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Crypto-tobiano horses in Hucul breed

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ABSTRACT: The aim of the study was to verify the hypothesis that the crypto-tobiano horses which do not exhibit the full tobiano white-spotting pattern nonetheless transmit the dominant *To* gene to the progeny. The study was conducted on the Hucul population. Seven horses with white patches on their limbs, and with or without a small patch on the head, neck or trunk, were selected. At least one of the parents of the horses had to be tobiano or crypto-tobiano. Occasionally such horses produced a tobiano foal from a crossing with a solid-coloured horse. The white patches of the horses used in our study were divided into categories. To identify the *To* gene, DNA was tested in the Animal Genetics Laboratory (UK). The ratio of non-tobiano, crypto-tobiano, and tobiano foals born from 36 matings of the studied horses was analyzed. The study substantiates the occurrence of crypto-tobiano horses in the Hucul breed. Horses that have patches on their limbs as well as a small white or intermingled patch on the head, neck or trunk, are crypto-tobianos, although crypto-tobianos may have no white on the head, neck, and trunk. The crypto-tobiano pattern occurs in horses which have a tobiano parent or grandparent. The results suggest that the activity of an inhibitor gene suppresses the *To* allele penetrance in the crypto-tobiano horses. As in the case of many white-spotting patterns, the presence of the *To* gene in a horse's genotype should be documented by DNA testing or test matings.

Keywords: coat-colour phenotype; marking; *To* gene; white-spotting pattern

INTRODUCTION

Coat colour importance. Horse breeders have always believed that coat colour was associated with a horse's performance. Such a connection would result from genes which control the coat colour and simultaneously are linked with genes responsible for the performance. Pleiotropic effects of genes are also possible and they do occur. For instance, some unpigmented phenotypes in the horse are associated with lethal or semi-lethal traits (Rieder et al. 2008). A study on Thoroughbreds and Purebred Arabians, however, demonstrated that racing scores were not related to alleles in *MC1R* and *STX17* loci responsible for the expression of eumelanin and progressive greying in hairs, respectively (Stachurska et al. 2007). In indigenous horse breeds, the coat colour is important mainly for aesthetic breeders' preferences and traditions. In breeds included in conservation programmes, conducted according to the Global Strategy for the Management of Farm Animal Genetic Resources (www.bioroznorodnosc.izoo.krakow.pl), the animal gene pool should be preserved in an unaltered state. Horses included in conservative breeding are a sort of gene bank. The coat colours are a distinct, characteristic trait, mostly inherited in a simple way. The colours can be used as a kind of a marker of possible undesirable trends in the conserved breeds, prior to molecular analyses. Detailed documentation of the changes can be provided by molecular analyses (Stachurska et al. 2012).

Hucul horse breed. The primitive Hucul horse breed derives from the East Carpathian Mountains. It is believed that Hucul ancestors originated early on from many types of horses, and from the extinct Tarpans (Hackl 1938; Komosa and Purzyc 2009). The Huculs are bred in the Czech Republic, Hungary, Slovakia, Romania, the Ukraine, and Austria, and more than one-half of the entire Hucul population is bred in Poland. The breed is included in the above-mentioned conservation

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programme. Presently, the Huculs registered in the Polish studbook are bay, black, yellow dun, blue dun (also called mouse dun or grulla), and some are chestnut. In the total studbook, 21.5% of the horses recorded had the tobiano white-spotting pattern. In the latest volume (the 8th volume) of the studbook, the number of tobianos equaled 30.4% (Stachurska et al. 2012). According to the current breeding programme, both the chestnut colour and white markings are undesirable (www.bioroznorodnosc.izoo.krakow.pl, 2013).

