THE OCCURRENCE OF SILVER DILUTION IN HORSE COAT COLOURS

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Abstract. The *MC1R* allele "e" in the homozygous state leads to the production of pheomelanin and is responsible for inhibition of expression of the silver dilution gene (*PMEL17* "Z" allele). Horse coat colour is one of the traits breeders select for. A total of 133 horses representing Estonian Native (48), Estonian Heavy Draught (40) and Tori (45) breeds were genotyped for key polymorphisms at C901T in *MC1R,* the 11 bp deletion in *ASIP* and C1457T in *PMEL17* to determine horse coat colour variation and selection possibilities to increase silver-diluted colours. Our genotyping results showed the "ee" genotype frequency in the *MC1R* gene to be as follows: Estonian Native 45.8%, Estonian Heavy Draught 65.0%, and Tori 77.8%, and the "Z_" genotype in *PMEL17* to be 10.4%, 12.5%, and 0.0%, respectively. Six of total 133 horses with silver dilution were examined for MCOA. No eye abnormalities were detected. Considering the *PMEL17* gene singly, silver coat colour could be expressed phenotypically in 12% of genotyped Estonian Heavy Draught horses, but due to unfavourable covariation with the *MC1R* "e" allele, it only occurred in two per cent of horses.

Keywords: *ASIP*, horse coat colour, *MC1R*, MCOA, *PMEL17*, silver phenotype.

SIDABRINIO ŠVIESINANČIO GENO PAPLITIMAS SPALVINĖJE ARKLIŲ KAILIO GAMOJE

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Santrauka. MC1R elelis e genų kombinacijoje lemia gamybą feomelanino, atsakingo už sidabrinio šviesinančio geno (*PMEL 17* Z alelis) slopinimą. Arklių kailio spalva – viena savybių, dominanti arklių veisėjus. Buvo tiriami 133 arklių (Estijos kleperių – 48, Estijos sunkiųjų – 40 ir Estijos Tori – 45) genotipai ir pagrindiniai genų polimorfizmai (C 901 T *MC1R* gene, 11 bp *ASIP* gene ir C 1457 T *PMEL 17* gene*),* norint nustatyti arklių kailio spalvos įvairovę ir galimybę su selekcijos pagalba sustiprinti sidabrinį šviesinantį geną. Tyrimų rezultatai parodė, kad ee genotipo dažnis *MC1R* gene buvo toks: Estijos kleperių – 45,8 proc., Estijos sunkiųjų – 65,0 proc. ir Estijos Tori – 77,8 proc. Z genotipo *PMEL 17* gene buvo atitinkamai 10,4 proc., 12,5 proc. ir 0,0 proc. Vertinant tik *PMEL 17* geną, galima buvo tikėtis, kad sidabrinio kailio spalvos fenotipas pasireikš 12 proc. Estijos sunkijų arklių, tačiau dėl nepalankios kovariacijos su *MC1R* e aleliu jis pasireiškė tik 2 proc. arklių.

Raktažodžiai: *ASIP*, arklių kailio spalva, *MC1R*, *MCOA*, *PMEL 17*, sidabrinis fenotipas.

Introduction. The basic coat colours of horses are: black – regulated by the *ASIP* (agouti-signalling-protein) gene, chestnut – regulated by *MC1R* (melanocortin-1-receptor) gene and bay regulated by the same gene. *MC1R*, also known as Extension locus (E), is responsible for the production of eumelanin (dominant allele – E) – black pigment, and pheomelanin (recessive allele – e) – yellow pigment. The difference between bay and chestnut horses results from one nucleotide change in the *MC1R* gene at position 901 (Marklund *et al*. 1996).

The *ASIP* gene produces a protein which causes secondary production of pheomelanin. The recessive allele "a" at the *ASIP* locus leads to production of the non-functional protein. Non-functionality is caused by an 11 bp deletion in the *ASIP* gene (Rieder *et al*. 2001). According to Lu *et al.* (1994) and Siracusa (1994), the *ASIP* gene also controls the amount of both melanins. Jackson (1994) and Thiruvenkadan *et al.* (2008) determined that the *ASIP* gene is associated with the regional distribution of eumelanine and pheomelanin pigment in the coat.

One of the dilution genes is *PMEL17* (pre-melanosomal protein 17), which causes silver dilution in coat colours. The SNP responsible for the effect is in the eleventh exon (position 1457). According to Sponenberg (2003) the *PMEL17* gene dilutes only eumelanin. Therefore the "ee" genotype does not result in silver dilution in horse coat colour. In some breeds, such as the Rocky Mountain and the Mountain Pleasure Horse, strong correlations between the Multiple Congenital Ocular Anomalies (MCOA) and the silver phenotype (Ramsey *et al.*1999) have been found. No such abnormalities have been reported in Estonian local breeds.

