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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

Table 1
Primers used to amplify the llama MC1R and ASIP genes.

Name	Primer 5′- 3′	Primer 3′- 5′	T° annealing	Size (bp)
MC1R-A	GCTGCGAAGTGACCAGACTC	GCAGTGCATAGAAGATGGAGATG	57 °C	626
MC1R-B	TGTCCAGCCTCTGCTCTCTG	CTCCTCATTGCCAAGTAACTGC	57 °C	671
ASIP Ex2	TCCCTCCCTTCCTTGTCTTT	CCACCAGGATTGTTTTGAGG	53 °C	541
ASIP-Ex3	TTGCTTTCCGTCAGAGGACT	GGGAAACACTGCATTCATCC	53 °C	579
ASIP-Ex4	GGATATCTGGTCGGGAACCT	GAAACCCCTCCTCCTGAAAG	55 °C	400

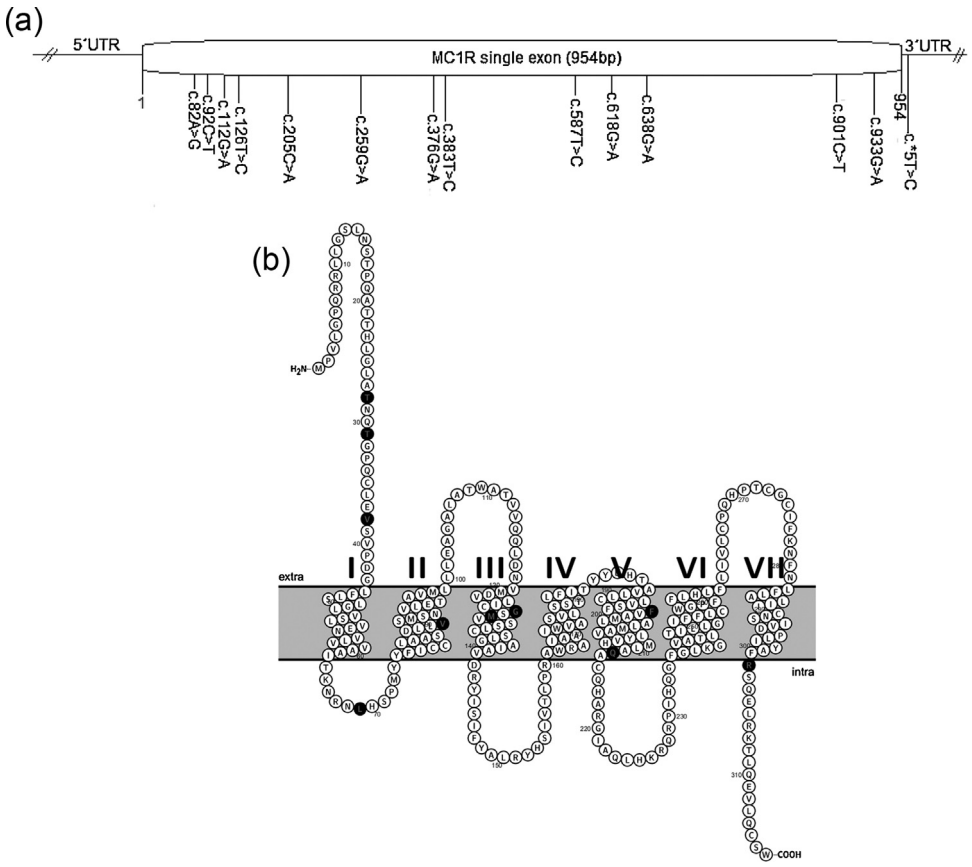

Fig. 1. (a) Structural organization of the llama MC1R gene. Position 1 was asignated to the translation initiation site (ATG codon). SNP numbering is relative to the coding DNA sequence. (b) Predicted secondary structure of llama MC1R amino acid sequence. The seven transmembrane domains are numbered I–VII. Amino acid replacements are shown in black. Extra = extracellular. Intra = intracellular.

individuals were excluded because haplotype phases could not be resolved (phase probability < 0.9).

H1, 2 and 3 were observed at frequencies 42.3%, 17.9% and 17.9% respectively, while the rest were found in only one or two individuals. The alignment with the guanaco sequence revealed that H3 is identical to the wild-type allele.

