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IDENTIFICATION AND ANALYSIS OF SINGLE NUCLEOTIDE POLYMORPHISMS IN THE MYOSIN VA (*MYO5A*) GENE AND ITS EXCLUSION AS THE CAUSATIVE GENE OF THE *DILUTE* COAT COLOUR LOCUS IN RABBIT

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Abstract: Classical genetic studies have identified different coat colour loci in rabbit and comparative analyses have established corresponding loci across species. In particular, the rabbit *dilute* locus is determined by a recessive coat colour mutation that modifies the basic colours influenced by the *agouti* and *extension* mutations. In mice, similar phenotypic effects are determined by a similarly named locus. This locus encodes the myosin VA (*Myo5a*) gene, whose protein product is an unconventional myosin that plays an essential role in melanosome transport in the melanocytes. We selected the same gene as a strong candidate for explaining the *dilute* coat colour in rabbit. To this end, 1399 bp were re-sequenced, spanning 4 exons out of 41 exons and portions of intronic regions of the rabbit *MYO5A* gene to identify polymorphisms that could be useful to confirm or exclude this gene as causative of the rabbit *dilute* locus. Nine polymorphisms were identified, one of which was used to follow the segregation of the blue and black colours in a Checkered Giant F1 family. The single nucleotide polymorphism (SNP) analysed did not co-segregate with the 2 colours. These results excluded the *MYO5A* gene as determinant of the *dilute* locus in rabbit. The 2 alleles of this SNP were also present in several other breeds with different coat colours, further indicating that this marker is not associated with the *dilute* mutation in rabbits. Other candidates should be investigated to identify the causative gene of this locus in rabbit.

Key Words: rabbit, candidate gene, coat colour, *dilute* locus, *MYO5A*, SNP.

INTRODUCTION

Pigmentation in mammals is determined by 2 types of melanin pigments, eumelanins (black/brown pigments) and pheomelanins (yellow/red pigments), which are synthesised in lysosome-related organelles (melanosomes) of specialised cells, the melanocytes. The presence or absence, distribution, morphology and structure of the melanocytes and the biochemical activity and regulation of their enzymatic machinery determine differences in animal coat colours and patterns. In mice, more than 300 loci have been shown to affect these phenotypes (Lamoureux *et al.*, 2010). Key roles are played by the *agouti* and *extension* loci that regulate the production and relative amount of the 2 melanin types. These loci encode the agouti signalling protein (*ASIP*) and melanocortin 1 receptor (*MC1R*) genes, respectively (Bultman *et al.*, 1992; Robbins *et al.*, 1993). We recently studied the rabbit *ASIP* and *MC1R* genes and identified causative

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mutations determining the main alleles described by classical genetic studies at their respective loci (Fontanesi *et al.*, 2006, 2010a, 2010b). In addition to our studies, only another coat colour locus (*albino*) was analysed at the DNA level in rabbits, and mutations in the tyrosinase (*TYR*) gene have been suggested as the cause of its corresponding *Chinchilla*, *Himalayan* and *Albino* alleles (Aigner *et al.*, 2000).

Several other coat colour loci remain to be characterised at DNA level in this species. Among them, the *dilute* locus is determined by a recessive coat colour mutation that modifies the basic colours influenced by the *agouti* and *extension* mutations. This allele dilutes the black to blue (grey) and the yellow to beige coat colours of several rabbit breeds and lines (Castle, 1930; Robinson, 1958; Searle, 1968; Fox 1994). In other species, similar effects on coat colour are determined by mutations in a few genes including the myosin VA (heavy chain 12, myoxin) (*MYO5A*) gene, also known as dilute myosin heavy chain, non-muscle (Mercer *et al.*, 1991; Huang *et al.*, 1998a, 1998b; Futaki *et al.*, 2000; Brooks *et al.*, 2010). MYO5A is an actin-based motor that belong to the large myosin superfamily (Mermall *et al.*, 1998). This unconventional myosin is part of a multiprotein complex that is essential for melanosome transport from the perinuclear region of melanocytes to their actin-rich cell periphery (Marks and Seabra, 2001; Barral and Seabra, 2004). In mice, the *dilute* locus is coded by the *Myo5a* gene, which accounts for hundreds of different alleles, most of which have been produced in large-scale mutagenesis screens (Mercer *et al.*, 1991; Huang *et al.*, 1998a, 1998b). Some of them cause a lightened coat colour only, whereas others, together with the coat colour effect, determine neurological defects similar to those observed in patients with Griscelli syndrome type 1 who carry mutations in the *MYO5A* gene (Pastural *et al.*, 1997; Van Gele *et al.*, 2009).

