Allelic frequencies of MC1r and ASIP genes in Iberian horses

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Abstract

Coat colour affects differentiation of populations in breeds. In horses, two different alleles have been described in the MC1r gene, the wild type allele (E+) and the recessive allele (C901T; e), which determines the chestnut coat colour when homozygous. In the agouti gene also two alleles have been described, the wild type (A+) and the black recessive allele (ADEx2; Aa), determining the black coat colour when homozygous. The MC1r gene is epistatic to agouti gene.
Iberian horses are classified in two different groups 'Celtic' and 'Iberian' that can be well differentiated by means of morphological traits. Here, we show the allelic frequencies of MC1r and ASIP genes in a representative sample of breeds belonging to 'Celtic': *Asturcón* (45), *Caballo de Corro* (8), *Mérens* (19), *Losino* (12) and *Garrano* (10); and 'Iberian': Carthusian (10), Andalusian (7), *Marismeño* (10) and *Mallorquí* (14). The black recessive allele is predominant in Celtic horses, being all *Asturcón*, *Mérens* and *Losino* samples homozygous, and also the Iberian *Mallorquí*. The chestnut allele is mostly found in Iberian horses and *Garrano* (>30%), and at a very low frequency (<10%) in the *Asturcón* and *Mérens* breeds.

Introduction

Iberian horses have been classically divided in two different native groups (Aparicio, 1944): 'Celtic' ponies and 'Iberian' horses. The first group, mainly settled along the Cantabrian Range in the Northwestern Atlantic Iberian areas (Figure 1) is subdivided into breeds or populations such as the *Garrano* in Portugal (which is called *Faco galego* on the Spanish side of the border) or the *Asturcón* and *Pottoka* in Spain. The second group is located in the Southern Iberian Peninsula, and includes the two lines of the Spanish purebred horse (Andalusian and Carthusian) and *Marismeño* (an ancient semi-feral horse population located in Doñana Natural Park) in Spain and the *Lusitano* and the *Sorraia* in Portugal. Both Celtic and Southern Iberian strains of horses can be well differentiated by means of morphological traits (Jordana et al., 1995; Jordana and Parés, 1999).

Coat colour in mammals basically depends on the relative amount of the two basic types of tyrosine-derived melanin: eumelanin (black/brown) and phaeomelanin (yellow/red) which are controlled, in turn, by the Extension (**E**) and Agouti (**A**) loci (Searle, 1968). Mutations at either locus may commit the melanocyte to exclusive synthesis of a single pigment. In a variety of mammals, dominant alleles at Extension locus act to produce a uniform black coat colour (Klungland et al. 1995; Jackson 1997), whereas recessive alleles at this locus extend the amount of red/yellow pigment. The wild-type state in many species involves synthesis of both melanin types. Conversely, dominant alleles at Agouti locus cause a yellow coat whereas homozygosity for the recessive allele would be associated with a uniform black coat (Jackson 1997). Molecular studies (Cone et al., 1996; Robbins et al., 1993) revealed that Extension locus encodes a melanocyte stimulating hormone receptor, also known as the melanocortin-1 receptor (MC1R) which is involved in the eumelanin and phaeomelanin production while the Agouti signaling peptide (encoded in the ASIP locus) acts as an antagonist of MSH by binding to MC1R and thereby preventing the MC1R-MSH interaction, resulting in phaeomelanin synthesis (Bultman et al., 1992).

In horses, polymorphisms in the MC1R and ASIP loci, affecting coat colour, have been identified (Marklund et al., 1996; Rieder et al., 2001). In the MC1R gene, a C901T mutation is responsible for the chestnut allele (Marklund et al., 1996). In the ASIP gene a 11 bp deletion in exon 2 (ADEx2) is responsible for the recessive black coat colour (Rieder et al., 2001). No other mutations have been described in this loci affecting coat colour.

In this work, we show the allelic frequencies of MC1r and ASIP variants in a representative sample of breeds belonging to the 'Celtic' and 'Iberian' groups.

Materials and Methods

Sampling and DNA isolation.

Blood samples were taken form a representative sample of breeds belonging to the 'Celtic': *Asturcón* (45), *Caballo de Corro* (60), *Mérens* (19), *Losino* (12) and *Garrano* (10); and 'Iberian': Carthusian (10), Andalusian (7), *Marismeño* (10) and *Mallorquí* (14), groups of horses in the Iberian Peninsula. Main locations of the sampled breeds are shown in Figure 1. DNA was isolated using standard procedures (Sambrook et al., 1989).

