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Identification and characterization of SNP in 5'UTR of *MLPH* gene for goats with different coat colours*

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Melanophilin (*MLPH*) gene has been characterized as one of the candidate genes for coat colour dilution in human, mice and dog, but little is known about it in goat. Part of the 5'UTR (650bp, JQ743911) was sequenced leading to identify four polymorphic *loci* (*g.G426A*, *g.A570G*, *g.G592T* and *g.612-614insCTC)*. The genetic diversity was analysed in 120 individuals of four breeds of goat. The possible transcription factors in the four *loci* were mined by online tools. The results show that the four *loci* were in Hardy-Weinberg equilibrium ($P>0.05$) and there was a significant difference in gene sequence between Jining Grey (JN) and Leizhou Black (LZ) breeds ($P < 0.05$). JN has the highest allele frequency of A, A, G and insertion CTC at the four *loci*; and LZ has the highest allele frequency of G, G, T and deletion CTC. JN has the lowest effective allele number and the lowest value of Shannon I at the four polymorphic *loci*. LZ has the highest effective allele number and the highest Shannon I at the four polymorphic *loci*. Moreover, JN has the highest Fst and Gst (0.08 and 0.12, respectively) and the lowest Nm (2.80) compared to LZ. *In silico* analysis showed that the possible transcription factors having a potential binding site in the sequence consisting of A, A and G and insertion CTC in the four polymorphic *loci* of 5'UTR of *MLPH* gene were BRN2, CCAT, P, STAT and USF. The sequence consisting of G, G, T and deletion CTC in the 5'UTR region of *MLPH* formed the possible binding sites of MyoD, c-Myb, v-Myb, AP-1, USF and NKX25. It could be concluded that JN has different gene sequence in the four mutation sites of 5'UTR of *MLPH* compared to LZ and the mutation caused the variation of possible transcription factors that may play a role in the *MLPH* gene expression regulation; so JN has dilute, ashen and grey coat colours and LZ is of solid black colour.

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Melanophilin, together with myosin VA and Rab27A in mammals, has been characterized to form a tripartite protein complex, transferring melanosome from the cell body to the tips of its dendrites by an actin-dependent movement [Matesic *et al*. 2001]. Defects in the transfer process can cause pigment dilution in skin and hair in human diseases (Griscelli syndrome) – [Fukuda *et al*. 2002, Ménasché *et al*. 2003, Kuroda *et al*. 2005] and the corresponding coat colour mutants in mice, dog and cat are dilute, ashen and leaden [Provance *et al*. 2002, Fukuda and Kuroda 2004, Philipp *et al*. 2005a, Drögemüller *et al*. 2007]. Among the three candidate genes, *MLPH*, Rab27a and Myo5a for the dilute coat colour phenotype, a mutation in the *MLPH* gene was responsible for colour dilution without any additional impairment in human GS3 patients [Philipp *et al*. 2005b] or in leaden mice [Ménasché *et al*. 2003]. It was considered to be the most suitable candidate gene for colour dilution [Ishida *et al*. 2006].

In goat breeds, part of the *MLPH* gene sequence was identified (19,289 bp) to contain the whole coding region, and three simple repeated sequence motifs were detected in the goat DNA sequences based on the SSR tool [Feng *et al*. 2009]. A missense mutation of g.11584A>G in exon 10 was determined and the allele G found in Nanjiang Brown goat and Chengdu Ma goat might be a candidate site for the particular dilute coat colour [Zhou *et al*. 2010]. The UTR does not encode proteins, but it is indispensable for the expression of gene. The insertion, deletion and substitution in the UTR are also gene mutational events, although most studies are limited to the mutations in the coding region. The 5'-UTR, from the mRNA methylation guanine nucleotide cap to the AUG initiation codon, contains the promoter of a gene. No paper made reference to concerning 5'UTR of the *MLPH* gene in goat. To find the correlation of mutation and coat colour of goat, we focused on studying the variation of 5' UTR in *MLPH* gene.

Material and methods

A total of 120 goats of four Chinese indigenous breeds with different coat colours were considered: Jining Grey (JN) of grey coat colour, Liaoning Cashmere (LN) with white coat colour, Leizhou Black (LZ) with solid black coat colour and Nanjiang Brown (NJ) with brown coat colour. Thirty animals of each breed were included.

