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PAPER

Identification of c.483C>T polymorphism in the caprine tyrosinase-related protein 1 (*TYRPI*) gene

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vs African breeds, being almost fixed in the latter. Moreover, the C-allele showed an increased frequency in white coated breeds (Girgentana, Grigia Molisana, Blanca de Rasquera and Saanen) when compared with those displaying a dark pigmentation (Cilentana Nera, Azpi Gorri and Murciano-Granadina). This could be due to genetic drift, migration and other factors associated with the demographic history of breeds under analysis or to a genetic hitchhiking event (c.483C>T frequencies would be shaped by a neighbouring causal mutation differentially selected in white and black goats). More refined studies will be needed to distinguish between these two alternative explanations.

Introduction

The molecular basis of coat colour inheritance in goats is very complex as demonstrated by recent studies targeting genes with known effects on pigmentation. Fontanesi *et al.* (2009) characterized the variability of the caprine melanocortin receptor 1 (*MC1R*) gene identifying one nonsense, three missense and one silent single nucleotide polymorphisms (SNP). Unfortunately, none of these mutations showed a clear association with coat colour when analysing their segregation in a pool of 317 goats belonging to six breeds with different pigmentation patterns (Fontanesi *et al.*, 2009). Moreover, sequence analysis of the caprine agouti signaling protein (*ASIP*) gene has evidenced the existence of a duplicated copy, as previously demonstrated in sheep (Norris and Whan 2008), and three missense SNP. Whilst the *ASIP* copy number polymorphism might be associated with white colour in the Girgentana and Saaanen breeds, the three missense SNP did not shown any significant effect on pigmentation (Fontanesi *et al.*, 2010).

Another molecule with known effects on the phenotypic variation of coat colour is tyrosinase-related protein 1 (*TYRPI*), a melanosomal enzyme deeply involved in eumelanin synthesis (Sarangerajan and Boissy 2001). Classical studies performed in mouse revealed that *TYRPI* loss-of-function alleles are associated with a brown coat (Bennett and Lamoreux 2003). In cattle, a non-conservative H434Y substitution at the bovine *TYRPI* enzyme has been related with the Dun coat of Dexter cows (Berryer *et al.*, 2003), while in Soay sheep there is strong evidence that replacement of a highly conserved cysteine at position 290 by phenylalanine results in a lighter colour

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(Gratten *et al.*, 2007).

Given its fundamental role in pigmentation, we have characterized the sequence variability of the coding region of the caprine *TYRPI* gene and investigated its segregation in a wide array of caprine breeds from the Iberian Peninsula and Balearic Islands, Italy, Canary Islands, Asia and North Africa.

Abstract

Tyrosinase-related protein 1 (*TYRPI*) has been shown to play a fundamental role in pigmentation both in human and mouse. In this work, we aimed to characterize the variability of the caprine *TYRPI* gene and investigate its segregation in a wide array of goat breeds. By partially sequencing the coding region of the *TYRPI* gene in 18 individuals from eight different breeds, we were able to identify a synonymous nucleotide substitution at exon 3 (c.483C>T). An extensive survey of Iberian and Balearic (N=175), Italian (N=99), Swiss (N=54), Asian (N=14), Canarian (N=92) and North African (N=117) goats with different coat colours was carried out. We found that the C-allele has a different distribution in European

Materials and methods

Nucleic acids isolation and complementary DNA synthesis

Genomic DNA extraction from hair follicles was performed with the DNeasy Blood and Tissue kit (Qiagen Iberia SL, Barcelona, Spain), while protocols reported by Zidi *et al.*, (2008) were employed to purify DNA from blood samples. A Nanodrop spectrophotometer ND-1000 (SG Servicios Hospitalarios, Barcelona, Spain) was used to measure the concentration of genomic DNA preparations. We also isolated total RNA, with the RiboPure kit (Applied Biosystems, Sant Andreu de Llavaneres, Spain), from skin samples

obtained from Palmera, Majorera and Tinerfeña goats at the slaughterhouse. Total RNA concentration was estimated with an Agilent 2100 Bioanalyzer equipment (Agilent Technologies, Barcelona, Spain) and reverse transcribed to complementary DNA with the ThermoScript RT-PCR System kit (Invitrogen, Barcelona, Spain). Both genomic DNA and cDNA were used as templates in sequencing experiments.