Translation of the nucleotide *MC1R* sequence produces a 317 amino acid protein. The 2D structure of the predicted llama protein and location of the amino acid substitutions is shown in Fig. 1b.

Substitutions p.T28A, p.T31M, p.V38M occur in positions that are variable across species (Fig. S2 in the online version at DOI: http://dx.doi.org/10.1016/j.smallrumres.2016.08.003). Thus, probably they have no effect on MC1R function. The R213 residue is conserved in mammals, but is occupied by an L in birds. Other amino acid replacements such as p.L69M, p.F196S and p.R301C that affect more evolutionarily conserved sites were observed at very low frequencies (<0.04) and they did not appear to correlate with a particular phenotype. The most frequent haplotype, H1, differs from the wild type (H3) by two amino acid substitutions p.V87M and p.G126S, respectively, while H2 differs by a single substitution (c.383T > C) that produces a p.M128T replacement in the protein.

Association of the major *MC1R* variants with coat color was then investigated by sequencing the fragment containing the SNPs c.259G > A (p.V87 M), c.376G > A (p.G126S) and c.383T > C (p.M128T) in 84 llama samples grouped as described in the 'Materials and Methods' section. Haplotype combinations (putative alleles) were designated as *MC1R*1* (c.259A/c.376A/c.383T), *MC1R*2* (c.259G/c.376G/c.383C) and *MC1R*3* (c.259G/c.376G/c.383T) to differentiate from the complete haplotype. The remaining SNPs are thought unlikely to be the main cause of coat color variation, so we excluded them from further analysis.

None of *MC1R* alleles studied showed a complete correlation with a particular color phenotype and in some cases such as the eumelanic and pheomelanic groups, the three haplotypes were present (Table 3). Nevertheless, genotype and allele distributions were significantly different among phenotypic classes and *MC1R*1* variant showed a significant association with pigmented coat ($P < 0.0001$). Of the 55 samples analyzed from animals with pigmented phenotype (eumelanic, pheomelanic and black face), 50 presented at least one copy of *MC1R*1* allele while this allele was not observed in any of the white llamas. In this last phenotypic group, *MC1R*2* was found at significantly higher frequency than

Table 2
Definition of MC1R haplotypes.

Haplotype	Variable sites											
	82[b] A > G T28A[c]	92C > T T31M	112G > A V38M	126T > C D42D	205C > A L69M	259G > A V87M	376G > A G126S	383T > C M128T	587T > C F196S	618G > A L206L	638G > A R213Q	933G > A E311E
H1(33)[a]	A	C	G	T	C	A	A	T	T	G	G	G
H2(14)	A	C	G	T	C	G	G	C	T	G	G	G
H3(14)	**A**	**C**	**G**	**T**	**C**	**G**	**G**	**T**	**T**	**G**	**G**	**G**
H4 (2)	A	C	G	T	C	G	G	T	T	G	A	G
H5 (2)	A	C	G	T	A	A	A	T	T	G	G	G
H6 (2)	A	T	G	T	C	G	G	T	T	G	G	G
H7 (1)	A	C	G	T	A	G	G	T	T	G	G	G
H8 (1)	A	C	G	T	C	A	A	T	T	G	G	A
H9 (2)	A	C	A	T	C	G	G	T	T	G	G	G
H10(1)	A	C	A	T	C	G	G	T	T	A	G	G
H11(2)	A	C	G	C	C	A	G	T	T	G	A	G
H12(1)	G	C	G	C	C	G	G	T	C	A	G	A
H13(1)	A	C	G	T	C	A	G	C	T	G	G	G
H14(1)	A	C	G	T	C	A	A	T	T	G	A	G
H15(1)	A	C	G	T	C	G	G	T	C	G	A	A

( )a= correspond to absolute frequencies (counts).
[b] Polymorphic site in the coding DNA sequence.
[c] Amino acid substitution.

Table 3
Genotypes obtained for MC1R gene.

