A new PCR-RFLP method for analyzing the *Cream* locus involved in the coat colour of horses

Sergiu Emil Georgescu, Andreea Toană, Anca Dinischiotu, Marieta Costache

University of Bucharest, Molecular Biology Center, ROMANIA

Abstract

In horses, basic colours such as bay or chestnut may be diluted to cream. A single point mutation in MATP gene is responsible for this. The mutation is localized in exon 2 of the MATP gene and determines the change of aspartic acid to asparagine in the encoded protein. Our objective is to develop an easy method to identify this mutation and then to examine the Cream locus in horse populations. We design primers to amplify only a 155bp fragment from the MATP gene with or without the single point mutation. The PCR products have been cut with Tsp509 I endonuclease and the restriction products were analysed by electrophoresis in agarose gel. Using the PCR-RFLP technique, we established an easy and efficient method that can be use to screen the Cream locus.

Key words: horse, cream colour, mutation, PCR-RFLP, sequencing.

Introduction

In mammals, coat colour is defined by two pigments present in the skin and the hair: the black eumelanin and red pheomelanin. The specific colour of an animal depends on the distribution and relative quantity of the two pigments on the body. If we do not consider the pattern of white, the basic colours of horses are bay, black, brown and chestnut, while the ones derived from these are diluted colours (Bowling, A. T., Ruvinsky, A., 2000).

The C^{cr} allele of the horse colour locus *Cream* is thought to control the cream coat colour dilution. When heterozygous the C^{cr} allele shows incomplete dominance, diluting pheomelanin (red pigment) to yellow, having little or no effect on eumelanin (black pigment). Heterozygotes for *Cream* (CC^{cr}) are palomino if the basic colour is chestnut and they are buckskin if it is bay. Blacks with a single copy of the dilution gene may show only subtle dilution effects on coat colour ("smoky" black) and on eye colour (amber or hazel). When the dilution allele is homozygous ($C^{cr}C^{cr}$), both eumelanin and pheomelanin are diluted to pale ivory, producing the colours known as cremello and perlino (Sponenberg, 1996).

In 2003, Mariat *et al.*, provide the molecular evidence that a single point mutation in *MATP* (membrane-associated transporter protein) gene is responsible for the cream coat colour of the horse. The mutation (a G to A transition) is localized in exon 2 of the *MATP* gene and determines the change of aspartic acid to asparagine in the encoded protein.

Our main objective was to develop an easy method to identify this mutation and then to examine the *Cream* locus in horse populations.

Materials and Methods

A group of 60 Romanian Sport Horses from Jegălia stud was analysed. The isolation of genomic DNA from fresh blood was performed with Wizard Genomic DNA Extraction Kit (Promega).

PCR-RFLP method

We used one set of primers which amplify a fragment of 155 pb from the *MATP* gene with or without the single point mutation. PCR conditions were optimised by varying the annealing temperature (50–60^{o}C) on a gradient thermocycler IQCycler (BioRad).

PCR was done using a GeneAmp 9700 PCR System (AppliedBiosystems). The reactions were carried out in 25-µl final volume containing PCR Buffer, $MgCl_2$, 200 µM of each dNTP, diluted DNA, 0.5 µM of each primer (F-TGTTGACCGAAGGAAGAAG; R-GGTGGTGGAGGCCCCTCTTCC), 0.5 units of AmpliTaq Gold DNA Polymerase and nuclease-free water. PCR amplifications were performed in 0.2 ml tubes using a programme with 40 cycles: denaturation at 95ºC (30 s), annealing at 58°C (30 s) and extension at 72°C (60 s). The first denaturation step was of 10 min at 95ºC and the last extension of 15 min at 72°C. PCR products were digested with restriction endonuclease *Tsp509 I* at 65°C for 2 hours. Restricted products were analysed by electrophoresis in 3% agarose gel stained with ethidium bromide.

PCR Amplification and Sequencing

We used the same set of primers. The amplified fragments were sequenced by ABI Prism 310 Genetic Analyzer, using the ABI Prism ® BigDye Terminator Cycle Sequencing Ready Reaction Kit after purification with the Wizard System Kit (Promega). The sequences were processed using DNA Sequencing Analysis 5.1 Software (AppliedBiosytems).

Results and Discussions

The *MATP* gene is responsible for the coat colour dilution. A single point mutation, a G to A transition, in exon 2, codon 153 resulting in an aspartic acid to asparagine substitution has been described as being responsible for hypo-pigmentation in the eye, hair and coat. The mutation appears at position 72, where a GAT codon (Asp) in normal horses is replaced by an AAT (Asn) in horses of cream colour. This mutation appears in a transmembrane domain of the transport protein and its substitution results in the disruption of the secondary local structure (Mariat *et al.*, 2003).