Sequencing of the goat *TYRP1* coding region

The goat *TYRP1* coding region was partially sequenced in 18 goats belonging to the Malagueña (N=2), Saanen (N=2), Palmera (N=3), Majorera (N=3), Tinerfeña (N=2), Garganica (N=2), Cilentana Nera (N=2) and Girgentana (N=2) caprine breeds. The coverage of the *TYRP1* coding region was 96% for the Canarian breeds and around 40% for the remaining ones. For all PCRs, the thermal profile consisted of 35 cycles of 94°C for 45 sec, Tann (Table 1) for 45 sec, and 72°C for 1 min. Amplification reactions had the following composition: 1.5 mM MgCl₂, 200 μM of each dNTP, 0.2 μM of each primer, 50-80 ng of genomic DNA (PCR₁, 2 and 3) or 1.5 μL cDNA (PCR₄, 5, 6 and 7), and 0.5 U of Taq DNA polymerase (Ecogen, Barcelona, Spain) in a final 20 μL volume.

Sequencing reactions were carried out with the BigDye Terminator v1.3 Cycle Sequencing Kit (Applied Biosystems, Sant Andreu de Llavaneres, Spain) and primers listed in Table 1. Genotyping was performed in a Sequenom MassARRAY iPLEX platform at the Spanish National Genotyping Centre (CeGen, Santiago de Compostela, Spain). A total of 551 individuals were genotyped. These goats belonged to the following geographical areas:

Italy (N=99): Cilentana Nera (N=26), Garganica (N=41), Grigia Molisana (N=13) and Girgentana (N=19)

Iberian Peninsula and Balearic Islands (N=175): Malagueña (N=43), Murciano-Granadina (N=81), Azpi-Gorri (N=13), Eivissenca (N=12) and Blanca de Rasquera (N=26).

Canary Islands (N=92): Tinerfeña (N=38), Palmera (N=38) and Majorera (N=16).

North Africa (N=117): Moroccan (N=32) and Tunisian (N=85).

Switzerland (N=54): Saanen (N=54).

Asia (N=14): Cashmere (N=14).

TYRP1 frequencies were compared taking into consideration the geographical distribution of each breed (population analysis). A second comparison was made between white (Girgentana, Grigia Molisana, Blanca de

Rasquera and Saanen) and black breeds (Cilentana Nera, Azpi Gorri and Murciano-Granadina). Instead of taking pictures from each one of the 551 goats under analysis and

defining colour on an individual basis, we considered that each breed is represented by a single coat colour that corresponds to the one defined in the racial standard or, in the lack of

Table 1. Annealing temperatures (Tann), amplified fragment sizes and sequences of primers used in the characterization of the goat *TYRP1* gene.

PCR	Primer	Sequence (5'-3')°	Tann (°C)
PCR1	TYRP-FW-5'UTR_G	CAAAAGGGAGTTGGAGTATCCA	60
	TYRP-REV-5'UTR_G	CCACCCACTCCCGTAGAG	
PCR2	TYRP1_FW_G	TGCTAGAAATCTGCCCCAA	59
	TYRP1_REV_G	GAGAACCCTCTGGTCACAGGC	
PCR3	TYRP_int2-FW	CTGCCTGTGACCAGAGGGTT	61
	TYRP_int2_REV	AATGCTGGTCCCTCGTGAGA	
PCR4	TYRP1-cDNA_FW1	GAATCTCCTACACTCCTCTCTC	60
	TYRP1-cDNA_RV1	GAAGGTTTCTCTGACTGTG	
PCR5	TYRP1-cDNA_FW2	CACGCTTCTTCAACAGGACA	60
	TYRP1-cDNA_RV2	CATCAAGTCATCGGTGCAAA	
PCR6	TYRP1-cDNA_FW3	GAGGGACCAGCATTTCTCAC	60
	TYRP1-cDNA_RV3	ATCAGCATTGTATCGCCTCA	
PCR7	TYRP1-cDNA_FW4	AGGTTACAGTGATCCCACAG	62
	TYRP1-cDNA_RV4	CAACCTATTATTTCTAAGCATGTGGTTT	

°Primers were designed using the following templates: bovine *TYRP1* genomic sequence (Ensembl entry: ENSBTAG00000020985) for PCR1, PCR2 and PCR3; bovine *TYRP1* cDNA sequence NM_174480.3 for PCR7. Primers for PCR 4, 5 and 6 were taken from Gratten et al. (2007).

