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Identification of genetic variants at *tbx3* locus associated with primitive coat colour traits in purebred Arabian horses*

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Purebred Arabian horses are one of the oldest and most influential breeds in the world. In historical presentation, the beginnings of the purebred Arabian horses date back to the VII–VIIIth century AD on the territory of the Middle East, mainly on the Arabian Peninsula (Głażewska, 2010). They are considered the noblest and the most perfect horses in terms of movement and correctness of structure. They are also characterized by high endurance and resistance to difficult environmental conditions, excellent health, high fertility rate and foal rate. Due to their characteristics, Arabian horses participated in the formation of many contemporary horse breeds (Kucera, 2013; Lange, 2016).

Purebred Arabian horses are characterized by the appearance of basic coat colours, i.e. bay and its shades, chestnut and the rarest black coat, which are variants of the E allele (*Extension*) and the A allele (*Agouti*). Melanocortin-1 receptor (*MC1R*) controlling the amount of eumelanin in body hair as well as mane and tail hair is localized at the E loci. However, at the A locus there is an agouti signalling protein (*ASIP*) blocking the MC1R receptor and controlling the zonal distribution of eumelanin and pheomelanin in hair (Rieder, 2009). According to the literature, in purebred Arabian horses the Sabino type coat colour patterns are described as well and they are characterised by white markings of various shapes including limbs, head and barrel (Brooks i Bailey, 2005). The Rabicano type pattern whose characteristic feature are white hairs appearing between the dark ones – white hairs on the belly, flanks and along the chest – is reported as well. The appearance of single white strands of hair at the dock is also possible (Brooks, 2006). Nevertheless, the most common colour is grey G determined by the duplication of 4.6 thousand nucleotides in intron 6 of the *STX17* gene encoding syntaxin 17 protein (Pielberg et al., 2008). The G allele is dominant and epistatic to the other variants of horse coat colour. In phenotype, it is demonstrated in greying that is progressive with age. This process is faster when an individual is a dominant homozygote G/G (Rieder, 2009). However, piebald horses are eliminated from breeding (Stachurska and Bruśniak, 2003).

In the process of horse domestication, the coat colour pattern was one of elements of selection of specimens for mating. These preferences have significantly changed depending on the period and the culture. Piebald horses occurred much more frequently at the beginning of the domestication process (i.e. from the end of the Stone Age to the Iron Age). On the other hand, in the Middle Ages the piebald phenotype became less popular, whereas the basic coat colours: bay, chestnut and black started to dominate (Wutke et al., 2016). An increase in the number of horses with diluted coat colour, including the DUN dilution, was demonstrated from the Iron Age to the Middle Ages, i.e. from 900 BC to 400

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AD (Ludwig et al., 2009). The molecular background of the dun D gene (*DUN*) was described relatively recently. It was demonstrated that two polymorphic changes in single nucleotides of SNP type (*Single Nucleotide Polymorphism*) together with a deletion of 1.6 thousand of base pairs in the proximity of the *TBX3* gene encoding the T-box3 transcription factor were responsible for the occurrence of the DUN phenotype (Imsland et al., 2016). The DUN dilution is a wild type of coat colour that is characteristic for Equidae, such as kiang, onager or African wild ass as well as the close relative of domesticated horse – Przewalski's horse (Imsland et al., 2016). It can be also observed in primitive horses, such as Icelandic horses or Polish konik horses (Stefaniuk-Szmukier et al., 2017). Their colour is one of the basic selective criteria, because the studbook of this breed has been closed since 1984 which means that breeding is conducted with maintenance of breed purity and only some specimens with DUN dilution may gain entry into the studbook (Stachurska and Brodacki, 2003). The DUN phenotype is characterised by lightening of chestnut, bay and black coat colour, thanks to which dun and mouse-gray colours are becoming visible. In lighter horse hair, the amount of dark pigment is reduced from by 25–50%. The dilution does not include mane and tail hair as well as the region of dorsal stripe; markings on the limbs often occur as well (Imsland et al., 2016). Three phenotype variants in the analyzed loci of the *TBX3 gene – Dun (D)*, which is dominant over *non-dun1 (d1)* and *non-dun2 (d2),* have been described to date. The non-dun phenotype is encoded by two allele versions: non-dun1 associated with primitive markings on limbs and back and non-dun2 without markings, in which a deletion of 1617 base pairs is present. It was observed that in some specimens of purebred Arabian horses, both dorsal stripe and primitive markings on legs occur. On this basis it was concluded that purebred Arabian horses can also exhibit diversity in the DUN loci.

