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SHORT COMMUNICATION

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DMRT3 is associated with gait type in Mangalarga Marchador horses, but does not control gait ability

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Summary

The Mangalarga Marchador (MM) is a Brazilian horse breed known for a uniquely smooth gait. A recent publication described a mutation in the *DMRT3* gene that the authors claim controls the ability to perform lateral patterned gaits (Andersson *et al.* 2012). We tested 81 MM samples for the *DMRT3* mutation using extracted DNA from hair bulbs using a novel RFLP. Horses were phenotypically categorized by their gait type (batida or picada), as recorded by the Brazilian Mangalarga Marchador Breeders Association (ABCCMM). Statistical analysis using the PLINK toolset (Purcell, 2007) revealed significant association between gait type and the *DMRT3* mutation ($P = 2.3e\text{-}22$). Deviation from Hardy–Weinberg equilibrium suggests that selective pressure for gait type is altering allele frequencies in this breed ($P = 1.00e\text{-}5$). These results indicate that this polymorphism may be useful for genotype-assisted selection for gait type within this breed. As both batida and picada MM horses can perform lateral gaits, the *DMRT3* mutation is not the only locus responsible for the lateral gait pattern.

Keywords Associação Brasileira dos Criadores do Cavalo Mangalarga Marchador, batida, equine, gaited horse PCR-RFLP, marcha, natural gait, picada, triple support

The Mangalarga Marchador (MM) is a Brazilian gaited horse breed known for its smooth and unique gaits. It is also the national horse of Brazil and is the most numerous of the Brazilian horse breeds with over 600 000 registered animals as stated by ABCCMM in 2013. In Brazil, MMs must pass rigid standards for conformation, gait, performance and endurance (USMMA 2013). Those standards are set by a regulation book and are supervised by the Associação Brasileira dos Criadores do Cavalo Mangalarga Marchador (ABCCMM) and the Brazilian Department of Agriculture (MAPA) (ABCCMM, 1998; MAPA, 2000). While traveling at a moderate speed (5–10 m/s), a horse using the 'batida' gait places the feet in diagonally coupled footfalls more frequently than laterally, although moments of triple-limb support exist (Video S1). If footfalls are instead more often laterally coupled, rather than diagonally, without loss of triple support moments, the gait is termed 'picada' (Video S2, Hendricks 2007). The quality of gait in the MM is certified by a system of official registration inspections and organized competitions that have been in place for more than three decades in Brazil. To be registered, the MM is inspected twice by a technician (veterinary or animal science professional) trained and authorized by the ABCCMM and MAPA. The first provisory inspection is carried out before 2 years of age to validate parentage and health. In a second inspection, horses over 3 years of age are ridden by the inspector and must fulfill all requirements set by the breed standard, for example, no moments of suspension or excessive lateral movement within the gait. Only horses passing the second inspection are admitted to the registry, allowing for competition in ridden classes and registration of offspring. These regulations do not permit the trot or pace (ABCCMM and MAPA 2011).

A recent publication described a mutation in the *DMRT3* gene which the authors claim controls the ability of a horse to perform lateral patterned gaits (Andersson *et al.* 2012). The MM breed, with its distinct gait types, presents a good model to better describe the effect of this locus on gait pattern. Samples were collected from 120 registered MM horses at the annual national competition and from several farms in Brazil. All horses were categorized as either batida or picada gait type based on ABCCMM registration records. DNA was extracted from hair bulbs using the Puregene DNA isolation kit (Gentra Systems Inc.) according to the

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manufacturer's published protocol. DNA was amplified by PCR using FastStartTaq DNA Polymerase and included all reagents per the manufacturer's recommended conditions (Roche Diagnostics), with the primers obtained from Andersson *et al.* (2012) to produce a product of 681 bp. Thermocycling on an Eppendorf Mastercycler Ep Gradient was also according to the manufacturer's recommendations with an annealing temperature of 58 °C and a total of 40 cycles. For convenience, we devised a novel RFLP test for the causative polymorphism reported in Andersson *et al.* (2012), using DdeI (1.0 U per reaction; New England Biolabs Inc.) incubated at 37 °C overnight. The resulting products were visualized by electrophoresis following standard conditions on a 3% agarose gel (Omnipur Agarose; EMD Chemicals Inc.). The wild-type allele produces 31-, 73- and 577-bp fragments following digestion, whereas the novel allele produced 31-, 73-, 145- and 432-bp fragments.

Pedigree information, as contained in the registry, was recorded for each horse. A total of 81 horses unrelated by a single generation were selected for genotyping, of which 44 were classified phenotypically as batida and 37 as picada. PLINK v1.07 (Purcell, 2007) was used to test allelic association between the *DMRT3* genotype and picada/batida gait type as well as deviation from Hardy–Weinberg equilibrium (Table 1). The *DRMT3* genotype is associated with batida/picada gait type, as determined by ABCCMM inspectors ($P = 2.3e\text{-}22$) (Fig. 1). However, this population is not in Hardy–Weinberg equilibrium ($P = 1.0e\text{-}5$) and exhibits an excess of homozygous genotypes. This excess of homozygous individuals may be the result of selective breeding for gait phenotypes or could reflect a genome-wide trend resulting from excessive inbreeding. Therefore, we evaluated the ABCCMM four-generation pedigree for inbreeding within the 81 sampled MM horses (PEDIGRAPH v2.4; Garbe & Da 2008). Pedigree inferred inbreeding coefficients were low in both batida and picada horses, 1.2% (range 0.56–1.8%) and 0.16% (range −0.50% to 0.80%) respectively, and differed between the two groups (Student's *t*-test, $P = 0.029$). Higher inbreeding in the batida may contribute to elevated homozygosity across the genome, including the *DMRT3* locus. However, this does not explain the mirrored elevation for the opposite homozygous genotype in the picada, which may reflect the action of selective breeding for gait type.

