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First detection of the DMRT3 "Gait Keeper" mutation in horse breeds in Bulgaria

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Abstract

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The present study aimed to identify the genotype and the allele variety of the DMRT3 gene highly linked SNP with the horse breed. It aims as well as to determine the effective alleles, influencing the ability to perform alternate gaits, which causes a favorable effect on the harness racing performance (all that being based on the results discussed). The animals were selected via genotyping based on their breed, gaits, and racing performance. Some of these horses showed good racing performance, so we've used their performance traits.

The RFLP analysis was carried out with specific endonuclease DdeI. The whole group of horses was genotyped for SNP (BIEC2_620109) via Sanger sequencing. In the study, the mutation was observed in horses from the following 5 harness breeds (Germany, Italian, Standardbred, French and Bulgarian trotters). In the breeds for a flat race, only the wild type was observed. SNP (BIEC2_620109) is highly linked with the A and C alleles of DMRT3_Ser301STOP mutation, except for only one horse of the Fresian breed. Both groups: young horses of 2 and 3 yo (years old), incl. a qualification test and horses older than 4 yo (years old) /of heterozygous genotype CA showed a better placing. The heterozygous genotypes (CA) have a positive effect on the winning results of the four-year-old ones. The heterozygous genotype (CA) has a negative effect on the qualification and the racing career of the young horses. These results indicate that this polymorphism may be useful for assisted selection for the gait type of the gaited breeds.

Keywords: harness horse; racing performance; gaits; genetic marker; PCR-RFLP

Introduction

Most of the horse breeds are used for racing, jumping, show, demonstration, etc. One of the racing performances is the flat race (Thoroughbred, Purebred Arabian) and another one – the harness racing (trotter racing). The horses used for harness racing possess the ability to trot or to pace at a high speed. The trotter racing is considered an economical important equine sport event, which is ranked in second place compared to the Thoroughbred racing (Thiruvenkadan et al., 2009). The three naturally occurring gaits of all equids, as per the increase of the speed, are: walk, trot/pace and canter/gallop (Andersson et al., 2012). The trot is diagonally symmetrical, where the diagonal front and hind legs move forward and backward together. The pace is laterally symmetrical, is a two-beat gait in which the horse moves the two legs on the same side of the body. The trot and the pace have a "flying" moment when all legs are in the air. After that one pair of legs lands at the same time. These horses are named to 'gaited' horse (Staiger et al., 2017). Andersson et al., 2012

reported a mutation in the *double-sex and mab-3-related transcription factor 3 (DMRT3).* The mutation (Cytosine – C to Adenine – A) was denoted as a "*Gait keeper"* but the stop mutation effects on the facility of the horses to gait (Andersson et al., 2012). The authors used thirty Icelandic horses categorized as four-gaited (walk, tölt, trot and gallop) and forty horses categorized as five-gaited (walk, tölt, trot and gallop and pace). Icelandic horses are characteristic with a regular ambling gait, named tölt. Many Icelandic horses also have an ability to pace (Andersson et al., 2012).

Additional studies confirm the results – for the stop mutation, the A allele is nearly fixed in gaited breeds such as the Tennessee Walking Horse, Paso Fino, Missouri Fox Trotter, Rocky Mountain Horse, etc. The heterozygous genotype is observed in the harness racing breeds such as the Standardbred, the trotters, and the Icelandic (Andersson et al., 2012; Jäderkvist et al., 2014; Promerová et al., 2014; Ricard, 2015; Regatieri et al., 2016).

The present study aimed to identify the genotype and the allele of the DMRT3 gene highly linked SNP in horse breeds to determine effective alleles influencing the ability to perform alternate gaits which has a favorable effect on harness racing performance.

Materials and Methods

Breads of horses and sample collection

This study included a total of 118 horses of 11 breeds, located in Bulgaria. Some of the horses are imported from Italy, France, Sweden, and Germany. The other part of the horses is born in Bulgaria. All horses are from private farms situated in different regions of the country: – at Pazardzhik, St. Zagora, Pleven, Balchik, etc. The number of the samples per breed varied from one (Danubian and Pura Raza Espanola) to 28 (Bulgarian trotter). The animal`s selection for genotype is based on their breed, gaits, and racing performance. The investigation includes Thoroughbred, Arabian, trotter horses, Trakehner, Fresian and Danubian horse breeds from both genders (63 male and 55 female) and over 6 months old. All horses were in good health and duly registered in their studbooks. Some sport breed horses have never performed. The details of the origin breeds and the sample sizes are presented in Table 3. We are considering the performances by the qualification and the racing career of the French trotters (n = 15). The first step of a racing career in the French trotter is to pass a "qualification" test. That is to loop of 2 000 meters distance. The qualifying time for the 2000-meters race depends on the age of the candidate (from 2 years) and has been modified over the years as the performance of racing horses has improved. A small proportion of the 2-year-old trotters could continue racing after the qualification test in the same year. Most of them could race between 3 and 4 years. Only the best horses continue racing between 5 and 10 years of age. The other horses stop racing during their fourth year. Horses that break stride are disqualified (gallop, pace, etc.) Horses without racing experience were excluded from data analysis.