The aim of the present study was to verify the genotype of purebred Arabian horses in *TBX3* locus responsible for the DUN type dilution.

Material and methods

Study material consisted of peripheral blood collected from 50 purebred Arabian horses that were kept in the stables in Janów Podlaski and Białka. Peripheral blood in the amount of 5 ml was kept at 4°C on EDTA anticoagulant until assayed. Genomic DNA was isolated with the use of Sherlock kit (A & A Biotechnology), according to manufacturer's instructions. Qualitative and quantitative assessment of the obtained DNA was conducted using a Nanodrop 2000 device. The presence of in/del deletion of 1.6 kbp (chr8: 18,227,267) was assessed with the use of the PCR reaction using Kappa HiFi PCR Kit (Kapa Biosystems), according to the protocol. Mutations SNP1 chr8: 18,227,267+1,066G>T and SNP2 chr8: 18,226,905A>G at locus *TBX3* (EquCab2.0). SNP1 and SNP2 were assayed by Sanger-based sequencing method with the use of Genetic Analyzer 3500xl capillary sequencer (Applied Biosystems). Sequences of primers used for amplification and size of the amplified regions were presented in table 1.

Table 1. Sequences of 5' →3' primers used for amplification of DNA fragments in the TBX3 gene region

Regiony	**SNP1 chr8: 18,227,267+1.066**	**SNP2 chr8: 18,226,905**	**IN/DEL chr8: 18,227,267**
Methods	*Sanger-based sequencing*	*Sanger-based sequencing*	*Length analysis of PCR fragments*
Produkt PCR ***PCR product***	240 pz/bp	155 pz/bp	IN 1,837 pz/bp [a] DEL 215 pz/bp
5'-3' primer	F: TAAGCCTCCA GACACCCAAG R: CAGCTCCCGT CTCCCTAGAT	F: TTCCAGGAAC CTGAGCAAAT R: ATAACCAGGC ACCCCTTCTC	F: CAAGACGCAA GGCTCTTTCT R: CGTTTCTTTA AGGGCTCGTG

SNP-single nucleotide polymorphism; PCR-polymerase chain reaction; [a]based on NCBI gbKT896509.1 sequence.

Results and discussion of results

In the process of horse domestication, the coat colour was the basis of selection of specimens for mating. Currently living horses show various types of coat colour, from basic ones to the diluted or piebald (Wutke et al., 2016; Eken and Mikko, 2009). Not only the effect of genes responsible for synthesis of pigments in hair, but also the genes encoding the proteins involved in the distribution of melanocytes in hair follicles are significant in the dynamism of the types of coat colour. As an example serves the described effect of the *TBX3* gene on the expression of the *KITLG* gene (KIT ligand) (Imsland et al., 2016) encoding the protein that participates in melanocyte migration in the skin and hair follicles (Miller et al., 2007). It was demonstrated that the modified expression of the *TBX3* gene in hair follicles of horses of Dun phenotype can be observed in the manner of distribution of coloured pigment. It is located in the developing keratinocytes forming hair cortex. In the non-dun specimens, the expression of the *TBX3* gene in keratinocytes of hairs collected from the croup was not observed as well as in the horses of Dun phenotype whose samples consisted of dorsal stripe hairs. The activity of this gene was noted in the outermost parts of hair, i.e. in the cuticle of hair follicles. Based on the above it was concluded that the *TBX3* gene functions in the selected regions of hair cortex in order to inhibit the synthesis of pigment in diluted hair in Dun phenotype horses (Imsland et al., 2016). Moreover, in non-dun horses the *TBX3* gene has the effect on the expression of the *KITLG* gene causing the accumulation of melanocytes only in one half of the hair follicle. It is thought that the *TBX3* affects keratinocytes in hair follicles, inhibiting the synthesis of coloured pigment in diluted hair in horses with Dun phenotype. In conclusion, diluted hair contains pigment that is lighter and asymmetrically distributed in a hair follicle due to the influence of the *TBX3* gene on the altered expression of the *KITLG* gene, whereas in non-dun phenotype horses, a free migration of melanocytes occurs (Imsland et al., 2016).

