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GENETIC STRUCTURE OF THE LOCAL YAKUTIAN HORSE POPULATION FOR GENES *MC1R*, *ASIP*, *DMRT3*, AND *MSTN*

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Abstract

The Yakutian horse is believed to be one of the oldest breeds. The breed has unique morphological characteristics and is well adapted to survive within the Arctic Circle. Yakutian horses have compact body conformation and extremely thick winter coats with long mane and tail. In the Yakutian breed dominate light coat colours: gray and dun. The gray and dun coat colours of Yakutian horses are their natural camouflage. The Yakutian horse is multipurpose breed, because the local horses have been used by people not only for the production of milk and meat, but also as transport animals. In this paper, the genetic structure of the native Yakutian breed was characterized using markers of four genes that are associated with important selected traits in different modern populations of domestic horses (Equus caballus). The aim of our study was to investigate the polymorphism of the *ASIP* and *MC1R* genes that determine skin and hair pigmentation, as well as to assess the occurrence of mutations in the *MSTN* (g.66493737C>T) and *DMRT3* (g.22999655C>A) genes associated with athletic performance and locomotion in domestic horses. Hair samples were collected from 45 adult purebred Yakutian horses (*Equus caballus*), including 11 samples from animals of the indigenous type and 34 samples from animals of the Yana type. DNA was isolated using ExtraGene™ DNA Prep 200 reagents (Isogen Laboratory, Russia). Genotyping for the SNP marker C>T of the *MC1R* gene was carried out using the PCR-RFLP (PCR-restriction fragment length polymorphism) method according to L. Marklund et al. (1996). Detection of 11 bp deletion in the *ASIP* gene was carried out according to the method described by S. Rieder et al. (2001). Allele nomenclature was used according to M. Reißmann (2009): *E* — dominant wild-type allele, *e* — recessive (mutant) allele (*MC1R*); *A* — dominant wild-type allele, *a* — recessive (mutant) allele (*ASIP*). The SNP mutation in the *MSTN* gene (g.66493737C>T) was detected by the amplification-created restriction site-PCR (ACRS-PCR) method described by M. Gábor et al. (2014). Genotyping of DNA samples for the SNP marker of the *DMRT3* gene (g.22999655C>A) was performed by PCR-RFLP method, C>A polymorphism was detected using restriction endonuclease HpyF3I (Thermo Scientific, Lithuania). Frequencies of alleles, frequencies of genotypes in the population and observed heterozygosity were calculated. Polymorphism of the *ASIP* and *MC1R* genes observed in Yakutian horses demonstrated a predominance of allelic variants that determine the synthesis of eumelanin, the darker type of the pigment. In the studied group of horses the frequency of the dominant *E* allele of the *MC1R* gene that determines the production of the black pigment eumelanin, was 0.711. The number of homozygous carriers of the recessive mutation of the *MC1R* gene (*e* allele) that determines production of red pigment pheomelanin was 13.3 %. The frequency of the dominant *A* allele of the *ASIP* gene that limits the synthesis of the black pigment eumelanin and affects the character of its distribution was 0.400. The number of homozygous carriers of the recessive mutation of the *ASIP* gene (*a* allele) among the tested Yakutian horses was 40 %. This is relatively high value, because in the most of modern horse breeds, the recessive *a* allele of the *ASIP* gene is rather rare. In total, eight different genotypes were identified for two key genes affecting skin and hair pigmentation. The most typical genotypes for Yakutian horses were *E/E-A/a* and *E/E-a/a*. The character of skin and hair pigmentation in the Yakutian horses could have an adaptive meaning for survival within the Arctic Circle. The frequency of the mutant variants of genes *DMRT3*

(g.22999655C>A) and *MSTN* (g.66493737C>T) in the tested horses were 0.011 and 0.022, respectively. Obviously, being presented in the population at a low frequency, the mutant variants of the *DMRT3* and *MSTN* genes have no selection value, because historically, the Yakutian horse has served people as a transport animal in the forest and swampy areas, where only riding is suitable and the most convenient gait is walk.

