IGM Publication
Journal of Medical Science and Clinical Research
Volume||1||Issue||2||Pages71-80||2013
Website: www.jmscr.igmpublication.org ISSN (e): 2347-176X

Study on Variation of Exon 8 and 16 of Goat *MLPH* Gene

Liying ZHANG[*], Qiang FU[*], Xianglong LI[**], Lanhui LI and Rongyan ZHOU
(College of Animal Science and Technology, Agricultural University of Hebei, Baoding, 071001, China)
E-mail: zhangliying86@163.com

Abstract

The variation of exon 8 and 16 of MLPH gene in different Chinese indigenous goat breeds with different color and locations was investigated in order to understand its genetic diversity. The mutation sites g.G9109A and g.G9125A in exon 8 and g.G18951A and g.A18955C in exon 16 were found. The g.G18951A and g.A18955C in exon 16 were in complete linkage disequilibrium and formed two haplotypes, GA and AC. Meanwhile the complete linkage mutation sites in exon 16 were in second structure domain of MLPH protain, which might affect its function. Leizhou black goat, Jining grey goat and Liaoning Cashmere goat were in Hardy-Wenberg disequilibrium at g.G18951A and g.A18955C in exon 16 except for Nanjiang Brown goat. In exon 16, Jining grey goat and Liaoning Cashmere goat had a closer relationship while Leizhou black goat was the farthest. The location of goat breeds affected the gene flow.

Key words: *goat, MLPH gene, exon, genetic diversity*

Introduction

Melanophilin, together with myosin Va and Rab27A in mammals, are characterized to form a tripartite protein complex, taking responsibility for transferring melano-somes from the cell bodies to the tips of their dendrites by an actin-dependent movement (Matesic and Yip, 2001). Defects in the transfer process can cause pigment dilution in the skin and hair in human diseases (e.g. Gris celli syndrome) (Kuroda and Fukuda, 2002; Ho and Menasche, 2003; Itoh and Kuroda, 2005) and the corresponding coat-color mutant mice (e.g. dilute, ashen and leaden) (Provance and James, 2002; Fukuda and Kuroda, 2004). Among the three candidate gene (*MLPH*, Rab27a and Myo5a) for dilute coat color phenotype, mutation in *MLPH* gene was responsible for color dilution without any further impairment in human GS3 patients or leaden mice, thus it was considered as the most suitable candidate gene for color dilution (Philipp and Hamann, 2005; Matesic and Yip, 2001; Ho et al., 2003).

The pigmentation of mammalian hair and skin is a multistep process involving the synthesis of pigments (melanins) within melanosomes, the specialized, pigment-producing organelles of melanocytes; long-range, bidirectional, microtubule-dependent melanosome transport from the cell body to the melanocyte's extensive actin-rich dendritic arbo; capture and short-range actin-dependent melanosome transport in the cell

[*] Author: Liying ZHANG. E-mail: zhangliying86@163.com. Qiang FU contributed equally to this work.

[**] Corresponding author: Xianglong LI. Present address: College of Animal Science and Technology, Agricultural University of Hebei, Baoding, 071001, China. Tel.: +86 312 7528451, Fax: +86 312 7528451, E-mail: lixianglongcn@yahoo.com.

periphery; and melanosome transfer from the dendritic tips to the neighboring keratinocytes and eventually into the hair shaft (Wu et al. 1998).

The association between *MLPH* gene mutation and coat color dilution has been reported in mice (Ho et al. 2003), cats (David and Ishida, 2006) and dogs (Philipp and Hamann, 2005; Quignon and Philipp, 2005; Philipp and Drogemuller, 2007). Recently, in cats, a single base pair deletion in exon 2 of the feline *MLPH* gene affecting both eumelanin and phaeomelanin pigment distribution was shown to cause the feline dilute coat color phenotype (Ishida et al. 2006). Feng et al. (2009) sequenced 19289bp including 1764bp CDS of *MLPH* gene (EU316218), and found *g.11584G>A* was relevant with brown goat color of NJ and Chengdu Ma goats. Zhou et al. (2010) showed that the allele G at *g.*11584A>G in exon 10 of goat *MLPH* gene might