Genotyping

We have developed a diagnostic protocol, which allowed to simultaneously identify the presence of the chestnut allele (C901T) in the MC1R gene and the black allele in the ASIP gene. For the identification of the chestnut allele a fluorescent PCR-RFLP protocol was used (Marklund et al., 1996; Rieder et al., 2001). For the black allele a fluorescent PCR protocol based on the sequence of the exon 2, including the deleted fragment, was used. The two alleles can be differentiated depending on the size of the fragment. Basically, the PCR protocol consisted of a 20 µl reaction mix containing approximately 50 ng of total horse DNA, 0.2 µM of each primer (2 couples), 2.5 mM Cl_2Mg, 0.2mM of dNTPs and 0.5 U of Taq polymerase (Biotools). The PCR consisted of an initial denaturation step at 94º C for 5 min followed by 35 cycles of 30 s at 94º C, 30 s at 60º C and 1 min at 72º C, with a final elongation step of 10 min at 72º C after the

last cycle. Sequences of used primers are listed in Table 1. After the PCR finalization, 10 µl of the PCR product were digested with 3 units of TaqI restriction enzyme (New England Biolabs). After PCR and digestion, 0.5 µl of each product were mixed, loaded into an acrylamide gel and electroforesed in an Alf Express II semiautomatic sequencer. Figure 2 shows the image of the diagnostic fragments.

Results and Discussion

A total of 187 horses belonging to 9 breeds were genotyped for the MC1R and ASIP genes. Number of sampled individuals per breed, description of the accepted coat colour in the corresponding studbook and the allelic and genotypic frequencies found are given in Tables 2 and 3, respectively. Three of the Celtic horse breeds sampled only accept black coats while the other two breeds (*Caballo de Corro* and *Garrano*) admit any thru colour with bay coats in high frequency. Regarding to the Iberian breeds there are more differences: Carthusians (all of them) and Andalusians are mainly grey while the other breeds are black (*Mallorquî*) or bay (*Marismeño*).

As expected, the black recessive allele (**a^b**) of the ASIP gene is fixed the *Asturcón*, *Mérens*, *Losino* and *Mallorquí* populations. In all those breeds, black coat colour is selected for. The lowest frequency of the black recessive allele is in the Carthusian population. The wild allele (**E^+**) of the MC1R gene exceeds a frequency of roughly two thirds in all the sampled breeds. The chestnut allele (**e**) was below 0.1 in three black coated breeds (*Asturcón*, *Mérens* and *Mallorquî*) while this allele is in a frequency of 0.25 in the *Losino* breed.

Only one animal (Andalusian) was homozygous for the chestnut allele. This genotype is not present in the Carthusian individuals sampled. Regarding the genotypic frequencies for the ASIP gene in Andalusians and Carthusians we can suggest that the grey factor is independent to the ASIP genotype but is dependent on the **E^+** allele of the MC1R gene.

In some Celtic horses an in the *Mallorquí* breed a selection for black coat colour has been being carried out for years. The black recessive allele is predominant in Celtic horses, except *Mallorquí*, being all *Asturcón*, *Mérens* and *Losino* samples homozygous. In these four breeds only black coated individuals are allowed to be included in the herdbook. Consequently, a selection against chestnut coats (appearing in low frequency in these breeds) is also carried out in these breeds especially in *Asturcón* and *Mallorquí* breeds. The chestnut allele is mostly found in Iberian horses (except *Mallorquî*) and *Garrano* (>30%), and the lowest frequency is found in the *Mérens* breed (0.05). Anyway, due to the recessive inheritance of chestnut coat colour, we could find some chestnut alleles in all the sampled populations. Among the Celtic horses some populations do not select animals based on its coat colour, then in *Caballo de Corro* and *Garrano* populations allelic frequencies in the ASIP gene are around 0.5.

Chestnut allele merits a special attention. Many herdbook associations do not like chestnut allele carrier animals in its populations. Then a rapid test based on the PCR can help them to choose preferential matings based on the genotype, to correctly manage the chestnut allele. Nowadays, the test presented here is being used for this purpose by the *Asturcón* pony breeders association (ACPRA), *Caballo de Corro* breeders association (García-Dory) and *Cavall Mallorquí* breeders associations.