Keywords: horses, Yakutian breed, DNA markers, polymorphism, *MC1R*, *ASIP*, *DMRT3*, *MSTN*, eumelanin, pheomelanin, performance traits

The Yakut horse is the northernmost breed in the world, which differs from others in its unique morphological and physiological characteristics [1]. This ancient aboriginal breed was formed in the extreme natural and climatic conditions of Yakutia, one of the coldest places on Earth. Since time immemorial, the indigenous population of Yakutia has been breeding herd horses, which were kept on pastures, independently extracting pasture [2]. As a result of centuries of selection, local horses have successfully adapted to year-round open-air keeping on natural pastures, using vegetation that is under snow cover during the long winter period. The compact physique of the Yakut horses, their extremely dense hairline and metabolic features contribute to survival in the extreme conditions of the Subarctic. The Yakut horse received the status of an independent breed in 1987 [3]. The breed is universal, Yakut horses are used in agricultural work, as transport animals, in sports, as well as for the production of meat, koumiss, leather and fur raw materials. Several intrabreed types are distinguished in the breed [4].

There are several hypotheses about the origin of the native Yakut horse [5]. P. Librado et al. [6] sequenced and analyzed the complete genomes of 11 Yakut horses, including nine modern animals and two fossils (one sample dated to the early 19th century, the second to 5200 BC). The authors came to the conclusion that, most likely, modern Yakut horses are the descendants of horses brought by the Yakut people who migrated to this region in the 13th-15th centuries. The main mechanism that ensured the relatively rapid adaptation of animals to existence in the subarctic conditions was cis-regulatory changes in the genome. Unlike mutations in the coding region, which can lead to a change in the structure of the encoded protein, cis-regulatory changes contribute to the adaptation of the animal population to extreme conditions by fine-tuning gene expression [6].

The development of molecular genetics technologies has provided new opportunities for a detailed study and comparative analysis of the genomes of modern and fossil horses, making it possible to reconstruct the history of animal domestication and the formation of individual breeds. A study of ancient genomes on the polymorphism of genes affecting hair pigmentation showed that during the process of domestication of the horse, the diversity of colors found in animal populations rapidly increased [7]. Genotyping of DNA samples of fossil horses for 8 mutations in 6 genes that determine the color showed that wild horses of ancient populations were characterized by the same type of hair color. On the contrary, a rapid and significant increase in the diversity of colors among ancient horses was observed both in Siberia and in Eastern Europe, starting from the 5th millennium BC, which is associated with a period of domestication [7].

Color is one of the most significant morphological features of domestic horses [8]. The color of hair and skin is determined by the pigment melanin, represented by two main forms - eumelanin (black pigment) and pheomelanin (red-yellow pigment). The color of a horse is determined both by the amount of melanin and by the distribution of its types in the covering and guard hairs [9]. Skin and hair pigmentation in mammals is a polygenic trait determined by the combined action of a large number of genes, with the genes of the *MC1R* and *ASIP* loci playing a key role [10].

The *MC1R* gene encoding the type 1 melanocortin receptor is localized on chromosome 3 of the horse and has two main alleles: the dominant allele *E* determines the production of the eumelanin pigment, the recessive allele e in the homozygous state suppresses the synthesis of eumelanin and causes the synthesis of predominantly red-yellow pigment pheomelanin [9]. Animals homozygous for the recessive allele of the *MC1R* gene have a red color. L. Marklund et al. [11] found that the recessive mutation that determines the red color in domestic horses is a C>T single nucleotide substitution in the *MC1R* gene [11].

The *ASIP* gene encoding the agouti-signaling protein affects the production and distribution of the black eumelanin pigment, while the wild-type dominant allele *A* limits the synthesis of eumelanin and allows it to accumulate only in certain parts of the body (in the hair of the legs, mane and tail), determining the bay color [9]. In 2001, S. Rieder et al. [12] found that the recessive mutant allele a of the *ASIP* gene, which does not affect the distribution of eumelanin and determines the black suit, is an 11 bp deletion.