be a candidate site for the particular dilute coat color found in Nanjiang Brown goat and Chengdu Ma goat. Nine linked SNPs were found in goat *MLPH* gene by sequencing a total of 108 individuals from 5 goat populations. No homozygous mutation of the linked SNPs was detected (Li et al. 2010). Though the melanophilin (*MLPH*) gene has been characterized as one of the candidate genes for coat color dilution in human, mice and dog, little is known about this gene in goat. In order to extend knowledge of *MLPH* gene and provide some useful information for coat and hair research in goat, the mutation in exon 8 and 16 of goat *MLPH* gene was determined according to the sequence EU316218 previously obtained, and its variation in different Chinese indigenous goat breeds with different color and locations was investigated.

Methods and Materials

Samples and primer design

In this study, 120 blood samples were taken from Jining Grey goat (JN, 30), Liaoning Cashmere goat (white, LN, 30)，Leizhou black goat (LZ, 30)，Nanjiang Brown goat (NJ, 30), respectively. They were distributed in Shandong province, Guangdong province, Liaoning province and Sichuan province in China (Fig.1).

Fig. 1 Geographic distribution of four goat breeds in China.
A: Liaoning Cashmere goats; B: Jining Grey goats; C: Nanjiang Brown goats; D Leizhou black goats

The primers P1 and P2 were designed on exon 8 and 16 by Primer 5.0 based on the *MLPH* coding sequence of *Capra hircus* (EU316218). They were synthesized by Sango Biotech (shanghai) Co., Ltd. The annealing temperature, extension time and cycle number were shown in Table 1.

Table1 Primers used for amplification of goat *MLPH* gene

Primer	Site	Primer sequence (5’ → 3’)	PCR products	Ta (℃)	Extension time (s)	Cycle Number
P1	exon 8	L 5’- AGTCCAGAGCCCTTTG -3’ R 5’- CAAACTGAGCGAACCC -3’	800bp	57.5℃	60s	32
P2	exon 16	L 5'- TGACCCACCAGCCCTAA -3' R 5’-ACTGCCAAAGCCCAAG-3'	285bp	58.0℃	30s	30

PCR amplification and sequencing of exon 8 and exon 16

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 MgCl2, 0.8 mM each forward and reverse primer, 1 U *Taq* DNA polymerase (TaKaRa Biotechnology Co., Dalian, China) and 34.4 μl distilled water. After predenaturation for 5 min at 94°C, 32 cycles for P1 (30 cycles for P2) of 94°C for 30 s, 57.5℃ for P1 （58.0℃ for P2) for 30 s, and an elongation step at 72°C for 60 s for P1 （30 s for P2), followed by a final extension of 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 Co., Shanghai.

Sequencing analysis

The mutation sites found in exon 8 and 16 analyzed in 120 individuals. The BioEdit (Version 7.0.5.2) and the BLAST were used to analyze the sequencing results. POPGene32 was used to analyze the distribution of the genotypes, allele of frequency and heterozygosis. Phylogenetic tree was constructed for all breeds by Mega 4.0 software using Neighbor Joining method based on genetic distance (Gst) between breeds. The haplotypes and linkage disequilibrium of variant sites were analyzed by SHEsis software (http://analysis.bio-x.cn/myAnalysis.php).

Results

PCR results of exon 8 and 16 of goat *MLPH* gene

1% agarose gel electrophoresis was used to detect the PCR amplification products of primer P1 and P2. The results were consistent with the expected amplification fragment length (800bp and 285bp) (Fig. 2 and Fig. 3).