Tobiano white-spotting pattern. The white-spotting patterns, white markings included, occur on any base colour. The patches of white result from the lack of melanocytes in the hair follicles and the skin. The lack is caused by aberrations in the migration, proliferation, differentiation, and survival of the melanoblasts (Thomas and Erickson 2008; Hauswirth et al. 2012). The white-spotting patterns are present at birth and except for the leopard pattern do not alter throughout life. The tobiano white-spotting pattern is favourable and selected by many horse breeders and owners. Unpigmented patches are vertical in character (Stachurska 2002; Ussing 2000; Haase et al. 2008; Arriens 2009). They usually cross the dorsal midline, whereas the belly remains coloured. The patches have sharp, definite edges, although the edges sometimes form a narrow shadow of pigmented skin covered with white hairs. The limbs are generally white or have extensive markings. The head is dark with the usual markings. The eye is usually blue when a marking extends on it. Main and tail hairs are white, dark or mixed, depending on the colour of the skin area on which the hairs grow. Sometimes half of the length of the tail is pigmented and the other half is unpigmented. Secondary spotting may occur on the white patches. These "ink spots" are small coloured spots. Bigger patches with smudgy edges are called paw prints (Bowling and Ruvinsky 2000). The hooves are usually white, since the limbs are white. According to Sponenberg (2009), when the tobiano pattern is present in a breed, white patches do not become progressively larger through the generations. The author distinguishes maximally marked tobianos that are white, sometimes have a small coloured patch in the flank or on the chest, and have coloured heads. Minimally marked tobianos have four white lower parts of limbs, usually from the knees and hocks down, with a small spot of white somewhere along the topline, and this frequently in the mane or tail. The head is solid coloured usually without markings or only minimally marked. Breeders sometimes call the minimally marked horses crypto-tobianos.

Tobiano inheritance. The tobiano spotting pattern is inherited as a dominant trait (Crew and Buchanan-Smith 1930; Klemola 1933). Brown (1970) postulated that there was a dominant gene *T* (also called *S* in the USA), further termed *To*, responsible for the tobiano pattern. Jones and Bogart (1971) hypothesized that several modifier genes operated in conjunction with the gene to cause variable white patches on particular body areas. The *To* allele is completely dominant which means that dominant homozygous and heterozygous horses are phenotypically indistinguishable. However, the secondary spotting often indicates a homozygous *ToTo* status (Bowling and Ruvinsky 2000). In the Hucul population registered in the studbook, from the 3rd volume of the studbook (0.006) to the latest 8th volume (0.199), the frequency of the *To* allele has been increasing, though the increases between successive subpopulations were not significant (Stachurska et al. 2012). For breeders producing tobiano foals, it is important to distinguish the homozygous horses. The first tests to identify the *ToTo* and *ToTo* horses were based on the linkage with albumin and vitamin D binding factor-S loci on ECA3 (Trommershausen-Smith 1978; Andersson and Sandberg 1982; Bowling 1987). There were exceptions to this linkage phase in some breeds. Further studies revealed a stronger association of the *To* with a base substitution in intron 13 of the *KIT* gene (Brooks et al. 2002). The dominant and recessive alleles were referred to as *KM1* and *KM0*, respectively. All dominant homozygotes and heterozygotes were tobiano, however, some solid-coloured horses also carried the *KM1* allele (Thiruvenkadan et al. 2008). The latest investigation showed a chromosomal inversion on ECA3. One of the inversion breakpoints is approximately 70 kb downstream of the *KIT* gene (Brooks et al. 2007; Rieder 2009). Studies on 204 German horses provided evidence of the inversion being the causative mutation for the tobiano spotting pattern (Haase et al. 2008). The *KIT* locus shows a remarkable allelic diversity. Plenty of mutant alleles at or near the *KIT* locus were reported to cause a large repertoire of either completely white horses or more or less extensive white-spotting patterns. Apart from the tobiano, the *KIT* causes sabino-1, roan,

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and a few dominant white variants (Haase et al. 2009a; Hauswirth et al. 2012, 2013).

