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An equine chromosome 3 inversion is associated with the tobiano spotting pattern in German horse breeds

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Summary

The tobiano white spotting pattern is one of several known depigmentation phenotypes in horses and desired by many horse breeders and owners. The tobiano spotting phenotype is inherited as an autosomal dominant trait. Horses that are heterozygous or homozygous for the tobiano allele (*To*) are phenotypically indistinguishable. A single nucleotide polymorphism (SNP) associated with the *To* had previously been identified in intron 13 of the equine *KIT* gene and was used for an indirect gene test. The test was useful in several horse breeds. However, genotyping this sequence variant in the Lewitzer horse breed revealed that 14 % of horses with the tobiano pattern did not show the polymorphism in intron 13 and consequently the test was not useful to identify putative homozygotes for *To* within this breed. Speculations were raised that an independent mutation might cause the tobiano spotting pattern in this breed. Recently, the putative causative mutation for *To* was described as a large chromosomal inversion on equine chromosome 3. One of the inversion breakpoints is approximately 100 kb downstream of the *KIT* gene and probably disrupts a regulatory element of the *KIT* gene. We obtained genotypes for the intron 13 SNP and the chromosomal inversion for 204 tobiano spotted horses and 24 control animals of several breeds. The genotyping data confirmed that the chromosomal inversion was perfectly associated with the *To* allele in all investigated horses. Therefore, the new test is suitable to discriminate heterozygous *To/+* and homozygous *To/To* horses in the investigated breeds.

Different white spotting patterns such as overo, sabino, and tobiano exist in horses. The tobiano white spotting pattern is one of the known depigmentation phenotypes in horses and desired and selected by many horse breeders and owners. The tobiano spotting pattern is characterized by regular and distinct oval or round patches of depigmented skin and coat that extend down over the neck and chest, giving the appearance of a shield. In tobiano animals the distal limbs are typically depigmented and one or more depigmented patches cross the dorsal midline. The extent of depigmentation can approximately range from 20% to 80% of the body surface. The tobiano coat pattern is inherited as an autosomal dominant trait and the mutant allele, *Tobiano (To),* can be found in various horse breeds (described in Sponenberg 1996). The spotting pattern is present at birth and stable throughout life. It can occur on any basic coat color and in combination with any other spotting pattern. Homozygote and heterozygote tobiano horses are phenotypically indistinguishable. For breeders interested in producing tobiano offspring, homozygous *To/To* horses are particularly valuable. *To* was previously shown to be linked to *albumin* (Trommershausen-Smith, 1978). Later a phase conservation to the Albumin-B (*ALB-B)* and Vitamin D binding factor-S (*Gc-S*) was demonstrated (Bowling, 1987). This was the first test to identify putatively homozygous *To/To* animals. However, exceptions have been reported and therefore, identification of a better marker or the causative mutation for tobiano spotting was sought for. A strong candidate for the tobiano gene was the equine homologue of the proto-oncogene *c-kit* (*KIT*). KIT is a member of the receptor protein-tyrosin kinase subclass III family and its gene is closely linked to the *albumin* and *Gc* loci (Sandberg & Juneja, 1978). Mutations in the *KIT* gene are known to cause pigmentation disorders in various species like human, mouse, pig, and horse (Giebel *et al.* 1991; Chabot *et al.* 1988; Fleischman *et al.* 1991; Spritz RA, 1994; Geissler *et al.* 1988; Marklund *et al.*

1998; Haase *et al.* 2007). A subset of sabino-spotted horses is also caused by a mutation within the *KIT* gene (Brooks and Bailey, 2005).

Sequence comparisons between solid-colored and homozygous *To/To* horses revealed a base substitution in intron 13 of the *KIT* gene, which was stronger associated with *To* than the *Alb-B:Gc-S* haplotype (Brooks *et al.* 2002). This substitution introduced an additional *Msp*I restriction site into the DNA fragment. The allele with the restriction site was termed *KM1* while the allele without the *Msp*I site was referred to as *KM0*. Every tested tobiano horse had the *KM1* allele. However, the *KM1* allele was also rarely found in solid-colored horses, which demonstrated that this polymorphism is not the causative mutation for the tobiano spotting pattern. Recently, a large chromosomal inversion on the equine chromosome 3 was described as the potentially causative mutation for *To*. The inversion was only found in horses with the tobiano spotting pattern. The inversion begins approximately 100 Kb downstream of the *KIT* gene and does not interrupt any annotated genes. The inversion was hypothesized to disrupt a regulatory element of the *KIT* gene.

