A Mutation In The Equine *SLC24A5* Gene Is Associated With A Dilution Of Black Horses

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Introduction

Several genes are known, which reduce the intensity of coat color. Two of these genes leading to cream and champagne dilution are members of the Solute Carrier family, *SLC45A2* (Solute Carrier 45 family A2) gene (Mariat et al. (2002)) and *SLC36A1* (Solute Carrier 36 family A1) (Cook et al. (2008)), respectively. Recently, Lamason et al. (2005) identified a mutation in a homologue of *SLC24A5* (Solute Carrier 24 family A5) which is causative for the golden phenotype in zebrafish. They also reported that human *SLC24A5* gene showed linkage to variation in skin colour. Later studies (Stokowski et al. (2007); Ginger et al. (2008)) confirmed the important role of *SLC24A5* on differences in skin pigmentation of humans. Therefore, *SLC24A5* is an important candidate gene for horse colour variations.

Material and methods

Horses. For comparative sequencing of the *SLC24A5* gene, 15 horses of different shaded colours, which also belonged to different breeds, were used. Moreover, a large variation of coat colour was available for sequencing. All horses were genotyped for known dilutions to exclude that the effect of a faded phenotype is originated by such a dilution. For further validation of the first results 31 black horses and 23 fading black horses (figure 1) were genotyped additionally. DNA preparation was performed from hair bulbs using NucleoSpin®Tissue-KIT (Macherey-Nagel) following manufacturer's instructions.

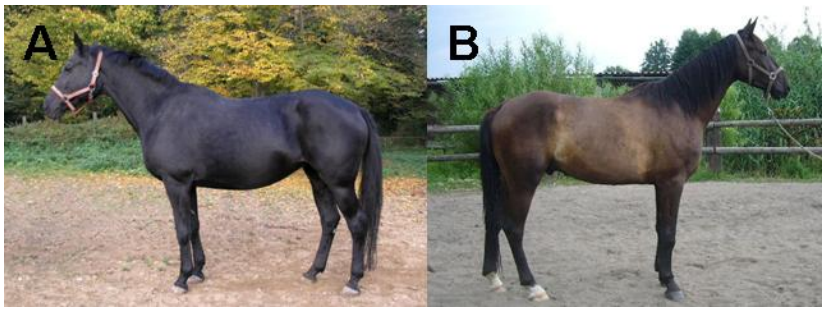

Figure 1: A shows a black horse; B depicts one with a fading black pattern

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Sequencing. Templates for sequencing of all *SLC24A5* exons were amplified by PCR. The PCR reaction was carried in a total volume of 25 µl under following conditions: 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 5 pmol of forward primer, 5 pmol of reverse primer, 0.5 U Go-Taq flexi polymerase (Promega) and 50 ng DNA. Eight primer pairs and three additional sequencing primers were designed. The sequencing reaction was done by using BigDyeTerminator chemistry (Applied Biosystems) according to standard protocol and was analyzed by an ABI PRISM^{TM} 310 Genetic Analyzer (Applied Biosystems). Exon 5 of *SLC24A5* was sequenced with the following primers: forward (5' ACC CAC AAA TAG CTC TTG GTC AA 3') and reverse (5' AGT TAG CTC AGA GAC CCA CTG GA 3') at an annealing temperature of 57°C.

Genotyping. Further genotyping for SME5 was performed by KASPar method (KBioscience). Following primers were used following a standard PCR protocol: forward primer allele 1 (5' GAA GGT GAC CAA GTT CAT GCT AAG CAT ATA CTT GAA GTT ACA GTG 3'), forward primer allele 2 (5' GAA GGT CGG AGT CAA CGG ATT CGC TAA GCA TAT ACT TGA AGT TAC AGT T 3') and a common reverse primer (5' CCT GTG AAA TAG TTA ATT TTG TGT GTC CAT 3').

Statistical analyses. Pearson's Chi-squared test was performed using R 2.10.0 (R Development Core Team, 2009).

Results and discussion

Here we show six novel mutations in the equine *SLC24A5* gene (figure 2). The SME1 mutation (ENSECAG00000018641:g.875C>T) was detected only within an Icelandic horse family. So SME1 can simply be a marker for this specific family. Furthermore, we were not able to find any association between horse colour and the mutations SME3 (g.13152delT), SME4 (g.13562T>A) as well as SME9a (g.21222G>C) and SME9b (g.21252A>G. Remarkable is SME5 (g.13858G>T). In the sequenced horse panel, the SME5 mutation shows complete association with the fading black phenotype.

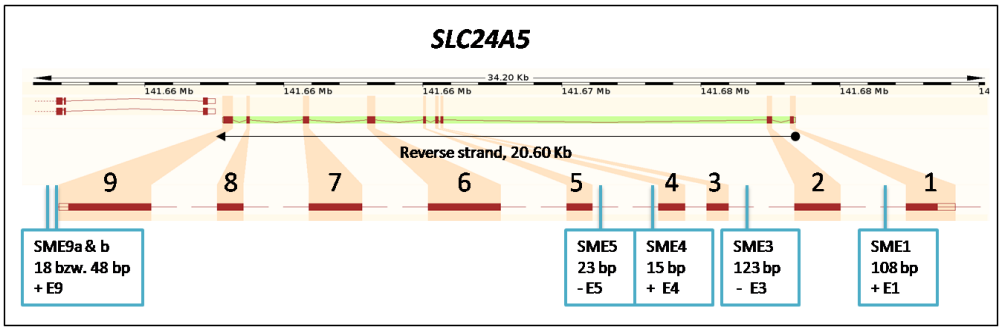

Figure 2: Relative positions of five novel SNP's and one single thymine deletion (SME3) in the equine *SLC24A5* gene referring to Ensembl (ENSECAG00000018641, 12.12.09).