Prevalence of the white markings and their heritability. Wild horses drawn in Paleolithic cave paintings were differently coloured and even had a white-spotting leopard pattern. Domestication and breeding led to new mutations responsible for the present great differentiation in coat colours. White markings are also thought to appear in the horse as a result of domestication (Rieder et al. 2008; Stachurska and Ussing 2012). Nowadays they are present in many breeds. The markings occur on areas of the unpigmented skin of the head and limbs. A hypothesis on the multifactorial mode of inheritance of white markings was put forward by Nebe (1984). According to Woolf (1990), the heritability of the markings on the head is 0.69, on the limbs 0.68, and combining the facial score and four limb score, the heritability amounts to 0.77. As mentioned, additional stochastic events in the development of melanocytes are the non-genetic components responsible for the markings. Studies performed on horses of various coat colours showed that *MC1R* (*E*) and *ASIP* (*A*) loci were associated with the occurrence of the markings (Woolf 1992). *AAee* genotypes which produce pheomelanic colours have more extensive markings than *aaEE* genotypes of eumelanic horses. The dominant *A* allele shows a similar although weaker function compared to the *E* allele. The *ASIP* locus was mapped to ECA22q15 (Rieder et al. 2001). The *MC1R* locus was mapped to ECA3p12 in a group linked with the *KIT* locus (Marklund et al. 1996; Brooks et al. 2007; Haase et al. 2009a), in the above-mentioned region important for the melanogenesis in horses. Molecular studies performed by Rieder et al. (2008) showed a putative major locus mapped to ECA3q at or near the *KIT* locus. A recessive single gene at that locus could account for 20–80% of the total heritability for the trait (Rieder 2009). Haase et al. (2009b) revealed the *MITF* gene on ECA16q. The gene is also responsible for the expression of white markings.

Tobiano Hucul horse breeding. For Hucul breeders, it is particularly important to discriminate whether white patches on the extremities of the horse's body are produced by the *To* gene or by genes responsible for white markings. Horses with white patches on their extremities are not always registered in the studbook, whereas the tobianos are completely allowed. Hucul breeders claim that some horses which have a tobiano parent and exhibit only white markings on the limbs sometimes produce tobiano foals when mated *inter se* or to solid-coloured horses. Jansen and Jansen (2012) reviewed the eight volumes of the Hucul horse studbook. They found 421 foals from matings of both parents registered as solid-coloured. The foals had at least one tobiano grandparent. Of those foals, 414 were solid-coloured and seven foals were tobiano. The parents of the seven tobiano foals fitted with the term "crypto-tobiano". However, those parents were assumed by studbook authorities not to be tobiano but solid with markings. As mentioned, the solid horses with markings do not comply with the studbook breeding goal. Presumably, more such horses occurred but were not registered. The lack of registration could have contributed to the low number of cases found. To date, studies have not used molecular tests to document whether such horses carry the *To* gene.

The aim of the study was to verify the hypothesis about the crypto-tobiano horses which do not exhibit the full tobiano white-spotting pattern but transmit the dominant *To* gene to their progeny. The study was conducted on the Hucul population.

MATERIAL AND METHODS

The study did not require approval of animal care and experimental methods since it consisted of the horse description and mane hair collection for DNA testing. In horse management, DNA testing is a routine procedure. The investigation was performed on the material delivered by Hucul breeders in Poland. We searched for horses having usual white markings, with or without a small spot on the head, neck or trunk. The horses were recorded by the Polish Horse Breeders Association as solid-coloured with white markings. At least one of the parents or a grandparent of the horses had to be tobiano. Occasionally the horses produced a tobiano foal from a crossing with a solid-coloured horse.

According to the above-described means of identification, seven horses were selected for further analysis. Two categories were developed for the head description: 0 – no spots, 1 – a spot. For the limbs, a 4-point scale was developed: 0 – no patches, 1 – a patch up to the fetlock joint, 2 – a patch including the fetlock joint up to the carpus or hock joint, 3 – a patch including the carpus and hock joint.

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Table 1. Results of DNA testing and colours in parents of the studied Hucul horses

Studied horses	Molecular result	Sire	Sire's colour	Dam	Dam's colour
Bajka mare	*ToTo*	Rewir	dark bay	Beocja	bay-tobiano
Gejsza mare	*ToTo*	Rewir	dark bay	Gawęda	bay-tobiano
Gejzer stallion	*ToTo*	Len	black	Gejsza	bay-crypto-tobiano
Grażda mare	*ToTo*	Szafir	bay-tobiano	Gniewoszówka	black
Laszka mare	*ToTo*	Tuhaj-Bej	bay-tobiano	Luna	black
Pelargonia mare	*ToTo*	Sokół	black	Petunia	bay-tobiano
Wega mare	*ToTo*	Jasmon	bay-tobiano	Wierchomla	light bay

The parentage of the horses was controlled by microsatellite sequences. In each case, the pedigree turned out to be correctly assigned. To identify the *To* gene, DNA was extracted from the roots of mane hairs and tested in the Animal Genetics Laboratory (Truro, Cornwall, UK). In the *To* test, the chromosomal inversion downstream the *KIT* gene on ECA3 was analyzed according to Brooks et al. (2007). The ratio of non-tobiano (solid-coloured), crypto-tobiano, and tobiano foals born from 36 matings of the studied horses was considered on the basis of data provided by the Polish Horse Breeders Association. According to the formal description of the phenotype and in some cases photographs, the crypto-tobiano pattern in the second parent and foals was hypothesized.

