SHORT COMMUNICATION

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

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Summary

The aim of this study was to ascertain the role of the Agouti signaling peptide (ASIP) gene coding region in the Agouti locus variation within wild-type coat colour in cattle. We determined the Extension genotype in 241 individuals from six Spanish and three French brown cattle breeds representative of wild-type coat variation. Polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP) analysis was carried out in individuals of each Extension genotypes within the same breed in an attempt to identify variants in the three coding exons of the ASIP gene. No SSCP variants were found. Results were confirmed by sequencing the coding exons of the ASIP gene in 20 individuals. Our results suggest that the ASIP coding region does not play a central role in coat colour variation in cattle.

Introduction

Coat colour in mammals basically depends on the relative amount of the two basic types of tyrosine-derived melanin: eumelanin (black/brown) and pheomelanin (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 pheomelanin 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 pheomelanin 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 E^D (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 E^D, 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: A^{bp} (patterned blackish agouti) modifying the wild-type to nearly entirely black and not influenced by the sex; a^w (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 aⁱ (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 E^D 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 (ARGI218-219ins) 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 E^D allele (L99P) was diagnosed using the enzyme *Acil*

Table 1 Description of breeds, wild-type Extension genotypes and coat patterns

Breed	N	Coat type	Coat variation	Extension genotype					
				E ⁺ /E ⁺	E ⁺ /E ¹	E ⁺ /E ²	E ¹ /E ¹	E ¹ /E ²	E ² /E ²
Asturiana de los Valles	64	Jersey type	From fawn to mahogany	1	3	9	–	12	32
Asturiana de la Montaña	35	Jersey type	From fawn to mahogany	1	–	2	–	4	28
Parthenais	16	Jersey type	From fawn to mahogany	4	8	–	4	–	–
Tarantaise	16	Jersey type	From fawn to mahogany	1	–	–	14	–	1
Parda Alpina	18	Brown Swiss type	From brownish grey to light grey	9	6	–	2	1	–
Negra Serrana	22	Black	Black with different degrees of brown dorsal stripe	17	1	–	1	–	–
Sayaguesa	18	Black	Black with different degrees of brown dorsal stripe	3	–	3	–	1	–
Tudanca	36	Grey	Dark grey in dams and blackish in sires	–	–	–	–	–	36
Normande	16	Brown	High frequency of brindle individuals	16	–	–	–	–	–

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

	Primer sequence
Exon 2 – 342 bp	forward 5'-CTTCTCTGTCTCACTCAGGCCTC-3' reverse 5'-CTTGATTCTCTCCAGAATTGTCT-3'
Exon 3 – 401 bp	forward 5'-CTGTCTCCAGGTCTGCAGCCAG-3' reverse 5'-TTCAGTGCTTGAGGTGAGGAC-3'
Exon 4 – 311 bp	forward 5'-GACGTCTAGTCCGAGGAGTTC-3' reverse 5'-AGCATCGGGTCTTTCAAATGA-3'

(NewEngland Biolabs, Hertfordshire, UK). Presence of the recessive *e* allele (310delG) 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% Glycerol, 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⁺*, *E¹/E¹* and *E²/E²*) 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 heterozy-

gous at nt163(G-A) in intron 2. This polymorphism is located one nucleotide after the donor (5') splice site (GT) 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.