Acknowledgements

This research was partially funded by a grant from the INIA No. RZ03-011. The authors are indebted to the Breeders Associations especially ACPRA (http://www.asturcones.com/) and *Asociación García-Dory* for their kind collaboration.

References

Aparicio, G., 1944 *Zootecnia especial. Etnología compendiada, 3ª edición*. Imprenta Moderna, Córdoba, Spain.

Bultman, S. J., Michaud, E. J., and Woychik, R. P. 1992: Molecular characterization of the mouse agouti locus. Cell. **71**:1195-1204.

Cone, R. D., Lu, D., Koppula, S., Våge, D. I., Klungland, H., Boston, B., Chen, W., Orth, D. N., Pouton, C., and Kesterson, R. A. 1996: The melanocortin receptors: agonist, antagonists, and the hormonal control of pigmentation. Rec. Progr. Horm. Res. **51**:287-318.

Jackson, I. J. 1997: Homologous pigmentation mutation in humans, mouse and other model organisms. Hum. Mol. Genet. **6**: 613-1624.

Jordana, J., P. M. Parés, and A. Sánchez, 1995 Analysis of genetic relationships in horse breeds. Journal of Equine Veterinary Science **7**: 320-328.

Jordana, J., and P. M. Parés, 1999 Relaciones genéticas entre razas ibéricas de caballos utilizando caracteres morfológicos (prototipos raciales). AGRI **26**: 75-94.

Klungland, H., Våge, D. I., Gómez-Raya, L., Adalsteinsson, S., and Lien, S. 1995: The role of melanocyte-stimulating hormone (MSH) receptor in bovine coat colour determination. Mamm. Genome **6**: 636-639.

Marklund S., Moller M.J., Sandberg K., Andersson L. 1996. A missense mutation in the gene for the melanocyte-stimulating hormone receptor (MC1R) is associated with the chestnut coat color in horses. Mamm. Genome **7**: 895-899.

Rieder, S., Taourit, S., Mariat, D., Langlois, B., and Guérin, G. 2001. Mutations in the agouti *(ASIP*), the extension *(MC1*R) and the brown *(TYRP*1) loci and their association to coat color phenotypes in horses *(Equus caballu*s). Mamm. Genome **12**: 450-455.

Robbins, L. S., Nadeau, J. H., Johnson, K. R., Kelly, M. A., Roselli-Rehfuss, L., Baack, E., Mountjoy, K. G., and Cone, R. D. 1993: Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function. Cell **72**: 827-834

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989: Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press. U.S.A.

Searle, A. G. 1968: Comparative genetics of coat colour in mammals. Logos Press, London, United Kingdom.

Table 1. Primer name and sequence.

Primer name	5’-3’ Sequence
Chestnut-for	5’-CCCATGTACTACTTCATCTGCTGCC-3’
Chestnut-rev	5’-GCACATCAATGATGTTGTCCAGCTG-3’
Recessive_Black -for	5’-TCCTCCCCAATTCTCTGCAGTTCATG-3’
Recessive_Black-dn	5’-TCCAAGGCCTACCTTGGAAGATCTC-3’

Table 2. Number of individuals sampled, description of the coat colour accepted in the studbook and allelic frequencies of the MC1R and ASIP genes for nine Iberian horse breeds .

			MC1R		ASIP	
Population	N	Coat Colour	Chestnut(e)	E^+	Recessive-Black(a^b)	A^+
Asturcón	45	Black	0.07	0.93	1	0
Caballo de Corro	60	Any thru colour; mainly Bay	0.2	0.88	0.42	0.58
Merens	19	Black	0.05	0.95	1	0
Losino	12	Black	0.25	0.75	1	0
Garrano	10	Any thru colour; mainly Bay	0.35	0.65	0.55	0.45
Carthusian	10	Grey	0.2	0.8	0.15	0.85
Andalusian	7	Any thru colour; mainly Grey	0.36	0.64	0.43	0.57
Marismeño	10	Any thru colour; mainly Bay	0.35	0.65	0.3	0.7
Mallorquí	14	Black	0.07	0.93	1	0

Table 3. Number of individuals sampled, description of the coat colour accepted in the studbook and genotypic frequencies of the MC1R and ASIP genes for nine Iberian horse breeds.