Phenotype	Genotypes					
	MC1R*1/MC1R*1	MC1R*1/MC1R*2	MC1R*1/MC1R*3	MC1R*2/MC1R*2	MC1R*2/MC1R*3	MC1R*3/MC1R*3
White	0	0	0	12	11	6
Black face	10	0	5	0	0	4
Pheomelanic	6	6	5	0	0	0
Eumelanic	6	5	7	0	1	0

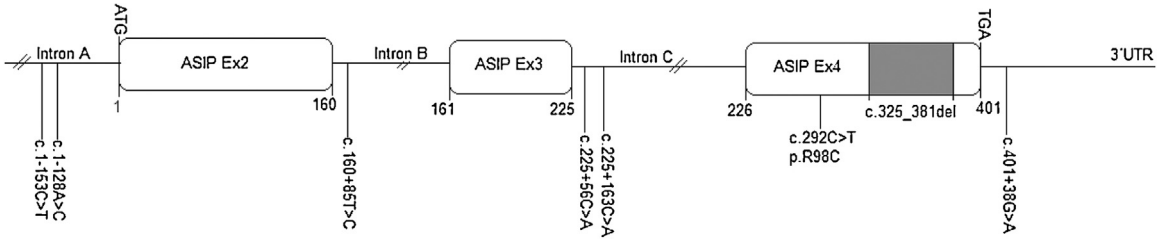

Fig. 2. Structural organization of the llama *ASIP* gene. Position 1 was asignated to the translation initiation site (ATG codon). SNP numbering is relative to the coding DNA sequence. Deletion in exon 4 is shaded in grey.

in colored animals ($P < 0.0001$). It was also observed that all llamas with the *MC1R*2* allele in homozygous state (12/29) were white, but only 41% of this phenotype was explained by the *MC1R*2/MC1R*2* genotype. This haplotype was not present in any of the llamas with black face and trims phenotype.

The wild genotype (*MC1R*3/MC1R*3*) was observed in ten individuals, four "black face" and six white animals, revealing the influence of other genes that produce those phenotypes.

3.2. *The* ASIP *gene*

We also sequenced the coding exons of *ASIP* and their flanking regions in order to find mutations responsible for pheomelanic versus eumelanic coat pigmentation. Organization of the coding region and polymorphisms distribution is shown in Fig. 2.

Most *ASIP* polymorphisms were located in intronic regions showing a variability pattern clearly different from that observed in the *MC1R* gene. However, two polymorphisms where found within exon 4, a 57 bp deletion (c.325_381del) and a non-synonymous SNP (c.292C > T). In the guanaco samples, five polymorphisms were identified, all located in intronic regions (c.1-51T > C, c.160 + 139G > A, c.160 + 85T > C, c.225 + 56C > A and c.401 + 38G > A). The non-deleted allele found in llamas corresponded to one of the guanaco variants.

Table 4
Genotypes obtained for ASIP gene.

Phenotype	Genotypes		
	−/−	−/D	D/D
White	19	9	1
Black face	6	9	4
Pheomelanic	11	6	0
Eumelanic	4	6	9

− = allele without deletion.
D = deletion.

To explore the potential association with coat color, the exon 4 deletion was genotyped in the same samples analyzed for the *MC1R* gene. Initially, we discarded c.292C > T for further analysis since it was detected only in two animals.

Almost half (47%) of the llamas with eumelanic coat were homozygous for the deletion (*D/D*) (Table 4). This genotype was not found in any of the pheomelanic animals, but was observed

in four animals with a mixed coat (BF) and in a white individual. Association between *D/D* genotypes and eumelanic coat was detected (P< 0.016) although this was not significant after applying the Bonferroni correction.

In order to identify additional polymorphisms that might allow us to explain the 10 remaining eumelanic phenotypes (-/- and -/*D*), the complete exon 4 was sequenced. We found that all but one animal carried the T variant at the SNP c.292C > T of exon 4. Thus, re-analyzing genotypes, we observed that eumelanic animals were homozygous for the deletion, homozygous for the T variant of c.292C > T or heterozygous for the combination of deletion and c.292C > T polymorphism.

4. Discussion

We found 10 nonsynonymous substitutions in the coding region of the llama *MC1R* gene. Two of them, c.205C > A and c.638G > A, are novel polymorphisms that had not been previously described in alpacas.

Combinations of c.259G > A; c.376G > A and c.383T > C defined the major haplotypes found in llamas: *MC1R*1, MC1R*2* and the wild haplotype *MC1R*3*.