The Estonian Native horse breed (EN) is an indigenous breed, which has the widest coat colour variation, from solid black to albino. The most frequent colours are bay, chestnut, black and grey. Tori (ET) and Estonian Heavy Draught (EH) were bred at the end of the 19th century and the beginning of the 20th century. The ET was established by crossing EN with warm-blooded horses, and the EH by crossing EN with cold-blooded horses. The main colours for both breeds are chestnut and bay. According to the stud book, silver coloured horses have been reported in EN and a few cases in the EH breeds. Despite the EN having influenced both EH and ET breed development, silver dilution has not been reported in the ET.

Silver dapple is currently a popular horse coat colour

for horse breeders in Estonia. The aim of this study was to determine the potential for silver dilution in Estonian horse breeds and describe Estonian local horse breeds in terms of variation among the coat colour genes *MC1R*, *ASIP* and *PMEL17*.

Material and methods. Blood and hair samples from 133 horses (48 EN, 40 EH and 45 ET) were collected by random sampling. Twelve different horse phenotypes were included (11 EN, 5 EH, 6 ET; Table 1). The coat colours were reported by the Estonian Horse Breeders' Society. Due to inconsistent colour definition in the database, dark and light shades were reduced to the basic colours (Table 1).

Table 1. **Distribution of coat colour phenotypes in Estonian horse breeds**

		Breed		
Phenotype	N	EN	EH	ET
Bay	27	10	12	5
Bay dun	2	2	-	-
Black	6	3	-	3
Blue silver	3	3	-	-
Buckskin	4	5	-	-
Chestnut	63	8	25	30
Grey	8	5	-	3
Palomino	10	7	-	3
Red chestnut	5	3	1	1
Roan bay	1	-	1	-
Silver bay	2	1	1	-
Silver on bay dun	1	1	-	-
Total	133	48	40	45

DNA was extracted from hair samples using a Puregene® Genomic DNA Purification Kit (Gentra Systems, USA) or from blood using the whole blood method (Miller *et al*. 1988). The studied polymorphisms and primer sequences are presented in Tables 2 and 3. All PCRs were performed in a total volume of 15 µl, containing 1x PCR buffer, 2.3 mM $MgCl_2$, 12 µM primers, 100 µM dNTP-s, 2.5 U Taq and 30 ng DNA. PCR programs are presented in Table 4. After SAP and ExoI (MBI Fermentas, Lithuania) treatment, the BigDye v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) was used for the sequencing reaction. Amplified fragments were sequenced with a 3130 Genetic Analyzer (Applied Biosystems, USA). For alignment of the analysed gene regions the BioEdit v7.0.9 program was used, and for testing the Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD), Arlequin v3.1 software was used. Genotypic differentiation among EN, EH and ET was evaluated by θ-value (Fstat 2.9.3) and the significance of differentiation by the exact test using the Markov chain method (Genepop 4.0.7) The eyes of the silver horses were examined with a slit-lamp biomicroscope SL-15 (Kowa Company LDT, Japan) a direct ophthalmoscope Beta 200 (Heine, Germany) and an indirect ophthalmoscope Omega 180 (Heine, Germany) to detect ocular anomalies.

Results. Our analysis showed that phenotype records corresponded to the genotype data, except in two EN horses, probably due to subjectivity of the colour definition by a colour-defining specialist. The distribution of genotypes is presented in Table 5.

Table 2. **SNPs chosen for genotyping *ASIP, MC1R, PMEL17* genes**

Gene	Polymorphisms	Reference
ASIP	11 bp del pos 2174-2184	Rieder *et al.* 2001
MC1R	Pos 901 C/T	Marklund *et al.* 1996
	Pos 903 A/G	Wagner and Reissmann 2000
	Pos 1140 C/T	Rieder *et al.* 2001
PMEL17	Pos 697 A/T	Brunberg *et al.* 2006
	Pos 858 C/T	Reissmann *et al.* 2007
	Pos 1457 C/T	Brunberg *et al.* 2006

Table 3. **Primers used for amplification of the coat colour genes**

Gene	Forward	Reverse
ASIP	5'-ctatccagccaatccctcct-3'	5'-cagcaaacatcagctccctgag-3'
MC1R	5'-gctggtgagcctagtggaa-3'	5'-agcaccctcttcatcgctta-3'
PMEL17	5'-cagctaggatcaaggccaag-3'	5'-gatgccatgatttacccagg-3'
PMEL17 (p1457)	5'-tgcaccaggtactgaagagtg-3'	5'-ttaccaccactcactcttcctcaa-3'