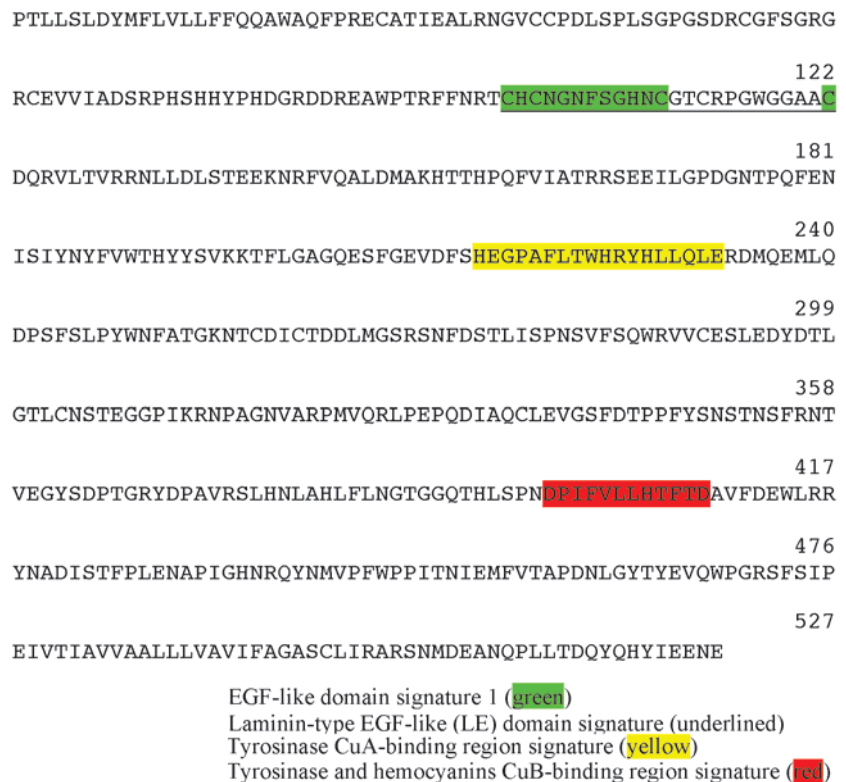


Figure 1. Functional domains of the goat *TYRP1* protein (GenBank accession number: HQ391904).

it, to the one that is more typically observed. Although this procedure simplifies the obtaining of phenotypes, it also diminishes the power of the statistical analysis because we did not take into account that a certain level of coat colour heterogeneity exists in each breed.

Results and discussion

In the current work we have characterized the near-complete sequence of the goat *TYRP1* coding region (GenBank accession number: HQ391904). Analysis with the Prosite software (<http://expasy.org/prosite>, Figure 1) showed that the goat *TYRP1* protein sequence has a conserved epidermal growth factor (EGF)-like domain represented by two hits (amino acid positions are numbered according to the *TYRP1* coding sequence, with position 1 indicating the first nucleotide of the translation initiation codon): *EGF-like domain signature 1* [99-110] and *laminin-type EGF-like (LE) domain signature* [99-122]. Furthermore, two histidine rich copper-binding sites, *i.e.* *tyrosinase CuA-binding region signature* [215-232] and *tyrosinase and hemocyanins CuB-binding region signature* [397-408], were found to be evolutionary conserved. According to Jackson *et al.* (1992), the EGF-like domain might allow the interaction of *TYRP1* with other melanoso-

mal proteins to form a multienzymatic complex, while the copper-binding domains might have an important catalytic role (Furumura *et al.*, 1998)

Partial sequencing of the *TYRP1* coding region in 18 individuals from diverse breeds allowed us to identify a synonymous c.483C>T SNP mapping to exon 3 (Figure 2). It is necessary to stress that our sequencing approach targeted a limited number of exons, so additional variability might be found by analysing

other *TYRP1* regions, such as introns and 3'UTR, as well as by sampling other goat populations. We investigated the segregation of the c.483C>T polymorphism in 551 goats belonging to 16 breeds by using a Sequenom MassARRAY iPLEX platform (Figure 3, Table 2). This analysis evidenced that the C-allele is almost fixed in Moroccan and Tunisian goats, and fixed in Canarian (Palmera, Tinerfeña and Majorera) breeds. This strong similarity in allelic frequencies between African and