Our own analyses corroborated the association between the *KM1* mutation and the tobiano spotting pattern in German ponies, Paint and Pinto horses, whereas the association was very weak in Lewitzer horses. Therefore it was speculated that there might be an independent tobiano causing mutation segregating in Lewitzer horses (unpublished data). In this study we compare the genotypes at the previously reported *KIT* intron 13 SNP and the recently reported chromosomal inversion to investigate whether the reported association of the chromosomal inversion on ECA3 with the tobiano spotting pattern also exists in German horse breeds, especially the Lewitzer horses.

Altogether 228 horses were tested for the *KIT* intron 13 *Msp*I polymorphism and the chromosomal inversion on ECA3. The samples consisted of 155 Lewitzer horses (148

tobiano, 7 solid-colored), 15 Pinto Pleasure horses (11 tobiano, 4 solid-colored), 7 German ponys (1 tobiano, 5 solid-colored, 1 sabino), 4 Pinto ponys (4 tobiano) and 47 Tinker horses (38 tobiano, 8 solid-colored, 1 roan). DNA was isolated from blood using the Nucleon BACC2 kit (GE Healthcare, Munich, Germany) according to the manufacturer's protocol.

Initially, the DNA samples were genotyped at the *KIT* intron 13 SNP. An *Msp*I PCR-RFLP was performed as described previously (Brooks *et al.* 2002). Briefly, PCR was carried out in a total volume of 20 µl containing 20 ng DNA, 1 unit AmpliTaq Gold (Applied Biosystems, Rotkreuz, Switzerland) 10 pmol of each primer, 5 mM dNTPs (Roth, Reinach, Switzerland) and 1.5 mM $MgCl_2$ in the buffer supplied by the manufacturer. The amplification was performed using an initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. Finally an extension step at 72°C for 5 min was performed. PCR products were digested using *Msp*I (New England Biolabs, Allschwil, Switzerland) according to the manufacturer's directions. Products were separated and visualized on 2% agarose gels stained with ethidium bromide.

Subsequently, the DNA samples were genotyped for the chromosomal inversion as published by Brooks *et al.* 2007. PCR was carried out in a total volume of 20 µl containing 20 ng DNA, 1 unit AmpliTaq Gold, 10 pmol of primer P3_F, 0.75 pmol of primer P3xR and 0.25 pmol of primer P3toR, 5 mM dNTPs, and 1.5 mM $MgCl_2$ in the buffer supplied by the manufacturer. The amplification was performed using an initial denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 95°C for 45 s, annealing at 57°C for 45 s and extension at 72°C for 45 s. Finally an extension step at 72°C for 5 min was performed. Products were separated on 3% agarose gels.

A total of 228 horses of five breeds were genotyped for this study. For most horses, pedigree information was made available by the breeders. Table 1 shows the genotypes of the animals and their association to the tobiano phenotype.

The association of the *KM1* allele at the *KIT* intron 13 mutation with *To* was found to be strong for German ponies, Pinto ponies and Pinto Pleasure horses. Within these breeds only one solid-colored German pony did not show the expected genotype as it was typed *KM0/KM1*. However, it had already previously been observed that the *KM1* allele rarely is found in solid-colored horses (Brooks *et al.* 2002).

In the Tinker and Lewitzer breeds we observed tobiano horses that were typed homozygous *KM0/KM0*. So far, only solid-colored horses carrying the *KM1* allele had been reported to cause misinterpretations of the intron 13 gene test. In Tinker and Lewitzer horses now also the opposite haplotype was observed, where tobiano spotted horses are homozygous for the *KM0/KM0* allele. In the Lewitzer population the frequency of such animals was 14%, which renders this test useless for marker-assisted selection decisions. As the previously reported strong association of the intron 13 mutation was not observed in Lewitzer horses, it was hypothesized that in this breed a second independent mutation causing the tobiano spotting pattern might segregate.

