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STANDARDIZATION OF METHODOLOGY FOR POLYMORPHISM DETECTION IN *ASIP*, *MC1R* AND *MATP* GENES IN RELATION TO COLOUR IN HORSES

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Abstract

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The basic colour in horses, such as black, brown and chestnut is affected by only two genes *MC1R* and *ASIP*. Other colours in horses are affected by modifying genes, such as dilution gene *MATP*. In this paper genotypes of 133 horses were determined using PCR-RFLP for *MATP* (membrane-associated transporter protein) gene and duplex PCR-RFLP for *MC1R* (melanocortin 1 receptor) and *ASIP* (agouti signaling protein) genes. The allele and genotype frequencies were detected in a group of horses of 6 different breeds. In animals, which was not influenced by modification allele of another colour gene, we found the concordance between detected genotypes and phenotypes observed in summer and in winter. But in some animals, it was difficult to derive genotypes from their colour and therefore DNA analysis could be necessary.

Keywords: horse, coat colour, *MATP*, *MC1R*, *ASIP*

INTRODUCTION

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

Horses with the same colour description may not have the same genotype (Bowling, 1996). Mostly colour classification of horses is based on combinations of body colour and colours of "points" (manes, tails, lower legs and ear rims). The correct determination of these regions is usually critical to identify a particular colour. Black mane and tail may get brighter and brown by sunlight. In these cases, is the most accurate indicator the colour of distal extremities (Sponenberg, 2003). Problems in colour recognition are often caused by season, age of the animal or different climatic conditions. There are differences in colour shades of coat colour in winter and summer of the same animals, which is influenced by exposition of the sun in summer months. In the spring, after moulting horses are usually darker. Sun, wind and rain contribute to fading. Well fed, healthy horses tend to have a darker shade. Another obstacle of the correct colour identification is the fact that each colour comes in many shades, so you can always find the horses on the border of two distinct colours (Sponenberg, 2003).

In many cases, it is difficult to derive genotype for colours in horses from their phenotypes and therefore it is necessary to use methods based on the DNA analysis. Complex analysis is not easy, because about 20 genes in relation to horse colours have been identified so far.

The melanokortin 1 receptor gene (*MC1R*) in Extension locus is responsible for distributing eumelanotic and phaeomelanotic areas which are capable of producing eumelanin (Sponenberg,

2003). The mutation in the gene in horses was first described by Marklund *et al.* (1996). They revealed SNP in the codon 83 of the *MC1R* (ECA3p) gene – the substitution of TCC for 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.

Polymorphism in the *ASIP* (agouti signaling protein) gene in Agouti locus was described by Rieder *et al.* (2001), who reports that the recessive allele *a* in horses is characterized by the deletion of 11 bp in exon 2 of the *ASIP* gene. Normal allele *A* reduced depositing of eumelanin to peripheral body parts, recessive allele *a* causes that eumelanin is formed within the entire body (Sild *et al.*, 2012).

MATP (membrane-associated transporter protein) gene on Cream locus codes MATP protein responsible for transports of molecules, across the melanocytic membrane (Costin *et al.*, 2003). Normal allele *N* participate in the production of pigments. A mutation leads to formation of allele *Cr* which causes the dilution of the colour called cream coat colour (Mariat *et al.*, 2003; Georgescu *et al.* 2007). The authors revealed a mutation in position 72 on exon 2, where GAT codon is replaced by AAT codon. *Cream* allele is incompletely dominant, which means that if only one allele is present, the colour is partially diluted, if both alleles are present, colour will be completely diluted (Sponenberg, 2003). An overview of the expected phenotypes for genotypes of tested genes is given in Tab. I.

The aim of this work was to modify and simplify the methodology for genotyping of *MC1R*, *ASIP* and *MATP* genes and verify the concordance between polymorphisms in the genes and the colour of horses in the set of selected animals in the Czech Republic.

MATERIAL AND METHODS

Animals

The total of 133 individuals in 6 breeds (78 Kinsky horse, 12 Hucul horse, 11 Quarter horse, 8 American miniature horse, 9 Welsh cob and 13 crossbreeds) were analysed.

DNA

Isolation of DNA was carried out from about 20 hair bulbs from the mane or tail of differently coloured 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.

Genotyping

We analysed two single nucleotide polymorphisms and one deletion – C to T substitution in the codon 83 of the *MC1R* gene (Marklund *et al.*, 1996), G72A substitution in exon 2 of the *MATP* gene (Mariat *et al.*, 2003) and a 11 bp deletion in exon 2 of *ASIP* gene (Rieder *et al.*, 2001).

The analysis consisted of two PCRs; each was performed in 12.5 µl of reactions mix and contained 20 pmoles of each primer, 11× HotStarTaq Master Mix (Qiagen, Hilden, Germany) and ultrapure H_2O. First PCR mix contained primers for the *MC1R* gene (Janova *et al.*, 2013), second duplex of primers for *ASIP* gene (Rieder *et al.*, 2001) and for *MATP* gene (Brooks *et al.*, 2005).