Due to the combined action of the *MC1R* and *ASIP* genes, as well as a number of modifier genes that can cause a decrease in pigmentation and the appearance of an admixture of white or dark hair in the coat, the colors of modern domestic horses are characterized by exceptionally wide variability [10]. In some modern commercial breeds, the color is one of the main traits selected by man, while animals of rare original colors are in high demand among buyers. The genes that determine the colors of native horses, bred for centuries by year-round herd keeping, were significantly influenced by natural selection. In the Yakut breed of horses, as a result of natural selection, gray and savras colors predominate [4, 13]. M.F. Gabyshev [13] notes that under polar conditions, the light gray color serves as a natural protection for animals, making them less noticeable to predators against the background of winter nature. Gray color in horses is an autosomal dominant trait in which there is a progressive "graying" of the integumentary and guard hairs, while the skin remains pigmented [10]. In 2008, it was found that the gray coat phenotype in the domestic horse is determined by a 4.6 bp duplication in intron 6 of the *STX17* gene, which is a cis-regulatory mutation [14]. Savrasaya color is caused by the dominant *TBX3* gene, which causes a decrease in the intensity of pigmentation of the hairline [15]. The phenotype of saurian colors also contributes to the effective visual camouflage of animals against the background of natural landscapes.

The use of horses as working and transport animals has had a huge impact on the development of human civilization. For thousands of years, domestic horses have been used for riding, and in many cultures, animals that are able to move in comfortable gaits (pacing) are especially valued [16, 17]. Nowadays, in many countries of the world, breeds of horses capable of alternative gaits are very popular.

L. Andersson et al. [18] found that the single nucleotide substitution C>A (chr23:22999655) in the *DMRT3* (doublesex and mab-3 related transcription factor 3) gene has a key effect on locomotion characteristics in domestic horses [18]. A high frequency of occurrence of the *DMRT3* gene mutation is observed in stud and native breeds, which are characterized by alternative gaits [19]. According to E.A. Staiger et al. [20], the *DMRT3* gene mutation (g.22999655C>A) could have appeared either immediately before domestication, or, more likely, some time after horse domestication and subsequently spread widely throughout the world due to intensive artificial selection [20]. It was established that in a highly specialized standardbred breed, bred exclusively for participation in hippodrome races of pacers and trotters, the mutant allele was completely fixed by selection [19].

Unlike the Standardbred breed, the Thoroughbred Saddlebred is a highly

specialized factory breed, the evolution of which took place under the pressure of intensive artificial selection of animals for the ability for outstanding gallop agility [21]. Exploring the genome of thoroughbred riding horses, E.W. Hill et al. [22] revealed a mutation — a single nucleotide substitution in the first intron of the myostatin gene (*MSTN*, g.66493737C>T), associated with high agility of racehorses over short distances. To win in races over short distances, the horse is required to develop maximum speed right from the start. It has been established that thoroughbred riding horses with the *C/C* genotype are predisposed to the manifestation of outstanding sprinting abilities, and the *T/T* genotype is characteristic of stayer horses [22]. M.A. Bower et al. [23] showed that the *C* allele became widespread in Thoroughbred horse breeds in the second half of the 20th century, which is explained by the growing popularity of sprint races during this period. It has been established that under the influence of one-sided artificial selection for agility over short distances, the mutation of the *MSTN* gene (g.66493737C>T) was completely fixed among racehorses in the American quarter-mile breed [24]. Interestingly, the *C* allele occurs not only in half-bred breeds bred using thoroughbred sires, but also in most aboriginal horse breeds of various geographic origins [23], which indicates the antiquity of the origin of this allele.

The obtained data on DNA polymorphism in horses of various breeds and directions of use provide basic insight into the mechanisms of evolution of breeds and intrabreed groups of horses. Over the centuries, human activity has selectively affected different populations of horses. Studies of the horse genome have shown that populations of ancient animals were characterized by significant genetic diversity. Artificial selection has shifted the average characteristics of different populations over time to form breeds [25].

In this paper, for the first time, the genetic structure of the native Yakut breed is characterized by four DNA markers that are of breeding importance in specialized breeds of horses for various purposes.

Our goal was to study the polymorphism of the *ASIP* and *MC1R* genes that determine skin and hair pigmentation, as well as to assess the occurrence of mutations in the *MSTN* (g.66493737C>T) and *DMRT3* (g.22999655C>A) genes associated with the working qualities of domestic horses.

