Advances in Fibre Production Science in South American Camelids and other Fibre Animals

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Interaction between ASIP and MC1R in Black and Brown Alpaca

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Abstract. Agouti (ASIP) and Extension (MC1R) are genes known to be involved in coat colour through pigmentation pathways by regulating type, amount and distribution of eumelanin and pheomelanin pigments in melanocytes. In alpaca genotype of ASIP and MC1R genes have already been analysed distinctly in previous studies, but so far their epistatic interaction have not been evaluated. In this study have been assessed their segregation more insights on black and brown phenotypes. In several mammals MC1R is epistatic over ASIP, id est recessive allele in Agouti (a) and dominant allele in Extension locus (E) produces black phenotype. That is confirmed in alpaca where black coat has aH/aΔ57 and aH/ahT genotype on ASIP and E/E or E/e genotype on MC1R locus. Otherwise ASIP and MC1R in brown, have a dominant profile at least in one allele as A/A, A/ahT on Agouti and E/e on Extension. Genotype and phenotype comparison clears that receptor and ligand are in concordance to produce pheomelanin and eumelanin in alpaca. Segregation analysis of 12 alpaca families genotyped by coat color, confirm the dominance of brown over black and could be helpful for coat colour classification and genotyping.

Resumen. Se sabe que los genes Agouti (ASIP) y Extension (MC1R) están implicados en el color de la capa al intervenir en las vías de pigmentación regulando el tipo, la cantidad y la distribución de los pigmentos de eumelanina y feomelanina de los melanocitos. Los genotipos disponibles para estos genes ASIP y MC1R ya han sido analizados en profundidad previamente, pero no se ha estudiado su interacción epistática hasta le fecha. En este estudio se estudia la segregación de estos genes en la aparición de fenotipos negro y marrón. MC1R es epistático sobre ASIP en varios mamíferos, produciendo el fenotipo negro la combinación del alelo recesivo del Agouti (a) con el alelo dominante del gen Extensión (E). Esto se confirma en la alpaca ya que la capa negra tiene un genotipo aH/aΔ57 o aH/ahT en el locus ASIP y E/E o E/e en el locus MC1R. Por el contrario, ASIP y

MC1R producirán capa de color marrón cuando el individuo porte al menos un alelo dominante siendo A/A o A/ahT para el locus Agouti y E/e en el locus Extension. La comparación entre genotipos y fenotipos permite concluir que el receptor y el ligando están en concordancia para producir feomelanina y eumelanina en alpacas. El análisis de segregación de 12 familias de alpacas genotipadas para el color de la capa, confirma el predominio del marrón sobre el negro y podría ser útil para la clasificación del color de la capa y el genotipado.

Keywords: Alpaca, Genetic interaction, ASIP, MC1R, Black, Brown.

Introduction

Fiber harvested from alpaca (Vicugna pacos) is sold as luxury yarn. Peru hosts the largest biological reserve of alpaca in the world. Alpaca are raised in a variety of coat phenotypes and, recent studies have investigated various candidate genes possible involved in coat color variation in alpaca (Feelay et al., 2011). Recently a scheme for coat colour classification was defined by the International Committee for Animal Recording (ICAR) (Antonini, 2009). As regards potential colour, it could be affected by the pigmentation process. The synthesis of both eumelanins and pheomelanins are under control of a group of genes acting at the melanosomes level. The principal genes are Extension (MC1R protein) and Agouti (ASIP protein). Melanocortin 1 receptor (MC1R) and its peptide antagonist agouti-signaling-protein (ASIP) are well known in the regulation of the eumelanin/ pheomelanin switch involved in the base color expression. As in other animals, the epistatic interaction of MC1R and ASIP, could be visible as hues palette of phenotypes, although genotype segregation patterns is not revealed. In alpaca, three significant mutations in the Agouti locus causing black and brown phenotypes were identified. In particular the SNPs (g.3836C>T and g.3881G>A) and an in-frame 57 bp deletion (g.3866_3923del57) in exon-4 are predicted to independently cause functional changes to ASIP protein. Missense mutation g. 3836 C>T involve in an amino acid substitution in R98C, would predict a change of arginine (R) to Cysteine, which seems to have minimal/ partial effect on the functional property. This is evident in one of the allele ahT associate with g.3836C>T mutations seems to have partial/minimal functionality. Missense mutation at g.3881G>A suggests that it may produces a substitution at the amino acid position R118H changes the R to histidine (H) in the cystine-rich domain, which disrupt the highly conserved Arg-Phe-Phe (R-F-F) motif in the protein. Other analysis with cloning experiment showed that one of the allele marked with g.3896G>A (aH) seems to be associated to non-functional ASIP due to the R-F-F motif disruption. Deletion of 57 bp. would result in a short 114 amino acid containing agouti protein, which lacks 19 amino acids from the cysteine (C) rich domain, which is critical in agouti function and ASIP is supposed to be not functional (Table 1). For the locus Extension (MC1R) in alpaca, four missense

