The coding sequence of the ASIP gene is identical in nine wild-type coloured cattle breeds

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Introduction

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 the E 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 the A 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 E locus encodes a melanocyte-stimulating hormone receptor, also known as the melanocortin-1 receptor (MC1R) which is involved in eumelanin and phaeomelanin production while the Agouti signaling peptide (encoded in the ASIP locus) acts as an antagonist of melanocyte-stimulating hormone (MSH) by binding to MC1R and thereby preventing the MC1R–MSH interaction, resulting in phaeomelanin synthesis (Bultman et al. 1992). In mice, the E locus is epistatic to A locus (Bateman & Sombre 1961; Wolff et al. 1978), but in fox it has been demonstrated that the E locus is not epistatic to the A locus (Adalsteinsson et al. 1987; Vage et al. 1997).

As the E locus is responsible for most of the variation in cattle coat colour, many studies have examined the MC1R locus in cattle. Initial studies identified the dominant black E0 (L99P) and the recessive red e (310delG) alleles (Klungland et al. 1995; Joerg et al. 1996). Any coding sequence without these mutations was considered as the wild-type allele (E+) responsible for most combinations of red or reddish brown and black coat variants (Olson 1998). Recent studies have identified two more missense mutations in the base E locus sequence: allele E1 (ARGI218-219ins) found in Brown Swiss and some light brown-coated French cattle breeds (Graphodatskaya et al. 2000; Rouzaud et al. 2000;
Kriegesmann et al. 2001) and allele E² (R223W) also found in Brown Swiss cattle and many Italian breeds (Graphodatskaya et al. 2000; Maudet & Taberlet 2002). All three E⁺, E¹ and E² alleles, present in cattle breeds that are wild type in colouration and thus would allow the Agouti variation. The E⁺, E¹ and E² alleles are hormonally activated receptors but both, the recessive red allele, e, and the dominant black allele E²D, have been characterized at the cell level as unresponsive to increased intracellular cAMP stimulation by a wide range of alpha-MSH concentrations (Graphodatskaya et al. 2002).

In contrast with the E locus (Adalsteisson et al. 1995), the A locus in cattle has not been completely studied. Mutants at the A locus modify the expression of the wild-type pattern. Reviewing the literature Olson (1998) describes the following A alleles: A⁰⁰ (patterned blackish agouti) modifying the wild-type to nearly entirely black and not influenced by the sex; a⁰⁰⁰ (white-bellied agouti) removing red pigment and a part of the black pigment while causing a more uniform distribution of black pigmentation especially across the sides of the animal; and a (fawn) removing red and black pigmentation and, particularly red along the underline and along the back (dorsal strip) resulting in tan to fawn colour. Adalsteisson et al. (1995) also proposed the existence of a wild allele of Agouti (A⁺) that allows the expression of the combination of red and black pigmentation of the wild-type coat colour, and a recessive black (a), which, when homozygous, would modify the wild-type coat to solid black. Girardot et al. (2005) found no evidence of coding region sequence variation in ten animals belonging to eight cattle breeds, most of them showing the red recessive or black dominant coat colour. This limited sampling do not permit to ascertain the possible influence of the coding region sequence of the ASIP gene on the whole wild type coat colour variation in cattle, especially for the recessive black coat pattern.

The objective of this work is to ascertain the role of the ASIP gene coding region in the A locus variation of wild-type coat colouration in cattle. To do this, we will study a sample of individuals showing most of phenotypic variation of this coat type described in the literature. Individuals within breeds will be first genotyped for the E locus. Animals carrying e or E²D alleles will be excluded from subsequent analysis. Homozygotes showing the same Extension genotype and different phenotypes will be selected as candidates to search for allelic variants in the ASIP coding region.

Materials and methods

A total of 241 animals belonging to six Spanish (Asturiana de los Valles, Asturiana de la Montaña, Negra Serrana, Parda Alpina, Sayaguesa and Tudanca) and three French cattle breeds (Parthenais, Tarantaise and Normande) were sampled. The nine populations were representative of the wild-type phenotype in coat colour. DNA was isolated from semen or blood using standard procedures (Sambrook et al. 1989). A list of chosen breeds, number of individuals and coat characteristics are detailed in Table 1. Polymerase chain reaction–restriction fragment-length polymorphism (PCR-RFLP) assays to identify E locus genotypes were conducted using primers and PCR protocols described by Graphodatskaya et al. (2000). E¹ allele (ARG1218-219ins) was simply identified by electrophoresis in acrylamide gels based on its size. E² allele (R223W) was identified by means of the HapII enzyme (Amersham Biosciences, Buckinghamshire, UK). Dominant E²D allele (L99P) was diagnosed using the enzyme AciI.

### Table 1

<table>
<thead>
<tr>
<th>Breed</th>
<th>N</th>
<th>Coat type</th>
<th>Coat variation</th>
<th>Extension genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asturiana de los Valles</td>
<td>64</td>
<td>Jersey type</td>
<td>From fawn to mahogany</td>
<td>E⁺/E⁺</td>
</tr>
<tr>
<td>Asturiana de la Montaña</td>
<td>35</td>
<td>Jersey type</td>
<td>From fawn to mahogany</td>
<td>E⁺/E¹, E⁺/E²</td>
</tr>
<tr>
<td>Parthenais</td>
<td>16</td>
<td>Jersey type</td>
<td>From fawn to mahogany</td>
<td>E⁺/E¹, E⁺/E²</td>
</tr>
<tr>
<td>Tarantaise</td>
<td>16</td>
<td>Jersey type</td>
<td>From fawn to mahogany</td>
<td>E⁺/E¹, E⁺/E²</td>
</tr>
<tr>
<td>Parda Alpina</td>
<td>18</td>
<td>Brown Swiss type</td>
<td>From brownish grey to light grey</td>
<td>E⁺/E¹, E⁺/E²</td>
</tr>
<tr>
<td>Negra Serrana</td>
<td>22</td>
<td>Black</td>
<td>Black with different degrees of brown dorsal stripe</td>
<td>E⁺/E¹, E⁺/E²</td>
</tr>
<tr>
<td>Sayaguesa</td>
<td>18</td>
<td>Black</td>
<td>Black with different degrees of brown dorsal stripe</td>
<td>E⁺/E¹, E⁺/E²</td>
</tr>
<tr>
<td>Tudanca</td>
<td>36</td>
<td>Grey</td>
<td>Dark grey in dams and blackish in sires</td>
<td>E⁺/E¹, E⁺/E²</td>
</tr>
<tr>
<td>Normande</td>
<td>16</td>
<td>Brown</td>
<td>High frequency of brindle individuals</td>
<td>E⁺/E¹, E⁺/E²</td>
</tr>
</tbody>
</table>