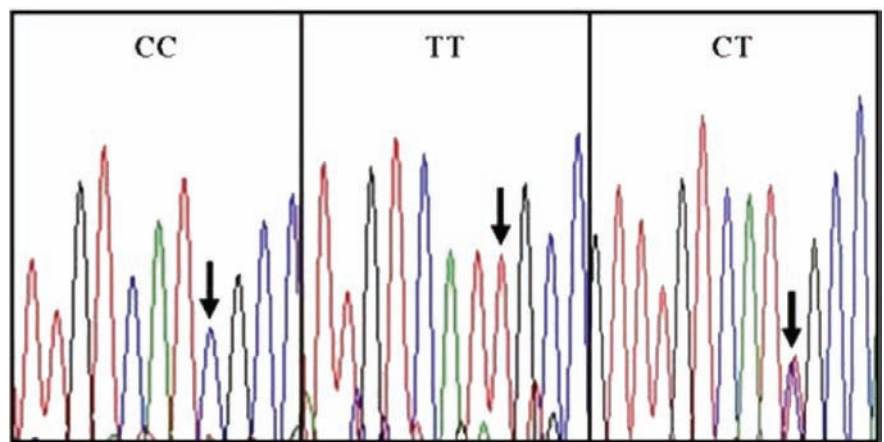


Figure 2. Partial view of sequencing electropherograms corresponding to individuals displaying CC, TT and CT c.483 C>T *TYRP1* genotypes (the polymorphic site is indicated with an arrow).

Table 2. Genotypic frequencies of the *TYRP1* c.483C>T polymorphism in goat breeds from five geographical areas and diverse coat colours.

Breed	Coat colour	Location ^o	N	CC	CT	TT
Cilentana Nera	Black	IT	26	0.38	0.54	0.08
Garganica	Black, sometimes with reddish areas	IT	41	0.17	0.49	0.34
Girgentana	White, with brownish or brown areas in the front and cheeks	IT	19	0.95	0.05	0.00
Grigia Molisana	White, sometimes gray or brown	IT	13	0.69	0.23	0.08
Overall (IT)		IT	99	0.44	0.38	0.18
Azpi-Gorri	Black, reddish tone in the belly and knees	IP + BI	13	0.38	0.38	0.24
Blanca de Rasquera	White, often with black spots, sometimes with reddish/cream spots	IP + BI	26	0.46	0.50	0.04
Eivissenca	Polychromous	IP + BI	12	0.42	0.42	0.16
Malagueña	Cream to dark red	IP + BI	43	0.44	0.51	0.05
Murciano-Granadina	Black or dark brown	IP + BI	81	0.20	0.60	0.20
Overall (IP + BI)		IP + BI	175	0.32	0.54	0.14
Majorera	Polychromous	CAN	16	1.00	0.00	0.00
Palmera	Red	CAN	38	1.00	0.00	0.00
Tinerfeña	Black or dark brown	CAN	38	1.00	0.00	0.00
Overall (CAN)		CAN	92	1.00	0.00	0.00
Moroccan	Polychromous	NA	32	0.94	0.06	0.00
Tunisian	Polychromous	NA	85	0.93	0.07	0.00
Overall (NA)		NA	117	0.93	0.07	0.00
Saanen	White	SW	54	0.72	0.24	0.04
Cashmere	Polychromous	AS	14	1.00	0.00	0.00

^oIT, Italy; IP + BI, Iberian Peninsula and Balearic Islands; CAN, Canary Islands; NA, North Africa; SW, Switzerland, AS, Asia.

