INVESTIGATION OF COAT COLOUR AFFECTING GENES IN SEVERAL EUROPEAN RABBIT BREEDS AND OTHER LEPORID SPECIES

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ABSTRACT

Pigmentation in mammals is mainly determined by the distribution of pheomelanin and eumelanin pigments which produce red/yellow and dark phenotypes, respectively. The relative amount of eumelanin and phaeomelanin in the melanocytes is controlled primarily by two loci, the *Extension* and *Agouti* loci. *Extension* locus encodes the melanocortin 1 receptor (MC1R). *MC1R* mutations have been identified to alter coat colour and pigment synthesis in several mammals. Analysing almost the complete coding region of the *Oryctolagus cuniculus MC1R* gene, we recently identified two mutations associated with red (recessive allele *e* of the *Extension* locus) or black (E^D or E^S, dominant black or steel, weaker version of E^D) coat colours in different European rabbit breeds. Here we completed the sequence of the 953 bp coding region of the *MC1R* gene in *O. cuniculus* excluding the presence of additional common disrupting or functional mutations. *Agouti* locus encodes for the agouti signalling protein (ASIP). In European rabbit, classical studies have suggested the presence of three alleles at the *Agouti* locus: *A* (wild type allele), a^t (black and tan) and *a* (non-agouti). We sequenced the *O. cuniculus ASIP* exon 2 region and identified three mutations. Two were synonymous substitutions and one was an insertion of 1 bp. This insertion causes a frameshift of the translation suggesting that this mutation might be the molecular basis of the recessive black non-agouti allele at the *Agouti* locus (*a* allele). Genotyping this mutation in a larger number of animals confirmed the fixation of the insertion in all animals of breeds with black/dark coat colour. In addition, *MC1R* gene and *ASIP* exon 2 were sequenced in other Leporid species obtaining useful information to study these two coat colour genes from an evolutionary point of view.

Key words: Coat colour, Agouti signaling protein, Melanocortin 1 receptor, Mutations, *Leporidae.*

INTRODUCTION

Pigmentation in mammals is mainly determined by the distribution of pheomelanin and eumelanin pigments which produce red/yellow and dark phenotypes, respectively. The relative amount of eumelanin and phaeomelanin in the melanocytes is controlled primarily by two loci, the *Extension* and *Agouti* loci (Searle, 1968).

Extension locus encodes the melanocyte-stimulating hormone receptor (Robbins *et al*., 1993), also known as melanocortin 1 receptor (MC1R). Mutations of the single exon *MC1R* gene causing a constitutively active receptor are dominant and produce black coat colour, whereas inactivating mutations are recessive and result in red/yellow pigmentation. *MC1R* mutations have been identified to alter coat colour and pigment synthesis in several mammals, like mice (Robbins *et al*., 1993),

human (Valverde *et al*., 1995), cattle (Klungland *et al*., 1995), horse (Marklund *et al*., 1996), fox (Våge *et al*., 1997), pigs (Kijas *et al*., 1998), sheep (Våge *et al*., 1999), dogs (Newton *et al*., 2000) and other species. Analysing almost the complete coding region of the *Oryctolagus cuniculus MC1R* gene, we recently identified two mutations associated with red (recessive allele *e* of the *Extension* locus) or black (E^D or E^S, dominant black or steel, weaker version of E^D) coat colours in different European rabbit breeds (Fontanesi *et al*., 2006). These mutations were caused by the deletion of 30 bp (c.304_333del30) or 6 bp (c.280_285del6) of the *MC1R* gene determining the production of a putative non functional or activated transmembrane receptor, respectively. From our previous study it was not possible to define if the c.280_285del6 deletion was the causative mutation of the E^D or E^S alleles. Two other synonymous mutations, organized in two haplotypes, were also identified.

Agouti locus encodes for the agouti signaling protein (ASIP) that is a paracrine signaling molecule antagonist of MSH in binding to MC1R and thereby preventing the MC1R-MSH interaction, resulting in pheomelanin synthesis instead of black/brown eumelanin (Bultman *et al*., 1992). In mice as well as in other species, loss-of-function mutations (recessive) of the *Agouti* gene determine only the production of eumelanin while gain-of-function mutations (dominant) lead to pheomelanin production (i.e.: Bultman *et al*., 1992; Kuramoto *et al*., 2001; Kerns *et al*., 2004). A variety of coat colours appear as a result of these alterations that show also epistatic or partial epistatic interactions with the *Extension* locus (Searle, 1968; Våge *et al*., 1997). In European rabbit, classical studies have suggested the presence of three alleles at the *Agouti* locus: *A* (wild type allele), a^t (black and tan) and *a* (non-agouti) (Robinson, 1958; Searle, 1968).

