other important implications because the classification of gait type in animals of this breed is done empirically by visual assessment. An inadvertent error in the classification of gait type can cause animals with genotypes related to marcha batida be classified as animals with marcha picada.

The stratification analysis results showed that individuals with different gait patterns are not from distinct populations. It is instead a single population in which animals may be born with one phenotype or another (marcha picada or marcha batida). These results are in agreement with Study Book information from the MM breeders association.

In contrast to the marcha picada gait that is apparently determined by variation (g.22999655C>A) in a single gene (*DMRT3*) in the MM breed, the results of GWAS obtained here indicate that the marcha batida gait is controlled by a larger number of genes. As SNPs in *DMRT3* gene region were significantly associated with the marcha picada gait, a similar situation would be expected for the other phenotype (i.e., significant associations from other markers with the marcha batida gait) if this trait were governed by a gene with great influence. However, because of the small number of animals used in the two groups compared, it was not possible to identify SNPs and genomic regions associated with smaller effects on the marcha batida gait. Future studies with larger animal samples and resequencing of points of interest in the genome will show chromosomal regions related to this type of gait.

Another interesting finding in this study was that genotype GG of SNP BIEC2_690834 (ECA 26) of the Equine SNP70 BeadChip was only detected in the marcha batida group, in 100% of its individuals. This marker was near-significant associated with marcha picada and marcha batida ($P = .054$) and was found to be very close (only 71.06 kb considering equine genome assembly EquCab2.0) to amyloid beta (A4) precursor protein gene (*APP*), whose protein product plays an important role in neurological and locomotor functions [11,12]. This was the unique gene found in a 400 kb window surrounding the SNP BIEC2_690834.

5. Conclusion

In contrast to the marcha picada phenotype, which is apparently controlled by only one gene (*DMRT3*), the results of the present study indicate that the marcha batida gait may be controlled by a larger number of genes in the Brazilian Mangalarga Marchador breed. Because of the small number of animals used in the two groups compared (marcha picada and marcha batida), SNPs and genomic regions associated with smaller effects on the marcha batida gait could not be identified.

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