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

Eliška Horecká1, Čeněk Horecký1, Aleš Knoll1

1Department of Animal Morphology, Physiology and Genetics, Faculty of AgriSciences, Mendel University in Brno, Zemědělská 1, 613 00 Brno, Czech Republic

Abstract


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,
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).

**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, 1× HotStarTaq Master Mix (Qiagen, Hilden, Germany) and ultrapure H₂O. 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 20 s, 58 °C for 20 s, 72 °C for 20 s), 72 °C for 7 min for MC1R 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 Msel for MATP gene or TaqI for MC1R/ASIP gene duplex (Thermo Fisher Scientific Inc. Waltham, USA), 5 µl of appropriate PCR product and ultrapure H₂O. 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 \( \chi^2 \) 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 TaqI. 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.

![Image](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.
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. 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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Contact information

Aleš Knoll: knoll@mendelu.cz