RESULTS

The results of the DNA testing are presented in Table 1. All seven horses turned out to carry the single *To* gene (Figures 1–7). The amount and localization of the white spots on the trunk of particular horses are specified in Table 2 (Figures 8 and 9). One of the horses had some white hairs on the forehead due to advanced age (21 years old) and a white spot on the lower lip (Bajka mare) (Figure 1). The horses had a maximum of one limb without a white patch. The white patches reached over the fetlock joint on at least one limb. On the trunk, three horses had no patches, one horse had a white spot, and three horses had indistinct intermingled patches of a few cm up to several dozen cm in length. The base colour of all the crypto-tobianos and their parents was bay or black.

The appearance of white patches on limbs of a sire which covered the studied crypto-tobiano horses, and three foals born from the studied crypto-tobiano horse matings, suggested their crypto-tobiano status. The mating results of the studied horses are presented in Table 3. From all of the horses which were subject to test matings, a tobiano or crypto-tobiano foal was obtained. In total, 12 tobiano and crypto-tobiano foals of crypto-tobiano *inter se* matings or crypto-tobiano × non-tobiano matings were found. The ratio of progeny of crypto-tobiano test crossings was 14 non-tobianos : 3 crypto-tobianos : 8 tobianos.

Table 2. White patches in the studied Hucul horses

Studied horses	Base colour	Head	Left front limb	Right front limb	Left hind limb	Right hind limb	Neck and trunk
Bajka mare	light bay	1	3	3	2	3	weak intermingled patches on left shoulder and both flanks
Gejsza mare	bay	0	0	1	2	2	–
Gejzer stallion	black	0	3	2	2	2	weak intermingled patch on girth
Grażda mare	dark bay	0	2	2	3	3	weak intermingled patch on belly
Laszka mare	bay	0	2	2	2	3	–
Pelargonia mare	bay	0	1	0	2	2	–
Wega mare	bay	0	1	1	1	2	white spot behind elbow

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Figure 1. Bajka mare

Figure 2. Gejzer stallion

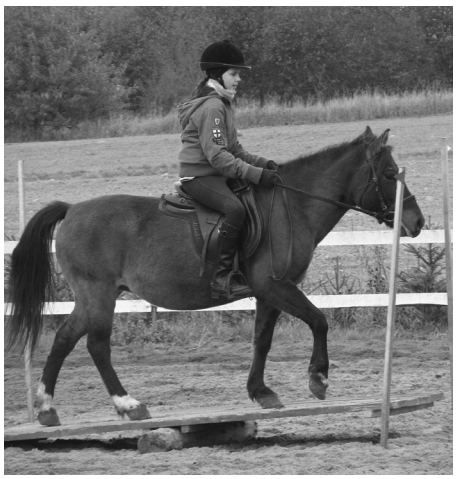

Figure 3. Gejsza mare

Figure 4. Grażda mare

Table 3. Offspring of the studied Hucul horses

Studied horses	Second parent	Offspring			
		non-tobiano	crypto-tobiano	tobiano	total
Bajka mare	non-tobiano	2	0	1	3
	crypto-tobiano	1	0	1	2
	tobiano	1	0	2	3
Gejsza mare	non-tobiano	2	1	0	3
Gejzer stallion	non-tobiano	2	0	4	6
	tobiano	1	0	4	5
Grażda mare	non-tobiano	2	1	1	4
Laszka mare	not covered				
Pelargonia mare	tobiano	0	0	1	1
Wega mare	non-tobiano	6	1	2	9
Subtotal	non-tobiano	14	3	8	25
	crypto-tobiano	1	0	1	2
	tobiano	2	0	7	9
Total		17	3	16	36