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References

- Adalsteinsson S., Hersteinsson P., Gunnarsson E. (1987) Fox colors in relation to colors in mice and sheep. *J. Hered.*, **78**, 235–237.
- Adalsteinsson S., Bjarnadottir S., Våge D.I., Jonmundsson J.V. (1995) Brown coat colour produced by the loci Extension and Agouti. *J. Hered.*, **86**, 395–398.
- Bateman N., Sombre A. (1961) Viable dominant mutant in the house mouse. *J. Hered.*, **52**, 186–189.
- Bultman S.J., Michaud E.J., 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., Kesterson R.A. (1996) The melanocortin receptors: agonist, antagonists, and the hormonal control of pigmentation. *Rec. Progr. Horm. Res.*, **51**, 287–318.
- Dinulescu D.M., Cone R.D. (2000) Agouti and Agouti-related protein: analogies and contrasts. *J. Biol. Chem.*, **275**, 6695–6698.
- Fernández A. (2003) *Estudio de la base genética del color de la capa y aplicaciones prácticas en porcino*. PhD Thesis, Universidad Complutense de Madrid, Madrid.
- Girardot M., Martin J., Guibert S., Leveziel H., Julien R., Oulmouden A. (2005) Widespread expression of the bovine *Agouti* gene results from at least three alternative promoters. *Pigment Cell Res.*, **18**, 34–41.
- Graphodatskaya D., Joerg H., Stranzinger G. (2000) Polymorphism in the MSHR gene of different cattle breeds. *Vet. Med. Czech.*, **45**, 290–295.
- Graphodatskaya D., Joerg H., Stranzinger G. (2002) Molecular and pharmacological characterization of the MSH-R alleles in Swiss cattle breeds. *J. Recept. Signal. Transduct. Res.*, **22**, 421–430.
- Jackson I.J. (1997) Homologous pigmentation mutation in humans, mouse and other model organisms. *Hum. Mol. Genet.*, **6**, 1613–1624.
- Joerg H., Fries H.R., Meijernik E., Stranzinger G.F. (1996) Red coat colour in Holstein cattle is associated with a deletion in the MSHR gene. *Mamm. Genome*, **7**, 17–318.
- Jörg H., Rieder S., Fellay E., Lauvergne J.J., Gaillard C. (2002) Coat colour and polymorphism in the MC1R gene of the Hérens cattle breed. ISAG Göttingen D, poster no. D037.
- Klungland H., Våge D.I., Gómez-Raya L., Adalsteinsson S., Lien S. (1995) The role of melanocyte-stimulating hormone (MSH) receptor in bovine coat color determination. *Mamm. Genome*, **6**, 636–639.
- Kriegesmann B., Dierkes B., Leeb T., Jansen S., Brenig B. (2001) Two breed-specific bovine Mc1-r alleles in brown swiss and salers breeds. *J. Dairy Sci.*, **84**, 1768–1771.
- Leeb T., Deppe A., Kriegesmann B., Brenig B. (2000) Genomic structure and nucleotide polymorphisms of the porcine agouti signalling protein gene (ASIP). *Anim. Genet.*, **31**, 333–346.
- Lewin B. (2000) *Genes VII*. Oxford University Press and Cell Press, New York.
- Maudet C., Taberlet P. (2002) Holstein's milk detection in cheeses inferred from melanocortin receptor 1 (MC1R) gene polymorphism. *J. Dairy. Sci.*, **85**, 707–715.
- Miltenberger R.J., Wakamatsu K., Ito S., Woychik R.P., Russell L.B., Michaud E.J. (2002) Molecular and phenotypic analysis of 25 recessive, homocigous-viable alleles at the mouse agouti locus. *Genetics*, **160**, 659–674.
- Mountjoy K.G., Robbins L.S., Mortrud M.T., Cone R.D. (1992) The cloning of a family of genes that encode the melanocortin receptors. *Science*, **257**, 1248–1251.
- Olson T.A. (1998) Genetics of colour variation. In: R. Fries, A. Ruvinsky (eds), *The Genetics of Cattle*. CABI Publishing, Wallingford, pp. 33–53.
- 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.
- Robbins L.S., Nadeau J.H., Johnson K.R., Kelly M.A., Roselli-Rehffuss L., Baack E., Mountjoy K.G., Cone R.D. (1993) Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function. *Cell*, **72**, 827–834.
- Rouzaud F., Martin J., Gallet P.F., Delourme D., Goulemot-Leger V., Amigues Y., Menissier F., Leveziel H., Julien R., Oulmouden A. (2000) A first genotyping assay French cattle breeds based on a new allele of the extension gene encoding the melanocortin-1 receptor (MC1R). *Genet. Sel. Evol.*, **32**, 511–520.
- Sambrook J., Fritsch E.F., Maniatis T. (1989) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Searle A.G. (1968) *Comparative Genetics of Coat Colour in Mammals*. Logos Press, London.
- Vage D.I., LU D., Klungland H., Lien S., Adalsteinsson S., Cone R. D. (1997) A non-epistatic interaction of agouti and extension in the fox, *Vulpes vulpes*. *Nature Genetics*, **15**, 311–315.
- Wolf G.L., Galbraith D.B., Domon O.E., Row J.M. (1978) Phaeomelanin synthesis and obesity in mice: interaction of the viable yellow (A^{VY}) and sombre (E^{So}) mutations. *J. Hered.*, **69**, 295–298.

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