Intra-chromosomal recombination of *agouti* gene in white alpaca

Bathrachalam Chandramohan, Carlo Renieri, Vincenzo La Manna, Antonietta La Terza

School of Environmental Science, University of Camerino, Via gentile III da Varano, 62032-Camerino (MC), Italy. Fax: +39- 0737-403290, Phone: +39-0737-403272.
Carlo.renieri@unicam.it

Abstract
The role of the *agouti* gene in white phenotype was explored in mice. The agouti signalling protein (ASP) can inhibit the differentiation of melanoblasts through the inhibition of the alfa-MSH-induced expression of microphtalmia (MITF) and its binding to a M box regulatory element. The level of microphtalmia in the cells is reduced. To investigate the role of agouti in this phenotype we characterised the transcript structures an relative mRNA expression levels in 13 white alpaca. The reverse transcription analysis of mRNA purified from skin biopsies revealed the presence of three transcripts with different 5'untranslated regions (UTRs) and color specific expression. One of the transcripts, possibly originating from a duplication event (intra-chromosomal recombination) of the agouti gene is characterised by a 5'UTR containing 142 bp of the NCPOA6 gene sequence. Furthermore, the raltive level expression analysis of mRNA demonstrates that the agouti gene has up-regulated expression in white skin, suggesting a pleiotropic effect of agouti gene in the white phenotype.

Resumen : Recombinación cromosómica del gen *agouti* en alpacas blancas.
Fue explorado en ratones el rol del gen *agouti* en el fenoripo blanco. La protein señalarizadora agouti (ASP) puede inibir la diferenciacion de los melanoblastos a traves de la inhibición de la expresion alfa-MSH inducida de macroftalmia (MITF) y su union al elemento regulador Mbox. El nivel de macroftalmia en las células se reduce. Para investigar el rol del agouti en este fenotipo, caracterizamos las estructuras transcriptas a una relativa expresion del mRNA en 13 alpacas blancas blancas. El analisis de transcripción reversa del mRNA purificado a partir de las biopsias de piel, revelaron la presencia de tres transciptos con diferentes regiones no traducidas del extremo 5' (5'UTR) y expresiones de color específicas. Uno de los transcriptos, posiblemente procedente de un evento de duplicación (recombinación intra-cromosómico) del gen agutí se caracteriza por un 5' UTR que contiene 142 pb del gen NCPOA6. Por otra parte, el análisis del nivel relativo de expresión del mRNA demuestra que el gen agouti tiene sobre expresión reguladora en piel blanca, lo que sugiere un efecto pleiotrópico del gen agouti en el fenotipo blanco.

Keywords: agouti, duplication, gene expression, intra-chromosomal recombination

Introduction

White indicates absence of pigmentation. Because the melanocytes, the cells that produce pigments, are found not only in the hair but also in the eyes, the inner ear and in other internal organs, a first distinction is required between a complete absence of pigmentation and a partial presence of pigmentation, confined to the hair follicle. The first type of white is refferred to as Oculocutaneous Albinism (OCA), which is present which is present in animals

with hearing as well as vision problems, can result from two genetic mechanisms: “tirosinase negative” albinism (OCA 1): derives from a mutation and loss of functions of the wild alleles at the tirosinase structural locus (albino), which is the key gene for the synthesis of all pigments (from thyroxine to DOPA quinone); “tirosinase positive” albinism derives from a mutation of associated loci, such as pink eyed (OCA 2), TYRP1 (OCA3) and MATP (OCA4) (King and Oetting, 2006).
The non-albino uniform white, typical of several species of mammals is obtained through a mutation with loss of function of genes involved in prenatal migration of melanoblasts, from the neural crest to the hair follicle of the animals and compromising local development (Montoliou et al., 2013). Twenty two of these genes have been identified and cloned in mammals so far (Baxter et al 2004). Most mutations associated with white in these loci are fatal. On the other hand, in the cases for the loci MITF (microftalmia), C-Kit (Dominant White Spotting) and Steel it is possible to obtain completely white live animals. These could be the genes responsible for uniform white in mammals.
Agouti gene encodes a 131-amino acid protein containing a signal sequence, the agouti-signal protein (ASP). ASP, which is produced in the dermal papilla cells within the hair follicle, acts on follicular melanocytes to switch them from eumelanin to pheomelanin production.
Some studies suggest that ASP can regulate melanoblast differentiation acting through a receptor distinct from the Mc1r. ASP inhibits a specific melanogenic transcription factor, microphthalmia, and its binding to an M box regulatory element. Besides ASP inhibits alfa-MSH-induced expression of microphthalmia gene and reduces the level of microphthalmia in the cells (Aberdam et al., 1998)
In mice, it has been reported that the opposite orientation of an inverted duplication of *agouti* and its association with difference in the ventral pigmentation (Chen et al., 1996). A duplication encompassing the ovine *agouti* and *AHCY* coding sequence and the *ITCH* promoter causes the white phenotype in sheep (Norris and Whan 2008). In pig a large duplication involving the *KIT* locus causing dominant white coat phenotype has originated by homologous recombination (Marklung et al., 1998; Giuffra et al., 2002).
In this study, we report on the *agouti* genomic and transcripts structures and relative mRNA expression levels that probably affects white phenotype in native Peruvian white alpaca (Bathrachalam et al., 2013).
Skin biopsies from white and colored (brown and black) alpacas were collected from ILPA-Puno, Quimsachata Experimental Station, Instituto Nacional de Innovacion Agraria (INIA), Peru which is located 4300m above to the sea level.