To assess whether the *MYO5A* is the causative gene of the *dilute* locus in rabbit, DNA markers of this gene were identified and analysed in rabbits of different breeds and the linkage between a single nucleotide polymorphism (SNP) and the mutated *dilute* allele was evaluated.

MATERIAL AND METHODS

Animals and DNA

Six rabbits were used for re-sequencing of parts of the *MYO5A* gene. This group of animals consisted of 1 Belgian Hare, 1 Giant Grey and 4 unrelated Checkered Giant rabbits. Of the 4 Checkered Giant rabbits, 1 was a buck with blue spots, expected to be homozygous for the mutated *dilute* allele. Another was a doe expected to be heterozygous for the mutated *dilute* allele, as it was obtained by crossing a black spotted buck with another blue spotted doe which were not possible to sample. The other 2 Checkered Giant animals were 1 black buck and 1 black doe, which were expected to be homozygous for the normal allele according to pedigree information, subsequently confirmed by the results of crossbreeding with heterozygous or homozygous mutated *dilute* rabbits (data not shown). An F1 family with 8 rabbits (3 females and 5 males) was produced by crossing the blue Checkered Giant buck with the heterozygous doe used for re-sequencing of the *MYO5A* gene. In addition, another 165 rabbits of 19 breeds with different coat colours (Table 1) were used for allele frequency analysis of a selected *MYO5A* SNP.

Genomic DNA was extracted from blood (for the rabbits used for re-sequencing and the F1 animals) using the Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, WI) or from hair roots (for the rabbits used for allele frequency analysis) using the protocol already described by Fontanesi *et al.* (2007).

Table 1: Rabbits of different breeds genotyped for the g.20122039G>A SNP.

		g.20122039G>A genotypes (No.)		
Breeds[1]	No. of animals	GG	GA	AA
Alaska	3	3	-	-
Belgian Hare	3	3	-	-
Blue Vienna	17	16	1	-
Burgundy Fawn	9	8	-	1
Californian (black markings)	22	17	4	1
Champagne d'Argent	16	4	8	4
Checkered Giant (black spotted)	21	4	4	13
Dutch (black markings)	6	2	4	-
Dwarf (several colours)	5	-	2	3
English Spotted (black markings)	7	7	-	-
Fairy Pearly	3	1	1	1
Giant Chinchilla	12	10	2	-
Giant Grey	9	6	2	1
Giant White	3	-	2	1
Lop	3	1	2	-
New Zealand White	7	4	3	-
Rhinelander	7	4	3	-
Silver	7	7	-	-
Thuringian	5	3	1	1
Total	165	100	39	26

[1] Complete coat colour description of the breeds is reported in Fontanesi *et al.* (2010a). For breeds in which different marking colours are possible, only animals with black markings were genotyped, as indicated.

Identification and analysis of polymorphisms

Four PCR primer pairs were designed on the rabbit *MYO5A* gene sequence (Ensembl accession number ENSOCUG00000007669; located on chromosome 17 of the oryCun2.0 version) to amplify the coding regions of 4 exons and parts of downstream or upstream intronic sequences (Table 2 and Figure 1). Genomic DNA used for PCR amplification and sequencing was from the 6 rabbits already described above. PCR was carried out using a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA) in a 20 µL reaction volume containing ~50 ng genomic DNA, 1 U DNA Euro Taq DNA polymerase (EuroClone Ltd., Paignton, Devon, UK), 1×Euro Taq PCR buffer, 2.5 m*M* dNTPs, 10 pmol of each primer and 2.0 m*M* of $MgCl_2$. PCR profile was as follows: 5 min at 95°C; 35 amplification cycles of 30 s at 95°C, 30 s at 57-58°C, 30 s at 72°C; 5 min at 72°C (Table 2). The amplified fragments were sequenced after treatment with 1 µL of ExoSAP-IT® (USB Corporation, Cleveland, Ohio, USA) for 15 min at 37°C. Cycle sequencing of the treated PCR products was produced using the same PCR primers and the Big Dye v3.1 kit (Applied Biosystems, Foster City, CA, USA). Sequencing reactions, after precipitation with EDTA in ethanol 100% and ethanol 70%, were loaded onto an ABI3100 Avant capillary sequencer (Applied Biosystems). Sequences were edited and aligned with the help of the CodonCode Aligner software (CodonCode Corporation, Dedham, MA, USA).

