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Polymorphisms in *MC1R* and *ASIP* genes and their association with coat color phenotypes in llamas (*Lama glama*)

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ABSTRACT

The melanocortin 1-receptor (*MC1R*) and the *agouti* signaling protein (*ASIP*) are the major genes controlling the type and location of pigments produced in mammals. In recent years, polymorphisms in these genes have been associated with coat color variation in a number of species. Llamas (*Lama glama*) are characterized by a great diversity of coat colors. However, the genetic basis of coat color determination is still unknown. Here, we sequenced the *MC1R* and *ASIP* genes in llamas and studied the association between the polymorphisms identified and the coat color. Sequence analysis revealed ten nonsynonymous single nucleotide polymorphisms in the *MC1R* gene. Three main haplotypes were identified, none of which were completely associated to a particular color phenotype. However, significant association was detected between the *MC1R*1* haplotype and the presence of pigmented coat ($P < 0.0001$). Compared to the wild allele, *MC1R*1* carried two amino acid substitutions, p.G126S and p.V87 M. This last replacement occurs at a highly conserved residue among mammals and the same substitution has been previously associated to melanic phenotypes in avian species. Furthermore, two polymorphisms in *ASIP* exon 4, a 57 bp deletion (c.325_381del) and c.292C > T that are both predicted to have a deleterious effect on the protein, were found in homozygous state or combined in most llamas with eumelanic coat.

1. Introduction

South American camelids are currently represented by the domestic species llama (*Lama glama*) and alpaca (*Vicugna pacos*), and the wild species, guanaco (*Lama guanicoe*) and vicuña (*Vicugna vicugna*). After years of intense debate about the origin of the two domestic species, molecular studies have shown that the llama originated from the domestication of the guanaco and the alpaca from the vicuña (Stanley et al., 1994; Vidal-Rioja et al., 1994; Kadwell et al., 2001).

Domestication is a complex process that involves, among other changes, genetic modifications of the species by directed selection. This selection involves modifications of phenotypic characteristics and their underlying genotypes (Grandin, 1998). While many of the wild species are uniformly colored, a variety of colors and patterns are found in domestic animals. Coat color is one of the most noticeable phenotypic differences between llamas and their wild ancestor. Guanacos show a characteristic wild type phenotype, with reddish dorsal region, white belly and a dark grey head. Whereas, llamas exhibit a wide variety of colors.

Despite some differences between species, pigmentation in mammals is a highly conserved process. The basic coat colors are defined by the relationship between two pigments: eumelanin (black or brown) and pheomelanin (yellow or red). Eumelanin/pheomelanin ratio is regulated mainly by the ligand-receptor system of the *agouti* signaling protein (ASIP) and the melanocortin 1-receptor (MC1R). The binding of alpha-melanocyte stimulating hormone (α-MSH) to MC1R leads to eumelanin synthesis, while binding of ASIP inhibits signal transduction, causing the melanocyte to produce pheomelanin (Lu et al., 1994). Therefore, gain-of-function mutations in the *MC1R* gene produce eumelanic pigmentation, whereas those that cause loss-of-function lead to pheomelanic phenotypes (Barsh, 1996). *MC1R* has a characteristic allelic hierarchy with the dominant allele *(E)* producing black color and the recessive allele (*e*) responsible for yellow color. Such mutations have been associated with color variation in species such as dogs (Schmutz et al., 2003), pigs (Kijas et al., 1998) and horses (Marklund et al., 1996), among others. The dominant *ASIP* allele *(A)*,

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produces a yellow-red coat while the recessive allele *(a)* is responsible for uniform black color. Loss-of-function mutations in *ASIP (a)* resulting in eumelanic phenotypes have been described in several species (Rieder et al., 2001; Eizirik et al., 2003; Kerns et al., 2004; Royo et al., 2008). Although MC1R is primarily responsible for the pigment type produced, both *MC1R* and *ASIP* can act locally, influencing the distribution of eumelanin and pheomelanin in different regions of the body (Cieslak et al., 2011). The dominant black allele (p.M73 K and p.D121N) and the putative recessive *e* allele (p.R67C) from the *MC1R* gene have been identified in sheep, (Våge et al., 1999; Fontanesi et al., 2010a). The variability of *MC1R* and *ASIP* has been also reported in goats. Fontanesi et al. (2009) found mutations in the *MC1R* gene that may be involved in the determination of eumelanic and pheomelanic phenotypes in this species. Moreover, copy number variation of *ASIP* gene has been also associated to light and dark coat in both goats and sheep (Norris and Whan 2008; Fontanesi et al., 2011; Dong et al., 2015)

Several polymorphisms have been identified in the alpaca *MC1R* and *ASIP*. The c.901C > T substitution resulting in the p.R301C amino acid change in the *MC1R* has been associated with pheomelanic and non pigmented phenotypes (Powell et al., 2008; Feeley and Munyard 2009; Guridi et al., 2011; Chandramohan et al., 2015). Furthermore, different polymorphisms within exon 4 of *ASIP* have been associated with recessive black color in alpaca (Feeley et al., 2011; Chandramohan et al., 2013).