Table 1 Allele frequencies for each gait type and inbreeding levels in the Mangalarga Marchador.

	CC	*CA*	*AA*	*N*	*F*	Range
Picada	0	16	21	37	0.0016*	0.005–0.018
Batida	41	3	0	44	0.012*	0.0056–0.018
Total	41	19	21	81		

CC, homozigous wild type; *CA*, heterozygous mutant; *AA*, homozygous mutant; *N*, number of horses for each gait type; *F*, inbreeding coefficient mean within gait type; Range, range of inbreeding coefficient within gait type; inbreeding between the two groups was significantly different: *$P = 0.029$.

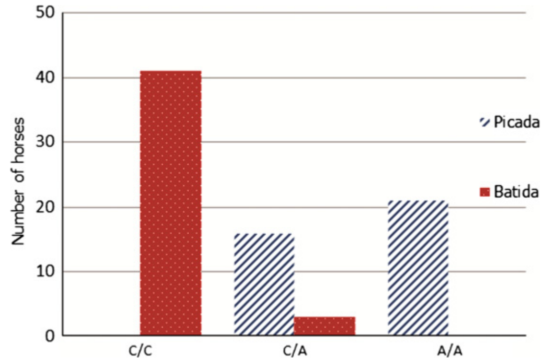

Figure 1 Genotype distribution by gait type within the analyzed population of Mangalarga Marchadors.

We demonstrate that the *DMRT3* mutation is segregating in a gaited horse breed exhibiting lateral-coupled footfalls. Although it has been previously reported that six other gaited breeds are nearly fixed for the homozygous mutant (Andersson *et al.* 2012), additional genotyping of 141 breeds from throughout the world identified the *DMRT3* mutation at various frequencies in both gaited and non-gaited breeds (Promerová *et al.* 2014). However, breeds in this report were classified as gaited based on a 50% or greater frequency of the mutation and not based on observation of the gait phenotype. In this latest study, the MM breed was classified as only partially gaited based on heterogeneity at the *DMRT3* locus in a small sampling of 22 horses (Promerová *et al.* 2014). The *DMRT3* mutation is likely fixed in the cited US breeds for two reasons: First, these breeds share a common ancestry, and second, the US breeds used are not often evaluated for quality of the three-beat canter. Thus, possessing the *DMRT3* allele could lead to more stance time spent in lateral-coupled footfall, which could be an advantage in US competitions. Both the Paso Fino and Peruvian Paso derived their ambling gait from the Spanish Jennet, a breed introduced to the Americas in 1493 (Hendricks 2007). All of the American gaited breeds are likely descendants of these Spanish horses. The MM also traces its ancestry back to the Spanish Jennet (Hendricks 2007).

In the *DMRT3* mouse knockout, gait analysis revealed increased stride length and swing times as well as uncoordination between front and hind limbs (Andersson *et al.* 2012), but no instances of lateral-coupled footfalls were observed by the authors. In the Icelandic horse, the pace has a tendency for significant asymmetry between subsequent flight phases, resulting in a broader distribution of footfall ratios (Robilliard *et al.* 2007). For this reason, the fifth gait of the Icelandic is often called the 'flying pace' Five Natural Gaits. United States Icelandic Horse Congress (USIHC). as it can be a broken two-beat gait where one foot lands before the other and not together, as occurs in a true pace. Both four- and five-gaited Icelandic horses exhibit the 'tölt', a four-beat lateral gait (Ziegler 2005), yet only

31% of the four-gaited horses possess a homozygous *AA* genotype at *DMRT3* (Andersson *et al.* 2012). Homozygous *AA* genotypes are observed in both trotting and pacing Standardbred racehorses (Andersson *et al.* 2012), suggesting that the mutation likely controls transitioning gait into the canter or the ability to coordinate the three-beat diagonal-coupled footfall pattern at high speeds. This hypothesis is supported by the observation by Andersson *et al.* (2012) that 'Icelandic horse homozygous mutants had inferior scores for gallop', and examination of the supplementary tables of that study also reveals differences ($P = 0.07$) between *C/–* and *AA* horses for 'slow gallop'. Most of the American gaited breeds can perform their intermediate gait at speeds up to 28 mph (Speed Racking Horse Association 2011) and have trouble performing the canter (Ziegler, 2005). In the MM, batida horses have an even three-beat canter, whereas picada horses show uncoordination at the canter (de Rezende Garcia T. & Coelho Naves R., personal communication, 2012), correlating with the different *DMRT3* genotype frequencies observed.

Deviation from Hardy–Weinberg equilibrium suggests that selective pressure for gait type is altering allele frequencies in this breed, with selection within batida horses concentrating on homozygous wild-type (*CC*) and picada horses on homozygous mutant (*AA*). It is important to note that, prior to 2007, there was no separation at the national competition level based on gait type and that past studies report that all MMs analyzed by video footage have both lateral and diagonal support in the gait (Hussni *et al.* 1996; Procópio, 2004). Both batida- and picada-type MM horses can perform lateral gaits; therefore, the *DMRT3* mutation is not solely responsible for controlling the lateral gait pattern or for the ability to perform a four-beat gait in this breed.

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Supporting information

Additional supporting information may be found in the online version of this article.

Video S1. Video displaying the batida gait in real-time and slow-motion.

Video S2. Video of a foal displaying the picada gait in real-time and slow-motion.