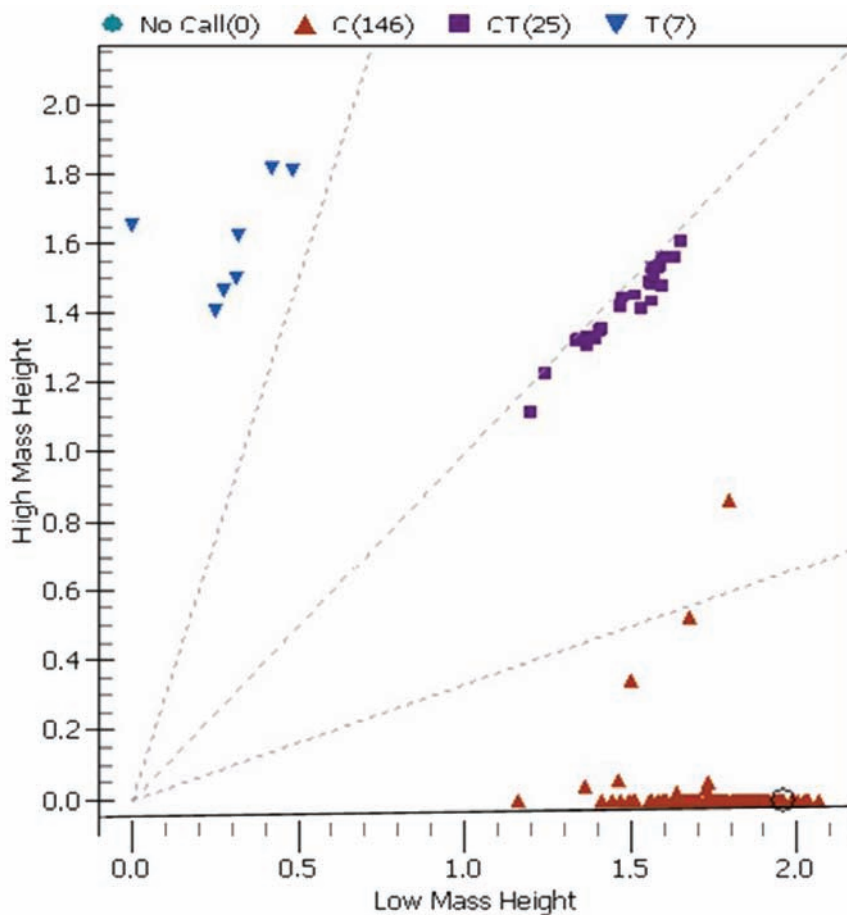


Figure 3. Cluster plot of caprine *TYRP1* genotypes inferred with the Sequenom MassARRAY iPLEX technology.

Canarian goats is expectable given the close relationship between both geographical areas. Remarkable coincidences have been found between the different dialects spoken by the indigenous populations of the Canary Islands and the Amazigh language (Farrujia 2004). Moreover, analysis of mitochondrial and Y-chromosome markers have revealed the presence of Berber haplotypes in the gene pool of Canary islanders at higher frequencies than in that of Iberian colonizers, a feature that suggests that these haplotypes entered the Canary islands as a consequence of a migratory movement originated at North Africa (Maca-Meyer et al., 2004; Fregel et al., 2009). In summary, our result is consistent with the hypothesis of a North African ancestry for Canarian goats but this interpretation still needs to be proved by surveying a representative number of genetic markers.

We also observed that the C-allele has an increased frequency in white (Girgentana,

Grigia Molisana, Blanca de Rasquera and Saanen) vs black (Cilentana Nera, Azpi Gorri and Murciano-Granadina, Tinerfeña was excluded because the C-allele is fixed) breeds. In this way, the frequency of the C-allele was around 0.57 and 0.86 in black and white goats, respectively (Table 2). This result can be explained in two ways. First, demographic and evolutionary forces might have differentially shaped *TYRP1* allele frequencies in these two groups of goats. In this case, we would assume that the *TYRP1* polymorphism is neutral and that differences (0.57 vs 0.86) observed in the frequencies of the C-allele are fortuitous. Alternatively, the increased frequency of the C-allele in the white goat group might have been caused by genetic hitchhiking i.e. the synonymous polymorphism we have analysed is linked to an unknown causal mutation affecting melanogenesis. There are several factors that make difficult to discriminate between these two alternative hypotheses. In the first

place, the goat groups we have analysed are composed by different breeds i.e. there is a substantial population substructure, a feature that might favour the emergence of spurious associations. Moreover, several of the analysed breeds do not display homogeneous coat colours limiting the power of our analysis. Finally, epistatic interactions between pigmentation genes occur very often, implying that the specific genetic background of each breed can differentially affect the penetrance of the *TYRP1* genotype. By these reasons, trying to infer associations between pigmentation genes and coat colour based on comparing allelic frequencies between breeds is not expected to be a very fruitful approach. A more informative strategy would be to examine the co-segregation of the C- and T-alleles and pigmentation in within-breed crosses between individuals with divergent phenotypes for this trait. Once associations were confirmed, it would be necessary to sequence the 16.8 kb transcription unit of *TYRP1* in related individuals with different coats to identify the causal mutation.

Conclusions

We have identified a synonymous c.483C>T SNP in the goat *TYRP1* gene. Although this substitution is not expected to produce a functional change, we have observed an increased frequency of the C-allele in white vs black goats. Additional studies should be performed at the within breed level to evaluate the relative impact of demographic vs selection factors on the variability of the goat *TYRP1* locus.

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