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Genotyping of coat color genes *(MC1R, ASIP, PMEL17* and *MATP)* polymorphisms in cold-blooded horses bred in Poland reveals sporadic mistakes in phenotypic descriptions

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Equine coat color is an important phenotypic trait, predominantly within the context of correct animal identification. Due to the increased import of horses to Poland during the last years, uncommon coat colors seem to occur more often. In this study, we have analyzed the genotypes of cold-blooded horses representing four breeds (Polish Cold-blooded Horse, Belgian Draft Horse, Percheron Horse and Ardennes Horse) for genes determining basic coat colors *(MC1R* and *ASIP)* as well as silver and cream dilutions *(PMEL17* and *MATP).* We have also compared the results of our molecular study with the coat color of each horse listed in the breeding documents. Uneven distribution of genotypes between the investigated groups was observed. Moreover, we have identified several mistakes in coat color descriptions of horses, which justifies the necessity for genetic testing, particularly in case of coat colors difficult to categorize "by eye". We also suggest to extend the list of horse coat colors recognized by the Polish Horse Breeders Association.

KEY WORDS: cold-blooded horses / coat colors genes / genotype frequency / SNP

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The horse coat colors are considered to be an important phenotypic trait for several reasons. Firstly, they play a role in animal identification and are a significant element of a horse breed phenotype. Some of the coat colors are also associated with several diseases occurring in horses, e.g. leopard spotting – related with congenital night blindness, and overo frame with the lethal white foal syndrome [Bellone 2010]. Rapid development of the horse genome sequence information allowed identifying the majority of mutations responsible for the horse coat color variability, and simple molecular tests for genotyping are now available [Rieder 2009]. The first important Single Nucleotide Polymoprhism (SNP), described in 1996, was a missense mutation resulting in 83 Ser→Phe substitution in the MC1R (melanocortin 1 receptor) protein which revealed the complete association with the chestnut coat color [Marklund *et al.* 1996]. The molecular background of the remaining basic coat colors (black and bay) was discovered in 2001 when causative 11 bp deletion in exon 2 of the *ASIP* (agouti signaling protein) gene was found by Rieder et al. [2001]. Till date, the molecular basis of many other coat colors in horses was identified, including cream and silver dilutions as an effect of the 153 Asp→Asn mutation in the MATP (solute carrier family 45, member 2) and 618 Arg→Cys substitution in the PMEL17 (melanocyte protein 17 precursor) proteins, respectively [Mariat *et al*. 2003, Brunberg *et al*. 2006].

The genetic description of the horse coat color is important especially in the case of colors difficult to distinguish "bye eye" (e.g. the difference between perlino and cremello or between chestnut and bay with silver dilution). Taking into consideration that during last several years, many horses were imported to Poland, it is likely that uncommon coat colors can occur in the Polish horse population at increased frequencies. The aim of this study was to analyze the distribution of genotypes in four horse coat color genes *(MC1R, ASIP, PMEL17* and *MATP)* on sampled population of the cold-blooded horses bred in Poland (Polish Cold-blooded Horse, Belgian Draft Horse, Percheron Horse and Ardennes Horse). Results of molecular studies were compared with phenotype description of horses to identify the putative inaccuracies.

Material and methods

Genomic DNA of 402 horses was isolated from the peripheral blood samples or hair follicles. All of DNA samples used for genotyping (Polish Cold-blooded Horse, n=184; Belgian Draft Horse, n=96; Ardennes Horse, n=94 and Percheron Horse, n=28) derived from the Horse Genetic Markers Laboratory database at the Poznań University of Life Sciences (Poznań, Poland) where blood or tissue samples have been collected for routine parentage control tests. The group of Ardennes horses (n=94) consisted of Swedish Ardennes (n=37) French Ardennes (n=21), and the remaining horses (n=36) described in breeding documentation as Ardennes, with the exact origin unknown. We have also divided the Polish Cold-blooded Horse breed into two groups with the criterion of Polish origin (n=96) or foreign (n=98; at least one foreign parent). Distribution of coat colors in the investigated horses (based on their breeding

documents) was as follows: bay (n=245), chestnut (n=74), black (n=53), gray (n=28) and palomino (n=2).

DNA from the blood samples was isolated with the MasterPure® kit (Epicentre, USA) according to manufacturer's instructions. DNA isolation from hair follicles was carried out using the following procedure: 6 follicles were incubated in 40 µl of 200 mM NaoH (75°C/1 min, 80°C/2 min, 90°C/1 min, 97°C/10 min). Afterwards, 40 µl of solution consisted of 200 mM HCl and 100 µM Tris-HCl (pH 8.5) was added. Samples were mixed and stored in -20°C.