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Figure 5. Laszka mare

Figure 6. Pelargonia mare

Figure 7. Wega mare

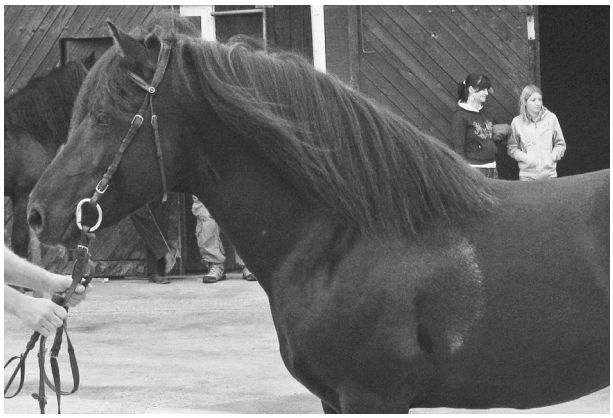

Figure 8. White patch on the trunk in Gejzer stallion

Figure 9. White patch on the elbow in Wega mare

Photographs taken by A. Deszczyńska and A. Stachurska

DISCUSSION

Crypto-tobiano horses documented. In the case of most patterns, it is often not possible to identify the exact genotype of a white or partially white horse from its phenotype (Haase et al. 2009a). Breeders report that it may be particularly difficult to reveal the *To* allele in horses which look like they are solid-coloured with markings. The horses subject to this study have turned out to be heterozygous *ToTo*. Their phenotypes were different from the typical tobiano pattern. They had no white vertical patches crossing the dorsal midline and their limbs were not white. Taking both the genotype and phenotype into account, we consider the horses to be crypto-tobianos.

Some authors have previously reported tobiano foals that descended from non-tobiano parents, in other breeds. Salisbury (1941) found 2.4% of tobiano foals produced in 7000 matings of non-tobiano American Shetland ponies, and of ponies with small white markings. Brown (1970) reported cases of two solid-coloured horses which produced tobiano offspring, suggesting the heterozygote parent might not always show white spotting. Bowling (1987) also reported four examples of tobianos which had non-tobiano parents, but a tobiano grandparent. These cases may be explained by a wrong colour identification of the parents which had to be crypto-tobianos and transmitted the *To* allele to their foals. Duffield and Goldie (1998)

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analyzed the colour of foals born from matings of homozygous tobiano horses. The authors described minimally spotted foals with the midline white expressed only around or on the tail and on one side or the other of the dorsal line.

How may the crypto-tobiano pattern be inherited? The *To* gene penetrance in some horses is very weak, in spite of the fact that the gene usually behaves as completely dominant (Haase et al. 2008). The foals produced in test matings of the crypto-tobiano horses were typical tobiano, crypto-tobiano, and solid. The appearance of tobiano and crypto-tobiano foals indicated that activity of the *To* gene in the crypto-tobiano parents was partially inhibited and in the foals it was fully or partially expressed. The results suggest the penetrance of another gene which may interact with the *To* gene in producing the tobiano or crypto-tobiano pattern. The unknown gene, let us term it *Cp*, might suppress the *To* activity, resulting in the crypto-tobiano pattern. The matings considered were not numerous enough to enable speculation on the inheritance mechanism of the gene. However, if the *Cp* gene was completely dominant, the actual ratio of foals born from the crypto-tobiano test matings would be similar to the expected ratio: 2 non-tobianos : 1 crypto-tobiano : 1 tobiano.

The base colour of the crypto-tobianos and their parents shows that all the genotypes were *E_* at *MC1R* locus. It is known that the recessive *e* gene increases the amount of white markings (Woolf 1992). Considering the low frequency of the *e* allele in Hucul population (Stachurska et al. 2012) and the linkage between the *MC1R* and *KIT* loci (Penedo et al. 2005), there would be little probability of a chestnut crypto-tobiano Hucul. Hence, at least in the case of the crypto-tobianos found in the study, the white patches could not have been increased by the *e* allele activity.