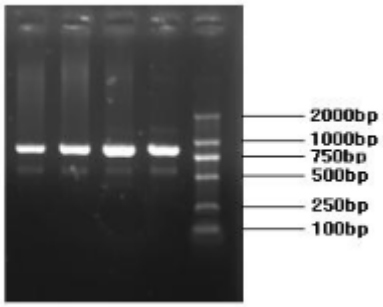

Fig. 2 PCR result of exon 8

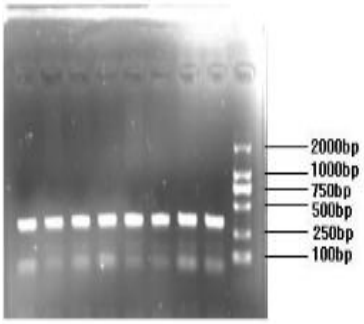

Fig. 3 PCR result of exon 16

Sequencing of exon 8 and 16 of goat *MLPH* gene

PCR products were sequenced with cutting the target fragments by Beijing liuhe huada Gene (BGI). The Blast (Basic Local Alignment System Tools) software was used to compare the sequencing results with the sequences in GenBank（http://www.ncbi.nih.nlm.gov）. The sequencing result of P1 has 100% and 97% homology with part of the *MLPH* gene in *Capra hircus* (EU316218.1) and *Ovis aries* (EU316219.1) respectively. The sequencing result of P2 has 95% and 86% homology with part of the *MLPH* gene in *Capra hircus* (EU316218.1) and *Bos Taurus* (NM001081597.1) respectively. The results were consistented with bioinformatics standards (Zhang et al. 2002).

Mutation sites in exon 8 and 16

The sequencing results in exon 8 and 16 of 120 goats showed that, in exon 8 we detected two SNPs (*g.G9109A* and *g.G9125A*). Three genotypes (GG, GA and AA) were found in *g.G9109A* and also in *g.G9125A* (Fig.4). In exon 16 we detected two SNPs (*g.G18951A* and *g.A18955C*). Three genotypes (GG, GA and AA) were found in *g.G18951A* and three genotypes (AA, AC and CC) were found in *g.A18955C* (Fig.5).