The PCR primers for the amplification of *MC1R*, *PMEL17* and *MATP* gene fragments, were designed with the use of Primer3 program (http://frodo.wi.mit.edu/) (Tab. 1). For the *ASIP* gene amplification, we used the primers described before by Rieder et al. [2001]. The PCR amplification was conducted in MyCycler® thermocycler (BioRad, USA) using the Taq DNA Polymerase (EURx, Poland). Amplification of all fragments was conducted in the following conditions: 95°C/5 min (initial denaturation); 35 cycles of: denaturation (95°C/30 s), primers annealing (45 s, temperature for each of amplified fragments is shown in Table 1); elongation (72°C, 1 min) and final extension (72°C, 10 min).

Table 1. PCR conditions for *MC1R*, *ASIP*, *PMEL17* and *MATP* genes amplification

Primer sequence	PCR product size	Annealing temperature	Source sequence (GenBank) or reference
MC1R-F-CCTCGGGCTGACCACCAACCAGACGGGGCC MC1R-R- CCATGGAGCCGCAGATGAGCACAT	350	60	NM_001114534.1
ASIP-F- CTTTTGTCTCTCTTTGAAGCATTG ASIP-R- GAGAAGTCCAAGGCCTACCTTG	94/105	55	Rieder *et al*. [2001]
PMEL17-F- CAGCTAGGATCAAGGCCAAG PMEL17-R- CTCTCACCAAAGGGGGAAG	229	60	NC_009149
MATP-F- TTTGATTGCTGACCGAAGGAAGAA MATP-R- GAGACTGAGCCCGCGTGATGAGAG	320	65	AH012460.2

Genotyping of the *MC1R, PMEL17* and *MATP* gene polymorphisms was performed using the PCR-RFLP technique and the following restriction endnonucleases (Fermentas, Lithuania) were applied: *Taq*I (*MC1R*), *Hha*I (*PMEL17*) and *Tas*I (*MATP*). After digestion, the PCR products were separated in 2% agarose gel (45 min/120 V). An 11 bp InDel polymorphism of the *ASIP* gene was genotyped by separation of the received PCR product in 2.5% agarose gel (60 min/130 V). The genotypes were tested for Hardy-Weinberg equilibrium in each analyzed horse breed group. Additionally, we referred the identified genotypes to the information regarding the coat color of the horses available in the official breeding documentation, although the full comparison was impossible, because we did not tested for some of white color patterns (e.g. gray, roan, appaloosa, tobiano, overo and sabino).

Results and discussion

The genotyping of polymorphisms in four coat color genes revealed an uneven distribution of genotypes in the examined horse breeds (Tab. 2). The differences were observed predominantly for the *MC1R* and the *ASIP* polymorphisms. The highest frequency of the recessive TT genotype in the *MC1R* (determining the chestnut color) was observed in the Polish Cold-blooded horses of the Polish origin. In this case, a statistically significant deviation ($P<0.01$) from the Hardy-Weinberg Equilibrium (HWE) was observed. This result remains significant also when Polish Cold-blooded horses (of the Polish and foreign origin) are considered as one group. The difference in the TT genotype frequency between the Polish Cold-blooded horses of the Polish and foreign origin (0.23 *vs*. 0.13, respectively) may indicate that traditional coat color of the native Polish cold-blooded horses was chestnut and thus, this coat color is still preferable by horse breeders. A similar *MC1R* genotype distribution was reported by Rendo et al. [2009] for the two native cold-blooded horse breeds (Euskal Herriko Mendiko Zaldia and Burguete) from the Iberian Peninsula, which may suggest that within the context of the coat color, the population of the cold-blooded landrace horse breeds in Europe is homogenous (with relatively high frequency of the chestnut allele). Interestingly, in case of the Belgian Draft Horse, a low frequency (0.04) of the TT genotype *(MC1R)* was observed which may indicate that majority of the horses representing this breed imported to Poland have the bay or black coat color.