The normal allele, with G in position 72 was named C while the recessive one, with G replaced by A, C^{cr}.

In our experiment the set of primers was designed to amplify only a 155bp fragment from the *MATP* gene with or without the single point mutation (G ¦ A). This mutation modifies the recognition site for *Tsp509 I* restriction endonuclease (GATT → /AATT).

After digestion with *Tsp509 I* endonuclease the profile may be: i) normal genotype (CC) has shown one band of 155bp (Figure 1); ii) carrier heterozygote

(CC^{cr}) three bands of 155, 91 and 64bp; iii) recessive genotype ($C^{cr}C^{cr}$) two bands of 91 and 64bp.

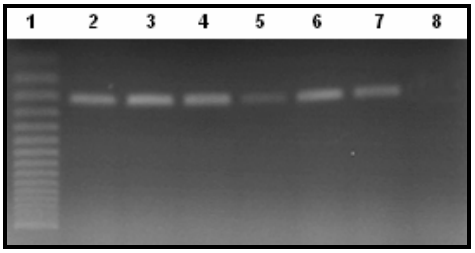

Figure 1: Electrophoresis pattern of *Cream* locus after digestion with *Tsp509 I* enzyme. Line 1 - 50 bp DNA Step Ladder, Line 2 – uncut PCR product, Line 3-7 - one fragment of 155bp indicate homozygous horses with C allele, Line 8 – negative control.

The next step was to obtain the sequence of our PCR products. The profile of this region that may contain the single point mutation is shown in Figure 2.

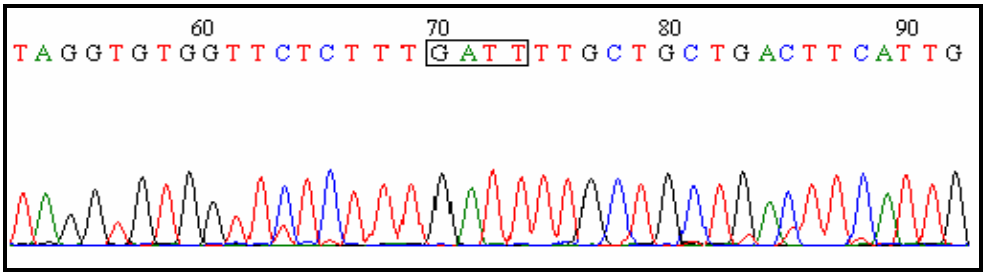

Figure 2: The sequence of the region from the PCR product that may contain the single point mutation inside the recognition site for *Tsp509 I*.

In our study all the analysed horses present the normal genotype (CC). We did not find any carrier or recessive horse with C^{cr} allele.

The *MATP* mutation is the fourth causal mutation discovered for coat colour described in horses, after the mutation responsible for chestnut (Marklund *et al.*, 1996), lethal white (Metallinos *et al.*, 1998) and black (Rieder *et al.*, 2001).

Using the PCR-RFLP technique, we established for the first time in Romania, an easy and efficient method that can be use to determine the normal or recessive genotypes for the *Cream* locus.

Therefore, these new methods increase the panel of molecular tools available to horse breeders for improving horse identification and artificial selection.

References

1. Bowling, A. T. and Ruvinsky, A. (2000) - The genetics of the Horses, CABI Publishing, ISBN 0 85199 101 7, p. 53-70.

2. Locke, M. M., Ruth, L. S., Millon, L. V., Penedo, M. C. T., Murray, J. D. and Bowling A. T. (2001) - The cream dilution gene, responsible for the palomino

and buckskin coat colours, maps to horse chromosome 21. Animal Genetics, 32, p. 340-343.

3. Mariat, D., Taourit, S., Guérin, G. (2003) - A mutation in the *MATP* gene causes the cream coat colour in the horse. Genetics, Selection and Evolutionism 35, p. 119-133.

4. Marklund L., Johansson Moller M., Sandberg K., Anderson L. (1996) - A missense mutation in the gene for melanocyte-stimulating hormone receptor (MC1R) is associated with the chestnut coat color in horses, Mammalian Genome 7, p. 895-899.

5. Metallinos D.L., Bowling A.T., Rine J. (1998) - A missense mutation in the endothelin-B receptor gene is associated with Lethal White Foal Syndrome: an equine version of Hirschsprung disease, Mammalian Genome 9, p. 426-431.

6. Rieder S., Taourit S., Mariat D., Langlois B., Guérin G. (2001) - Mutations in the *agouti* (ASIP), the *extension* (MC1R), and the *brown* (TYRP1) loci and their association to coat color phenotypes in horses (*Equus caballus*), Mammalian Genome 12, p. 450-455.

7. Sponenberg, D.P. (1996) - Equine Coat Color Genetics. Iowa State University Press, Ames.