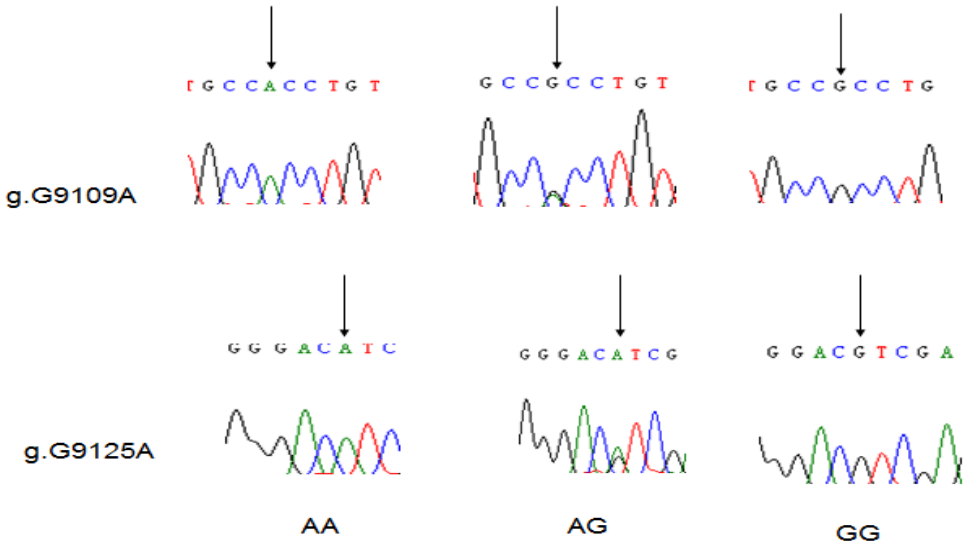

Fig.4 The sequencing peak drawing of exon 8

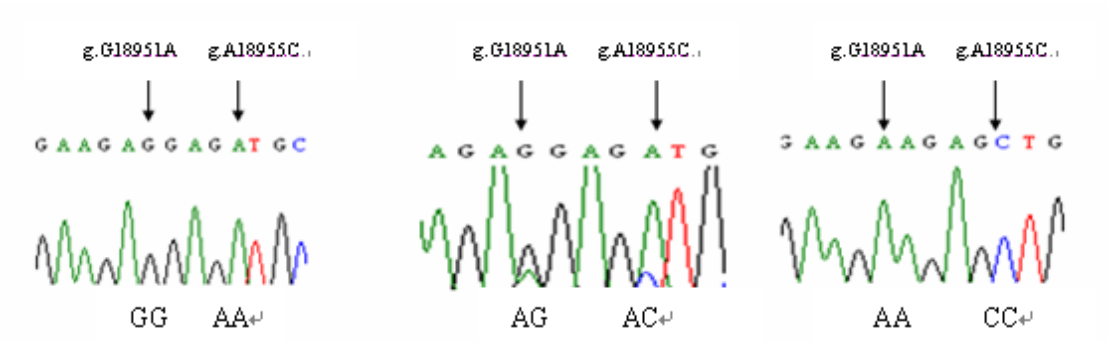

Fig.5 The sequencing peak drawing of exon 16

Distribution of mutation sites in different populations

The haplotypes and linkage disequilibrium analysis of these four variant sites showed that in *g.G18951A* and *g.A18955C* of exon 16 there were two haplotypes on the premise of the default value (0.03) of the lowest frequency threshold (LFT), which were GA (69.5%) and AC（31.5%）. From Table 2 we can see that the two haplotypes were in strong linkage disequilibrium. For population genetics analysis, we defined GA as C and AC as D.

Table 2 Results of Linkage Disequilibrium

D'	*g.G18951A*	*g.A18955C*

r^2		
g.G18951A	-	1.000
g.A18955C	1.000	-

Distribution of the genotypes, allele frequency and heterozygosis of *g.G9109A*, *g.G9125A*, *g.G18951A* and *g.A18955C* in different goat breeds was shown in Table 3. It was obvious that at *g.G9109A*, the main genotype in JN was AA and the main genotype was heterozygous in other three breeds. At *g.G9125A*, the GG was existed in all the four breeds except three individuals of JN. The CD was the main genotype in the four breeds at *g.G18951A* and *g.A18955C* of exon 16. The highest ratio of CD was in JN (97.77%) and there were no DD in LZ and JN. The LZ, JN and LN were in Hardy-Wenberg disequilibrium at *g.G18951A* and *g.A18955C* in exon 16 except for NJ.

The allele A, G and C was the advantage allele in four breeds at *g.G9109A*, *g.G9125A*, *g.G18951A* and *g.A18955C*, respectively. The results of Hardy-Weinberg law test showed that the four breeds were in equibrium state ($p > 0.05$) at the three sites except LZ, JN and LN at *g.G18951A* and *g.A18955C* ($p < 0.01$). It was also obvious that the genetic diversity has no obvious difference. The results of Shannon information index (I) was that there was a highest I in LZ and LN at *g.G9109A* site, which indicated that these breeds has a bigger genetic diversity at this site. At *g.G9125A* site, I was all lower in the four breeds, range from 0.2449 to 0.5799. The genetic diversity can be ordered as JN > NJ =LN >LZ at *g.G9109A* and *g.G9125A* of exon 16. The bigger difference between Obs Het (0.9677 and 0.8000) and Exp Het (0.5006 and 0.5079) at *g.G9109A* and *g.G9125A* of exon 16 in JN and LN were found due to the higher heterozygote frequency, which indicated that the two breeds had higher heterozygosity in this site.

