POLYMORPHISM IN *ASIP, MC1R* AND *MATP* GENES IN RELATION TO COLOR IN HORSES

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ABSTRACT

The basic color in horses, such as black, brown and chestnut is affected by only two genes *MC1R* and *ASIP*. Other colors in horses are affected by modifying genes, such as dilution gene *MATP*. In this thesis genotypes of 130 horses were determined using PCR-RFLP for *MATP* (membrane transport protein association) gene and multiplex PCR-RFLP for *MC1R* (melanocortin 1 receptor) and *ASIP* (agouti signaling protein) genes. Subsequently, the allele and genotype frequencies were detected in a group of horses of different breeds using SAS programe to assess the association between polymorphisms in the *MATP* gene and phenotype. Statistically, it was evaluated that the genotype was highly significantly associated with phenotype.

Key words: horse, coloring, *MATP*, *MC1R*, *ASIP*

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INTRODUCTION

All horses have an ability to produce pigment on the entire body. When identification is necessary to first identify the basic color, white patches or badges are described subsequently. For "pseudoalbinos" and a completely white horses the basic color is usually not possible to determine (Sponenberg, 2003).

Horses with the same color description may not have the same genotype (Bowling, 1996). Mostly color naming of horses is based on combinations of body color and colors of "edges" of the body such as the mane, tail, distal limbs and ears hem. The correct determination of these regiones is usually critical to identify a particular color. Black mane and tail may get brighter and brown by sunlight . In these cases, is the most accurate indicator the color of distal extremities (Sponenberg, 2003). Problems in color recognition are often caused by season, age of the animal or different climatic conditions. In the spring, after moulting horses are usually darker. Sun, wind and rain contribute to fading (Giddings, 2013). Well fed, healthy horses tend to have a darker shade. Another obstacle of the correct color identification is the fact that each color comes in many shades, so you can always find the horses on the border of two distinct colors (Sponenberg, 2003).

Since it is difficult to correctly identify the color of the horse at the level of phenotype methods based on the DNA level are used. Currently there is 11 genes identified to determine color of horses.

The mutation in *MC1R* gene in horses was first described by Marklund *et al.* (1996). They revealed in the *MC1R* (ECA3p) genes codon 83 the substitution in TCC to TTC leading to substitution of serine for phenylalanine in the final protein which was associated with the recessive allele *e*. Genotype *ee* was completely associated with the chestnut phenotype. Rieder *et al.* (2001) states that heterozygotes *Ee* are responsible for light shades of brown, while dominant homozygotes are responsible for dark shades of brown. No health problems associated with allele *E* were reported yet.

Abdel-Malek *et al.* (2001) reported that ASIP competes with α-MSH for binding to the MC1R. Functional MC1R is essential to the response of mammalian melanocytes to agouti signaling protein (Abdel-Malek *et al.*, 2001). Therefore, it is responsible for distributing eumelanotic and feomelanotic areas which are capable of producing eumelanin (Sponenberg, 2003). Polymorphism in the *ASIP* gene described Rieder *et al.* (2001), which in their results suggest that the deletion of 11 bp in exon 2 of the *ASIP* gene causes the recessive allele *a* in horses. Allele *a* causes a loss of protein function, signal process is not blocked and eumelanin is formed within the entire body (Sild *et al.*, 2012).

MATP protein transports a variety of molecules across the melanocytic membrane including, but not limited to, tyrosinase (Costin *et al.*, 2003). Mutations in the *MATP* gene were first described by Mariat *et al.* (2002), which revealed a mutation in position 72 on exon 2 of *MATP* gene, where GAT codon is replaced by AAT codon, which was also confirmed by Brooks *et al.* (2005) and Georgescu *et al.* (2007). *Cream* allele is incompletely dominant, which means that if only one allele is present, the color is partially diluted, if both alleles are present, color will be completely diluted (Kostelnik, 2000-2009). Amino acid substitution probably leads to disruption of secondary transmembrane domain structure of transport protein. The gene encodes a transport protein, which may be partially or completely disrupted. The situation is in conformity with the status of incomplete dominance, which is also illustrated by phenotype. (Newton *et al.*, 2001).

MATERIAL AND METHODS

Isolation of DNA was carried out from about 20 hair bulbs torn from the mane or tail of differently colored horses of different breeds. For isolation of DNA a commercially available tissue kit QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and GenElute ™ Mammalian Genomic DNA Miniprep Kits (Sigma-Aldrich, St. Louis, USA) were used. The isolation proceeded according to the attached protocol.

Most of the PCR reactions was stirred at volume 12.5 µl, with 10 pmoles of each Primer (IDT Inc., Coralville, USA), 2 x HotStarTaq ™ MasterMix (Qiagen, Hilden, Germany), ultrapure H_2O (Qiagen, Hilden, Germany). For the primers and mathods of *MC1R* gene design was based on Marklund *et al.* (1996) work. Primers and methods for the *ASIP* gene were taken from the work of Rieder *et al.* (2001) and *MATP* gene primers were adopted from Brooks *et al.* (2005) work.

Most RFLP reactions were carried out in a volume of 15 µl, containing 10x Buffer for restriction endonuclease (Thermo Fisher Scientific Inc.. Waltham, USA) restriction enzyme *Mse*I for *MATP* gene or *Taq*I for *MC1R* gene (Thermo Fisher Scientific Inc.. Waltham, USA), PCR product, ultrapure H_2O. Incubation of the reaction mixture was carried out at 65 ° C. After the incubation period, the samples were immediately spotted on 3% electrophoretic gel for genotype determination.