PrimerP(L:5'-TGATGCGAAGACCCGAC-3',R:5'-CAAAGTTGGCGACGAGG -3') was designed using Primer 5.0 based on the *MLPH* gene sequence of *Bos taurus* (NM_001081597) and was assembled by SANGON Co., Shanghai. PCR was carried out in a BIOMETRA personal PCR instrument with a total volume of 50.0 µl reaction containing 2.0 µl (75 ng/µl) goat genomic DNA, 5.0 µl 10×PCR standard reaction buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.8 mM each forward and reverse primer, 1 U *Taq* DNA polymerase (TaKaRa Biotechnology Co., Dalian, China) and 34.4 µl distilled water. The programme of PCR is as follows: denaturation for 5 min at 94°C, 35 cycles at 94°C for 30 s, an annealing step at 57.0°C for 30 s and an elongation step

at 72°C for 60 s. And the final step was an extension for 10 min at 72°C. The PCR products were detected on an ethidium bromide (EB) gel (1.0% agarose gel including 0.5 μg/ml EB) and sequenced by SANGON BIOTECH (Shanghai) Co., Ltd.

The sequences were aligned and linked using Seqman software in a DNASTAR software package (Version 6.0). The Seqman software was also used for identification of SNPs. PopGene32 was used to analyse genetic diversity, heterozygosis analysis, gene flow (Nm) and genetic differentiation index (Fst). The Hardy-Weinberg equilibrium and the difference of genotype frequency of the four polymorphic *loci* in four goat breeds were tested with chi – square test.

In silico analysis for the possible binding site of transcription factors was conducted on line by PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html), MultiSearch Promotor Sites (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3), TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html), TFBIND (http://tfbind.hgc.jp/) and MATCH (http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi).

Results and discussion

Polymorphic sites and polymorphism at g.G426A, g.A570G, g.G592T and g.612-614insCTC

The amplification fragment obtained with primer P was 650 bp long. Four polymorphic sites were found by alignment of 120 individuals, which were *g.G426A*, *g.A570G*, *g.G592T* and *g.612-614insCTC* (Fig. 1). The insertion of CTC was defined

Fig. 1. The sequencing peak drawing of *g.G426A, g.A570G, g.G592T* and *g.612-614insCTC.*

Table 1. The distribution of genotypes and alleles of the mutational site in goats of four breeds

Site	Breed	Genotype frequency			Allele frequency		Hardy-Weinberg equilibrium test	
		GG	GA	AA	G	A	χ^2	P
g.G426A	LZ	0.40	0.37	0.23	0.58	0.42	2.08	0.15
	JN	0.03	0.60	0.37	0.33	0.67	3.36	0.07
	NJ	0.17	0.33	0.50	0.33	0.67	2.16	0.14
	LN	0.13	0.47	0.40	0.37	0.63	0.00	0.95
		GG	GA	AA	G	A	χ^2	P
g.A570G	LZ	0.13	0.53	0.33	0.40	0.60	0.27	0.61
	JN	0.00	0.03	0.97	0.02	0.98	0.00	1.00
	NJ	0.10	0.40	0.50	0.30	0.70	0.13	0.72
	LN	0.00	0.13	0.87	0.07	0.93	0.11	0.74
		GG	GT	TT	G	T	χ^2	P
g.G592T	LZ	0.30	0.37	0.33	0.48	0.52	2.40	0.12
	JN	0.47	0.50	0.03	0.72	0.28	1.40	0.24
	NJ	0.50	0.33	0.17	0.67	0.33	2.16	0.14
	LN	0.40	0.47	0.13	0.63	0.37	0.01	0.95
		AA	AB	BB	A	B	χ^2	P
g.612-614insCTC	LZ	0.33	0.40	0.27	0.53	0.47	1.37	0.24
	JN	0.43	0.53	0.03	0.70	0.30	1.95	0.16
	NJ	0.50	0.33	0.17	0.68	0.33	2.16	0.14
	LN	0.40	0.47	0.13	0.63	0.37	0.01	0.95

Table 2. Results of Pearson chi-square test for the genotype frequency in the mutational site between pairs of goat breeds

Breed	LZ	JN	NJ	LN
LZ		11.89 (0.00) 26.49 (0.00)	5.84 (0.05) 1.71 (0.42)	5.68 (0.06) 18.31 (0.00)
JN	9.07 (0.01) 6.41 (0.04)		5.59 (0.06) 16.76 (0.00)	2.34 (0.31) 1.96 (0.16)
NJ	3.21 (0.20) 1.87 (0.39)	3.70 (0.16) 4.15 (0.12)		1.11 (0.57) 9.95 (0.01)
LN	3.36 (0.19) 1.67 (0.43)	1.99 (0.37) 1.97 (0.37)	1.11 (0.57) 1.11 (0.57)	

Chi-square and P value at *g.G426A*, *g.A570G* (above diagonal), *g.G592T* and *g.612-614insCTC* (below diagonal).

as type A and the normal one as type B at *g.612-614insCTC.* The distribution of the genotypes of the four polymorphic *loci* in different goat breeds is shown in Table 1 and the difference of genotype frequency of the four polymorphic *loci* between pairs of breeds is presented in Table 2.