mutation are relevant with the black and brown phenotypes. In particular the g.901C>T nucleotide mutation resulting in the R301C amino acid change shows that all the brown animals were heterozygous to C901T (Powell et al., 2008). The interesting fact observed with our molecular segregation analysis is that the animals homozygous to the mutations A82/ A259/ A376/ C901combination expressed black skin phenotypes with an EE genotype. The brown phenotypes were observed to have the heterozygous condition for the observed mutations (A82G/ A259G/ A376G/ C901T) with Ee genotypes (Table 2). In alpaca mutations of this two genes were analysed separately in two previous studies performed by our research group (Bathrachalam et al., 2011; Bathrachalam et al., 2013), but so far their epistatic interaction have not been evaluated. In this regard, the present study aims to fill the knowledge gap on the genetic interaction between ASIP and MC1R in alpaca and, to provide novel information useful for both applicative and basic science issues. Our outcomes provide further insights into the mechanisms of pigmentation in alpaca as well as provide "molecular tools" for the development of an efficient marker assisted breeding program for coat colors in this animal.

Table 1: The ASIP geonotypes and phenotype of Peruvian alpacas.

Phenotype	g.3836C>T p.R98C	g.3896G>A p.R118H	g.3866-3923del57 p.C109_R127del	Allele
Black	C	A	-	a^{H}
	C	-	Yes	$a^{\Delta 57}$
Black	C	A	-	a^{H}
	T	G	-	a^{bT}
Brown	C	G	-	A
	T	G	-	a^{bT}
Brown	C	G	-	A
	C	G	-	A

Materials and Methods

Sampling and processing of the samples

Alpacas were sampled from the Quimsachata Experimental Station, Peru. The Station is located on the Andean Plateau at 4,300 m under the management of the Instituto Nacional de Innovacion Agraria (INIA). The trials have been organized in a hierarchical scheme. Five Black rams have been mated to 10 black dams, and for them and their crias (baby alpaca) the colors have been assigned. Brown animals have not been crossed in this mating plan. Skin biopsies were obtained after antisepsis, and local anesthesia was injected at the border of the sampling site. Samples have been collected from parents and crias by disposable biopsy punch

(8 mm diameter) and have been stored in All Protect (Qiagen). Then, the skin fragments have been removed from preservative reagents and stored in liquid nitrogen. For molecular genetic analysis all samples have been transferred to the laboratories at the School of Environmental Sciences, University of Camerino, Italy. The genomic DNA was isolated from the skin biopsies by using DNAeasy tissue kit (Qiagen S.A., Courtaboeuf, France) according to the instruction of the industry. The DNA samples with good quantity and quality have been stored at -80 °C for further analysis. The genotyping assays have been performed for ASIP and MC1R in all the informative phenotypes according to method reported in the previous Bathrachalam works (Bathrachalam et al., 2011; Bathrachalam et al., 2013). Then the genetic interaction between ASIP and MC1R have been analysed (Table 3).

Table 2: The MC1R genotypes and phenotype of Peruvian alpacas.

g.A82G (p.T28A)	g.A259G (p.V87M)	g.A376G (p.G126S)	g.C901T (p.R301C)	Phenotype	MC1R Genotype
A/A	A/A	A/A	C/C	Black	*EE*
A/G	A/G	A/G	C/T	Black	*Ee*
A/G	A/G	A/G	C/C	Black	*Ee*
A/G	A/G	A/G	C/T	Brown	*Ee*

Ethics statement

In agreement with the new European Directive on the protection of animals used for scientific purposes (Directive 2010/63/EU, Article 15, Annex VIII), all animal procedures used in the study are classified as 'mild' (i.e., procedures with no significant impairment of the well-being or general condition of the animals) and were preemptively approved by the Animal Ethics Committee of the University of Camerino.

Table 3: ASIP and MC1R genotype interaction and the following phenotype of Peruvian alpacas.

