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Genetic mapping of the (*G*)-locus, responsible for the coat color phenotype "progressive greying with age" in horses (*Equus caballus*)

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Color phenotypes may have played a major role during initial selection among domestic animal species. Even today, breeds are often distinguished by a typical pattern of coat or plumage color. It appears that most color phenotypes follow a relatively clear mode of Mendelian inheritance. Therefore, color phenotypes are among the first traits to be systematically analyzed and characterized on a molecular level (Jackson 1994). Genetic variation in coat color genes is supposed to be one of the tools to allow for breed-specific genotyping in order to identify individuals of unknown origin and to control the traceability of their products. Mutations with functional consequences, such as coat color alleles, would further provide a better idea of the effective genetic diversity in domestic animal species than estimations based only on non-functional genomic variation.

Progressive greying with age (*G*) is a coat color phenotype in the horse with a dominant mode of inheritance. The color inheritance of grey has also delineated what can be called the "grey rule": a grey horse must have at least one grey parent (Trommershausen-Smith et al. 1976). Greying (*G*), together with the basic horse coat colors chestnut, bay, and black, is widely spread among horse breeds all over the world. A grey horse is always born colored, and its skin remains pigmented, even after the coat has turned grey. Exceptions are white head and leg markings, as well as partial vitiligo due to aging. The four basic horse coat colors interact together in an epistatic manner, such that bay and black are not expressed in the presence of chestnut, and that grey is always expressed (Rieder et al. 2001). Grey horses are known to be particularly susceptible to skin melanoma (Fleury et al. 2000; Heinzerling et al. 2001). It is not known what sort of correlation exists between greying with age and melanoma development. However, a genetic impact on the disease on the basis of family data has been demonstrated (Rieder et al. 2000; Seltenhammer et al. 2002).

The *Franches-Montagnes* (*FM*) is a native Swiss horse breed with presently about 3500 registered mares. Among solid coat colors, greying with age (*G*) segregates in this light draft horse population. Based on stud-book entries from the *Swiss FM-Horse Breeding Association*, samples were collected from a total of 50 *FM* horses, and DNA was extracted according to standard protocols. The 50 horses belong to two distinct family groups (A and B; Fig. 1) segregating for *G*. Information about coat color phenotypes of non-living ancestors within these families was available from the breed registry. It was possible to trace back the pedigrees to the two "grey founder" horses of unknown direct common ancestry (for family A, Arabian stallion 1955; for family B, mare of unknown oriental-North African origin 1945). It is important to note that, owing to breeding policies of the *Swiss FM-Horse Breeding Association*, no male carriers of *G* were used for reproduction apart from the one founder stallion (Family A; Fig. 1). This particular situation allows us to confirm the dominant mode of inheritance for *G* in horses, since this phenotype segregates only through the dams in the *FM* population and, as a consequence, results in only heterozygous carriers (Fig. 1). This is especially helpful for the analysis of the trait, as it allows us to clearly define the genotype underlying the grey or solid colored phenotype of each horse in each family. Altogether, the study includes coat color segregation information of 96 *FM* horses, excluding 15 unrelated *Camargue* horses that were added at a later stage of the analysis. This very ancient, semi-feral French breed is known for its all-grey phenotype. No other coat colors are allowed to be registered to the *Camargue* stud-book. Therefore, *Camargue* horses are believed to be homozygous for *G* (Duncan. 1992).

The 50 sampled *FM*-horses from families A and B were genotyped with a series of microsatellite markers covering parts of ten different equine chromosomes (Lindgren et al. 1998; Shiue et al. 1999; Guérin et al. 1999; Swinburne et al. 2000). From comparative mapping (Raudsepp et al. 1996) or available horse physical and synteny mapping data (Godard et al. 2000; Mariat et al. 2001; Bowling and Ruvinsky 2000), those chromosomal regions were believed to contain candidate genes for coat color and/or melanoma susceptibility: *HMS007*, *HMS015*, *UM004*, *UM026* (ECA1q-MYO5A, PED); *ASB023*, *LEX057* (ECA3q–KIT); *COR070*, *TKY028*, *UCDEQ465* (ECA6q–PMEL17, PAX3); *LEX038*, *TKY012*, *SGCV028* (ECA7p&q–TYR); *AHT037*, *AHT038*, *HMS020*, *HTG003* (ECA16q–MITF); *COR007*, *COR105* (ECA17q–TYRP2, EDNRB); *COR001*, *COR022* (ECA22q–ASIP); *LEX063*, *UM022* (ECA23q–TYRP1, putative CDKN2A, putative MART1); *COR018*, *COR080*, *UCDEQ405*, *UCDEQ 464* (ECA25q–putative CDKN2A, putative MART1); *HTG030*, *IGF1*, *NVHEQ054*, *TKY333*, *UM003* (ECA28q–KITLG, putative APAF1). The detailed allocation of conserved chromosomal segments from human Chr 9 to ECA23q and ECA25q is not completely known yet.