The different coat color phenotypes in llamas were described by Frank (2001) and Frank et al. (2006). Based on these descriptions and by classical crossbreeding analysis, those authors studied the segregation of colored phenotypes and postulated that pigmented phenotypes are segregated by the *Agouti* locus. Nevertheless, the molecular basis of coat color determination in this species has not yet been established.

The aim of this work was to characterize the *MC1R* and *ASIP* genes in llamas, identify allelic variants and determine the association between them and the coat color.

2. Materials and methods

2.1. Samples

For initial characterization, polymorphism identification and haplotype analyses in the llama *MC1R* and *ASIP* genes, we used DNA samples stored in our lab (n = 41 and n = 19, respectively) representative of diverse llama populations in Argentina.

Additionally, seven guanaco samples previously collected for other research projects were utilized to determine the wild genotype for both genes.

New blood samples were collected from 84 unrelated llamas, from six different breeding establishments, for association studies between genetic variants and coat colors. The samples were taken by jugular vein puncture by trained personnel following the Argentinean Ethical References for Biomedical investigation in Animals from Laboratory, Farm or obtained from Nature (Resolution D N° 1047/05 from CONICET). Coat color phenotype of each individual was determined by visual inspection and corroborated by opening the fleece. Phenotypes were recorded and, whenever possible, photographs and fiber samples were taken.

Genomic DNA was isolated from blood following the procedure described by Gemmell and Akiyama (1996).

2.2. Primer design and PCR conditions

Two PCR primer pairs were designed to cover the entire coding region of *MC1R* based on the alpaca gene sequence (GenBank EU135880.1). For *ASIP* coding region amplification, the primers were designed on alpaca sequence obtained from Ensembl database (Version 84.1 GeneScaffold_575:110617:111376:1; GeneScaffold_575:112068:112732:1 and GeneScaffold_575:109885:127988:1) on the flanking regions of each exon. The design of all primer pairs was performed with the Primer 3 software (Rozen and Skaletsky, 2000).

Amplification reactions were carried out in 25 μl PCR mix containing 1× PCR Buffer (200 mM Tris-HCL (pH8.4), 500 mM KCl-Invitrogen, Carlsbad, CA, USA), 1.5 mM $MgCl_2$ (2 mM for *ASIP*-Ex4), 0.2 mM dNTPs, 0.65U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 0.25 μM of each primer and 75 ng of DNA. The cycling profile consisted of an initial denaturation step at 94 °C for 3 min, 30–35 cycles of 1 min at 94 °C, 1 min at 53–57 °C, 1 min at 72 °C and a 5 min final extension at 72 °C. Sequence and annealing temperature for each primer are listed in Table 1.

PCR products were checked on a 2% agarose gel stained with GelRed™ (Biotium, Hayward, Ca), purified by PEG precipitation, sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) and then analysed in an automatic sequencer 3730xl (Applied Biosystems, Foster City, USA).

2.3. Sequence analysis and polymorphism identification

Llama *MC1R* and *ASIP* gene sequences obtained were deposited in GenBank under the accession numbers KP715426-KP715432.

Single nucleotide polymorphisms (SNP) were identified by sequence alignment using Geneious (www.geneious.com), and confirmed by resequencing the whole fragment in the opposite direction. The gametic phase of each haplotype was determined with the software Arlequin 3.5 (Excoffier and Lischer, 2010) using the ELB algorithm with default options. Haplotype reconstruction with a phase probability >0.9 was considered reliable.

An association study of *MC1R* and *ASIP* variants with coat colors was performed on the samples of 84 animals classified in four phenotypic classes: (a) Pheomelanic (N = 17), including animals that present reddish brown coat (Fig. S1a in the online version at DOI: http://dx.doi.org/10.1016/j.smallrumres.2016.08.003). (b): Eumelanic (N = 19), black or dark brown coat (Fig. S1b in the online version at DOI: http://dx.doi.org/10.1016/j.smallrumres.2016.08.003). (c) Black face (N = 19), reddish brown animals with black face and extremities (Fig. S1c in the online version at DOI: http://dx.doi.org/10.1016/j.smallrumres.2016.08.003). (d) White (N = 29), non-albino animals with non pigmented coats, pink skin and pigmented eyes rims and snout (Fig. S1d in the online version at DOI: http://dx.doi.org/10.1016/j.smallrumres.2016.08.003). For this purpose, we sequenced the *MC1R-A* fragment containing the most informative polymorphisms of the *MC1R* gene. Moreover, the deletion in exon 4 of *ASIP* gene was genotyped by 2% agarose gel electrophoresis.

Association between genotypes and coat color phenotypes was determined by using Fisherís exact test (GraphPad Prism 6; GraphPad Software Inc., San Diego, CA, USA). Critical p values were corrected by applying the Bonferroni method to account for multiple hypotheses testing.

3. Results

3.1. The MC1R gene

We determined the entire coding sequence (954 bp) and part of the 5′and 3′untranslated regions (123 bp and 91 bp) of the llama *MC1R* gene. Screening for genetic variation revealed 13 SNPs, ten of which were non synonymous (Fig. 1a). In contrast, guanaco sequences revealed no variation in *MC1R*.

Fifteen haplotypes (H) could be inferred from genotype data considering all SNPs identified in llamas (Table 2). Data from two