Table 3 Distribution of genotypes, allele frequency and heterozygosis of *g.G9109A*, *g.G9125A*, *g.G18951A* and *g.A18955C* in different goat breeds

Sites	Breed	Genotype frequency			Allele frequency		Chi-square test	Observed Het	Expected Het	Shaoon I
		AA	AG	GG	A	G	*p*			
g.G9109A	LZ	0.3667	0.4000	0.2333	0.5667	0.4333	0.2671	0.4000	0.4994	0.6842
	JN	0.5000	0.3667	0.1333	0.6833	0.3167	0.3499	0.3667	0.4401	0.6243
	NJ	0.4000	0.5000	0.1000	0.6500	0.3500	0.6523	0.5000	0.4627	0.6474
	LN	0.3333	0.5000	0.1667	0.5833	0.4167	0.9492	0.5000	0.4944	0.6792
g.G9125A	LZ	0.0000	0.3667	0.6333	0.1833	0.8167	0.2441	0.3667	0.3045	0.4764

	JN	0.1000	0.3333	0.5667	0.2667	0.7333	0.3623	0.3333	0.3977	0.5799
	NJ	0.0000	0.1333	0.8667	0.0667	0.9333	0.7368	0.1333	0.1266	0.2449
	LN	0.0000	0.1667	0.8333	0.0833	0.9167	0.6586	0.1667	0.1554	0.2868
		CC	CD	DD	C	D	*P*			
g.G18951A and *g.A18955C*	LZ	0.3333	0.6667	0.000	0.6667	0.3333	0.0078	0.6667	0.4520	0.6365
	JN	0.0333	0.9677	0.000	0.5167	0.4833	0.0000	0.9667	0.5079	0.6929
	NJ	0.3000	0.5333	0.1667	0.5667	0.4333	0.7051	0.5333	0.4994	0.6842
	LN	0.1667	0.8000	0.0333	0.5667	0.4333	0.0007	0.8000	0.5006	0.6842

The gene flow (Nm) and genetic distance (Gst) of these four breeds were showed in Table4. The smallest Gst (0.0032 in exon 8 and 0.0009 in exon 16) and the biggest Nm (64.5000 in exon 8 and 99.0556 in exon 16) showed the closest relationship between LN and NJ. The largest Gst and the smallest Nm displayed the earliest differentiation between LN and JN in exon 8, between LZ and JN in exon 16.

Table 4 Gene flow (Nm) and Genetic Distance (Gst) of exon8 and 16 in goat breeds

exon 8					exon 16				
Nm / Gst	LZ	JN	NJ	LN	Nm / Gst	LZ	JN	NJ	LN
LZ		19.6351	16.6662	34.7773	LZ		10.4887	23.3889	23.3889
JN	0.0173		8.5338	8.3854	JN	0.0424		99.0556	99.0556
NJ	0.0125	0.0277		64.5000	NJ	0.0280	0.0121		99.0556
LN	0.0063	0.0323	0.0032		LN	0.0255	0.0078	0.0009	

Note: Gene flow Nm (above diagonal) and Genetic Distance (below diagonal)

Phylogenetic tree was constructed for all breeds by Mega 4.0 software using Neighbor Joining method based on Gst between breeds was shown in Fig. 6 (exon 8) and Fig. 7 (exon 16). From the Fig. 6 we could see that NJ and LN were together first, and then LZ, the last was JN in exon 8. The Fig. 7 showed that JN and LN were together first, and then NJ, the last the LZ in exon 16. The phylogenetic tree results were consistent with the Gst in Table4.

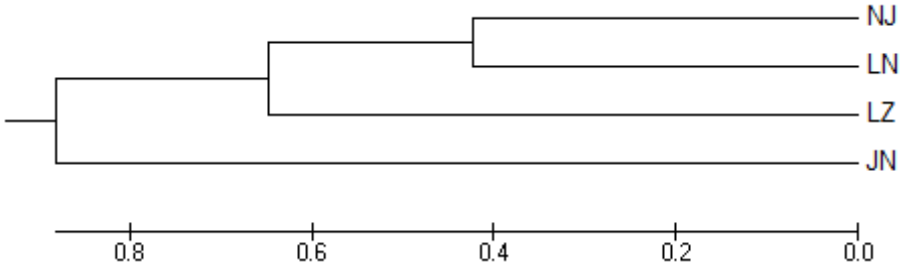

Fig 6 UPGMA dendrogram of the four Chinese indigenous goat breed of exon 8 based on Gst