PCR were performed on a thermal cycler PTC-200 (MJ Research Inc., St. Bruno, Canada) with the following cycling conditions: 95 °C for 15 min, 30 cycles of (95 °C for 30 s, 58 °C for 30 s, 72 °C for 20 s), 72 °C for 7 min for *MATP* gene and 95 °C for 15 min, 35 cycles of (95 °C for 20 s, 56 °C for 20 s, 72 °C for 30 s), 72 °C for 7 min for duplex of *MC1R* and *ASIP* genes. Absence of contamination has been confirmed using negative controls.

The RFLP reactions were carried out in the total volume of 15 µl, containing 1× buffer for restriction endonuclease (Thermo Fisher Scientific Inc. Waltham, USA), restriction enzyme *Mse*I for *MATP* gene or *Taq*I for *MC1R/ASIP* gene duplex (Thermo Fisher Scientific Inc. Waltham, USA), 5 µl of appropriate PCR product and ultrapure H_2O. In case of *MC1R/ASIP* duplex reaction, only *MC1R* amplicon contained polymorphic restriction site; *ASIP* gene contained ins/del polymorphism. Incubation of the reaction mixture was carried out at 65 °C. After incubation period, the samples were immediately analysed using 3% agarose gel electrophoresis stained with GelStar (LONZA, Basel, Switzerland) and genotyped. Fragment size was verified using a weight marker GeneRuler 50 bp (M50) DNA Ladder and GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific Inc., Waltham, USA).

For animals which colour was not influenced by another gene than *MR1C*, *ASIP* and *MATP* the relation between genotype and phenotype of winter and summer coat colour was evaluated using χ^2 test. A total of 17 animals whose colouration was obviously influenced by another gene were excluded from the analysis (probably effects of grey, silver, dun and flaxen alleles).

RESULT AND DISCUSSION

A duplex PCR-RFLP reaction was optimized for testing of polymorphisms in *MC1R* and *ASIP* genes. The PCR mixture was digested with restriction enzyme *Taq*I. The resulting fragments characteristic for both alleles of these SNPs were easily distinguishable from alleles of *ASIP* gene polymorphism (see Fig. 1). Genotype and allele frequencies of three analysed genes are shown in Tab. II and III, respectively.

If horses had at least one allele *E* and were recessive in *ASIP* gene, they should be black coloured, what

affirms the statement of Rieder *et al.* (2001) and Stachurska *et* Brodacki (2008). Horse of chestnut colouration had *ee* genotype in the *MC1R* gene, regardless of genotype in *ASIP* gene, as confirmed by Marklund *et al.* (1996), Rieder *et al.* (2001) and Andersson (2003). When *Cream* allele occurred in genotype, base colour was diluted to palomino, buckskin or smoky black. This finding has been confirmed by Mariat *et al.* (2003), Brooks *et al.* (2005) and Georgescu *et al.* (2007). Horses with two *Cream* alleles were diluted to pseudoalbinos. These animals are cremello, perlino and smoky cream depending on the basic colour (see Tab. I), but in real it is very difficult to distinguish between these pseudoalbino phenotypes.

Generally, there is no statistically significant difference between detected genotype and phenotype estimated in summer ($\chi^2 = 9.72$, $p = 0.137$), between genotype and phenotype estimated in winter ($\chi^2 = 5.58$, $p = 0.472$) and between phenotypes estimated in summer and winter ($\chi^2 = 2.92$, $p = 0.713$) (Tab. IV). We found full agreement between genotype and phenotype estimated by breeder in buckskin, chestnut, palomino and pseudoalbino. Breeders sometimes, especially in summer false estimated black horses as brown. They have also trouble to distinguish smoky black coat colour and horses are false determined as brown. These problems are probably due to close colour similarity, lighter shades of coat colours in summer due to sun exposition.

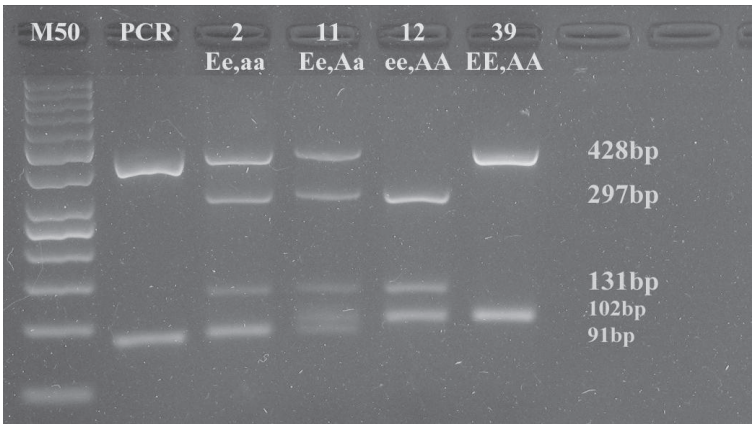
One horse with chestnut genotype had the colouring similar to palomino. It could be due to the fact, that the individual was a cross between Fjords and Haflinger. The Haflinger is not palomino colour, but chestnut with white mane (affected locus *flaxen*). Another horse of buckskin genotype was determined as palomino phenotype. It was an American miniature horse where *silver* allele is known. This allele may cause the colouring very similar to palomino (Thiruvenkadan *et al., 2008).*

The black genotype was determined in two young animals with brown colouring and in three with buckskin. The colour of these animals was change to black at the later age; with the exception of one animal (American miniature horse) where colour changed to "grey", presumably due to the presence of silver allele.