SME5 is an intronic mutation, which is localized 23 bp upstream of exon 5. Thus it is possible that SME5 can alter splicing of exon 5 and therefore change the structure of the NCKX5 protein (encoded by *SCL24A5* gene). The NCKX5 protein is presumed to have a feature to change the pH in the melanosomes (Lamason et al. (2005)) and therefore could influence the activity of tyrosinase. An acidification of the melanosome leads to a decrease of tyrosinase activity followed by a reduced eumelanin production (Ancans et al. (2001)). On that account, a diminishing of colour intensity could be possible. Changes of the pH-value can also contribute to a different structure of the eumelanin molecule that is more sensitive to degradation by solar radiation. For validation of the mutation effect, we have analysed the genotypes at SME5 of 60 horses with a black basic colour (table 1).

Table 1: Genotypes at the SME5 mutation (g.13858 G>T) of the *SLC24A5* gene in black and fading black horses.

	Black	Fading Black
G/G	30	5
G/T	3	17
T/T	0	5
Σ	33	27

A Cohen-Friendly association plot (figure 3) referring to allels of SME5 and the Pearson's Chi-squared test (χ^2 (1df) = 30.35; p = 3.611e-08) revealed a close association between the SME5 polymorphism and the fading black phenotype. As figure 3 shows, the T allele is associated with fading black, whereas the G allele is associated with the black phenotype.

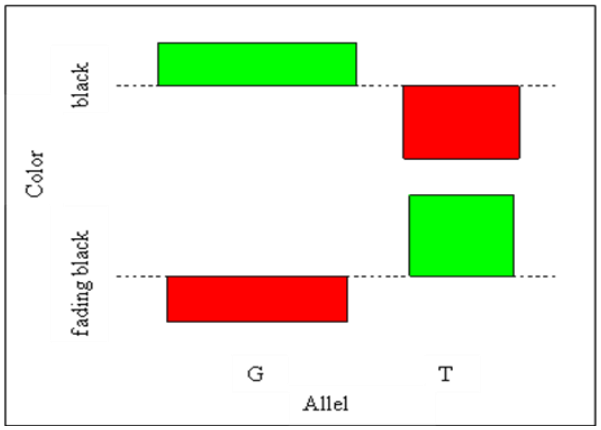

Figure 3: Cohen-Friendly association plot indicating deviations from independence. The area of the boxes is proportional to the difference in observed and expected frequencies of the alleles G or T in black or fading black horses. If the observed frequency is greater than the expected one, the box rises above the baseline and is shaded in green. Otherwise, the box falls below the baseline and is shaded in red.

In the enlarged subset, the complete association is defunct but a strong association remains. With the acceptation of about 150 genes involved in melanogenesis of mammals

(Yamaguchi et al. (2009)), the quality trait colour is highly complex and hence, an additive model of colouration becomes more likely (Stokowski et al. (2007)). In horses with an Arabian background, carrier of the SME5 mutation showed a clear pattern of fading black. So there might be an interaction of SME5 and an Arabian specific mutation. It is also interesting to test, if there is any effect of other known alleles on this trait. The black coat colour is determined by the Extension locus (E locus); therefore a strong influence on the colour intensity is possible. We observed that darker fading blacks had often an E/E genotype while horses with a distinct fading black phenotype carried mainly the E/e genotype. In previous studies, no correlation between the intensity of black and the allele constellation on E locus has been observed. Additional genes can disrupt the melanogenesis balance leading to a change in the importance of the E locus. Thus, the E locus genotype might be important for the penetrance of the fading black phenotype. But this result needs further validation.

In addition, it should be noted that it is difficult to distinguish the darker fading blacks from the appearance of a black horse. There are also environmental effects that complicate the precise classification. In particular, the sun by its colour fading UV effect must be mentioned here. According to this, it is difficult to compare stable and grazing horses. Thus, the no longer complete association between SME5 and fading blacks in the enlarged sample might be also possibly attributed to difficulties in phenotyping. Last but not least, SME5 mutation seems to be one of several mutations, which are responsible for fading black phenotype. It is possible that the five fading blacks, which carry the non-mutated genotype G/G could be caused by other mutations. Due to these aspects, it is possible that SME5 could be partly responsible for a dilution of the black phenotype.

Conclusion

Here we show for the first time that *SLC24A5*, a known gene involved in pigmentation of zebra fish, mouse and human, might also play a role in horse colouration. We detected six novel mutations in the *SLC24A5* gene. There is strong evidence that the mutation SME5 is partly responsible for the fading black phenotype. However, there is a close association between SME5 and the fading blacks. These findings are a step towards the possibility for horse breeders to select for truly blacks or for fading blacks as an interesting coat colour phenotype.

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