The experiment was conducted according to National Committee for Ethics and Welfare for Animal approved by BFSA (Bulgarian Food Safety Agency).

DNA extraction from samples

The genomic DNA was extracted from the hair bulb with the commercial kit Blood-Animal-Plant DNA Preparation Kit (Jena Bioscience) as per the provided instructions. The hair samples were collected from the manes of horses. DNA from the hair samples was extracted using 50–60 hair bulbs from each horse and stored at $+4^0$C the sterile containers. The quality of the extracted DNA was determined by NanoDrop 2000UV-via spectrophotometer (ThermoScientific).

PCR of the DMRT3 gene and SNP (BIEC2_620109) sequence

The target regions of DMRT3 gene and highly linked SNP were amplified with DMRT3 F × R primers (Regatieri et al., 2016) and two sets of specific SNP primers, presented in the (Table 1). These SNP primers were designed, using a Vector NTI program for efficient amplification of analyzed BIEC2_620109 sequence as well as a direct Sanger sequencing method application.

Table 1. Primers of PCR fragment of the DMRT3_ Ser301STOP mutation and SNP BIEC2-620109

Locus	Primers
DMRT3	F: 5`GGGAACAGAATCACCTCCTG3` R: 5`CGACTG GTTTCTTGCCAAAG3`
SNP	F1: TGAAGGCAAACTAAAATACTT R1: CGACTGGTTTCTTGCCAAAG F2: CATGAACTTTTCTCCTGAAACA R2: TCTTTTGGAATGGTTCACATTAAG

The reaction mix was prepared by a "ready to use" kit with a total volume of 25 µl (MyTaq HS Mix – Bioline contain all the necessary components as follows: 15 µl HS PCR mix supplied with Taq polymerase, buffer, dNTPs, $MgCl_2$ 10 pmol primers and ddH_2O. The PCR amplification was carried out in a thermal cycler QB-96 (Qianta Biotech) at specific conditions, presented in Table 2.

The obtained PCR products from all 118 samples with the expected length of 470 bp for DMRT3 gene, 521 bp and

Table 2. Condition of PCR amplification of the investigated locus

Locus	Primary denaturation	Denaturation	Annealing	Elongation	Repeat	Final extension	Store
DMRT3	94ºC/3 min	94ºC/15 s	59ºC/30 s	72ºC/5 s	35	72ºC/ 1 min	4ºC
SNP	94ºC/3 min	94ºC/45 s	51ºC/50 s	72ºC/15 s	40	72ºC/3 min	4ºC

332 bp for highly linked SNP fragments were amplified with the above-described reaction mixture and corresponding set of primers, presented in Table 1. A part of the samples did not reach a successful amplification and a second primer set, designed as SNP F2 × R2 was necessary to be used. The identification of the amplified fragments was performed via agarose electrophoresis in 1% agarose gels, stained by GelRed dye. Images were obtained in the UVP BioImaging documentation system (Cambridge, UK).

Restriction fragment length polymorphism (RFLP analysis)

The RFLP analysis of resulting PCR products from the amplification of DMRT3 gene was carried out with specific endonuclease DdeI, which restriction enzyme recognizes C^TNAG sites:

5′ C↓T N A G 3′

3′ G A N T↑ C 5′

The products from each digestion were separated by using 1.5% agarose gels and stained with GelRed dye. For more accuracy, the correct size bands were checked, using a DNA Ladder, 100 bp (Jena Bioscience). Images were obtained in the UVP BioImaging documentation system (Cambridge, UK) also.

Sanger sequencing and data analysis (SNP genotyping)

The amplified SNP-PCR products after agarose gel electrophoresis were purified with GeneJET Gel Extraction Kit (Thermo Scientific) and stored at -20°C until sent for sequencing. The representative products were sequenced by the Macrogen Sequencing service, using designed above SNP R1, R2, and F1 primers. The resulting nucleotide sequences were processed and analyzed by:

– Vector NTI software;

– Comparing to the known sequences, using BLAST searching in GenBank to determine their identity to studied sequences.

