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SHORT COMMUNICATION

Sequence and gene expression analysis of the agouti signalling protein gene in Rex rabbits with different coat colours

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Abstract

Rex rabbits have a unique fur structure with a variety of different coat colours. In this study, we analysed variability in the agouti signalling protein (*ASIP*) gene in Rex rabbits with different coat colours (castor, red, blue, chinchilla, otter and black). Three alleles at this locus were identified: *A*, light-bellied agouti (wild type), in castor and chinchilla Rex; *d*, black and tan, in otter, castor and chinchilla Rex; and *a*, black non-agouti, in black, red, blue, castor and chinchilla Rex rabbits. Two missense mutations, two synonymous substitutions and one indel were the identified polymorphisms associated to these three alleles, as already described by others. Gene expression of the *ASIP* gene was also evaluated in different tissues from animals of the six coat colours. *Agouti signalling protein* expression was always observed in all tissue/coat colour combinations.

Introduction

Coat colour is not only a basis for the identification of breeds, populations and individuals in domesticated species but it also has a special economic value and cultural significance. In recent years, many studies have examined coat colour genes, primarily focusing on mice and humans, and more than 378 loci that affected pigmentation have been identified in mice (Montoliu *et al.*, 2014). Among these, the *agouti* and *extension* loci interact to determine the relative amounts of two types of melanin in the coat, pheomelanin (yellow-red pigment) and

eumelanin (black-brown pigment). The *agouti* locus encodes the agouti signalling protein (*ASIP*), which is a 131 amino acid paracrine peptide that in wild type mice is produced only in specialised dermal cells at the base of the hair follicle (Bultman *et al.*, 1992). The *ASIP* protein regulates the production of eumelanin and pheomelanin, which determines agouti coat colour locus (Bultman *et al.*, 1994). Coloured Rex rabbits are economically important animals that produce a coat that is characterized by short, flat, thin, and dense hairs that is used for fur production. Rex rabbits have a rich variety of coat colours that contribute to offer a differentiated product for fur production. The coat colour determines in part the value of the coat of Rex rabbits. The variation in rabbit coat colour is primarily controlled by mutations in a few loci including the melanocortin 1 receptor (*MC1R*) gene (the extension locus; Fontanesi *et al.*, 2006, 2007, 2010b) and the *ASIP* gene (Fontanesi *et al.*, 2010a). In this study, we characterized by sequencing the coding region of the rabbit *ASIP* gene in Rex rabbits of different coat colours (castor, red, blue, chinchilla, otter and black) and evaluate the expression of this gene in a few tissues. Our results confirmed the presence of the *ASIP* gene polymorphisms reported by Fontanesi *et al.* (2010a) and provided some new evidences about gene expression levels in rabbits with different *ASIP* mutations.

Materials and methods

Resequencing and identification of mutations *agouti signalling protein* gene

Three PCR primer pairs, designed in intronic or noncoding regions of the rabbit *ASIP* gene (GenBank accession number AM748788.1), were used to amplify the coding regions of the three coding exons (exons 2, 3 and 4) using genomic DNA isolated from blood of 459 rabbits belonging to six coat colour types: 108 Castor Rex, 58 Otter Rex, 112 Chinchilla Rex, 69 Black Rex, 45 Red Rex, 67 Blue Rex. The three PCR primers pair were as follows: exon2, 5'-GCACCAACCACCTTCTCTGT-3', 5'-GGAATAAACCATGATGATGGCAA T-3'; exon3, 5'-GACACACAGGCAACCC-3', 5'-CCCTCTCAACACGACCC-3'; and exon 4, 5'-CTGAGGTGCCCTGTGTC-3', 5'-TCAGCAGTTGGGTTGAGCACGCGGCAGGT-3'. PCR was conducted in a 50 L reaction volume containing with 50 g of genomic DNA, 2.5 U of DNA Taq DNA polymerase, 1x Taq

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Key words: Agouti locus; Coat colour; Gene expression; Gene polymorphisms; Rex rabbit.

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PCR buffer, 10 mM dNTPs, and 10 M of each primer. The PCR profile was as follows: 5 min at 94°C; 35 amplification cycles of 30 s at 95°C, 30 s at 55°C (exon 2) or 52.5°C (exon 3) or 58°C (exon 4), and 30 s at 72°C; and 5 min at 72°C. The amplified fragments were sequenced at BIOMED Co. Ltd. The sequences were edited and aligned using DNAMAN (Woffelman, 2004) and BioEdit 7.0.1 (Hall, 2004) software.