			MC1R			ASIP		
Population	N	Coat Colour	E^+/E^+	E^+/e	e/e	A^+/A^+	A^+/a^b	a^b/a^b
Asturcón	45	Black	0.87	0.13	0	0	0	1
Caballo de Corro	60	Any thru colour; mainly Bay	0.77	0.23	0	0.25	0.67	0.08
Merens	19	Black	0.9	0.1	0	0	0	1
Losino	12	Black	0.5	0.5	0	0	0	1
Garrano	10	Any thru colour; mainly Bay	0.3	0.7	0	0.3	0.3	0.4
Carthusian	10	Grey	0.6	0.4	0	0.8	0.1	0.1
Andalusian	7	Any thru colour; mainly Grey	0.43	0.43	0.14	0.29	0.57	0.14
Marismeño	10	Any thru colour; mainly Bay	0.3	0.7	0	0.4	0.6	0
Mallorquí	14	Black	0.79	0.21	0	0	0	1

Figure 1. Main geographical location of the Iberian horse breeds sampled in the present study.

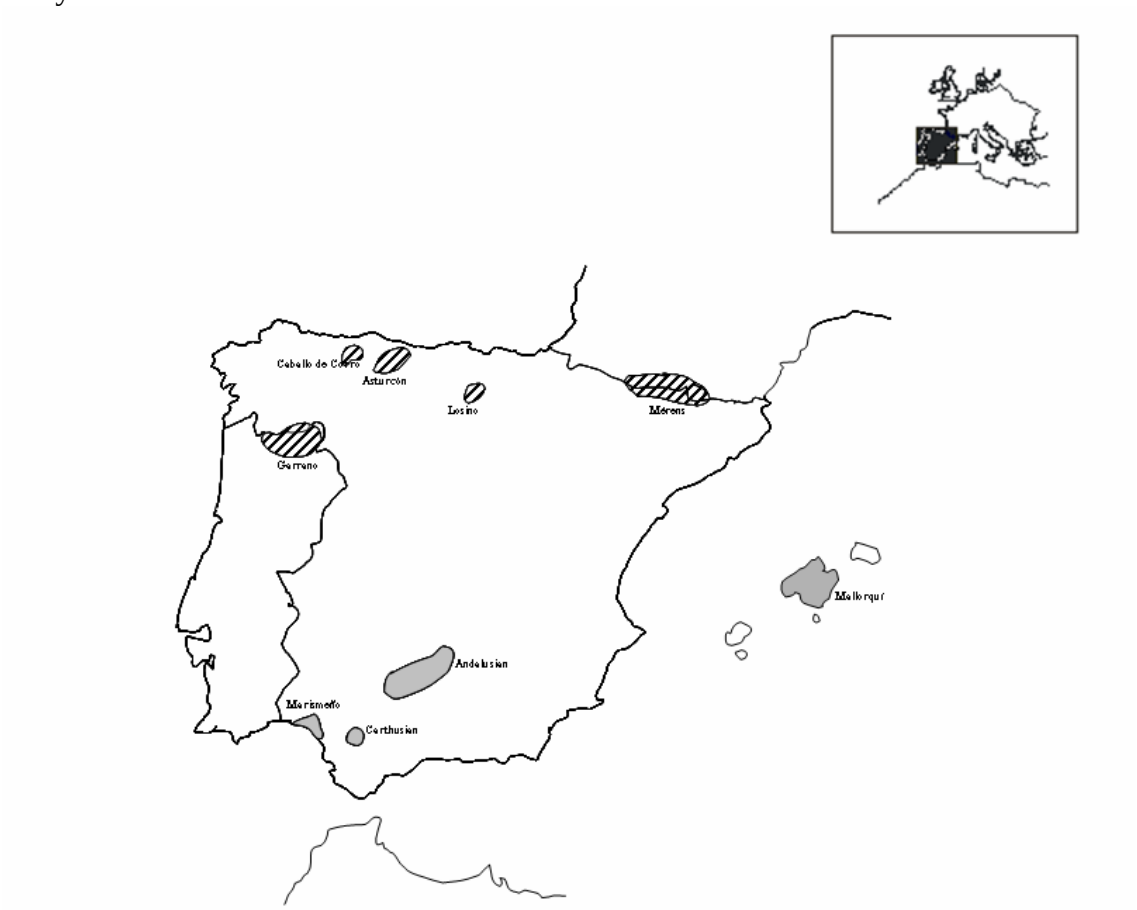

Figure 2. Genotyping of alleles affecting coat colour patterns in horses. The two first peaks represent the chestnut and wild-type (E^+) alleles at the MC1R locus. The two others peaks represent the black recessive and wild-type (A^+) alleles at the ASIP locus.