No complete correlation between *MC1R* alleles and coat color was found. However, a significant association between *MC1R*1* haplotype and pigmented coat (P < 0.0001) was observed. According to our results, one copy of *MC1R*1* (presumptively *E*) would be enough for the development of a pigmented (eumelanic, pheomelanic or mixed) coat. Compared to the wild type, *MC1R*1* carried two amino acid replacements p.V87M and p.G126S. Position 126 is indistinctly occupied by a G or S residue in other mammals; however p.V87 is highly conserved, including also in non-mammalian species. Interestingly, an equivalent mutation p.V85M has been associated to melanism in avian species (Mundy et al., 2004; Baião et al., 2007—See Fig. S3 in the online version at DOI: 10.1016/j.smallrumres.2016.08.003).

The pigmented phenotype of five animals could not be explained by the presence of a *MC1R*1* haplotype: one black llama, and four animals with black face pattern with genotypes *MC1R*2/MC1R*3* and *MC1R*3/MC1R*3*, respectively. However this finding would be in agreement with the fact that wild type genotypes (E^+/E^+ and E^+/e) are expected to allow the expression of both eumelanin and pheomelanin, depending on the alleles at the *ASIP* gene (Fang et al., 2009).

The mutations responsible for coat color in llamas appear to be different to those reported in alpacas. Feeley and Munyard (2009) found that alpacas with haplotype combination c.82A/c.126T/c.901C (*E/E* or *E/e*) in *MC1R* are able to produce eumelanin whereas animals which have the combination c.82G/c.126C/c.901T (*e/e*), only express pheomelanin. Contrarily, we found that SNP c.901C > T, c.82A > G and c.126T > C appeared in very low frequency, being almost fixed for the 'eumelanic' combination proposed by those authors in the alpaca. In contrast to what happens with alpaca and other species like goats (Fontanesi et al., 2009) and sheep (Våge et al., 1999; Fontanesi et al., 2010a, 2011). *MC1R* alleles were not associated with pheomelanic/eumelanic phenotypes in llamas. Nevertheless, we identified two different haplotype combinations c.259A/c.376A/c.383T and c.259G/c.376G/c.383C that were respectively associated with the presence or absence of pigment. This finding is not unexpected considering the different phenotypes of the respective wild ancestors of the llama and the alpaca. The vicuña has a pheomelanic coat, with pale belly and legs. The guanaco presents a reddish brown color on the back while the head and ears have a dark gray color, evidencing the expression of both pigments. However, hybridization between the llama and the alpaca is a well documented process that could have played a role in introducing coat color variation. Therefore, the ability to produce eumelanin could be an ancestral condition in the llama while in the alpaca it would have been gained during domestication or crossbreeding after domestication. Consistent with that, all pheomelanic llamas carried at least one copy of the non deleted variant of *ASIP* and one *MC1R*1* allele.

Frank (2001) and Frank et al. (2006) postulated that *ASIP* is the main determinant of coat color in llamas. In dogs, animals with at least one copy of the dominant allele a^y of the *ASIP* gene have a reddish or "beige" coat depending on the breed. Unlike red phenotypes determined by an *e/e* genotype at *MC1R*, where eumelanin expression is completely absent, dogs with the dominant a^y allele have black whiskers and some dark hairs intermixed in their coat (Schmutz, 2005). Similar to what occurs in dogs, pheomelanic llamas usually had residual eumelanin expression (grey or black pigmentation) in specific regions, such as eyelashes, around the eyes and snout (Fig. S4 in the online version at DOI: http://dx.doi.org/10.1016/j.smallrumres.2016.08.003). Deletions in coding region of *ASIP* have been associated to black coat in several species such as horse, cat and sheep (Rieder et al., 2001; Eizirik et al., 2003; Royo et al., 2008). In alpacas, the black coat has been proposed to be determined by a combination of three *ASIP* alleles. In two independent studies, Feeley et al. (2011) and Chandramohan et al. (2013) found that black alpacas were either homozygous for p.C109_R127del (*D/D*), the *T* allele of p.R98C, the *A* allele of p.R118H or were heterozygous for a combination of two of these mutations. The R118H variant was not observed in our data but, in agreement with that reported for alpacas, 17/19 llamas were homozygous for the deletion (*D/D*), homozygous for *T* allele of p.R98C (*T/T*) or heterozygous for the deletion and the *T*.