Characterization of the agouti full length transcript and genomic locus

The combined transcript sequence initially obtained from total RNA purified from lateral skin of brown alpaca is 822 bp long (excluding poly A tail). It is composed of 402 bp open reading frame (ORF), a 196 bp 5’UTR and a 224 bp 3’UTR (Fig.1). It encodes a putative 133 amino acid protein, which is 83 and 85% identical to the ovine and bovine proteins, respectively.
The amplification of genomic DNA displayed a 3945 bp fragment (GenBank accession no: HQ645014), which contains three coding exons (2, 3 and 4), in addition to intronic sequences (Fig.2A). The coding exons 2, 3 and 4 are separated by 1262 and 2281 bp intronic sequence, respectively. Each exon is flanked by consensus splice donor and acceptor sites, except for the exon 4, which has only a splice acceptor site (Fig.2).

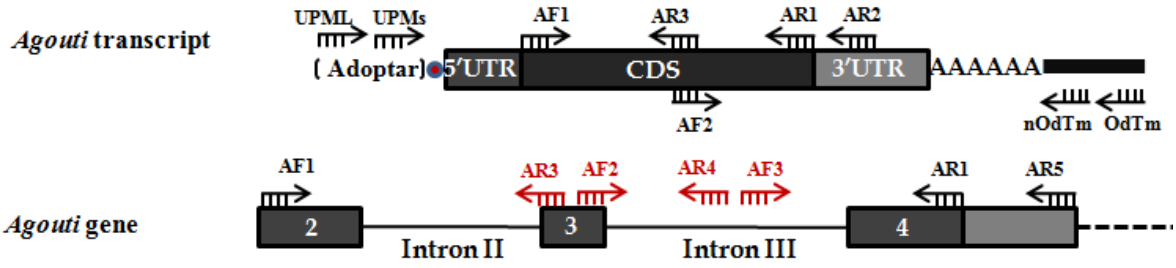


Figure.1. *Schematic view of agouti transcript and gene amplification. Agouti transcript: A schematic picture represents the strategy followed in the full length transcript amplification (CDS, 3'UTR followed by 5'UTR). The primers (forward & reverse) and its positions mentioned above to the cDNA structure are used in the CDS and 5'UTR amplification. Primers and it location mentioned below to the cDNA structure are used in the 3'UTR amplification. Agouti gene: A schematic picture of the agouti gene amplification. The primers mentioned in black colour are used in the amplification and the primers mentioned in the red colour are used in the sequencing of the amplified gene.*