Table 2: PCR primers, PCR conditions and use of the amplified fragments.

Primer pair name	Primer sequences (5'-3')[1]	Amplified fragment/position[2]	PCR[3]	Use[4]
Ex 2	TTCCTGGGGCTAGAGTACTTTTT GGACAAAAATTTAAGCTTGCTG	398 (part of intron 1, exon 2 and part of intron 2) /20112168-20112565	58/2.0	Re-sequencing
Ex 3	TTTGCCATTTCCTTTAGTTTCTG TTACTCTGGAGGACCTGCTTG	413 (part of intron 2, exon 3 and part of intron 3) /20121777-20122189	57/2.0	Re-sequencing and PCR-RFLP (*Rsa*I)
Ex 3 short[5]	CCTCACGGCTCTCAGCTATC TTACTCTGGAGGACCTGCTTG	187 (part of exon 3 and part of intron 3)	58/2.5	PCR-RFLP (*Rsa*I)
Ex 4	TGGCTCACTTTACCAGGTTTG AACAGTAATTTAGGCTATCTGAAACAA	375 (part of intron 3, exon 4 and part of intron 4) /20123617-20123991	57/2.0	Re-sequencing
Ex 5	GCAGAACTCACTGTGCCTCA TCCCCAATCTTAGTGCCTGA	390 (part of intron 4, exon 5 and part of intron 5) /20136785-20137174	58/2.0	Re-sequencing

[1] Forward and reverse primers. [2] Size in bp (including PCR primers). Amplified gene regions are according to the exon/intron organisation of the human *MYO5A* gene (GenBank accession number: NG_009887). Nucleotide positions (start and end) of the amplified fragments on chromosome 17 of the oryCun2.0 rabbit genome version is reported. [3] Annealing temperature (°C)/[$MgCl_2$] m*M*. [4] Use of the amplified fragments. [5] The reverse primer is the same of primer pair Ex 3. A shorter fragment containing the g.20122039G>A polymorphic site was used to amplify DNA extracted from hair roots.

PCR-RFLP (Table 2) was used to genotype the g.20122039G>A SNP (nomenclature of identified polymorphisms is based on system coordinates of rabbit chromosome 17 in the oryCun2.0 genome version: http://www.ensembl.org/Oryctolagus_cuniculus/Info/) in the F1 rabbits and in the 165 rabbits of different breeds. Briefly, the amplified fragment of 413 bp obtained using the same exon 3 primers used for re-sequencing or a shorter fragment of 187 bp (to facilitate amplification; Table 2) was digested with *Rsa*I. Digestion reaction was carried out overnight at 37°C in a 25 µL reaction volume including 5 µL of PCR product, 1× restriction enzyme buffer and 2 U of *Rsa*I (MBI Fermentas, Vilnius, Lithuania). The resulting DNA fragments were electrophoresed on 10% 29:1 polyacrylamide:bis acrylamide gel and visualised with 1× GelRed Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA, USA).

RESULTS AND DISCUSSION

Considering that the *Myo5a* gene is responsible for the *dilute* locus in mouse (Mercer *et al.*, 1991), we selected the same gene as a strong candidate to explain the coat colour phenotype that is fixed or segregates in several rabbit breeds. To this end, parts of the rabbit *MYO5A* gene were re-sequenced to identify polymorphisms that could be useful to confirm or exclude this gene as causative of the locus which, according to the classical genetic nomenclature, has been cited as such in mouse (Searle, 1968). On the whole, 1399 bp were re-sequenced, spanning 4 exons out of 41 exons that this gene might have according to the organisation of the human *MYO5A* gene (GenBank accession number: NG_009887), including parts of the contiguous intronic regions (Figure 1). Obtained sequences were combined and submitted to GenBank/EMBL under the accession number FR849714. Comparing the sequences obtained in the sequenced panel,