Table 3. Genotype and allele frequencies of the DMRT3_Ser301STOP mutation and SNP BIEC2-620109 in different horse breeds

Breeds	*n*	Country of origin	DMRT3 Ser301STOP Mutation			SNP 620109		BIEC2-	Freq. of A allele (%)	Freq. of C allele (%)	Freq.of T allele (%)	Freq. of C allele (%)	Chi-square values for HWE test	Gait and performance
			AA	CA	CC	TT	CT	CC						
Thoroughbred	26	Great Britain	–	–	26	–	–	26	0.0	1.00	0.0	1.00	NA	Not gaited (flat racing)
Purebred Arabian	18	Middle East	–	–	18	–	–	18	0.0	1.00	0.0	1.00	NA	Not gaited (flat)
Trakehner	12	Germany	–	–	12	–		12	0.0	1.00	0.0	1.00	NA	Not gaited (sport horse)
German trotter	4	Germany	4	–	–	4	–	–	1.00	0.0	1.00	0.0	NA	Harness
French trotter	15	France	9	6	–	9	6	–	0.80	0.20	0.80	0.20	0.938^{ns}	Harness
Italian trotter	7	Italy	7	–	–	7		–	1.00	0.0	1.00	0.0	NA	Harness
Bulgarian trotter	28	Bulgaria	27	1	–	27	1	–	0.98	0.02	0.98	0.02	0.009 ^{ns}	Harness-some pace
Standardbred trotter	3	Sweden	3	–	–	3	–	–	1.00	0.0	1.00	0.0	NA	Harness
Danubian	1	Bulgaria	–	–	1	–	–	1	0.0	1.00	0.0	1.00	NA	Not gaited (draft)
Friesian	3	Nether-lands	–	–	3	–	1	2	0.0	1.00	0.17	0.83	0.120^{ns}	Not gaited (sport horse
Pura Raza Espanola	1	Espania	–	–	1	–	–	1	0.0	1.00	0.0	1.00	NA	Not gaited (sport horse

Abbreviations: NA – not available; ns – not significant

Statistical analyses were performed using a programmer GenAlex and IBM SPSS Software program, version 21/Windows developer by IBM Corporation.

An established polymorphism of these two target regions was used to estimate the allele frequency and Hardy-Weinberg equilibrium (Table 3).

Results and Discussion

Figure 1 presents the results after the PCR-RFLP analysis of DMRT3gene, the segment digested with the restriction endonuclease. The size and the number of the fragments determined the alleles and the corresponding genotypes. The names of the alleles were given from the occurring mutations – transversion from C to A in the DdeI recognition site.

DdeI digestion resulted in one fragment (~220 bp) for homozygous mutant genotype AA; two fragments (~220 bp and ~470) for heterozygous genotype CA and one fragment (~470) for homozygous wild-type genotype CC (Figure 1).

DMRT3 gene RFLP analysis showed that there is polymorphism in the locus under study. In our study, the mutation was observed in horses from 5 harness breeds (German, French, Italian, Standardbred, and Bulgarian trotters). In the breeds for the flat race were observed only wild type alleles.

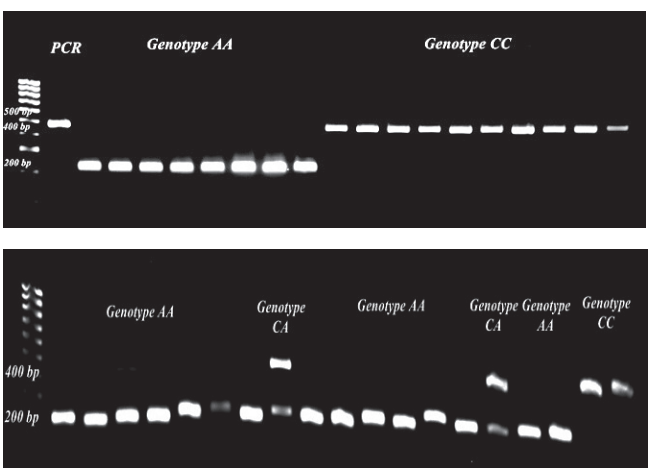

Fig.1. Agarose gel (1%) electrophoresis analysis of PCR-RFLP amplified DMRT3 gene fragments

All horses belonging to 11 breeds were genotyped for SNP BIEC2_620109 (118 horses) (Andersson et al., 2012; Promerová et al., 2014).

Three of the tested breeds (French trotter, Bulgarian trotter, and Friesian) showed deviations from HWE. In the other eight breeds, the two investigated regions were monomorphic.

The results from our study showed that the mutation of highly linked SNP overlaps those of genotyping DMRT3 _Ser301STOP except one horse from the Fresian breed.