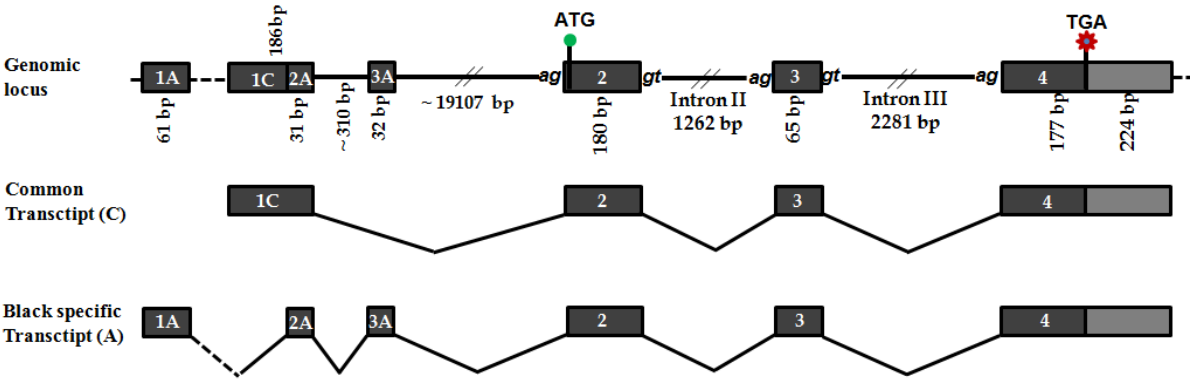


Figure.2. *The alpaca agouti genomic locus and its transcripts. Numbers above and below the gene structure are length of exons and introns. Consensus acceptor and donors splice sites are identicated. Boxes show coding exons (2, 3 and 4) and 5'UTR exons (1A, 2A, 3A and 1C). Transcript C and A are represented under the genomic locus. The position of coding exons (2, 3&4), introns (2&3) and 3'UTR are deduced from our experiment. The positions of non coding exons (2A, 3A and 1C) are deduced based on the ensemble 2X alpaca genome. The position of the non coding exon 1A is not known (---).*

Characterization of 5'UTR in black, brown and white alpaca

We identified in total three different 5'UTRs from the skin of alpaca i.e. 136, 142 and 196 bp long, respectively (Fig.1). Interestingly, among the transcripts identified, one is observed to be common in all the three phenotypes, which has the 5'UTR of 196 bp (GenBank accession no: HQ645017). Nucleotide analysis of the 5'UTR revealed the presence of 4 upstream open reading frame (uORF). In which, uORFs (1, 2 and 3) appear to be in the same reading frame as the start codon of the *agouti* ORF, whereas the codon 4 is not (Fig.1). Translation starting at these upstream start codons could potentially results in 4 short peptides, which range in size from 14 – 27 amino acids which terminates before the main *agouti* ORF, none of which

showed any identity with previously known proteins. The comparison of common 5'UTR with 2X genomic sequence (Ensembl) showed, the corresponding sequences are from the intron less exon (1C), which is located approximately 19.5 kb upstream to the main ORF (Fig.2). The second transcript which is specific to black has the 5'UTR of 136 bp (GenBank accession no: HQ645015) and predicted to have only one uORF (Fig.1). The predicted uORF is not in-frame with the *agouti* ORF and it could produce a short peptide of about 52 amino acids and doesn't show any identity with any previously reported proteins. Sequence comparison of black specific 5'UTR with the genomic sequence of alpaca revealed this 5'UTR results from three non coding exons (1A, 2A and 3A) splicing and thus designated as transcript A. We are unable to locate the non coding exon-1A on the available alpaca genome, non-coding exons 2A and 3A are separated by 310 bp intronic sequence and the non coding exon-3A and coding exon-2 are separated by approximately 19 kb intronic sequence (Fig.2). Apart from the common and black specific transcripts, a 5'UTR specific to white phenotype of 142 bp (GenBank accession no: HQ645016) and no uORF was predicted as in the common and black specific 5'UTRs. Furthermore, blast analysis of the white specific 5'UTR showed high identity (89%) with human *nuclear receptor co-activator 6* (*NCOA6*) mRNA and thus it was designated as transcript-D (*NCOA6-Agouti* chimera) (Fig.3). Interestingly, comparison with the published genome sequences of human and alpaca revealed the presence of an inversion in the 5'UTR of white specific transcript D. Further analysis with the alpaca genome confirmed the location of this 5'UTR sequence between *NCOA6* and *gamma-glutamyltransferase 7* (*GGT7*) which is located approximately 600 kb downstream to the *agouti* locus (Fig.2), this findings suggest that white alpaca may have *agouti* gene duplication as reported in the mouse (Chen et al., 1996) and sheep (Norris and Whan 2008). Thus, in accordance with the data suggesting that these three transcripts may represent different sites of transcription initiation evidenced by the differences in the 5' UTRs.