*Materials and method*s. The material for the study was hair samples with bulbs from the mane, taken in 2014 in the horse breeding farms of the Republic of Sakha (Yakutia) from 45 adult purebred Yakut horses (*Equus caballus* L.), including 11 samples from indigenous animals and 34 samples from animals yang type.

DNA was isolated from hair follicles using ExtraGene™ DNA Prep 200 reagents (Isogen Laboratory, Russia). Genotyping of biological samples was performed using commercial kits of reagents GenPak® PCR Core (Isogen Laboratory, Russia) in accordance with the manufacturer's recommendations.

Genotyping for the SNP marker C>T of the *MC1R* gene was performed using the PCR-RFLP (PCR-restriction fragment length polymorphism) method as described by L. Marklund et al. [11] using published primer sequences [26]: 5'-CCTCGGGCTGACCACCAACCAGACGGGGCC-3', 5'-CCATGGAGCCGC-AGATGAGCACAT-3'. Amplification was carried out in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Inc., USA) according to the following scheme: 10 min at 95 °C; 30 s at 95 °C, 40 s at 60 °C, 1 min 30 s at 72 °C (35 cycles); 30 min at 72 °C (final elongation). Detection of C>T polymorphism in the amplified DNA fragment was carried out using TaqI restriction endonuclease (Thermo Scientific, Lithuania) according to the manufacturer's recommendations with further separation of the resulting fragments by electrophoresis in 2%

agarose gel.

11 bp deletion detection in the *ASIP* locus was carried out according to the method of S. Rieder et al. (12) using primer sequences: 5'-CTTTTG-TCTCCTTTGAAGCATTG-3', 5'-GAGAAGTCCAAGGCCTACCTTG-3'). The amplification mode was as follows: 10 min at 95 °C; 30 s at 95 °C, 40 s at 55 °C, 1 min 30 s at 72 °C (35 cycles); 30 min at 72 °C (final elongation). The resulting amplicons were separated by electrophoresis in 3% agarose gel.

Designations of allelic variants of the *MC1R* and *ASIP* genes corresponded to the nomenclature of M. Reißmann [9]: *E* — dominant wild-type allele, *e* — recessive (mutant) allele (*MC1R*); *A* is the dominant allele of the wild type, *a* is the recessive (mutant) allele (*ASIP*).

The SNP mutation in the *MSTN* gene (g.66493737C>T) was detected by the amplification-created restriction site-PCR (ACRS-PCR) method proposed by M. Gábor et al. [27], using published primer sequences: 5'-GAGAAGG-CATGACACGGAAG-3', 5'-TTGATAGCAGAGTCATAAAGGAAAAGTA-3'. PCR was carried out according to the scheme: 10 min at 95 °C 30 s at 95 °C, 40 s at 56 °C 1 min 30 s at 72 °C (35 cycles); 30 min at 72 °C (final elongation). The polymorphism of the obtained fragments was detected using restriction endonuclease RsaI (Thermo Scientific, Lithuania) in accordance with the manufacturer's recommendations with electrophoresis in 3% agarose gel.

Genotyping of DNA samples for the SNP marker of the *DMRT3* gene (g.22999655C>A) was carried out by the PCR-RFLP method, as described earlier, using the original primers 5'-AGCTTGAAAGCCAACAGACC-3', 5'-CAAAGA-TGTGCCCGTTGGA-3'. They were designed using the Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/pri-mer-blast/) and PerlPrimer (http://perl-primer.sourceforge.net/) programs using the reference the DNA sequence of the domestic horse *Equus caballus* published in the NCBI database (NC_009166.2). Amplification was carried out according to the scheme: 10 min at 95 °C; 30 s at 95 °C, 40 s at 60 °C, 1 min 30 s at 72 °C (35 cycles); 30 min at 72 °C (final elongation). Detection of C>A polymorphism in the amplified DNA fragment was carried out using restriction endonuclease HpyF3I (Thermo Scientific, Lithuania) in accordance with the manufacturer's recommendations, followed by separation of the obtained fragments in 3% agarose gel [28].

Fig. 1. A stallion of the Yakut breed at the experimental stable of the All-Russian Research Institute of Horse Breeding (Ryazan Province).