RESULT AND DISCUSSION

In this work PCR-RFLP method was used to test two polymorphisms and one deletion - C248T substitution in the *MC1R* gene (Marklund *et al.*, 1996), G214 substitution in exon 2 of the *MATP* gene (Mariat *et al.*, 2002) and a 11 bp deletion in exon 2 of *ASIP* gene (Rieder *et al.*, 2001).

Results from PCR reaction were tested by electrophoresis on 3% agarose gel and visualized by EtBr. Fragment size was compared with a weight marker M50 and M100.

A multiplex PCR-RFLP reaction was optimized for testing polymorphisms in *MC1R* and A*SIP* gene due to savings . The PCR mixture was digested with restriction enzyme *Taq*I. The resulting fragments characteristic for both alleles of this polymorphism were easily distinguishable from alleles of *ASIP* gene polymorphism (see Figure 1)

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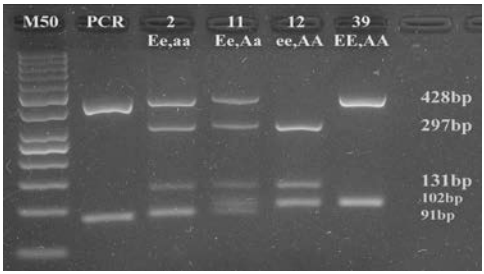

Fig. 1 deduction of genotypes after multiplex PCR-RFLP

From Table 1 it is clear that the phenotype did not match each genotype. The only phenotypes that fit 100% genotype were stained black and chestnut. Pseudoalbinos had two *Cream* alleles in 100% so genotypes were cremello, pelino and smoky cream. Horses with smoky black color seemed

phenotypically as dark brown horses or buckskin. The dapple gray phenotype was impossible to recognize whether they have or do not have the allele *Cr*.

If horses had at least one allele *E* and were recessive in *ASIP* gene, they were black colored, what affirms the statement of Stachurski *et al.* (2008), Rider *et al.* (2001). Horse of chestnut coloration had *ee* genotype in the *MC1R* gene, regardless of genotype in *ASIP* gene, confirmed by Marklund *et al.* (1996), Rieder *et al.* (2001) Anderssson (2003). When allele *Cream* occurred in genotype a horse base color was diluted to palomino, buckskin or smoky black, which confirmed allegations of Mariat *et al.* (2002), Brooks *et al.* (2005), Georgescuet *et al.* (2007). Horses with two *Cream* alleles were diluted to pseudoalbinos completely, or at cremello, perlino or smoky cream, it is also confirmed by Kostelnik (2000-2009) and Giddings (2013).

One horse of palomino phenotype had a chestnut genotype, that could be due to the fact that the individual was a cross between Fjords with Haflinger. The Haflinger is not palomino color, but chestnut with white mane (affected locus *flaxen*). Another horse of palomino phenotype came out as buckskin genotype, which may be due to breed as well or due to another gene. It was an American miniature horse and influencing locus *Buckskin silver*, which is phenotypically very similar to the palomino.

Phenotype brown corresponded with black genotype which was probably due to the fact that the hairs of horses were collected in the summer, and therefore these horses were sun lightened to brown. Genotype smoky black were always determined incorectly, confirmed by assertion of Kostelnik (2000-2009) that smoky black usualy do not differ from the black, but sometimes the are colored like dark brown or chestnut. There is usually not a good awareness of smoky black color among breeders. And smoky black horses are incorectly labeled as dark buckskin after birth.

The value of $\chi 2$ test fits tests for Table 1 is 463.2685 and $P < 0.0001$, implying that it is statistically very conclusive association between phenotype and genotype.

After comparing the $\chi 2$ test for the correlation of phenotype and *ASIP* gene, the null hypothesis was confirmed. After calculating the $\chi 2$ test for phenotype x *MATP* gene, and phenotype x *MCR1* gene, genotype x *MATP* gene and genotype x *MC1R* gene were values always $P < 0.0001$, which rejects the null hypothesis, and the association of these genes to genotype and phenotype is statistically highly significant.

Table 1 Frequency genotype x phenotype

Genotype / Phenotype	*black*	*brown*	*buckskin*	*chestnut*	*cremello*	*palomino*	*perlino*	*smoky black*	*smoky cream*	Overall
black	6									6
brown	2	19	1					2		24
buckskin	4	4	10					4		22
chestnut				18						18
palomino			1	1		40				42
pseudo-albinos					10		2		1	13
dapple gray		3	2							5
overall	12	26	14	19	10	40	2	6	1	130

CONCLUSIONS

The aim of this work was to verify the relationship between polymorphisms in the genes *MC1R* (melanocortin 1 receptor), *ASIP* (agouti signaling protein) and *MATP* (membrane transport protein association) with the color of horses. Polymorphisms were determined in all of 130 samples of horses of various breeds and different phenotypes. The association between polymorphisms in the *MATP* gene and phenotype was confirmed but only in smoky black horses phenotype is sometimes incorectly determined, because allele *Cream* is a so-called hidden and in adult horses appear to be bown colored, so I would recommend (especially when breeding animals and predicting color of foal) to test the *Cream* allele. In "pseudoalbinos" genotype *CrCr* occurs hundred percent certainty, but is overridden by the effects of genes that form the basic color (*MC1R* and *ASIP*) and so we do not know what genotype it is. In this work multiplex PCR-RFLP was oprimized to test polymorphisms in these two genes, which reduces the number of reactions necessary for the analysis and that can be routinely used to save time and mainly finances for reagents.

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