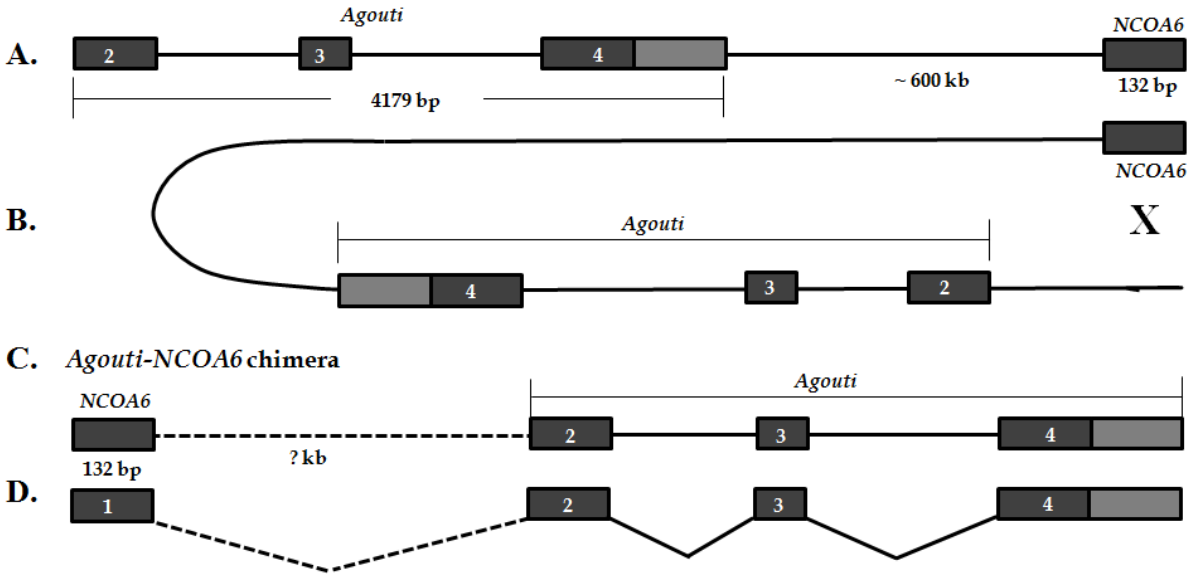


Figure.3. *Agouti and NCOA6 gene on alpaca genome and its homologous recombination.* ***A****. Structure and location of Agouti and NCOA6 gene on alpaca genome (2X).* ***B****. Possible mechanism of intra-chromosomal homologous recombination of agouti and NCOA6 gene.* ***C.*** *Schematic picture of agouti–NCOA6 chimera after the homologous recombination.* ***D****. Agouti transcript with 132 bp of NCOA6 as a 5'UTR and its possible location on genome after recombination, (--) unknown sequences.*

Agouti expression in white, black and brown phenotypes

Semi-quantitative RT-PCR was conducted to study the relative level expression pattern of *agouti* mRNA in different phenotypes. An *agouti* fragment of 230 bp was amplified with the primers AF3/AR4 in each of the total RNA (white, black, brown) and a 252 bp fragment of *GAPDH* gene was also amplified and the expression levels were compared between the phenotypes. Analysis of variance showed that the *agouti* expression significantly varies with coat color. The expression of *agouti* mRNA was high in white (0.93), moderate in brown (0.62), and low expression level was detected with the black (0.58) skin biopsy (Fig.4).

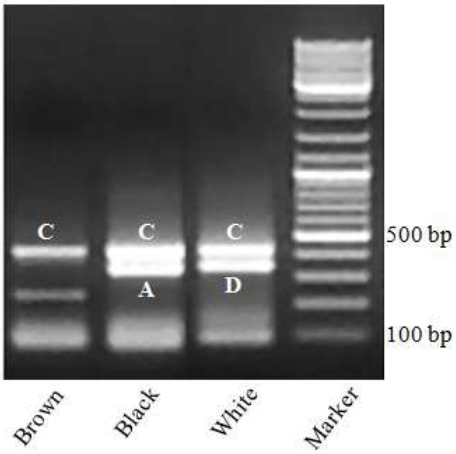


Figure 4. *5'RACE amplification of agouti transcripts from skin. A representative banding pattern of agouti transcripts resulting from 3 independent experiments on different colours i.e. brown, black and white. (C) Common transcript observed in all the three colours; (A) Black specific transcript; (D) white specific transcript.*

Discussion

The comparative sequence analysis with human and alpaca genome shows the alpaca *agouti* gene is embedded in a conserved syntenic block, which contains *RALY, IF2S2, XPOTP1, Agouti, AHCY, ITCH, PIGU, NCOA6* and *GGT7*.