Genetic and population analysis was carried out with the determination of the frequency of occurrence of allelic variants of the studied genes, the frequency of occurrence of genotypes in the population, and the observed heterozygosity (H_o). Statistical processing of the obtained results was performed using Microsoft Excel 2010 software.

Results. When genotyping Yakut horses (Fig. 1) for the *MC1R* gene, we identified three genotypes: 25 horses had the *E/E* genotype homozygous for the dominant wild-type allele, 6 horses were homozygous for the recessive mutant allele (*e/e* genotype), and 14 horses were heterozygous genotype *E/e*. Most of the studied animals (39 animals) turned out to be carriers of the dominant allele *E* which determines the production of the black eumelanin pigment. The frequency of occurrence of the C>T mutation in the *MC1R* gene (allele *e*) which determines the suppression of the synthesis of the eumelanin pigment, was 0.289 in the studied group (Table 1).

A study of Yakut horses for the ASIP gene showed that the frequency of occurrence of the mutant allele a in the population was 0.600, including 18 animals (40% of the population) were its homozygous carriers. This is a relatively high figure, since the recessive allele a in the *ASIP* gene is quite rare in most modern horse breeds [10]. For example, the frequency of allele a in horses of the Vladimir breed is 0.252, while in purebred Arabian horses of the Russian population it is 0.100 [29, 30]. Consequently, the genes that determine the synthesis of eumelanin predominate in the Yakut horse population.

1. Characteristics of the population of horses (*Equus caballus* L.) of the Yakut breed according to the frequency of occurrence of alleles of the *MC1R* and *ASIP* genes that determine skin and hair pigmentation (n = 45, Republic of Sakha-Yakutia, 2014)

Gene	Allele	Frequency	Observed heterozygosity
MC1R	*E*	0.711	0.311
	e	0.289	
ASIP	*A*	0.400	0.400
	a	0.600	

In general, we identified 8 variants of genotypes in 45 Yakut horses based on the two studied genes that determine pigmentation (Table 2). Interestingly, the most typical for Yakut horses were the E/E-A/a and E/E-a/a genotypes, which were not found in a group of 80 purebred Arabian horses (30) (Fig. 2).

2. Genotypes for the *MC1R* and *ASIP* genes encoding skin and hair pigmentation in horses (*Equus caballus* L.) of the Yakut breed (n = 45, Republic of Sakha-Yakutia, 2014)

Genotypes	Число лошадей		Frequency
	Yang type (n = 34)	root/native type type (n = 11)	
E/E-A/A	4	0	0.089
E/E-A/a	11	0	0.244
E/e-A/A	1	2	0.067
E/e-A/a	6	1	0.156
E/E-a/a	6	4	0.222
E/e-a/a	2	2	0.089
e/e-A/A	2	0	0.044
e/e-a/a	2	2	0.089

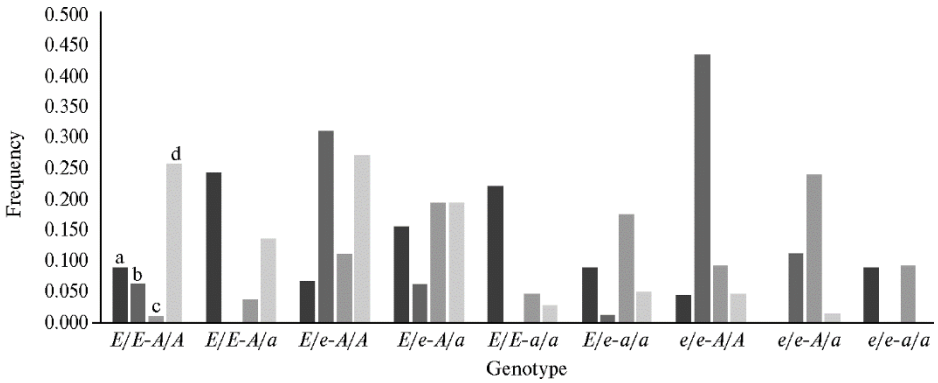

Fig. 2. Comparative characteristics of four breeds of horses (Equus caballus L.) according to the frequency of occurrence of different genotypes for the MC1R and ASIP genes: a — Yakut breed (n = 45, present study, Republic of Sakha-Yakutia, 2014); b — Arabian horses (n = 80) [30]; c — native horses from Jeju Island (Jeju) (n = 108) [31], d — Vladimir breed (n = 220) [29**].**