Discriminating the crypto-tobiano pattern from white markings. The results infer the only traits which may allow us to phenotypically distinguish the crypto-tobianos from the solid-coloured horses with markings – the absence of white spots on the head or a minimally marked head, combined with white patches on the limbs. Contrary to Sponenberg's (2009) finding, the limbs are not always extensively marked. In Gejsza, Pelargonia, and Wega mares in our study, the white patches were not considered to be extensive. Some crypto-tobianos had additive intermingled or white patch on the trunk. Such a patch accompanied either great, or rather small white patches on the limbs. On the other hand, horses that did not have any white on the trunk, had either less or more white on the limbs. Hence, no rule concerning the simultaneous amount of the white on the limbs and trunk in the crypto-tobianos can be defined. Presumably, horses that have patches on their limbs as well as a small white or intermingled patch on the head, neck or trunk, are crypto-tobianos, although crypto-tobianos may have no white on the head, neck, and trunk.

The occurrence of the usual face white markings and limb white markings is correlated. According to Woolf (1990), the correlation in Arabian dams equaled 0.42 which was the first indication of the same genetic mechanism influencing the appearance of white markings on the head and limbs. The correlation shows that large white markings on limbs should accompany large white markings on the head, whereas small markings on the limbs generally occur together with small face markings. An opposite combination, i.e. large patches on the limbs and no white on the head is not usual and may indicate the horse carries the *To* gene. Sponenberg (2009) postulated that such large patches on limbs with no white on the head were a subtle but important indication of the horse's true genetic makeup. However, as mentioned, the limb white patches in crypto-tobianos are not always extensive. Smaller patches may be misleading as they suggest the horse is solid-coloured with the markings. Thus, distinguishing whether a horse is crypto-tobiano or solid with markings on the limbs remains difficult. The presence of the *To* allele and crypto-tobiano pattern should always be documented by tobiano foals produced in test matings or by positive DNA testing results. As mentioned, the situation is similar in the case of many white-spotting patterns. Eliminating Huculs with white patches on the limbs from breeding, according to the selection criterion, is hasty when the genetic basis of the patches is unknown.

Possible genes producing white-spotting patterns in Huculs. Roan and the usual sabino white-spotting patterns caused by mutations in the *KIT* locus do not occur in Huculs. Those overlapping patches making an incorrect identification are, therefore, not a threat. However, as mentioned, there are several mutations at the *KIT*. At least one of the mutations accounts for most of the heritability of the markings seen at the head and limbs

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of horses (Rieder et al. 2008). Hauswirth et al. (2012) have reported a series of mutations in the *MITF* and *PAX3* loci that lead to milder depigmented splashed white and newly revealed macchiato patterns, which phenotypically overlap with the common white markings. The typical splashed white and the macchiato phenotypes are characterized by a large blaze and are not observed in Huculs. However, many genes produce various white-spotting phenotypes, e.g. *EDNRB* and *TRPM1*. New mutations, particularly at the *KIT* and *PAX3* loci, are continuously being revealed (Hauswirth et al. 2013). The Hucul horses have not been molecularly studied with regard to the genes responsible for the coat colour. It cannot be excluded that in some Huculs, white patches on the limbs (and head) may sometimes be due to a mutation different from the *To*.

Hucul selection criteria. When considering the breeding programme of Hucul horses, the simultaneous occurrence of the tobiano or crypto-tobiano patterns and the usual white markings should be discussed. The question is whether eliminating horses with markings according to the selection criterion may contradict the allowing of the patterns. Long-termed selection has resulted in rare markings, at least in solid-coloured Huculs. Probably, even if the *To* gene was closely linked with one of the most important genes responsible for the markings (Rieder et al. 2008), those genes would be inherited together, whereas the recessive *To* allele would pair with an allele not producing the markings. The amount of the markings in tobianos is not important. The solid-coloured horses would not inherit the undesirable trait. Thus, the breeding criteria would not contradict each other. However, those suggestions need further investigation on the molecular level.

CONCLUSION

The study displayed the occurrence of crypto-tobiano horses in the Hucul breed. The crypto-tobiano phenotype is different from the usual tobiano pattern. The crypto-tobiano pattern occurs in horses which have a tobiano parent or grandparent. Horses that have patches on their limbs as well as a small white or intermingled patch on the head, neck or trunk, are crypto-tobianos, although crypto-tobianos may have no white on the head, neck, and trunk. Like in the case of many white-spotting patterns, the presence of the *To* gene should be documented by DNA testing or test matings. The results suggest an activity of an inhibitor gene which suppresses the *To* allele penetrance in crypto-tobiano horses.

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