The presence of a "null-allele" of microsatellite marker *COR080* was circumvented by designing a new reverse primer sequence (5′AGCATGCATTGTAGCAACTCCA3′), based on the GenBank entry AF142617. To determine the birth-color of grey horses and the variation of known coat color alleles in the population, all *FM* horses were typed for *MC1R* (*Ee*) and *ASIP* (*Aa*) mutations, as described previously (Henner et al. 2002). PCRs were performed on a PTC 100 MJ-Research thermocycler (Watertown, USA) by using either

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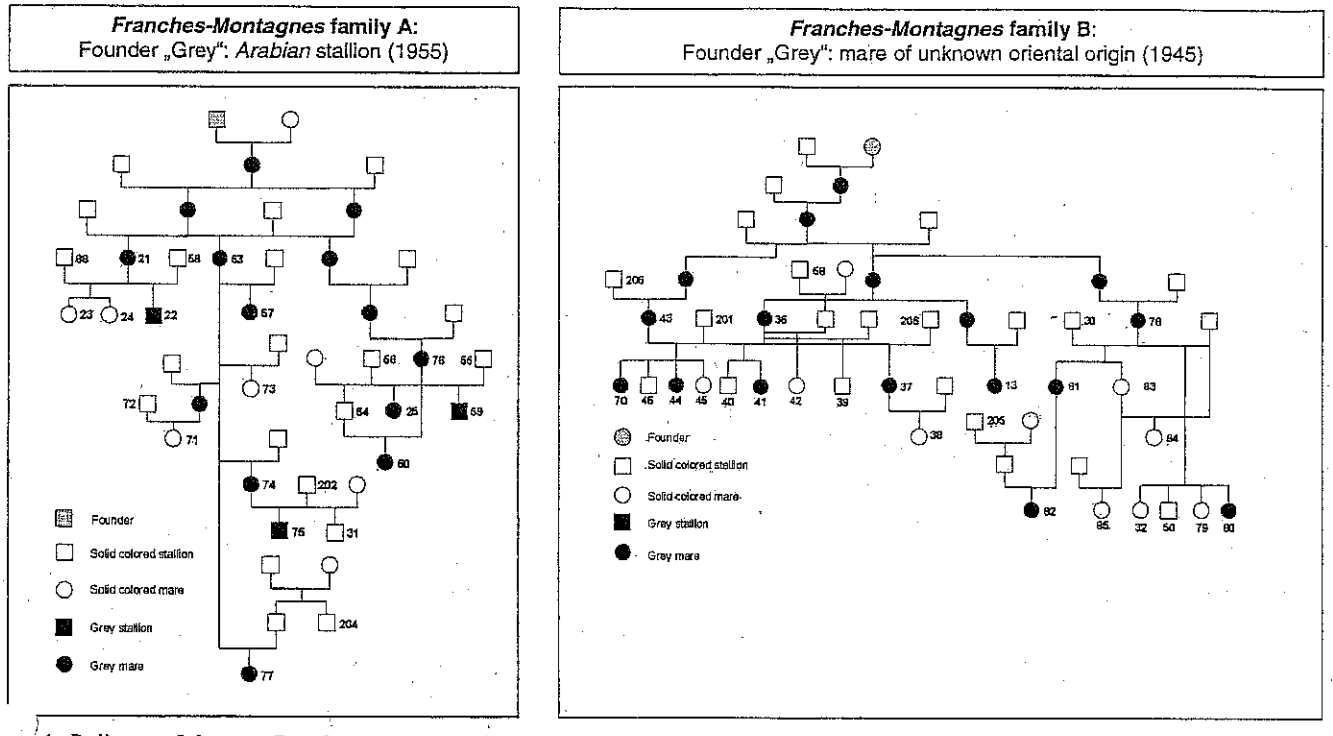

Fig. 1. Pedigrees of the grey *Franches-Montagnes* reference families A and B (ID-numbers indicate horses with a blood or hair sample).

Table 1. Overview of lod scores detected for a group of markers on ECA25q in two horse families segregating for grey coat color (MS = microsatellite markers; NA = number of alleles; IM = informative meioses; Z = lod score; θ = recombination fraction).

Family	MS	NA	IM	Z	θ
A	*UCDEQ405–G* locus	6	12	–	–
	COR18–G locus	5	9	–	–
	COR80–G locus	4	11	**2.20**	**0.00**
	UCDEQ464–G locus	4	8	–	–
B	*UCDEQ405–G* locus	6	22	2.30	0.06
	COR18–G locus	4	16	**3.20**	**0.00**
	COR80–G locus	4	20	**4.80**	**0.00**
	UCDEQ464–G locus	4	26	–	–
A ± B	*UCDEQ405–G* locus	6	34	1.90	0.15
	COR18–G locus	5	25	1.90	0.12
	COR80–G locus	5	31	**7.00**	**0.00**
	UCDEQ464–G locus	4	34	–	–
A + B	*UCDEQ405–UCDEQ464*	–	–	1.10	0.12
	UCDEQ405–COR018	–	–	2.40	0.09
	UCDEQ405–COR080	–	–	2.10	0.10
	COR018–COR080	–	–	2.80	0.05
	COR018–UCDEQ464	–	–	0.70	0.20
	COR080–UCDEQ464	–	–	1.50	0.08