Table 4. **PCR conditions used for amplifying coat colour genes**

Gene	I denaturation	II denaturation	Annealing	Elongation	Final elongation
ASIP	96 °C 3 min	96 °C 25 sec	61 °C 20 sec	72 °C 45 sec	72 °C 5 min
MC1R	96 °C 3 min	96 °C 25 sec	66 °C 20 sec	72 °C 45 sec	72 °C 5 min
PMEL17	96 °C 3 min	96 °C 25 sec	63 °C 20 sec	72 °C 45 sec	72 °C 5 min
PMEL17 (p1457)	96 °C 3 min	96 °C 25 sec	66 °C 20 sec	72 °C 45 sec	72 °C 5 min
		30 cycles			

Eighteen horses (11 EN, 4 ET, 3 EH) were heterozygous for the *PMEL17* gene at position 858. One EH horse showed polymorphism in the *MC1R* gene at position 903 (Wagner and Reissmann 2000). No polymorphism in *MC1R* at position 1140 was found (all horses had the C nucleotide in that position).

In our sampling, SNPs in *PMEL 17* (pos 649, 695, 795) not previously described and *ASIP* (pos 2171) genes were found (Table 6). In EN the deviation from HWE at the *ASIP* del locus ($P < 0.05$) was determined. Disequilibrium at *MC1R* pos 901 was found ($P < 0.05$) across the breeds. Deviations from HWE may be the result of planned mating and small sample size in analysis. Statistically significant differentiation among breeds ($P < 0.05$) was found at four of the ten loci (*MC1R* pos 901, *PMEL 17* pos 697*, PMEL 17* pos 858 and *PMEL 17* pos 1457), caused by different distributions of genotypes. The overall θ estimate was 0,092. Within breeds, the EN had the highest mean allele number (1.9) compared to both EH (1.7) and ET (1.3) and Nei's gene diversity (0.16, 0.12 and 0.08 respectively).

Table 5. **Genotype distribution** (%) **in *ASIP, MC1R* and *PMEL17*, detected by different coat colour phenotypes across breeds**

Phenotype	Gene								
	ASIP			*MC1R*			*PMEL17*		
	AA	Aa	aa	EE	Ee	ee	ZZ	Zz	zz
Bay	9.0	11.4	-	4.5	15.6	-	-	-	20.2
Bay dun	-	0.7	0.7	-	0.7	0.6	-	-	1.4
Black	-	-	4.6	1.5	2.9	-	-	-	4.4
Blue silver	-	-	2.3	-	2.2	-	-	2.3	-
Buckskin	-	3.8	-	1.5	2.2	-	-	0.7	3.0
Chestnut	16.5	20.4	10.4	-	-	47.0	-	3.0	44.3
Grey	0.7	2.5	3.0	-	3.7	3.7	-	-	6.7
Palomino	0.7	5.4	1.4	-	-	7.4	-	-	7.4
Red chestnut	1.5	0.7	1.4	-	-	3.7	-	-	3.7
Roan bay	0.7	-	-	-	0.7	-	-	-	0.7
Silver bay	-	1.5	-	-	1.4	-	-	1.5	-
Silver on bay dun	-	0.7	-	-	0.7	-	-	0.7	-
Sum over genotypes	29.1	47.1	23.8	7.5	30.1	62.4	0.0	8.2	91.8
Sum over genes	100			100			100		

Table 6. **New polymorphisms found in two coat colour genes**

Gene	Breed	Position	Nucleotide change	Accession no	Change effect
ASIP	EN, EH	Exon 2 pos 2171	G/T	ss405178461	Ser63Ile
PMEL17	EN	Exon 8 pos 649	C/T	ss405178462	Non
	EN	Intron 9 pos 695	A/C	ss405178463	Non
	EN	Exon 10 pos 795	C/T	ss405178464	Leu603Ser

All horses with silver dilution (six of total 133) were examined for MCOA. Based on the sampling, no eye abnormalities were detected.

Discussion. No dominant black E^D (Dreux 1966, Sponenberg and Weise 1997) horses were detected, despite the fact that the Arabian horse, which has been described as dominant black, has been used for upgrading the EN breed. All five black and three blue silver (black silver) horses were recessive black horses.