At the *locus* of *g.G426A*, the GG genotype had the highest frequency (40%) in LZ with solid black coat colour and the lowest frequency (3%) in JN with dilute grey coat colour. The GA genotype had the highest frequency (60%) in JN and the AA genotype had the highest (50%) in NJ with brown coat colour. JN and LZ were significantly

different in the genotype frequency tested with independence chi-square test (P = 0.00 < 0.01, Tab. 2) and there was no significant difference between other breeds (P = 0.05~ 0.57 >0.05). The frequency of allele A was higher in JN, NJ and LN than that of allele G, while G was the highest in LZ. A allele in JN, NJ and LN had the highest frequency, from 67% to 63%, and A allele in LZ had the lowest frequency of 42%.

At the *locus* of g.A570G, the genotype AA had the highest frequency in JN (97%) and the lowest in LZ (33%). A allele in the four breeds had the highest frequency (98%) in JN and the lowest in LZ goats (60%). The chi-square test indicated that JN and LN had no significantly different genotype frequency (P= 0.16 > 0.05), just like LZ and NJ with P = 0.42 >0.05. The other pairs of breeds were significantly different (P= 0.00~0.01<0.05; Tab. 2).

At the *locus* of *g.G592T,* JN had the highest frequency in GT genotype (50%) and the lowest in TT genotype (3%) while LZ had the lowest frequency in GG genotype (30%) and the highest in genotype TT (33%). Frequency of allele G was higher than that of allele T in JN, NJ, and LN whereas a frequency of allele T was higher than that of allele G in LZ. JN and LZ were significantly different in genotype frequency at the *locus* of *g.G592T* (P= 0.01 < 0.05). The other pairs of goat breeds showed no significant difference (P= 0.16~0.57 > 0.05) – (Tab. 2).

At the *locus g.612-614insCTC*, the deletion genotype of BB had the lowest frequency in JN (3%). The CTC insertion allele A occurred more frequently than allele B in all the four breeds, with JN as the highest frequency (70%) and LZ at the lowest (53%). The chi-square test showed that JN and LZ had significant difference in genotype frequency at *locus* (P = 0.04 < 0.05); the other pairs of breeds showing no significant difference (P = 0.12~0.57 > 0.05) (Tab. 2).

The four polymorphic *loci* in four breeds were all in Hardy-Weinberg equilibrium as tested with chi-square test (P = 0. 07~1.00 >0.05, Tab. 1).The Pearson's chi-square test of the polymorphic *loci* between pairs of breeds for the genotype frequency indicted that there was a significant difference in genotype frequency between LZ and JN at all the four polymorphic *loci*. JN had higher allele frequency of A, A, G and A at the four *loci,* respectively. LZ had higher allele frequency of G, G, T and B, respectively. It indicted that JN goats had the different gene sequence compared to LZ in the four mutational sites of 5'UTR of *MLPH* gene.

Genetic diversity of *g.G426A, g.A570G, g.G592T* and *g.612-614insCTC*

The genetic diversity of mutational sites in different goat breeds is shown in Table 3, including observed and expected Het, Ne's expected Het, effective allele number and Shannon I.

LZ had the highest effective allele number (1.95, 1.92, 2.00 and 1.99 at the four polymorphic *loci*, respectively) and the highest Shannon I (0.68, 0.67, 0.69 and 0.69, respectively); while JN had the lowest effective allele number (1.80, 1.03, 1.68 and 1.72 at the four mutational loci, respectively) and the lowest Shannon I (0.64, 0.09, 0.60 and 0.61, respectively). It might suggest that the mutational sites in 5'UTR in JN

Table 3. The heterozygosis of the polymorphic *loci* in four goat breeds

Site	Breed	Observed Het	Expected Het	Ne′s expected Het	Effective allele number	Shannon I
g.G426A	LZ	0.37	0.49	0.49	1.95	0.68
	JN	0.60	0.45	0.44	1.80	0.64
	NJ	0.33	0.45	0.44	1.80	0.64
	LN	0.47	0.47	0.46	1.87	0.66
g.A570G	LZ	0.53	0.49	0.48	1.92	0.67
	JN	0.03	0.03	0.03	1.03	0.09
	NJ	0.40	0.43	0.42	1.72	0.61
	LN	0.13	0.13	0.12	1.14	0.25
g.G592T	LZ	0.37	0.51	0.50	2.00	0.69
	JN	0.50	0.41	0.41	1.68	0.60
	NJ	0.33	0.45	0.44	1.80	0.64
	LN	0.47	0.47	0.46	1.87	0.66
g.612-614insCTC	LZ	0.40	0.51	0.50	1.99	0.69
	JN	0.53	0.43	0.42	1.72	0.61
	NJ	0.33	0.45	0.44	1.80	0.64
	LN	0.47	0.47	0.46	1.87	0.66

goat were more invariable and LZ was more variable. Meanwhile, we could see that the observed Het and expected Het or Ne's expected Het in all the four *loci* are nearly the same, which is in accordance with the finding that they are all in Hardy-Weinberg equilibrium.