GoldStar Taq-polymerase from Eurogentec (Seraing, Belgium) or Taq-polymerase from Amersham-Pharmacia Biotech (Piscataway, USA). Information about marker oligonucleotide primer sequences, amplification conditions, and polymorphism information content were taken from the original publications or from horse maps available on the web for all microsatellite markers (http://locus.jouy.inra.fr; http://www.thearkdb.org). The forward primer of each marker was 5′-labeled, with either a *FAM*, *JOE*, or *TAMRA* fluorescence tag, in order to perform fragment length analysis of obtained PCR products with an ABI 377 sequencer and the GeneScan software package (Applied Biosystems, Foster City, CA, USA). The analysis followed standard protocols and manufacturer's instructions. Marker genotypes, phenotypes, and pedigree data were analyzed for possible linkage with *MLINK* and *ILINK* software from the *LINKAGE* package (Terwilliger and Ott 1994). Multilocus linkage disequilibrium analysis was performed with the *DISMULT* software (Terwilliger 1995). All linkage computation was done on a server at the *UK HGMP Resource Centre in Cambridge, GB* (http://www.hgmp.mrc.ac.uk/).

Positive lod scores were found in family B between marker *COR018* and greying, and between marker *COR080* and greying from horse Chr 25q (Table 1). In family A, no linkage was found between marker *COR018* and greying, but a tendency could be shown between *COR080* and greying (Table 1). The additive and overall lod score found in families A and B between marker *COR080* and greying was $Z = 7.0$ for $\theta = 0$ (Table 1). This result indicates that, although the founder horses for greying in the *FM* families A and B are not closely related (even though both were of oriental origin), the (*G*)-locus appears to map to the same chromosomal region on ECA25q. Furthermore, these findings are in accordance with those of an independent full-genome scan study, analyzing the same coat color trait in the *American Quarter Horse*, presented very

recently by Locke et al. (2002). The *American Quarter Horse* (*QH*) is a breed particularly influenced by ancient *Iberian* horse breeds and *English Thoroughbred*. *Iberian* horses have a partial north African (*Barbe*) and oriental (*Arabian*) origin, just as the *English Thoroughbred* does. It is known that oriental-type horse breeds show an increased frequency of grey individuals; maybe a favorable adaptation to hot climates? The results from those two different studies indicate that the equine (*G*) locus maps in different horse populations to a common region on ECA25q.

Linkage analysis between microsatellite markers from ECA25q shows a tendency for linkage with insignificant lod scores (Table 1). It seems that in the two *FM* families, the markers proximal of *COR080* (*COR018*, *UCDEQ405*) keep a tendency for linkage, whereas the one marker distal of *COR080* (*UCDEQ464*) shows no relation with grey coat color at all (Table 1). However, this might be due to the structure of the pedigrees, the limited number of animals, and informative meioses.

Multilocus linkage disequilibrium analysis for fine mapping of the (*G*) locus in families A and B revealed significant LRT-chi-square-values and lod scores. Intermarker map distances were kept fixed according to the results shown in Table 1. Highest values were found for the map position *COR080* (LRT = 20.0; Z = 4.3). Thus, the linkage disequilibrium results suggest that the (*G*) locus is located very close to *COR080*. LRT-chi-square-values and lod scores increased (LRT = 28.4; Z = 6.2), but the position of the (*G*) locus did not differ, after a similar haplotype analysis was performed, including 15 unrelated homozygous grey *Camargue* horses to the *FM* sample. This suggests that the (*G*) locus maps in both breeds to the same chromosomal region. The *FM*, the *QH*, and also the *Camargue* horses were all influenced by "oriental blood" during their breeding history, and the hypothesis of a common oriental origin of the grey coat color can be put forward but needs further investigation.

According to comparative mapping, the (*G*) locus maps to an equine chromosomal region sharing homology with human Chr 9q22.3 to 9q34.3 (Raudsepp et al. 1996; Caetano et al. 1999; Godard et al. 2000; Swinburne et al. 2000), itself homologous to mouse Chr 2, 4, and 13. According to the horse full-sibling linkage map of Swinburne and colleagues (2000), thioredoxin (*TXN*) on human Chr 9q31 is closely linked to *COR018*. Furthermore, *UCDEQ464* maps to ECA25q18-19 (Lindgren et al. 2001) in the same region as dopamine beta-hydroxylase (DBH)—HSA9q34.3—and erythrocyte membrane protein band 7.2 (*EPB72*)—HSA9q34.1; the latter was mapped to ECA25q17, as shown by Godard and colleagues (2000). Finally, this human chromosome region also contains glucose-regulated protein (*GRP78*), synteny mapped to ECA25q (Caetano et al. 1999). Thus, a potential candidate gene homolog for the equine grey coat color locus (*G*) most likely belongs to these human or mouse chromosome regions.

Apart from applications in practical horse breeding programs, mapping of the progressive greying with age (*G*) locus is of particular interest as a first step towards a molecular explanation of aging grey horse melanoma. Whether greying with age (*G*) in horses is comparable to greying human hair remains to be analyzed in future studies.

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