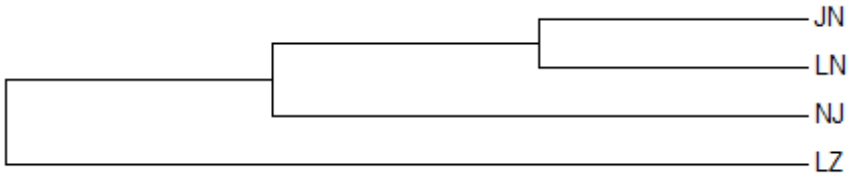

Fig 7 UPGMA dendrogram of the four Chinese indigenous goat breed of exon 16 based on Gst

Bioinformatics analysis of mutation sites

The MLPH protein structure domain was analysed by SMART software (http://smart.embl-heidelberg.de). We found MLPH protein was contained two structure domain (Fig. 8). The two mutation sites of exon 16 were in the second structure domain. The relation between mutations and structure domain needed further test.

Confidently predicted domains, repeats, motifs and features:

Name	Begin	End	E-value
low complexity	173	185	-
low complexity	390	401	-
low complexity	413	426	-
Pfam:MOBP_C-Myrip	462	554	4.90e-05

Fig. 8 Bioinformatics analysis of mutation sites

Discussion

The goat coat color is controlled by many genes, such as *Agouti*, *TYR*, *MSHR*, *MLPH* and so on. In *TYR* gene, Zheng (2010) found that allele A and genotype AC of *g.1264A>C* in intron1 were in favor of the synthesis of eumelanin. For *MSHR* gene, Wu et al. (2006) and Li et al. (2008) found that *259A>G* is the main reason for the formation of brownish red color on head and neak of Boer goat. For *agouti* gene, Tang et al. (2008) postulated that the T of *5700G>T* in exon4 might be associated with the black phenotype, or linked with the site controlling black coat color in goat after analysis of 677 individuals from 12 Chinese indigenous goat breeds and 1 imported Boer goat breed. Tang et al. (2009) also showed that AA of 128delT in intron 1 might have relationship with brown phenotype and BB might correlates with black phenotype.

In this paper, sequencing analysis results of exon 16 showned that the *g.G18951A* and *g.A18955C* in strong linkage disequilibrium. The reasons for linkage disequilibrium contain random genetic drift, founder effect, mutation, selection, gene conversion, group mixtion, and segregation and so on. The reasons of genetic drift and selection might disappear quickly in dislinkage loci. But the linkage disequilibrium

between closely linkage loci was disappeared slowly. The four breeds of this study were all China local goat breeds. So there was little influence factors with genetic drift, group mixtion and segregation, largely with mutations.

We obtained two haplotypes using the SHEsis software. Haplotype is a linear permutation and combination of more alleles which closely linkage on a chromosome. The linear combination could genetic to the offspring as a whole, won't be interrupted by genetic restructuring, and can keep stability in offspring chromosomes（Hu et al. 2011）.

In exon 8, there was no significant relation between mutations and colour. Cluster analysis result of exon 16 was mainly agreement with the result of Tang et al. 2007. Jining grey goat and Liaoning Cashmere goat were all north location breeds in China and they had a closer relationship while Leizhou black goat was in Guangdong province and had the farthest relation. The location of goat breeds affected the gene flow, the further the location was, the more significant difference exists. Cluster analysis result provided further proof that different geographical position effected gene communication of different goat breeds. *MLPH* gene is a colour dilution related gene (Quignon P et al. 2005, Provance DW et al. 2002). The relation between mutation and protein expression, mutation and colour association needed further experiment. Because the forming of coat color is controlled by many genes, even if so many variant sites affecting goat coat color were found, more works need to be done to study their interaction.

Acknowledgments This work was supported by Natural Science Fund of Hebei (No. C2010000775).

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