Coat colour genes are of particular interest to investigate phenotype evolution, signature of selection and adaptive variation in wild populations. A few studies in mice, primates, birds and fish have already reported evidences of the role of *MC1R* and *ASIP* genes in pigmentation evolution and adaptation (i.e.: Mundy and Kelly, 2003; Nachman *et al*., 2003; Mundy, 2005; Hoekstra *et al*., 2006; Selz *et al*., 2007). Moreover, phylogenetic evolutionary studies have used the *MC1R* gene to evaluate functional constrains of protein domains and to compare phylogenetic trees obtained with other molecular evidences (Klungland *et al*., 1999; Li *et al*., 2007; Selz *et al*., 2007). However, due to the lack of information on these two colour affecting genes in *Lagomorpha* species, similar studies cannot be carried out for species of this order.

Here, we investigated the *MC1R* and *ASIP* genes with a three fold objective: i) complete the sequence of the *MC1R* European rabbit gene and confirm the association of the reported deletions with coat colour phenotypes across breeds; ii) identify mutations in the *ASIP* gene that could be associated with coat colour phenotypes in European rabbit breeds; iii) obtain sequence information for the *MC1R* and *ASIP* genes in other Leporid species in order to provide the basic tools for evolutionary biology studies.

MATERIALS AND METHODS

Animals and DNA Isolation

Sixteen European rabbits (*O. cuniculus*) across 12 breeds or strains with different coat colour (Alaska, n. 1; Belgian Hare, n. 2; Blue Vienna, n. 1; Burgundy Fawn, n. 2; Californian, n. 1; Checkered Giant with black markings, n. 2; pale Siamese Coloured Dwarf, n. 1; English Spot with Madagascar markings, n. 1; Giant Grey, n. 2; Russian, n. 1; Silver, n. 1; white commercial hybrid, n. 1), and one animal for each of five different Leporid species (Riverine rabbit, *Bunolagus monticularis*; Amami rabbit, *Pentalagus furnessi*; Volcano rabbit, *Romerolagus diazi*; Eastern cottontail, *Sylvilagus floridanus;* Mountain cottontail, *Sylvilagus nuttallii*) were used to obtain the *MC1R* gene sequence. Eight European rabbits of different breeds with diverse coat colour (Belgian Hare, Black and Tan, Blue Vienna, Burgundy Fawn, Champagne Argent, Checkered Giant, Giant Grey and Rhinelander) and one animal for each of eight Leporid species (*B. monticularis*; Brown hare, *Lepus europaeus*; Mountain hare, *Lepus timidus;* Snowshoe hare, *Lepus americanus*; *P. furnessi*; *R. diazi*; *S. floridanus*)

were used to sequence exon 2 of the *ASIP* gene. Additional 124 European rabbits of 16 different breeds were used for genotyping by PCR-RFLP the *ASIP* exon 2 insertion. European rabbit DNA was isolated from blood and/or hair roots as previously reported (Fontanesi *et al*., 2006; 2007). DNA for the other Leporid species was isolated from cultured fibroblast cells, blood, muscle samples or ear notches using a standard phenol-chloroform protocol or the DNAeasy Tissue Kit (Qiagen).

Polymerase Chain Reactions (PCR)

Four PCR primer pairs were used to amplify and sequence the *MC1R* gene in the animals listed above. Three primer pairs have been already reported by Fontanesi *et al.* (2006). To complete the sequence of the European rabbit coding region, an additional primer pair was designed aligning the *MC1R* gene in different species. Two primers (forward: 5'-CAGGAAGGCACATCCTCTTT-3'; reverse: 5'-TTCCCAAACCAAAGAAGTCAA-3') were used to amplify and sequence part of intron 1, exon 2 and part of intron 2 of the *ASIP* gene in the animals reported above. PCR was carried out in 20 µl containing 1 U EuroTaq DNA polymerase (EuroClone Ltd.), 1X PCR Buffer, 2.5 mM dNTPs, 10 pmol of each primer and 1.0-2.0 mM of $MgCl_2$. PCR profile was as follows: 5 min at 95°C; 35 amplification cycles of 30 sec at 95°C, 30 sec at 56-64°C, 30 sec at 72°C; 10 min at 72°C. PCR was performed using a PT-100 (MJ Research) or a TGradient (Biometra) thermal cycler.

Sequencing Analysis

PCR products obtained from animals indicated above were sequenced on both strands using the same PCR primers and the BigDye v3.1 cycle sequencing kit (Applied Biosystems). Sequencing reactions, after purification steps to eliminate unincorporated labelled nucleotides, were loaded on an ABI3100 Avant sequencer (Applied Biosystem). Sequences were edited and aligned with the help of the CodonCode Aligner software (CodonCode Corporation) and inspected manually. Estimation of dN/dS ratios (dN: number of non-synonymous substitutions; dS: number of synonymous substitutions) was obtained using the codeml option of the PAML package (Goldman and Yang, 1994).