Table 2. Genotype frequencies of coat color genes *(MC1R, ASIP, PMEL17* and *MATP*) in cold-blooded horses bred in Poland

Gene		Polish Cold-blooded Horse (Polish origin)	Polish Cold-blooded Horse (Foreighorigin)	Belgian Draft Horse	Ardennes Horse	Percheron Horse
MC1R	CC	0.13	0.29	0.78	0.49	0.57
	CT	0.64	0.58	0.18	0.38	0.40
	TT	0.23	0.13	0.04	0.14	0.03
ASIP	Ins/Ins	0.40	0.54	0.30	0.71	0.00
	Ins/Del	0.45	0.33	0.41	0.26	0.31
	Del/Del	0.15	0.13	0.29	0.03	0.69
PMEL17	CC	1.00	0.98	1.00	1.00	1.00
	CT	0.00	0.02	0.00	0.00	0.00
	TT	0.00	0.00	0.00	0.00	0.00
MATP (SLC45A2)	AA	0.00	0.00	0.00	0.00	0.00
	AG	0.02	0.00	0.00	0.00	0.00
	GG	0.98	1.00	1.00	1.00	1.00

It should be underlined that in case of the basic coat color phenotypes (bay, black and chestnut) majority of the genotyping results were compatible with the coat color descriptions available in Horse Breeders Association database.

Missing HWE for the *MC1R* genotypes distribution was also observed in the Ardennes horses ($P<0.05$). Such deviation could be the result of the selection pressure

on coat color as an important phenotypic trait characterizing given horse breed. For example, low frequency of the TT genotype *(MC1R)* and the increased frequency of the Del/Del genotype *(ASIP)* observed in the Percheron horses, may reflect the fact that black coat color is preferable in this breed above other coat colors (e.g. chestnut which occur rarely). Unfortunately, due to technical difficulties we were not able to test for the grey coat color by detection of genotypes in the 4.6 kb intronic duplication of *STX17* gene [Rosengren Pielberg *et al.* 2008].

Additionally, we have performed an analysis of the genes causing silver and cream coat color dilutions (*PMEL17* and *MATP*) which revealed the presence of two palomino and two silver animals in the Polish Cold-blooded horses. Comparison of the results received from the molecular tests with breeding documentation of animals, showed that the description of the two silver horses could be incorrect, because they were described as chestnut but basing on genetic analyses their true coat color was bay with silver dilution. Despite the fact that the two palomino horses were described correctly in the breeding documentation, we have found several inconsistencies in their pedigrees. For example, some of their ancestors were described incorrectly as dun whereas their probable coat color was buckskin (Fig. 1).

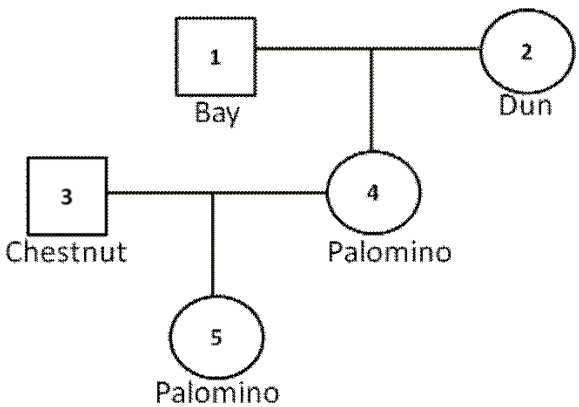

Fig. 1 . Pedigree analysis of sample palomino mare (based on breeding documentation). 5 – palomino mare (coat color confirmed by molecular tests). 1,3,4 – ancestors with putatively correct coat color identification. 2 – mare with incorrect coat color identification (probable pigmentation – buckskin).

The above inconsequence in the coat color description possibly aroused from the fact that some of the coat colors are difficult to distinguish without genetic analysis. Moreover, the list of the coat colors recognized by the Polish Horse Breeders Association does not include silver and buckskin coat colors, thus such cannot be used in the official breeding documents.

A retrospective research of the horse fossil samples has shown that variation in horse coat color increased rapidly after the domestication [Ludwig *et al*. 2009]. Centuries of the horse selection have produced a number of different coat colors observed in the present time. Thus, the management of the modern horse breeding

within the context of the offspring coat color planning is sometimes difficult without genetic testing.

Till date, studies on the coat color patterns of the horses bred in Poland were based on information included in studbooks. Such analyses were published for the Thoroughbred and Hucul horses [Stachurska, Brodacki 2008; Stachurska *et al*. 2012].

Our study is the first describing the genetic background the coat color in Polish horses using molecular techniques. The results confirm the utility of the molecular tests in accurate characterization of the horse coat color. This study indicates that if the correct phenotypic description of a horse is in doubt, then genetic testing should be employed to avoid mistakes in official breeding documents. It is also necessary to extend the existing list of the horse coat colors recognized by the Polish Horse Breeders Association, predominantly within the context of the colors affected by the dilution genes, and white color patterns (which were not included in this study).

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