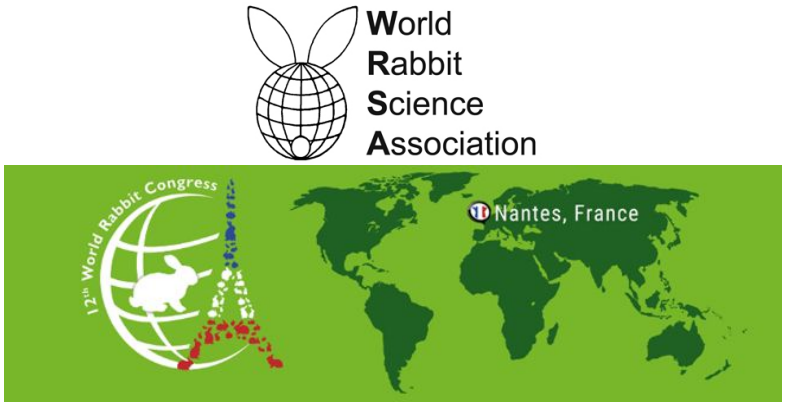

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Utzeri V.J. Ribani A., Fontanesi L.

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VARIABILITY IN THE TYROSINASE (*TYR*) GENE (THE *ALBINO* LOCUS) IN DOMESTIC AND WILD RABBITS

Utzeri V.J.[1], Ribani A.[1], Fontanesi L.[1,*]

[1]Department of Agricultural and Food Sciences, Division of Animal Sciences, University of Bologna, Viale G. Fanin 46, 40127 Bologna, Italy
[*]Corresponding author: luca.fontanesi@unibo.it

ABSTRACT

Disrupting mutations affecting the *TYR* gene cause different forms of albinism in mice, humans, and several other mammals. Classical genetic studies have already reported five alleles at the rabbit *Albino* locus, indicated to be part of the *C* series, each of them with different actions on pheomelanin and eumelanin production, as well as on the eyes. A few of these alleles have been already characterized at the DNA level by sequencing the coding region of the rabbit *TYR* gene in a few breeds or strains with specific alleles at this locus. In this study, we further characterized the *TYR* gene in rabbits by sequencing about 2000 bp encompassing all coding regions and flanking regions in a total of 25 rabbits from 11 domestic breeds (2 Belgian Hare, 2 Burgundy Fawn, 3 Californian, 3 Champagne d'Argent, 2 Giant Chinchilla, 1 Giant Grey, 1 Havana, 2 Leprino di Viterbo, 4 New Zealand White, 2 Silver and 3 White Vienna) and other 11 wild rabbits hunted in Sardinia. Sequencing data identified a total of 15 mutations. We confirmed five missense mutations already detected by other studies, three of which associated with different coat colour phenotypes: p.T373K determining the albino allele; p.E294G causing the Himalayan and the chinchilla allele; p.T358I observed only in Chinchilla rabbits. In addition to seven other synonymous mutations and one polymorphism in the 3'-untranslated region (UTR), two novel missense mutations (one identified only in wild rabbits), were identified. This study further contributed to disclose the variability in the *TYR* gene in different rabbit populations and confirmed the effect of functional mutations at this locus.

Key words: Albinism, Breed, Coat colour, Mutation, *Oryctolagus cuniculus*.

INTRODUCTION

Since the rediscovery of the Mendelian laws, coat colour in mammals has been the subject of a lot of genetic studies that, in mice, led to the identification of more than 300 loci affecting pigmentation. Coat colours largely depends on the amount and distribution of two types of melanin, pheomelanin (yellow-red pigment) and eumelanin (black-brown pigment) located in the skin, in the hair and in the pigmented cell layers of eyes. We recently identified mutations in several genes determining coat colour variability in different rabbit breeds (Demars et al., 2018; Fontanesi et al., 2006, 2010a, 2010b, 2014a, 2014b; Utzeri et al., 2014).

Tyrosinase (TYR) is the key enzyme involved in the first step of melanogenetic pathway, in which tyrosine is transformed in a metabolic intermediate shared with both eumelanin and pheomelanin. pathways. Disrupting mutations affecting the *TYR* gene cause different forms of albinism in mice, humans, and several other mammals (e.g. Schmutz et al., 2004; Rees, 2011; Utzeri et al., 2016).