The higher "ee" genotype frequency (ET 65.0, EH 60.0 and EN 40.0%) than expected from the allele frequency indicates heterozygote deficiency in the Estonian horse populations, which was confirmed by the HW test. The EN showed a HW disequilibrium at the *ASIP* del locus. For all breeds a significant LD between *ASIP* del and *PMEL17* pos 1457 was recorded. The other LDs between locus pairs were different for breeds. Significant LDs are presented in Table 7.

Table 7. **Occurrence of LD** ($P<0.05$) **between studied polymorphic sites analysed in Estonian horse breeds, within and across breeds**

Locus	*ASIP* del	*PMEL17* 585	*PMEL17* 1457
ASIP 2171	Across breeds, EH	-	-
ASIP del	-	ET	Across breeds, EH
MC1R 901	-	-	EN

Due to a lack of variability among analysed markers (EN had one, EH three and ET seven monomorphic loci) and the low number of analysed markers, the diversity study was not carried out in detail. A more complex, microsatellite-based diversity study will be published elsewhere at a later date.

All silver horses had the T nucleotide at positions 697 and 1457. No horse with silver dilution was of the homozygous genotype ("ZZ") and no dominant allele for silver dilution was found in ET horses (Fig. 1). Also, five chestnut EH horses had a silver dilution polymorphism without phenotypic expression, which corresponds to the findings of Sponenberg (2003), Brunberg *et al*. (2006), and Reissmann *et al*. (2007) that the extension genotype "ee" suppresses the appearance of silver dilution. Therefore, the EH has high silver dilution potential. No polymorphism in the *PMEL17* gene was found in the ET.

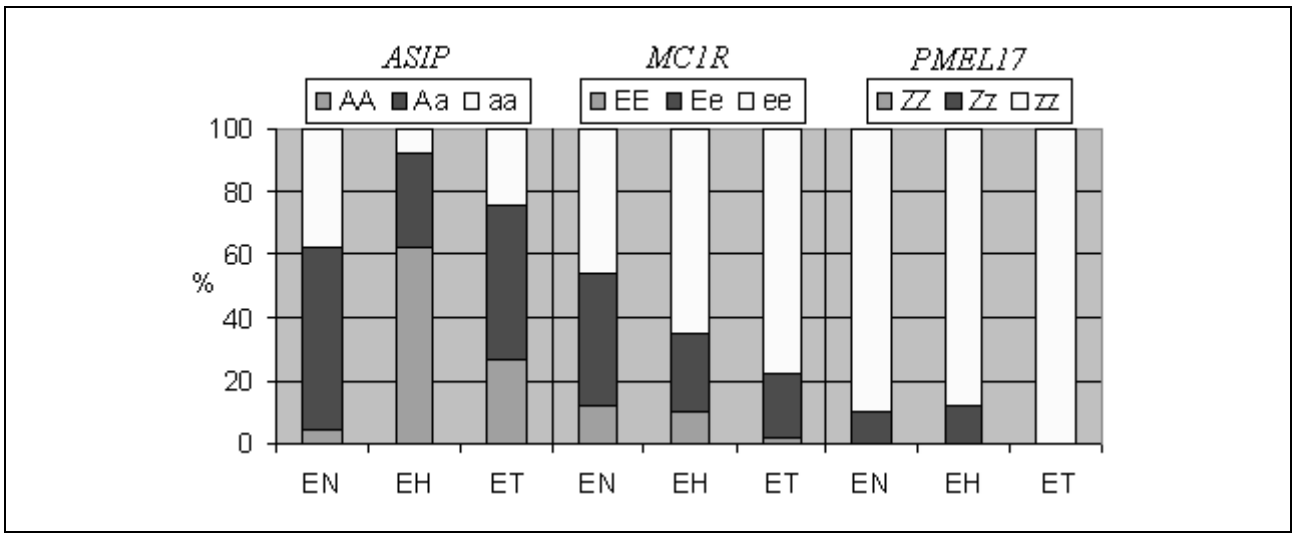

Fig. 1. ***ASIP, MC1R, PMEL17* genotype distribution in Estonian horse breeds**

No ocular abnormalities found in this study support the hypothesis that MCOA originates from a single stallion only (Ramsey *et al.* 1999).

The oldest local breed, EN, has the highest level of diversity in coat colour. The EH is the youngest local breed and only two classical basic colours occur, bay and chestnut. Genotyping three genes associated with coat colour (*ASIP*, *MC1R*, and *PMEL17*) showed that silver phenotype in EN horses equates with silver genotype. Despite EN horses having contributed to the ET breed, no "Z" allele was found in the ET samples. About ten per cent of the EH horses are silver dilution carriers due to the extension locus effect. Marker selection could possibly increase the number of horses with silver dilution in the EH breed.

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