Both allele frequency (allele A and T) in Bulgarian trotter, in two regions, were 0.98 for allele A (DMRT3_Ser301STOP) and allele T (SNP BIEC2_620109), respectively. The established frequency of mutant alleles for this breed was 0.02. For the French trotter, we observed a significantly higher frequency of mutant alleles (0.20). The frequencies of the A allele in the gaited breeds are higher in this study (0.8 – 1.0) compared to the ones, established in the previous publications (Regatieri et al., 2016; Promerová et al., 2014; Haoyuan et al., 2015).

Promerova et al., 2014 investigation is one of the first one to examine worldwide the horse population of the DMRT3 „Gait keeper" mutation in the horse and the nonsense mutation has worldwide located. That was detected in 68 of the 141 breeds included in the study. The relationship between this polymorphism and the closely linked BIEC2_620109 SNP in all tested breeds possibly appear only once and was circulation world-wide by a positive selection. The mutation is most common in harness breeds, as well as gaited breeds, such as Hokkaido and Icelandic horses. In part of the not gaited breeds, the mutation occurs frequently, but with a low frequency (New Forest Ponies and Welsh) (Promerova et al., 2014).

Regarding Ricard (2015) the mutant allele A is fixed in the American Standardbred trotter bred. According to Staiger et al. (2017) and Regatieri et al. (2016) likelihood in "gaited" breeds wild type allele C is lower but not impossible. Jäderkvist Fegraeus et al. (2017) investigate a proposed association between DMRT3 mutation and the early career performance in Swedish-Norwegian Coldblooded trotters. The CC horses had the highest number of disqualifications – 4.1. The frequency of the wild-type allele in French trotters is 24% (Ricard, 2015).

Most of the horses included in the study belong to the wild type, where allele C accounts for 53% (Figure 2). According to Regatieri et al. (2016) the allele C was also shown to be virtually fixed for not gaited breeds (Quarter horses and Purebred Arabians) and the not show the allele A in high frequency and could not determine its influence in the performance of either breed.

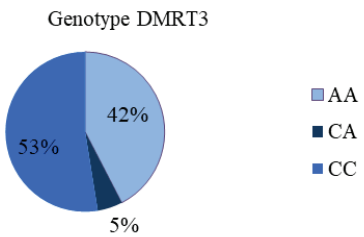

Fig. 2. *DMRT3* genotype frequency in all observed breeds (n = 11)

The detected genetic variation in the two target regions of the DMRT3 gene and highly linked SNP has not a significant impact but is influencing the ability and the effective alleles introducing alternate gaits and having a favorable effect on the harness racing performance.

The victories of the horses with genotype AA are better than heterozygotes genotype CA for 2 and 3 years old (yo) including qualification test. The reason may be that the young horse does not have enough racing performance. In our case, the group of young horses – 2 and 3 yo homozygous genotype AA has better results of placing than heterozygous. Heterozygotes genotype CA is better ranked in the racing career after 4 years old. The heterozygous genotype (CA) has a positive effect on the victories. The heterozygous genotype (CA) has a negative effect on the racing career of the young horses and qualification. However, some of the heterozygous CA horses overcome the gallop and managed to enter the placing (1-4) (Figure 3).

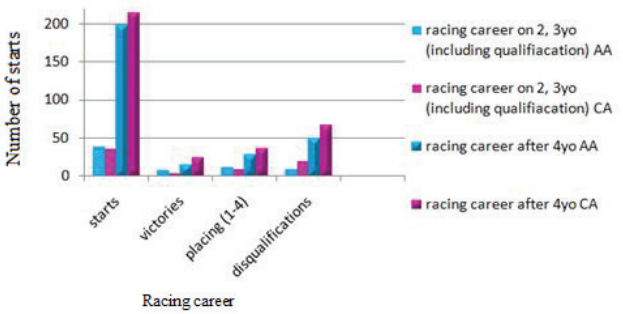

Fig. 3. The racing career of the 2 and 3 years old (including qualification test) and the racing career of the ones after 4 years for French trotters according to DMRT3 genotype

Conclusion

The mutation of the DMRT3 gene is observed in breeds with the ability to perform alternative gaits named "gaited breed". The mutant allele A occurs in harness racing trotters and pacers. The CA genotype is fixed mostly in the French trotter breed and their progeny. They have a positive effect on the victories at after 4 years old. The galloping of the harness horses is the major reason for their disqualification. It is due to the genetic conditionality to allele C and some outside factors like the: driver, horseshoes, racetrack, training, etc. The young horses of harness breeds are more sensitive to a gallop. The results suggest that the DMRT3 is not the only factor controlling the gait of the harness horses.

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