RT-PCR and RACE experiments show that in alpaca, the *agouti* gene produce three (A, D and C) approximately similar sized transcripts in skin. All the 3 transcripts posses a common 224 bp 3'UTR bearing the canonical polyadenylation signal indicating that this region may play a role in the regulation of *agouti* expression at transcriptional level. The main characteristic of Transcript-C resides in its 4 uORFs. This is quite an uncommon situation, as less than 10% of known mammalian genes have uORFs in their 5'UTR (Geballe and Morris 1994, Hinnebusch, 1990). The putative peptides encoded by the 4 uORFs have lengths ranging from 14-27 amino acids and show no homology with any known protein sequences. The black specific 5'UTR (transcript-A) has a single uORF that can produce a short peptide of about 52 amino acids and in the white specific 5'UTR none was predicted. This condition suggests that the different transcripts identified might play critical roles in regulating the expression of the gene at the translational level.

An inversion in 5'UTR observed with the white specific transcript and the over expression of *agouti* mRNA opens a new window for the white phenotypes. The genomic location of the white specific 5'UTR lies approximately 600 kb downstream to the *agouti* gene. This condition supports the fact that the white alpaca seems to have duplication of *agouti* gene

locus by means of a possible intra-chromosomal homologous recombination event; it is further evidenced by the presence of the common transcript-C observed in the white phenotypes.
In conclusion, the characterization of transcripts provides another level of information about agouti gene regulation in alpaca pigmentation. A novel duplication of agouti gene evidenced by *NCOA6-agouti* transcript could have pleiotropic role in white phenotypes as in mouse and sheep.

Acknowledgement

The authors would like to thank the Alpaca Research Foundation (ARF) for their financial support. We would also like to thank INIA, Peru and people who helped in the sampling.

References

Aberdam E., Bertolotto C., Sviderskaya E.V., de Thillot V., Hemesath T.J., Fisher D.E., Bennet D.C., Ortonne JP, Ballotti R., 1998. Involvement of Microphthalmia in the Inibition of Melanocyte Lineage Differentiation and of Melanogenesis by Agouti Signal Protein. J. Biol. Chem., 273 (31), 19560-19565

Bathrachalam, C., Renieri, C., La Manna, V., La Terza, A., 2013. The alpaca agouti gene: genomic locus, transcripts and causative mutations of eumelanic and pheomelanic coat color. Gene, 521 (2), 303-310.

Baxter, L.L., Ling Hou, Loftus S.K. and Pavan W.J., 2014. Spotlight on Spotted Mice: A review of White Spotting Mouse Mutants and Associated Human Pigmentation Disorders. Pigment Cell Res., 17 (3), 215-224.

Chen, Y., Duhl, D.M., Barsh, G.S., 1996. Opposite orientations of an inverted duplication and allelic variation at the mouse agouti locus. Genetics. 144, 265-77.

Geballe, A.P., Morris, D.R., 1994. Initiation codons within 5'-leaders of mRNAs as regulators of translation. Trends Biochem. Sci. 19, 159–164.

Giuffra, E., Tornsten, A., Marklund, S., Bongcam-Rudloff, E., Chardon, P., Kijas, J.M., Anderson, S.I., Archibald, A.L., Andersson, L., 2002. A large duplication associated with dominant white colour in pigs originated by homologous recombination between LINE elements flanking KIT. Mamm. Genome. 13, 569–577.

Hinnebusch, A.G., 1990. Involvement of an initiation factor and protein phosphorylation in translational control of GCN4 mRNA. Trends Biochem. Sci. 19, 159-164.