Genetic differentiation between pairs of goat breeds

The gene flow (Nm) and genetic differentiation index (Fst and Gst) between pairs of goat breeds are shown in Table 4. There is the smallest Nm (2.80) between LZ and JN, and the Nm between other goat breeds is 4.57~47.02. The genetic differentiation index Fst (0.08) and Gst (0.12) between LZ and JN are the biggest; and Fst and Gst between other goat breeds are 0.01~0.05 and 0.01~0.08, respectively. It shows that JN

Table 4. Gene flow (Nm) and genetic distance (Fst and Gst) between pairs of goat breeds

Breed	LZ	JN	NJ	LN
LZ	-	2.80	7.50	4.57
JN	0.08 (0.12)	-	9.11	47.02
NJ	0.03 (0.06)	0.03 (0.03)	-	14.16
LN	0.05 (0.08)	0.005 (0.01)	0.017 (0.02)	

Gene flow Nm (above diagonal) and genetic differentiation index Fst and Gst in bracket (below diagonal).

and LZ have minor gene flow and middle differentiation, and the other goat breeds have higher gene flow and nearly no differentiation in the 5'UTR of *MLPH*. JN goat has dilute, ashen and grey coat colour, while LZ goat is solid black. LN has white coat colour and NJ has yellow coat colour. In both humans and mice, it is postulated that the dilution phenotype is caused by the inability of MLPH to bind Rab27a and link it to MyoVa. From these, it might be suggested that the 5'UTR variation would play a role in the expression of *MLPH* and the forming of dilution colour in JN.

***In silico* analysis of transcription factors in the mutation of 5'UTR region of *MLPH* gene**

The possible different transcription factors having a potential binding site in the mutational sites of 5'UTR region of *MLPH* gene are shown in Figure 2. The allele G

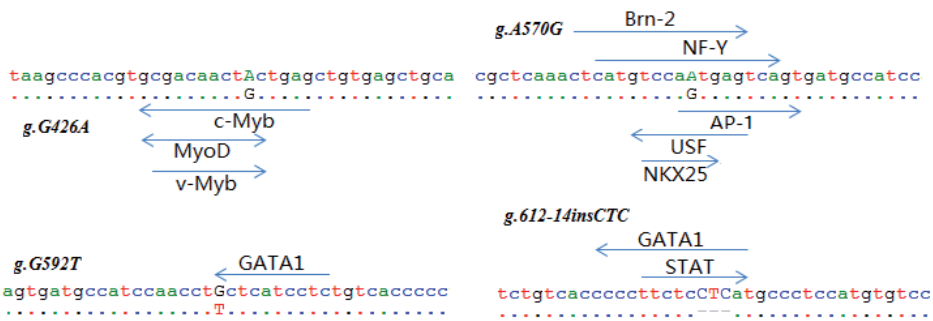

Fig. 2. Transcription factors of the polymorphic sites in the 5'UTR of *MLPH*.

in *g.G426A* formed the binding sites of c-Myb, v-Myb and MyoD, while the sequence of allele A in *g.G426A* could not form the possible binding sites of these transcription factors. The allele A in *g.A570G* contains the binding sites for BRN2 and NF-Y, while allele G for AP-1, USF and NKX25. The allele G in *g.G592T* formed the binding sites of GATA1 while allele T did not contain it. The sequence of CTC insertion in the *g.612-614insCTC* formed the possible binding sites of GATA1 and STAT. Transcription factors are essential for the regulation of genes in when, where and how the gene's DNA is transcribed into RNA [Vaquerizas *et al*. 2009]. The variation of transcription factors caused by the mutation in the 5'UTR of JN *MLPH* might have some effects on the expression of *MLPH*. JN has the higher allele frequency of A, A, G and CTC insertion in the 5'UTR region of *MLPH*, so the transcription factors of BRN2, NF-Y, GATA1 and STAT might regulate the *MLPH* gene expression in JN to have the dilute, ashen, and grey coat colour. LZ goat has the higher allele frequency of G, G, T, and CTC deletion in the 5'UTR region of *MLPH*, so the transcription factors of c-Myb, v-Myb, MyoD, AP-1, USF and NKX25 might regulate the *MLPH* gene expression in LZ to have the solid black coat colour. The biological contain it function should be further tested by the cell cultivation experiment.

It could be concluded that JN has the different gene sequence in the four mutation sites of 5'UTR of *MLPH* compared to LZ and the mutation causing the variation of possible transcription factors that may have a role in the *MLPH* gene expression regulation, making JN to have dilute, ashen and grey coat colour and LZ, solid black coat colour.

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