Genotype ASIP (Ligand)	MC1R(Receptor)	Phenotype
$a^{\Delta57}/a^{H}$,	E/E, E/e	Black
a^{H}/a^{hT}	E/E, E/e	Black
A/a^{hT}	E/e	Brown
A/A	E/e	Brown

Results

ASIP – MC1R combined Genotype

In alpaca, the black animals observed to have two agouti genetic backgrounds. One seems to have completely recessive alleles i.e. aH/aΔ57 and another with a recessive and a partial dominant allele i.e. aH/ahT. The MC1R genotypes of black with two agouti genetic background were completely in association, thus black phenotypes with aH/aΔ57 and aH/ahT had either E/E or E/e (MC1R) genotypes (Table 3), thereby the receptor and ligand genetic background is perfectly balanced to produce eumelanin in black animals. Furthermore, black animals were heterozygous to aΔ57 allele with ahT or aH, heterozygous to both ahT and aH, homozygous to the mutation C901 (EE) and heterozygous to C901T (Ee). Brown animals were heterozygous to ahT, homozygous for aH, homozygous to g.3836C, wild allele (A). The aΔ57 and the A were not observed. All the brown animals analysed in the present study were heterozygous to C901T (Ee).

Segregation Analysis

Segregation analysis was performed on 10 alpaca families constituted from 5 male (rams), 10 female (dam) and 10 crias (baby alpaca). All of them presented a black phenotype, and the genotype at the Extension and Agouti locus have been evaluated (Table 4). In all the families the allelic variants at the Agouti locus was aH/ahT or aH/aΔ57 and at the Extension locus was Ee or EE.

Discussion

Molecular identification of the Agouti and MC1R genes provided much of the molecular groundwork for understanding the role of melanocortin signaling in pigmentation. In alpaca, the extension locus encoding for MC1R is epistatic over locus Agouti which means that a fully functioning MC1R receptor is necessary for the Agouti to be expressed. If MC1R is not functional then it cannot be activated or inactivated by either of two alternate ligands. In most of the mammals the recessive allele in agouti (a) and dominant allele in MC1R locus (E) produces black phenotypes. In black alpacas MC1R is functional at the least in one allele, and ASIP is in recessive form due to possible explained missense mutations. In these cases, ASP lost the ability to block α-melanocyte-stimulating hormone (α – MSH). Improvement of the α-MSH receptor induced cAMP production and up-regulated the transcription of MITF that activates the expression of the melanogenesis-related enzymes and regulating the gene expression of tyrosinase, TRP-1, and TRP-2. These pathways enhance the pigmentary function of melanocytes to produce more eu- and pheo-melanin and hair darkening.

Table 4: The Agouti and Extension genotype segregation in 10 black alpaca families.

Family n	Code	Color	ASIP	MC1R	Family n	Code	Color	ASIP	MC1R
RAM	366203[4]	*Black*	a^H/a^{bT}	*E/e*		RAM	95101[2]	*Black*	$a^H/a^{\Delta 57}$	*E/E*
1 DAM	301204	*Black*	$a^H/a^{\Delta 57}$	*E/E*	2 DAM	386301	*Black*	$a^H/a^{\Delta 57}$	*E/E*
CRIA	138108	*Black*	$a^H/a^{\Delta 57}$	*E/E*		CRIA	81108	*Black*	$a^H/a^{\Delta 57}$	*E/E*
RAM	35104[1]	*Black*	a^H/a^{bT}	*E/E*		RAM	95101[2]	*Black*	$a^H/a^{\Delta 57}$	*E/E*
3 DAM	83104	*Black*	a^H/a^{bT}	*E/e*	4 DAM	275204	*Black*	$a^H/a^{\Delta 57}$	*E/e*
CRIA	78108	*Brown*	a^H/a^{bT}	*E/E*		CRIA	287208	*Black*	$a^H/a^{\Delta 57}$	*E/E*
RAM	35104[1]	*Black*	a^H/a^{bT}	*E/E*		RAM	237204[3]	*Black*	a^H/a^{bT}	*E/E*
5 DAM	348204	*Black*	a^H/a^{bT}	*E/E*	6 DAM	283205	*Black*	$a^H/a^{\Delta 57}$	*E/e*
CRIA	264208	*Black*	a^H/a^{bT}	*E/E*		CRIA	160108	*Black*	$a^H/a^{\Delta 57}$	*E/E*
RAM	244203[5]	*Black*	$a^H/a^{\Delta 57}$	*E/E*		RAM	237204[3]	*Black*	a^H/a^{bT}	*E/E*
7 DAM	75103	*Black*	$a^H/a^{\Delta 57}$	*E/e*	8 DAM	480100	*Black*	$a^H/a^{\Delta 57}$	*E/E*
CRIA	241208	*Black*	$a^H/a^{\Delta 57}$	*E/E*		CRIA	437308	*Black*	$a^H/a^{\Delta 57}$	*E/E*
RAM	244203[5]	*Black*	$a^H/a^{\Delta 57}$	*E/E*		RAM	244203[5]	*Black*	$a^H/a^{\Delta 57}$	*E/E*
9 DAM	2160200	*Black*	$a^H/a^{\Delta 57}$	*E/e*	10 DAM	130105	*Black*	$a^H/a^{\Delta 57}$	*E/e*
CRIA	262208	*Black*	$a^H/a^{\Delta 57}$	*E/E*		CRIA	89108	*Black*	$a^H/a^{\Delta 57}$	*E/E*