Genotyping

Two mutations in *ASIP* gene were genotyped by PCR-RFLP. The insertion of 1 bp identified in the *ASIP* exon 2 (c.5_6insA) was detected using *EcoRI* (Fontanesi *et al.*, 2010a), and c.266T > C mutation identified in exon 3 was detected using *Eco47III*. When the sequences changed, restriction sites are not present. Digestion of the amplified fragment was obtained overnight at 37°C in a 50 L reaction volume including 10 L of PCR product, 1X restriction enzyme buffer and 4 U of *EcoRI* and 4 U of *Eco47III*. The resulting DNA fragments were electrophoresed on 10% 29:1 polyacrylamide:bis acrylamide gel and visualised by ethidium bromide staining.

Total RNA extract and expression level of *agouti signalling protein* in Rex rabbits

Ten Rex rabbits (30 days old) for each of six different groups, defined according to their coat

colour (castor, chinchilla, otter, black, red and blue) were used for gene expression analysis. Blood, liver, ventral skin, hypophysis and spleen samples were collected from all animals. Total RNA was extracted using an RN03-RNAPure ultrapure total RNA extraction kit (BIOMED; Biomed Co. LTD., Beijing, China) according to the manufacturer's instructions. cDNA was synthesised from total RNA extracted from the aforementioned organ tissues of coloured Rex rabbits. Reverse transcription was performed with 1 g of DNase-treated RNA in a total volume of 20 L using 0.5 g of oligo (dT) primer and the M-MLV II First-Strand cDNA synthesis kit (BIOMED) according to the manufacturer's instructions. The reaction was incubated for 50 min at 42°C and then for 15 min at 70°C. The expression analysis of the *ASIP* gene was performed on cDNAs obtained as described above, the primer pairs: 5'-TAGCAGGTGTGGCTTTGT-3', 5'-GCTCATAACACTGTATTTTCG-3', and the amplified gene were located on 3'-UTR region. AB 7300 Real-time Quantitative PCR system (Applied Biosystems, USA). The PCR primer pairs, which were referenced to NCBI Gene Bank accession number AM748787.1. The combination of primers was used to amplify cDNAs obtained from all tissues listed above. PCR was conducted in a 50 L reaction volume containing 10 M of each primer, 25 L of 2× SYBR qPCR Mix, and 1 L of 50× ROX (2× SYBR Green qPCR Mix Kit; ZomanBio Co., Ltd., Beijing, China). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) cDNA amplification was used as a reference (primers: 5'-CCCCTCTCTACCTTCG-3', 5'-CGTCCTCTCTGGTCTCT-3'). The PCR cycling conditions for *GAPDH* and *ASIP* were as follows: 95°C for 2 min; 40 cycles at 95°C for 20 s, 55°C for 20 s, and 72°C for 30 s; and 72°C for 5 min.