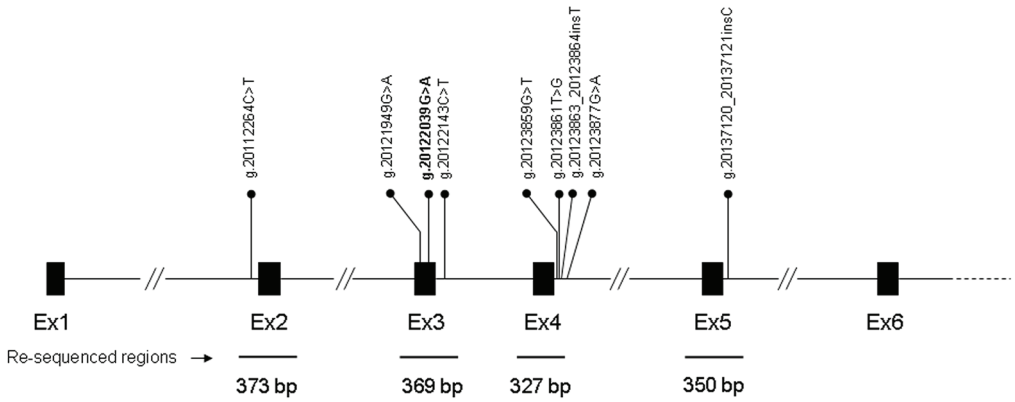

Figure 1: Partial structure of the rabbit *MYO5A* gene with indicated the re-sequenced regions (reported size does not include primers) and the polymorphic sites. Black boxes indicate the exons (Ex).

9 polymorphisms were identified, 7 of which were SNPs and 2 were insertions/deletions (indels) of 1 bp (Figure 1). Two synonymous SNPs were in exon 3, whereas all other polymorphisms were in intronic regions. These polymorphisms do not have any obvious functional or regulatory role, but can be used as DNA markers in linkage and association studies.

To identify the gene affecting the diluted coat colour, we produced a F1 family in which there was segregation of the blue and black coat colours (Figure 2). The animals were genotyped for a SNP in exon 3 (g.20122039G>A), which the sequencing results indicated to be homozygous AA in the parental blue buck and heterozygous GA in the parental black doe. It should be noted that the genotypes of the 2 founder rabbits was consistent, on the one hand, with a possible linkage between the A allele and the recessive *dilute* allele and, on the other hand, with a possible linkage between the G allele and the normal dominant allele. However, segregation of the g.20122039G>A SNP alleles and of the blue and black coat colours in the F1 rabbits clearly indicated that there was no linkage between this marker and the *dilute* coat colour (Figure 2). Blue and black F1 rabbits shared the same g.20122039G>A genotypes. This result excluded the *MYO5A* gene as the determinant of the *dilute* locus in rabbits.

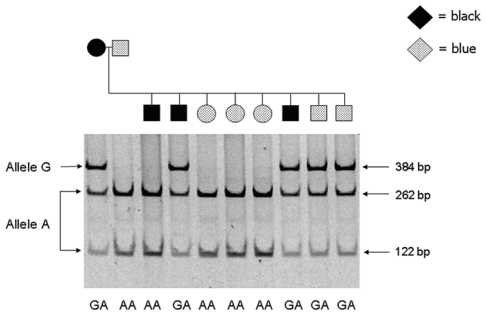

Figure 2: g.20122039G>A genotypes of the Checkered Giant rabbit family (segregating for the blue and black spotted coat colours) obtained by PCR-RFLP (fragment of 413 bp). The genotypes of the animals are at the bottom of each gel lane. A fragment of 29 bp derived by an additional *Rsa*I restriction site in the amplified fragment (present in all rabbits) is not evidenced in the gel.

To further confirm the lack of association of the *MYO5A* g.20122039G>A SNP with the *dilute* coat colour, this marker was genotyped in 19 rabbit breeds with different coat colours (Table 1). The 2 alleles and the 3 genotypes are shared by a large number of breeds having

different coat colours, suggesting that there is no association between this polymorphic site and coat colour as already evidenced in the linkage study with the F1 rabbit family. Differences in allele frequencies among breeds could be due to the low number of sampled animals for each rabbit, to random genetic drift. Moreover, we could not exclude that other mutations in this gene, not yet identified, might play a role as a modifier of the *dilute* locus determining different degrees of blue as observed, for example in Vienna Blue rabbits, where there are dark and pale blue lines.

However, the exclusion of the *MYO5A* gene as the determinant of the *dilute* coat colour in rabbit indicates that comparative coat colour genetics among species based on classical genetic studies (Searle, 1968) can sometimes be misleading, especially if similar phenotypes are determined by different genes involved in the same or close biochemical pathways or processes. In mice, for example, the *dilute*, *ashen* and *leaden* loci cause similar hypopigmentation or dilution of coat colour. They are determined by genes that code for proteins which interact together in defining melanosome transport (Wilson *et al.*, 2000; Marks and Seabra, 2001; Matesic *et al.*, 2001). In the case of the rabbit *dilute* locus, these remaining genes should be investigated to identify the causative gene affecting this coat colour locus.

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