[1]Ram1. [2]Ram2. [3]Ram3. [4]Ram4. [5]Ram5.

In alpaca, the black animals observed to have two agouti backgrounds that seems to have completely recessive alleles i.e. aΔ57/ aH, and aH/ ahT. The MC1R genotypes of black with two agouti backgrounds were completely in association. All the brown animals analysed in the present study were heterozygous at the extension locus E/e and present SNP g.901C>T in one allele. Furthermore, Agouti gene has the A/ahT, A/A genotype. In this case, at the least one allele of both genes have to be functional to express this phenotype that is the typical wild-type pigmentation pattern for the alpaca. This phenotype also occurs in domestic sheep (Våge et al., 2003), in which it is understood to be a product of positive artificial selection for novel colour variants during domestication. In fact when MC1R is bound by ASIP, eumelanin synthesis is inhibited because of the down-

regulation of several melanogenic factors, and pheomelanin production relies on the chemical status of the cell environment, i.e. the content of available tyrosine and tyrosinase activity. The depigmentation induced by ASIP results from the inhibited synthesis of both types of melanins concomitant with the dramatic decrease of tyrosinase activity. That leads preferentially to the suppression of eumelanin production during the synthesis of mixed-melanins than pheomelanin. Different shades of brown phenotypes observed in alpaca could be due to the interactive effect between agouti and MC1R followed by a mixed amount and type of melanins production. The dominance of brown phenotype over black coat was confirmed by segregation analysis. In fact occurrence of both black and brown baby alpaca (crias) in crosses of black parents could be explained by the absence of black offspring in crosses involving brown parents (Valbonesi et al., 2011). Furthermore black phenotype is under control of an allelic heterogeneity at ASIP locus. An effort of segregation about black phenotype was confirmed by genotype analysis in Agouti and Extension (MC1R) locus in 10 families. (Table 4)

Conclusions

Finally, clearly interaction of agouti and MC1R is in concordance with the black and brown phenotypes not the only genetic interaction through which black and brown pigments are synthetized in alpaca. As verified by the segregation analysis the black colour in alpaca is a recessive pattern. However, this research could have several implications: in fact standardize fibre production based on natural coat colour, could surely help herders farm. Breeders that perform mating program based on colour genotyping of their breeding alpacas could have a large amount of fleece with similar tone and shades. Select coat colour through biomarker assisted selection could increase breeders' income. That is considerable in Peru, where rear alpaca is popular and poverty prevails. Furthermore natural coloured fibre does not need to be dyed and that improve the performance of eco-friendly, low economic impact and compete with synthetically dyed products, that fade with time and sometimes causes harmfulness to human body. Further studies exploring other candidate genes, especially those with regulatory functions are likely to provide great insights into our understanding of the black and brown phenotypes in alpaca.

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Animal fibres from South American camelids and other fibre or wool bearing species provide important products for use by the human population. The contemporary context includes the competition with petrocarbon-based artificial fibres and concern about excessive persistence of these in the natural environment. Animal fibres present highly valuable characteristics for sustainable production and processing as they are both natural and renewable. On the other hand, their use is recognised to depend on availability of appropriate quality and quantity, the production of which is underpinned by a range of sciences and processes which support development to meet market requirements. This collection of papers combines international experience from South and North America, China and Europe. The focus lies on domestic South American camelids (alpacas, llamas) and also includes research on sheep and goats. It considers latest advances in sustainable development under climate change, breeding and genetics, repro-duction and pathology, nutrition, meat and fibre production and fibre metrology.

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