The deletion in exon 4 causes the loss of 19 amino acids in the C-terminal region of the protein, which is critical for maintaining the protein structure and function. It results in the loss of six conserved cysteine residues, five of which are essential for ASIP activity and receptor binding (Perry et al., 1996; Kerns et al., 2004). Thus, it is very likely that *ASIP* deleted allele results in a non functional protein in llamas.

The p.R98C mutation, is equivalent to p.R96C found in dogs (Feeley et al., 2011) which has been suggested to be the cause of completely black coat in German Shepherds (Kerns et al., 2004).This residue is highly conserved among mammals and it is located within the segment of ASIP (residues 92-131) that binds MC1R (Ollmann et al., 1998). The incorporation of an additional cysteine in the C-terminal region of ASIP is predicted to be disruptive and to affect the ligand-receptor binding (Kerns et al., 2004). Therefore, if the 57-bp deletion and p.R98C substitution are both loss of function mutations in llamas, it is expected that animals heterozygous for a combination of both polymorphisms will express a completely eumelanic coat.

We only found two eumelanic animals that did not have the expected 'black' genotypes. There are two possible explanations for such a discrepancy. The existence of mutations in other *ASIP* exons or polymorphisms in the promoter region affecting gene expression could explain the dark phenotype in these animals. Alternatively, it could be attributed to the incorrect assignment of the individual's phenotype. Since overlapping phenotypes cannot always be visually distinguished from each other, a reddish dark brown or a "black face" individual could have been erroneously classified as eumelanic. On the other hand, the existence of dominant inheritance of black in llamas and alpacas has been speculated (Sponenberg, 2001). Thus, we cannot rule out the possibility that rare dominant mutations in the *MC1R* not genotyped here may be responsible of the eumelanic coat in those animals that did not carried two putative black alleles *at ASIP*.

An interesting finding was the detection of association between *MC1R*2* haplotype and white coat. White phenotypes are caused

in most species by mutations in *MITF* and *KIT* genes (Haase et al., 2007; Pielberg et al., 2002). As mutations in those genes are dominant and epistatic to *MC1R* (Cieslak et al., 2011), any genotype could be expected at the *MC1R* gene. Contrary to expectations, frequency of *MC1R*2* allele was significantly higher in this phenotypic group than in the others ($p < 0.001$). Additionally, almost half of the white llamas were homozygous *MC1R*2/MC1R*2*, a genotype not observed in the pigmented llamas. *MC1R*2* bears a p.M128T change that occurs in a site that is highly conserved in mammals. The same substitution has been reported by Fernandez et al. (2007) as a rare variant associated with human malignant melanoma in a Spanish population. Later, Pérez Oliva et al. (2009) confirmed that this variant shows marked loss of function and reduced agonist binding affinity. Therefore, more research is needed to clarify the functional effect of that variant on the llama protein and its relationship with the white coat.

Finally, no association was found between *ASIP* or *MC1R* alleles and the "black face" pattern. It is possible that regulatory mutations located outside the coding region would be responsible for this pattern. In other species the distribution of both pigments types is controlled by the alternative use of different promoters and untranslated first exons of the *ASIP* gene (Vrieling et al., 1994; Drögemüller et al., 2006; Fontanesi et al., 2010b; Ciampolini et al., 2012; Chandramohan et al., 2013). *ASIP* transcripts with different 5′-untranslated regions having ventral or dorsal skin specific expression have been reported in mice (Vrieling et al., 1994). Ciampolini et al. (2012) found that black and tan areas in Doberman dogs express different *ASIP* transcripts, which differed by their 5′-untranslated exons. Although black face llamas exhibit the reverse pattern (red-brown body with black face and extremities), more similar to a "bay" horse, it is possible that differences in the expression of *ASIP* transcripts are responsible for this pattern.

5. Conclusion

This work is the first contribution to the understanding of the molecular basis of coat color determination in llamas. Coat color genetics in domestic camelids seems to be complex. The characterization of non coding regions of *ASIP* and other genes involved in the melanogenesis pathway will provide further insights into the mechanism of pigment production in these species.

Conflict of interests

The authors declare that there is no conflict of interests.

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