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(New England Biolabs, Hertfordshire, UK). Presence of the recessive e allele (310delIG) was detected with HapII. Primers were designed based on the published sequence X99691, except Exon2-forward and Exon4-reverse which are based on human–murine sequences (Table 2). PCR-single-strand conformation polymorphism (SSCP) analysis was carried out in at least one individual for each Extension genotypes within the same breed to identify variants existing into the three coding exons of the ASIP gene (Table 2). Five different electrophoretic protocols were used: (i) 0.5 X Mutation Detection Enhancement gels (MDE; Cambrex BioScience, Rockland, ME, USA), 6°C, 20 W, 3.5 h; (ii) 0.7 X MDE, 16°C, 20 W, 3.5 h; (iii) Acryl:Bis (37.5:1) 12%, 5% Glyceral, 4°C, 400 V, 12 h; (iv) Acryl:Bis (37.5:1) 12%, 5% glycerol, 20°C, 400 V, 12 h; and (v) Acryl:Bis (100:1) 12%, 5% glycerol, 4°C, 400 V, 20 h). The PCR products for the three exons were sequenced by fluorescent dye-terminator cycle sequencing on an automated sequencer (AlfexpressII; Amersham Biosciences).

**Results and discussion**

A total of 241 individuals were genotyped for the E locus. Individuals showing e (seven Asturiana de los Valles and one Asturiana de la Montaña) or E$^D$ (11 Sayaguesa and three Negra Serrana) alleles were excluded from subsequent analysis and they are not detailed in Table 1. After the five assays of PCR-SSCP, no SSCP variants were found in any of the three coding exons. Results were confirmed by sequencing both strands of the PCR fragments containing the coding sequence of ASIP gene in 20 individuals: one for each breed/homozygous extension genotype (E$^E$/E$^E$, E$^E$/E$^I$ and E$^I$/E$^I$) and two additional (randomly chosen) individuals for each of the black patterned breeds (Sayaguesa and Negra Serrana). The obtained sequences are available in the Genbank data base with the following accession numbers: AY348953, AY348954, AY352659. Two Sayaguesa individuals were found to be heterozygous at nt163(G-A) in intron 2. This polymorphism is located one nucleotide after the donor (5’ splicing site) of intron 2. Notice that the most frequent nucleotide in the donor splice site third position is adenine (62%) (Lewin 2000). Then it is considered unlikely to affect the ASIP mRNA.

The phenotypic coat colour variation in cattle can not be explained solely by the action of the Extension locus (Rouzaud et al. 2000; Jörg et al. 2002). Earlier studies suggest that the ASIP gene is responsible for the agouti variation (Bultman et al. 1992; Mountjoy et al. 1992; Robbins et al. 1993; Jackson 1997). However, evidence of the relationship between ASIP gene and colour has been obtained in mice and other laboratory rodents (Bultman et al. 1992; Miltenberger et al. 2002). In domestic animals this task has not been clearly stated so far. Rieder et al. (2001) found only one allelic variant in the ASIP gene (recessive black) in 22 different horse breeds that showed considerable variation in coat coloration. In pigs only one mutation has been found in the sequence of the exon 3 (E68K) and it failed to show an association with coat colour variation (Fernández 2003). Three mutations have been described in the untranslated region or introns of the agouti gene, obviously without affecting coat colour in pigs (Leeb et al. 2000). Even in humans, Dinulescu & Cone (2000) reported that the agouti gene does not appear to play a role in human pigmentation. Taking into account these findings and our results, the ASIP coding region does not play a central role in coat colour variation in cattle.

**Acknowledgements**

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**References**


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**Table 2** Primer sequence used ASIP for PCR-SSCP analysis and sequencing

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer sequence</th>
</tr>
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<tbody>
<tr>
<td>Exon 2 – 342 bp</td>
<td>forward 5’-CTCTCTGTCCTCAGGTGCT-3’ reverse 5’-CTGATTCCCTTGAGTAATTT-3’</td>
</tr>
<tr>
<td>Exon 3 – 401 bp</td>
<td>forward 5’-CTGTGCTCCAGTGCAGCCAG-3’ reverse 5’-TTCAGGCCTGGGAGCTGAC-3’</td>
</tr>
<tr>
<td>Exon 4 – 311 bp</td>
<td>forward 5’-GACGCTATGCGAGGAGTCT-3’ reverse 5’-AGCATCGGGGTCTTTCATGA-3’</td>
</tr>
</tbody>
</table>


Rieder S., Taourit S., Mariat D., Langlois B., Guérin G. (2001) Mutations in the agouti (ASIP), the extension (MC1R) and the brown (TYRP1) loci and their association to coat color phenotypes in horses (Equus caballus). Mamm. Genome, 12, 450–455.