Classical genetic studies have already reported five alleles at the rabbit *Albino* locus, indicated to be part of the *C* series, each of them with different actions on pheomelanin and eumelanin production, as well as on the eyes (Searle, 1968): *C*, full colour (with normal melanin production and dark eye-colour); c^{chd}, dark chinchilla, with reduced pheomelanin production, normal eumelanin production and dark eye colour); c^{chm}, medium chinchilla (without any pheomelanin production, slightly reduced eumelanin production and reddish-black eye-colour); c^{chl}, light chinchilla (no pheomelanin production, reduced eumelanin production and red eye-colour); c^{h}, Himalayan (no pheomelanin production,

reduced eumelanin production and located only at the extremities and pink eye-colour); *c*, albino (without any melanin production and pink eye).

The coding region of the rabbit *TYR* has been sequenced in a few rabbits with different alleles at the *Albino* locus (Aigner et al., 2000). A missense mutation causing the p.T373K amino acid substitution, identified in New Zealand White rabbits, has been indicated to cause the recessive *c* albino allele (Aigner et al., 2000). The same mutation in humans causes a type I oculocutaneous albinism (OCA1; Sanabria et al., 2012). Other two missense mutations (p.E294G, identified in Californian rabbits showing the Himalayan phenotype; and the p.E294G together with the p.T358I, identified in Chinchilla rabbits) associated to different alleles at this locus were identified, in addition to few other polymorphisms shared by several rabbits of different strains (Aigner et al., 2000).

In this work we further investigated the *TYR* gene in 11 rabbit breeds carrying different putative alleles at the *Albino* locus (Belgian Hare, Burgundy Fawn, Californian, Champagne d'Argent, Giant Chinchilla, Giant Grey, Havana, Leprino di Viterbo, New Zealand White, Silver and White Vienna) and in wild rabbits to identify additional variants in this gene.

MATERIALS AND METHODS

Animals

A total of 26 domestic rabbits from 11 different breeds (2 Belgian Hare, 3 Burgundy Fawn, 3 Californian, 3 Champagne d'Argent, 2 Giant Chinchilla, 1 Giant Grey, 1 Havana, 2 Leprino di Viterbo, 4 New Zealand White, 2 Silver and 3 White Vienna) and 11 wild rabbits hunted in Sardinia were used in this study. All domestic animals had standard breed coat colours and were registered to the corresponding breed herd book, managed by the Italian Rabbit Breeders Association.

PCR and Sanger sequencing

Genomic DNA was extracted from hair roots (domestic rabbits) or from liver specimens (wild rabbits) using a standard phenol-chloroform-isoamyl alcohol extraction protocol (Sambrook et al., 1989) or the Wizard® Genomic DNA Purification Kit (Promega Corporation). Using the sequence of the *TYR* gene annotated in the OryCun2.0 rabbit genome (Carneiro et al., 2014), seven primer pairs were designed on flanking or intronic regions to amplify all five exonic regions and parts of the introns. PCR amplifications were carried in a 20 μL of reaction volume containing 1 X reaction buffer 10X, 2.5 mM of $MgCl_2$, 1.5 mM dNTP mix, 10 pmol of each primer and 1 U of Taq DNA polymerase (EuroClone). PCR cycles were: initial denaturation step at 95 °C for 5 min; 35 cycles at 95 °C for 30 s, 30 s at the primer pair annealing temperature (50-60 °C), 72 °C for 30 s: final extension step at 72 °C for 5 minutes. After purification with 2 U of ExoSAP-IT (USB Corporation) for 15 min at 37 °C, PCR fragments were sequenced using the Big Dye v3.1 kit (Applied Biosystems). Sequencing reactions were then loaded on an ABI3100 Avant capillary sequencer (Applied Biosystems). Obtained sequences were visually inspected, assembled and aligned using MEGA 6 software (Tamura et al., 2013). The effect of each single nucleotide polymorphisms (SNPs) was evaluated using the Variant Effect Predictor (VEP) implemented in Ensembl Genome Browser (https://www.ensembl.org/info/docs/tools/vep/index.html), and the effect of all missense mutations was analysed using SIFT algorithm (Kumar et al., 2009).