King R.A. and Oetting W.S., 2006. Oculocutaneous Albinism. In Nordlung et al Eds, The Pigmentary System, Blackwell Publishing Ltd, 9600 Garsington Road, Oxford OX4 2DQ, UK

Marklund, S., Kijas, J., Rodriguez-Martinez, H., Rönnstrand, L., Funa, K., Moller, M., Lange, D., Edfors-Lilja, I., Andersson, L., 1998. Molecular Basis for the Dominant White Phenotype in the Domestic Pig. Genome Res., 8, 826-833.

Montoliou, L., Oetting, W.S., Bennett, D.C., 2013. Color Genes. European Society for Pigment Cell Research. World Wide Web (URL: http://www.espcr.org/micemut).

Norris., B.J., Whan, V.A., 2008. A gene duplication affecting expression of the ovine ASIP gene is responsible for white and black sheep. Genome Res., 18, 1282-93.

CARLO RENIERI
UNIVERSITY OF CAMERINO
SCHOOL OF ENVIRONMENTAL SCIENCES

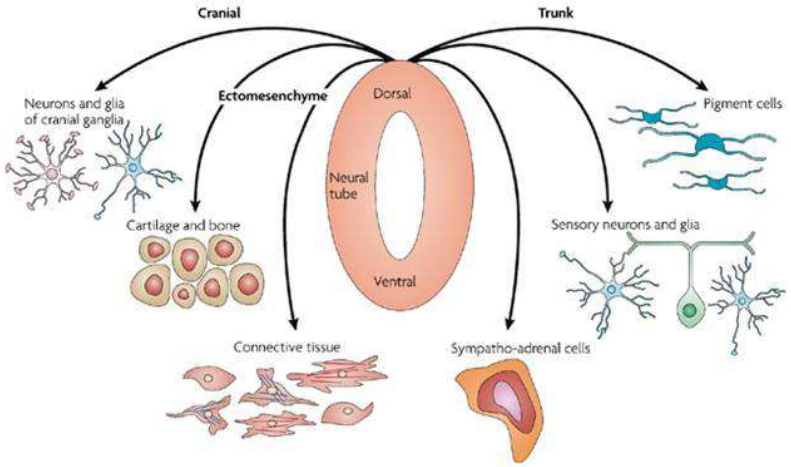
Intra-chromosomal recombination of *agouti* gene in white alpaca

Nantes
August 29, 2013

WHITE

White in mammals arise from improper melanoblast development or survival, reflecting absence of mature melanocytes.

White can be caused by defects at various stages of melanocytes development, including proliferation, survival, migration, invasion of the integument, hair follicle entry and melanocytes stem cell renewal (Baxter et al., 2004).



Neural crest population	Proposed FGF functions
Neural crest precursors	Specification
Emergent cranial neural crest	Regional specification (controversial)
Ectomesenchyme	Specification, migration into pharyngeal arches, survival
Cranial cartilage	Specification, proliferation, differentiation
Sympathetic neurons and glia	Proliferation of precursors, differentiation, neurite outgrowth
Adrenal chromaffin cells	Proliferation of precursors, differentiation
Sensory neurons and glia	Proliferation of precursors, differentiation (promotion of gliogenesis), survival
Pigment cells (melanocytes)	Migration, proliferation, differentiation

Nature Reviews | Neuroscience

AGOUTI
Melanoblast differentiation

inhibit expression of MIFT

Inhibit the binding of MIFT to on M-box regulatory element

Inhibit α – MSH stimulated expression of Tyrosinase, Tyrp 1 and Tyrp 2 through an inhibition of the transcriptional activity of their respective promoters

Inhibit α – MSH induced expression of the MIFT gene

Reduces the level of MIFT in the cells

Mc1r AND ASP GENEOTYPE IN WHITE ALPACA

- **W**hite animals were:
- - homozygous to g.3836C, wild allele;
- - heterozygous to a^{H},
- - homozygous to a^{H}.
- We did not observe $a^{\Delta 57bp}$.
- White animals were homozygous to T901 (***EE***) and heterozygous to C901T (***Ee***).