In contrast to the genotypes at the *KIT* intron 13 SNP, the genotypes at the recently reported chromosomal inversion were perfectly associated with the coat color phenotypes. Among the 204 animals registered as tobiano only two related Tinker horses did not have the chromosomal inversion. When the phenotypes of these horses were examined in more detail, it was observed that they had extended white markings and white belly spots but did not have depigmented areas across the dorsal midline (Fig. 1). Therefore, it seems likely that these horses were in fact incorrectly registered and do not represent tobiano spotted horses. Whereas all truly tobiano spotted horses

carried at least one chromosome with the inversion, none of the tested non-tobiano horses carried the inversion.

Thus the data presented here provide additional evidence that the reported inversion on equine chromosome 3 is indeed the causative mutation for the tobiano spotting pattern. Furthermore this mutation explains the phenotype of all investigated tobiano horses including all tobiano horses from the Lewitzer breed. Our study points to a common ancestor of tobiano spotted horses in diverse populations, such as e.g. American Paint horses, Pinto Pleasure horses, and Lewitzer horses, which most likely lived more than two hundred years ago. Finally, genotyping for the chromosomal inversion may help to resolve ambiguous phenotype classifications in addition to identifying the valuable homozygous *To/To* animals.

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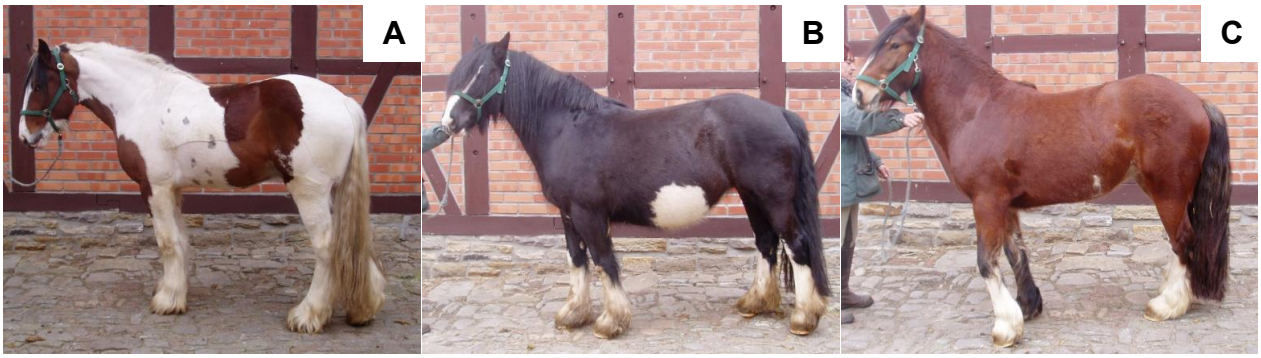

Figure 1 Phenotypes of investigates horses. (A) Tobiano spotted Tinker horse. Note the depigmented areas across the dorsal midline, which are characteristic for the tobiano spotting pattern. (B, C) Tinker horses registered as tobiano but without the chromosomal inversion on chromosome 3. The two horses are closely related (mother and daughter). As the phenotypes do not correspond to the tobiano spotting pattern, these horses are most likely incorrectly registered.

Table 1 Genotypes at the *KIT* intron 13 SNP and the chromosomal inversion.

		KIT intron 13			ECA 3 inversion		
		KM0/KM0	*KM0/KM1*	*KM1/KM1*	*+/+*	*+/To*	*To/To*
Tobiano							
	Lewitzer	21	97	30	0	107	41
	Tinker	3	26	11	2[1]	36	2
	German pony	0	1	0	0	1	0
	Pinto Pleasure	0	11	0	0	11	0
	Pinto pony	0	3	1	0	3	1
	total	24	138	42	2[1]	158	44
Non-tobiano							
	Lewitzer	7	0	0	7	0	0
	Tinker	7	0	0	7	0	0
	German pony	5	1	0	6	0	0
	Pinto Pleasure	4	0	0	4	0	0
	Pinto pony	0	0	0	0	0	0
	total	23	1	0	24	0	0

[1]These two Tinker horses were registered as tobiano horses. However, their phenotype did not fit the criteria for tobiano horses (Fig. 1). Therefore these two horses were most likely non-tobiano and registered with an incorrect coat color phenotype.