RESULTS AND DISCUSSION

A total of 2027 bp, including the complete coding region of the *TYR* gene (1593 bp) and non-coding regions (434 bp of 3'-untranslated regions and 5'-flanking regions) were sequenced in all investigated rabbits. Table 1 reports the mutations that were identified in this study.

Sequencing data confirmed the presence of five missense mutations already detected by Aigner et al. (2000), three of which strain-specific and associated with different coat colour phenotypes: p.T373K was homozygous in all New Zealand White rabbits; p.E294G was identified in homozygous state in all Californian and in all Giant Chinchilla rabbits; p.T358I was homozygous only in Chinchilla rabbits. The two other missense mutations (p.V31M and p.S287T), already detected in a few rabbit breeds by

Aigner et al. (2000), were present in homozygous and heterozygous conditions in several other breeds of our study, including wild rabbits.

In addition to these missense mutations, two novel missense mutations (Table 1) occurring in exon 1 were detected: p.T144S was found in a Burgundy Fawn rabbit in heterozygous condition; p.K224T was detected only in 6 wild rabbits, 3 were homozygous and 3 were heterozygous for the alternative allele. All these wild rabbits had the wild grey/brown coat colour.

Table 1: Polymorphisms identified by sequencing the *TYR* gene in different rabbits.

SNP position in OryCun2.0[1]	Position in the gene	SNP/effect (SIFT)	Reference
1:127667147	exon 1	G>A/p.V31M (0.27)	Aigner et al. (2000)
1:127666866	exon 1	C>T/synonymous	This study
1:127666862	exon 1	C>T/synonymous	This study
1:127666833	exon 1	T>C/synonymous	This study
1:127666807	exon 1	C>G/p.T144S (0.25)	This study
1:127666785	exon 1	C>T/synonymous	This study
1:127666662	exon 1	C>T/synonymous	This study
1:127666567	exon 1	A>C/p.K224T (0.13)	This study
1:127650986	exon 2	A>T/synonymous	This study
1:127650981	exon 2	G>C/p.S287T (1.00)	Aigner et al. (2000)
1:127650965	exon 2	T>C/synonymous	This study
1:127650960	exon 2	A>G/p.E294G (0.30)	Aigner et al. (2000)
1:127637042	exon 3	C>T/p.T358I (0.11)	Aigner et al. (2000)
1:127636997	exon 3	C>A/p.T373K (0.00)	Aigner et al. (2000)
1:127562872	3' UTR	A>G/-	This study

[1]Chromosome and nucleotide position on the coordinate system.
[2]The two alleles are reported/missense mutation or synonymous mutation if located in exons (SIFT score of missense mutations).

A total of seven novel synonymous polymorphisms located in exon 1 and exon 2 and one novel polymorphism in the 3'-untranslated region (UTR) of the *TYR* gene were identified in some domestic breeds and in wild rabbits. Wild rabbits carried most of these variants that were identified only in this population.

In mammals, *TYR* gene is known to be under natural selective pressure due to its importance in producing pigments for mimicry (Sturm and Duffy, 2012; Hubbard et al., 2010). In wild rabbits, the need to preserve a functional tyrosinase enzyme is crucial for the fitness of the animals. The new missense mutations (p.K224T) detected in wild rabbits is located between the two cupper binding domains (CuA and CuB) of the TYR enzyme but, according to *in silico* SIFT results, this amino acid substitution could be tolerated (P = 0.13) and it might be neutral.

CONCLUSIONS

Coat colour is considered one of the first traits on which domestication operated. The complete characterization of all coat colour genes in rabbit might provide additional hints on the domestication processes and on the constitution of the large number of fancy breeds. Coat colour genes might be also relevant in the adaptation of wild and feral rabbit populations to different environments. In this study we further contributed to disclose the variability of the *TYR* gene in different breeds and confirmed the presence of a few mutations already described by others to cause different coat colours. Further studies are needed to identify the mutation(s) causing other alleles predicted by classical genetic studies at the *Albino* locus in rabbit.

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