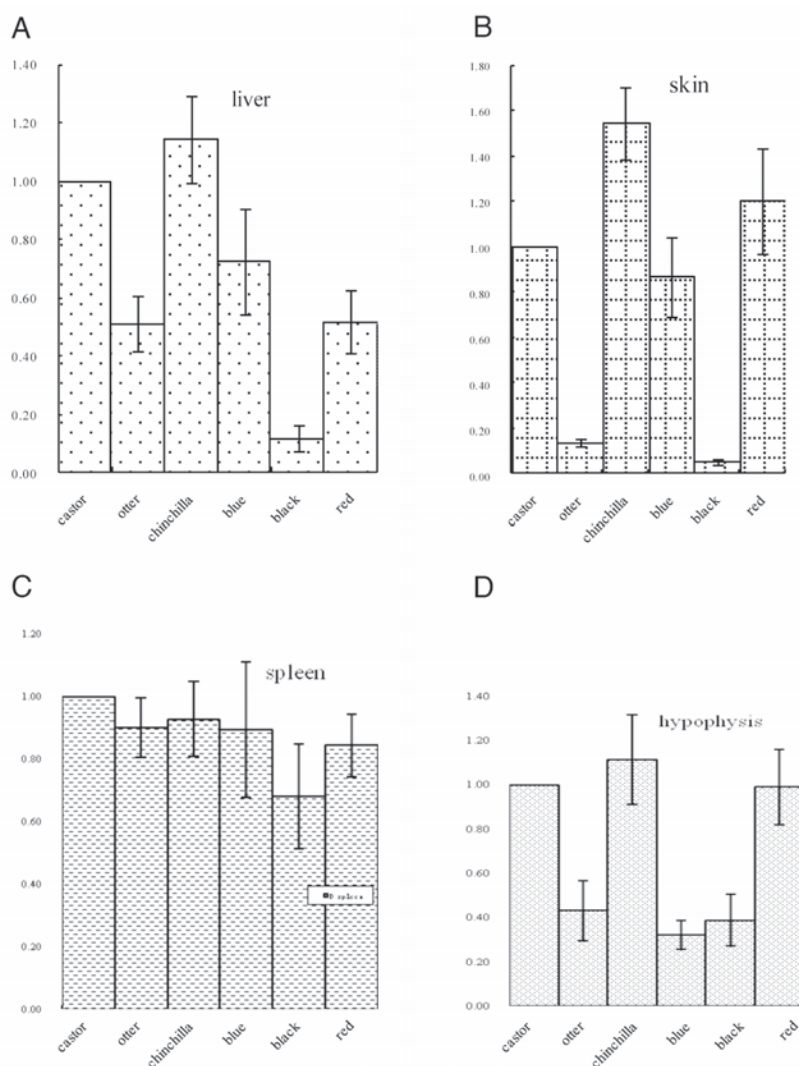


Figure 1. Expression levels of the *agouti signalling protein (ASIP)* gene in Rex rabbits having different coat colours. A, B, C and D present the expression levels of the *ASIP* gene in the liver, ventral skin, spleen, and hypophysis, respectively, of coloured Rex rabbits. The expression level of the *ASIP* gene was analysed by qRT-PCR using the $2^{-\Delta\Delta C_t}$ method, and the level of expression of the *ASIP* gene in castor Rex rabbits was used for comparison in each tissue. Note: every colour includes 10 Rex rabbits.

Table 1. *Agouti signalling protein* genotype and allele distribution in coloured Rex rabbits.

Rex coat colour types (no. of animals)	Genotype frequencies (no. of animals) ^o						Allele frequencies ^o		
	<i>AA</i>	<i>a'a'</i>	<i>aa</i>	<i>Aa</i>	<i>Aa'</i>	<i>a'a</i>	<i>A</i>	<i>a'</i>	<i>a</i>
Castor Rex (108)	0.824(89)	-	-	0.111(12)	0.065(7)	-	0.912	0.032	0.056
Otter Rex (58)	-	0.793(46)	-	-	-	0.207(12)	0.000	0.897	0.103
Chinchilla Rex (112)	0.812(92)	-	-	0.107(12)	0.071(8)	-	0.910	0.036	0.054
Black Rex (69)	-	-	1.000(69)	-	-	-	0.000	0.000	1.000
Red Rex (45)	-	-	1.000(45)	-	-	-	0.000	0.000	1.000
Blue Rex (67)	-	-	1.000(67)	-	-	-	0.000	0.000	1.000

^o Allele *a'* is determined by the following missense mutations (c.230A>G and c.266T>C) synonymous substitutions (c.147G>A and c.252G>A). Allele *a* is determined by the c.5_ginsA insertion (following Fontanesi et al., 2010a).

Results and discussion

Identification of gene polymorphisms in the rabbit *Agouti* locus

Three alleles at the agouti locus were identified in Rex rabbits of three different colours: the wild type agouti (*A*), the Tan agouti (*a'*), and the non-agouti (*a*) alleles. These alleles have been already characterized at the molecular level (Fontanesi *et al.*, 2010a). Polymorphisms were identified in exons 2, 3 and 4 as already reported by Fontanesi *et al.* (2010a) (Table 1): two SNPs were missense mutations (the c.230A>G mutation corresponding to the p.K77R amino acid substitution; the c.266T>C mutation corresponding to the p.L89P amino acid change), two SNPs were synonymous substitutions (c.147G>A and c.252G>A) and one polymorphism was the c.5_6insA insertion determining the *a* allele. The *d/d'*, *d'/a* and *a/a* genotypes were not present in castor and Californian Rex rabbits (Table 1). The most frequent genotypes were *A/A* (in Chinchilla rex and Castor Rex), *a'/a'* (in Otter Rex) and *a/a* (in Black Rex and Red Rex; Table 1).

ASIP gene expression level in different tissues

Total RNA was extracted from castor, chinchilla, otter, black, red and blue Rex rabbits. The differential expression of the *ASIP* gene in ventral skin, liver, hypophysis, and spleen of Rex rabbits was analysed using QRT-PCR with 2^{-Ct} method. The *ASIP* gene was expressed in all studied tissues (Figure 1). For the ventral skin, liver and hypophysis organs, the expres-

sion level of the *ASIP* gene was highest in the chinchilla coat skin, followed by castor, blue and red Rex rabbits, whereas *ASIP* expression in otter and black colour Rex rabbits was slightly lower. For the spleen tissues, little difference was observed in the expression level of the *ASIP* gene in rabbits of different colours.

Conclusions

This study confirmed the *ASIP* gene polymorphisms already identified by Fontanesi *et al.* (2010a), who evidenced three alleles (*A*, *a'* and *a*). All alleles were identified in Rex rabbits in which they might affect different coat colours. Gene expression analysis showed that the *ASIP* gene is expressed in all analysed tissues.

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