In the conducted studies, the mutation at the locus of *TBX3 gene:* chr8: 18,227,267+1,067 (SNP1: G in *Dun*, T in *non-dun1*); chr8: 18,226,905 (SNP2: G in *Dun*, A in *non-dun1* and *non-dun2*) as well as indel at the position chr8: 18,227,267 (IN/DEL) were assessed in all the animals. In the population of purebred Arabian horses, a frequency of the AA genotype of 100% in SNP2 was demonstrated. The IN/IN genotype was identified at the amount of 58.6% in mares and of 61.9% in stallions. The IN/DEL heterozygotes were the second most frequently occurring – 24.1% in mares and 33.3% in stallions. The DEL/DEL was the rarest genotype observed at the amount of 17.2% in mares and of 4.7% in stallions. SNP1 located in a fragment of 1.6 thousand base pairs appeared in 58.6% of mares and in 61.9% of stallions (table 2). The presence of SNP1 is enough to the appearance of the *non-dun1* phenotype, whereas the deletion of 1.6 thousand base pairs is a basis for the appearance of the *non-dun2* phenotype (table 3).

Table 2. Frequency and percentage of analysed genotypes at TBX3 loci in purebred Arabian horses

	DEL/DEL	**IN/DEL**	**IN/IN**	**SNP1**		**SNP2**
				TT	**T/DEL**	**AA**
***MARES* (29)**	17.2% (5)	24.1% (7)	58.6% (17)	58.6% (17)	24.1% (7)	100% (29)
***STALLIONS* (21)**	4.7% (1)	33.3% (7)	61.9% (13)	61.9% (13)	33.3% (7)	100% (21)

Table 3. Frequency and percentage distribution of analysed phenotype variants at TBX3 loci

	non-dun1 (d1/d1)	**non-dun2 (d1/d2;d2/d2)**
MARES (29)	58.6% (17)	41.4% (12)
STALLIONS (21)	61.9% (13)	38% (8)

The conducted studies revealed the presence of *non-dun1* and *non-dun2* alleles in the population of purebred Arabian horses. The *non-dun1* allele shows a weaker phenotypic effect than the *non-dun2*

allele (Imsland et al., 2016). The *non-dun2* phenotype is characterized by absence of primitive markings, such as dorsal stripe or markings on the limbs. The *non-dun1* phenotype illustrates an intermediate effect between the Dun and non-dun dilution, which means that dorsal stripe may be observed, but it is not as evident as in horses with D/- and D/D genotype. The *non-dun2* allele was not observed in other animal species than *Equus caballus*, which may prove its contemporary origin, on the contrary to the *non-dun1* allele, which was identified in the genetic material of an Equidae specimen from the period of approximately 43,000 years ago (Imsland et al., 2016). It was concluded that various coat colours were highlighted in the non-domesticated horses as well.

The type of a contemporary purebred Arabian horse was formed in the VIIth century AD, due to the developing Muslim religion and its leader - Muhammad. According to the legend, all the stocks of purebred Arabian horses originate from five mares of the Prophet, which he used during his famous escape from Mecca to Medina (Bielawski, 1971). Despite the fact that it was unquestionably determined that the VIIth century AD was the beginning of the breed, many centuries earlier nomadic Bedouin tribes had been successfully breeding horses on the Arabian Peninsula (Kucera, 2013). Due to the fact of a centuries-old tradition and breeding of this breed of horse, it can be expected that alleles which were discovered so early, such as *non-dun1* allele, had been present in the individual specimens of purebred Arabian horses and played an important role in the selection and choice of valuable breeders. Obtaining genetic material from horses of this period would allow to confirm this hypothesis and to conduct further studies on purebred Arabian horses.

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IDENTIFICATION OF GENETIC VARIANTS AT *TBX3* LOCUS ASSOCIATED WITH PRIMITIVE COAT COLOUR TRAITS IN PUREBRED ARABIAN HORSES

Abstract

Arabian horses are one of the oldest and most influential breeds in the world. They are characterized by the appearance of basic coat colours, i.e. bay, chestnut, black and seal brown, which are variants of the E allele (Extension) and the A allele (Agouti). Moreover, white patterns like sabino and rabicano occur. However, dominant and epistatic gray is the most common. It has been observed that some individuals of Arabian breed have both dorsal stripe and primitive markings on the legs. The aim of the study was to verify the Arabian horse genotype at the locus *TBX3*, responsible for DUN dilution. The performed research showed the presence of non-dun1 and non-dun2 alleles in the Arabian horse population. The non-dun2 phenotype is characterized by the lack of occurrence of primitive markings such as dorsal stripe or markings on the limbs. The non-dun1 phenotype illustrates the indirect effect between the dilution of Dun and non-dun, that is, it is possible to observe the dorsal stripe, although not as pronounced as in the genotype D / - and D / D horses.

Key words: Arabian horses, genetic variants, coat colour

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