Three animals with brown genotype (Hucul) were determined in the summer incorrectly as buckskin. In winter, the colour of this animals was darker, similar to brown. However, this colour is probably due to the presence of the dominant allele of the dun gene, which occurs in Hucul breed (Stachurska *et al.* 2012).

Animals of smoky black genotype was always determined as brown. This finding is confirmed by Mariat *et al.* (2002) which states that smoky black usually do not differ from the black, but sometimes that are coloured like dark brown. There is usually not a good awareness of smoky black colour among breeders. As in our study, smoky black horses are often incorrectly labelled as dark buckskin after birth. This colour can be hard to identify without a genetic test.

"Grey" colouring was observed in 10 animals of black, brown, buckskin and smoky black genotype, but this colour was fully presented in the later age and therefore the genotype could be derived incorrectly in young animals. The probable reason may be the presence of grey colouring allele on another locus that has not been analysed. This allele masks the effect of basic colour genes.



1: *Detection of MC1R (digested by TaqI) and ASIP (11 bp deletion) genotypes using duplex PCR-RFLP. 3% agarose gel electrophoresis stained with ethidium bromide.*

I: *An overview of the expected phenotypes for given genotypes of MC1R, ASIP and MATP genes.*

Phenotype expected	black	brown	buckskin	chestnut	palomino	pseudoalbino smoky cream	pseudoalbino perlino	pseudoalbino cremello	smoky black
Genotype	*EEaaNN* *EeaaNN*	*EEAANN* *EeAANN* *EEAaNN* *EeAaNN*	*EEAANCr* *EEAaNCr* *EeAaNCr* *EeAaNCr*	*eeAANN* *eeAaNN* *eeaaNN*	*eeAANCr* *eeAaNCr* *eeaaNCr*	*EeaaCrCr*	*EeAACrCr* *EeAaCrCr*	*eeAACrCr* *eeAaCrCr* *eeaaCrCr*	*EEaaNCr* *EeaaNCr*

II: *Genotype relative frequencies.*

MATP		***MC1R***		***ASIP***	
NN	0.43	***EE***	0.12	***AA***	0.20
NCr	0.46	***Ee***	0.36	***Aa***	0.57
CrCr	0.11	***ee***	0.52	***aa***	0.23

III: *Allele relative frequencies*

MATP		***MC1R***		***ASIP***	
N	0.66	***E***	0.3	***A***	0.48
Cr	0.34	***e***	0.7	***a***	0.52

IV: *Absolute frequencies of animals for specific observed colour (phenotype) and genotypes determined using genotyping of genes MC1R, ASIP and MATP.*

	Black	**Brown**	**Buckskin**	**Chestnut**	**Palomino**	**Pseudoalbino**	**Smoky black**
Genotype	11	19	10	17	39	15	5
Phenotype winter	11	24	10	17	39	15	0*
Phenotype summer	5*	30	10	17	39	15	0*

* phenotypes of all horses, that are not in accordance with genotype, was brown

CONCLUSIONS

We simplified and optimized the DNA test for genotyping of two basic colour genes (*MC1R* and *ASIP*) and one important dilution gene (*MATP*) in horses using two simple reactions. The new duplex PCR-RFLP was introduced to test polymorphisms in *MC1R* and *ASIP* genes, which reduce the time required for routine analysis and its costs. To verify our method, genotypes were determined in all of 133 samples of horses of various breeds and different phenotypes. The concordance between genotypes of the *MC1R, ASIP and MATP* genes and estimated phenotypes was confirmed, although in some cases the observed colour differed from the predicted. We found, that genotypically black horses can be often false determined as brown, especially in animals exposed by sun light. This problem can be reliably resolved only by genotyping of basic colour genes. The animals with smoky black genotype could be determined incorrectly as brown because allele *Cream* is a so-called hidden and so adult horses appear to be brown coloured – the basic colour genes override the effects of cream alleles and therefore the deduction of the genotype from phenotype is not reliable. We recommend *Cream* allele testing to improve prediction of the foal colour in the next generation of breeding animals.

The identification of coat colours is still subjected to uncertainties due to the existence of several modifying genes causing shades or to alleles, masking the effect of other alleles such as the cream allele. Other factors complicating the estimation of genotype from phenotype is the age of the animals and sun lightening especially during the summer. For this reason, it is appropriate to determine the genotype at least these three genes. Our method helps to simplify and reduce price of this important testing.

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