Mutation Analysis

A PCR-restriction fragment length polymorphism (RFLP) protocol with *Eco*RI as a restriction enzyme was set up to analyse the insertion identified in the exon 2 of the European rabbit *ASIP* gene. 5 µl of PCR product was digested overnight at 37°C with 2 U of *Eco*RI (Roche Diagnostics) in a final volume of 25 µl containing 1X enzyme reaction buffer. The resulting DNA fragments were separated by electrophoresis in 10% polyacrylamide:bis-acrylamide 29:1 gels with TBE 1X buffer and visualized with ethidium bromide on a UV apparatus.

RESULTS AND DISCUSSION

The complete coding sequence of the European rabbit *MC1R* gene excluded the presence of common additional disrupting or activating mutations except the two deletions already reported by Fontanesi *et al.* (2006). Then, *MC1R* gene sequence information was obtained for other five leporid species (Figure 1). According to the alignment of Figure 1, of the two wild type *O. cuniculus* sequences already described (Fontanesi *et al.*, 2006) the sequence that was originally obtained from Belgian Hare and other breeds (Oc1) is the ancestral form. dN/dS ratio between the Oc1 sequence and that obtained for the other species ranged from 0.0648 to 0.1596 suggesting a possible action of purifying selection on this gene.

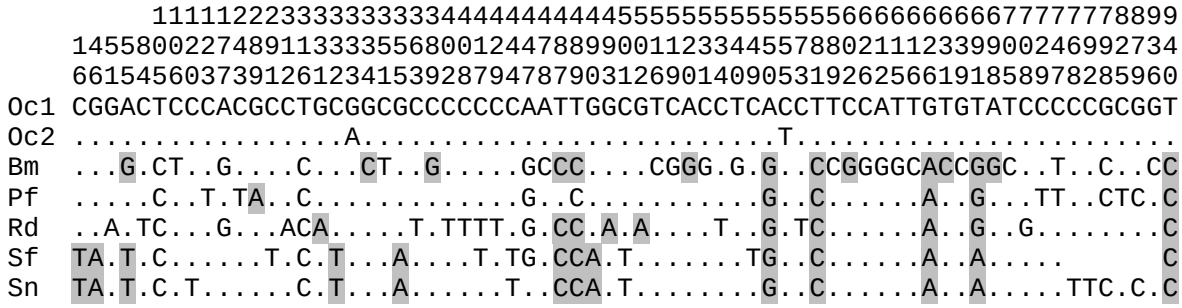

Figure 1: Comparison of *MC1R* variable sites among species (Oc1: *O. cuniculus*, EMBL accession number AM180879; Oc2: *O. cuniculus*, EMBL accession number AM180878; Bm: *B. monticularis*; Pf: *P. furnessi*; Rd: *R. Diazi*; Sf: *S. floridanus*; Sn: *S. nuttallii*. Numbers indicate the position of the sites in the coding region. Dots represent nucleotides identical to the Oc1 *MC1R* gene sequence. Grey highlighted positions denote non-synonymous substitutions. A small region was not sequenced in Sf (lack of dots)

Sequencing of the *O. cuniculus ASIP* exon 2 region revealed three mutations. Two were synonymous substitutions (G>A and G>A) and one was an insertion of 1 bp. This insertion causes a frameshift of the translation just after the start codon obtaining the production of a non functional ASIP protein. Disrupting or inactivating mutations in this gene produce recessive black non-agouti phenotypes (allele *a*) in other species (Bultman *et al*., 1992; Kuramoto *et al*., 2001; Kerns *et al*., 2004) suggesting that the insertion identified in exon 2 of the European rabbit *ASIP* gene is the molecular basis of the same allele in this species. This mutation was originally identified by sequencing the amplified product obtained from a Blue Vienna (homozygous) and a Checkered Giant (heterozygous) rabbit. Genotyping this mutation in a larger number of animals confirmed the fixation of the insertion in all Blue Vienna (20 animals), Champagne Argent (18), Alaska (5), Silver (4) and Russian (2) rabbits. These breeds have black/dark coat colour or black in the background and classical genetic studies have indicated that should be homozygous for the *a* non-agouti recessive allele at the *Agouti* locus (Robinson, 1958). Sequencing of the same region in other eight Leporid species did not identified this insertion. The *O. cuniculus* sequence differed from the three *Lepus sp.* sequences at 9 bp, but the sequences of species of *Lepus* did not diverge from each other. The sequence of *R. diazi* sequence was the most distant from *O. cuniculus* (27 bp different).

CONCLUSIONS

The complete coding sequence of the European rabbit *MC1R* gene confirmed that the two deletions already reported are involved directly in determining the recessive red and dominant black/steel coat colour phenotypes. However, biochemical and pharmacological studies will be important to investigate the functional role of these two naturally engineered mutations of the *MC1R* gene. Identification of a new functional mutation of the *ASIP* gene associated with the recessive-non agouti black phenotype in *O. cuniculus* opens new perspectives for the study of the interaction between the *Extension* and *Agouti* loci. Moreover, sequences obtained for the *MC1R* and *ASIP* genes in several other leporid species will provide important tools for evolutionary studies in the Order *Lagomorpha*.

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