According to N.-Y. Kim et al. [31], genotypes *E/E-A/a* and *E/E-a/a* are rare for Korean native horses from Jeju Island [31]. In the Vladimir horses, the *E/E-A/a* genotype is not frequent (0.136), and the *E/E-a/a* genotype is rare [29]. It is likely that the predominance of allelic variants of the *MC1R* and *ASIP* genes,

which determine the synthesis of eumelanin, in Yakut horses may be of adaptive importance for the survival of animals in Subarctic conditions.

Many genes that control skin and hair pigmentation in domestic horses are known to have a pleiotropic effect [32]. L.N. Jacobs et al. [33] investigated the dependence of temperament characteristics in Tennessee pleasure horses on *MC1R* and *ASIP* genotypes and found such a correlation for the *ASIP* gene. Interestingly, according to the results obtained by the authors, Tennessee horses with the *a/a* genotype were more independent and independent.

Yakut horses are adapted to year-round herd keeping, which suggests the selection significance of certain behavioral responses. Of great interest may be a detailed study of the dependence of the neurohumoral mechanisms that regulate the behavior of herd horses on the polymorphism of genes that control skin and hair pigmentation.

When analyzing the genetic structure of the Yakut breed using SNP markers associated with the working qualities of horses, one indigenous horse was identified, a heterozygous carrier of the *DMRT3* gene mutation (g.22999655C>A) and two horses of the Yang type with a heterozygous MSTN genotype (g .66493737C>T) (Table 3). The frequency of occurrence of mutant variants of the *DMRT3* and *MSTN* genes in the studied group of Yakut horses was 0.011 and 0.022, respectively. It is obvious that, being present in the population with a low frequency of occurrence, these mutations have no breeding value, since the Yakut horse historically served people as a transport animal in the forest and swampy areas [13].

3. Genotypes of Yakut horses (*Equus caballus* L.) for the *MSTN* and *DMRT3* loci (n = 45, Republic of Sakha-Yakutia, 2014)

Gene	Genotype	Number of animals	
		root/native type	Yang type
	T/T	11	32
MSTN (g.66493737C>T)	*C/T*	0	2
	C/C	0	0
DMRT3 (g.22999655C>A)	*C/C*	10	34
	A/C	1	0
	A/A	0	0

Numerous studies have shown that mutations in the *DMRT3* and *MSTN* genes associated with key breeding characteristics of today's highly specialized commercial breeds occur in many geographically distinct populations of native horses [19, 20, 23]. According to the hypothesis of P. Librado et al. [25], genetic polymorphisms associated with the desired phenotypes of modern prize horses existed in populations of ancient animals. In the process of horse domestication and subsequent breed formation, selection for the most important breeding traits occurred not by de novo mutations, but by genetic variations present in the domesticated stock of ancient populations [25].

Thus, the study of the polymorphism of the *MC1R* and *ASIP* genes which determine skin and hair pigmentation in the native Yakut breed showed that the frequency of occurrence of the dominant allele *A* of the *ASIP* gene and the dominant allele *E* of the *MC1R* gene was 0.400 and 0.711, respectively. In the studied population, allelic variants of genes that determine the predominant synthesis of eumelanin prevailed. In the tested horses, the most common genotypes were *E/E-A/a* (24.4%) and *E/E-a/a* (22.2%) where *E* is the dominant *MC1R* wild-type allele (no C>T mutation); *A* and *a* are the dominant wild-type allele and the recessive (mutant) *ASIP* allele (with an 11 bp deletion), respectively. The frequency of the mutant allele *A* of the *DMRT3* gene (g.22999655C>A) and the mutant allele *C* of the *MSTN* gene (g.66493737C>T) was 0.011 and 0.022,

respectively. Obviously, the mutant variants of the *DMRT3* and *MSTN* genes in Yakut horses have no breeding value.

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