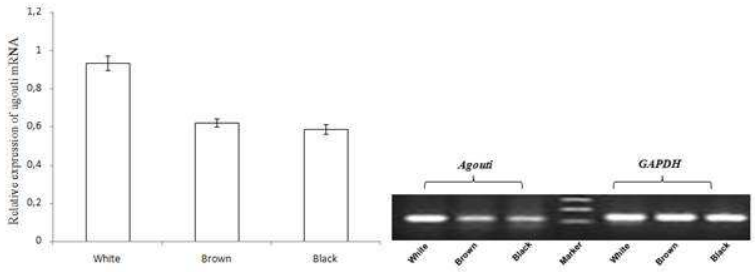
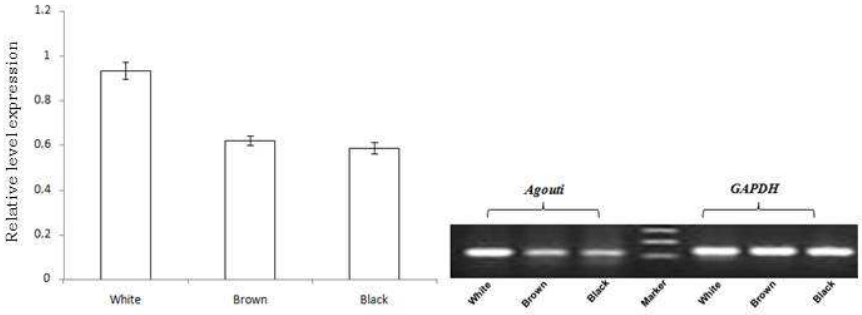
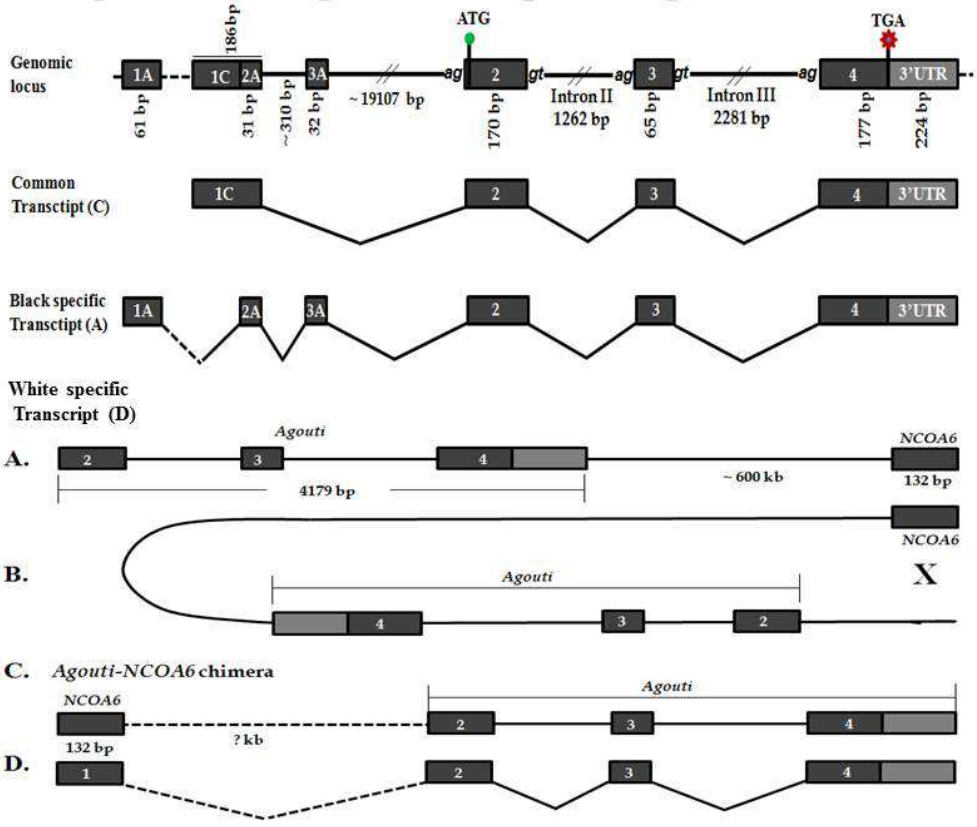


Figure.3. Relative expression levels of *agouti* mRNA in white, brown and black alpaca. **A.** Bar diagrams represents the expression changes of *agouti* relative to reference gene *GAPDH*. Data are shown as mean±SEM (n=4) ($p<0.05$). **B.** Ethidium bromide stained gel of *agouti* and *GAPDH*.

Relative level expression of *agouti* mRNA in white, black and brown alpaca



***Agouti* full length transcripts and genomic locus**




THANK YOU ALL
CAMERINO