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ASSOCIATION OF *MELANOPHILIN* (*MLPH*) GENE POLYMORPHISM WITH COAT COLOUR IN REX RABBITS

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Abstract: Rex rabbit, with multiple phenotypes and colourful fur, is an interesting model for assessing the effect of coat colour gene mutations on characteristic pigmentation phenotype. Based on previous study, the *melanophilin* (*MLPH*) gene is a positional candidate gene related coat colour dilution. The fur colours are a lighter shade, e.g. grey instead of black. We sequenced 1689 base pairs of the *MLPH* gene in Chinchilla and black Rex rabbit. A total of 13 polymorphisms were identified, including seven missense mutations. The rabbit *MLPH* gene has a very high GC content and the protein shows 64.87% identity to the orthologous human protein (lack of homologous amino acids encoded by human *MLPH* exon 9). Hardy-Weinberg test showed that, except for the g.606C>A single nucleotid polymorphism (SNP), all other SNPs were in Hardy-Weinberg equilibrium. Haplotype analysis revealed that the seven missense mutation SNPs of two strains of Rex rabbits formed 10 haplotypes, but there were only seven major types of haplotypes (haplotype frequency $P>0.05$). The major haplotypes of the Chinchilla and black Rex rabbits were H1/H2/H3/H4/H5 and H1/H2/H3/H6/H8, respectively. The special haplotypes of Chinchilla Rex rabbit (H4, H5, H7) were consistently associated with the Chinchilla phenotype. This study provides evidence that different coat colour formation may be caused by one or more mutations within *MLPH* gene in several Rex rabbit strains. The data on polymorphisms that are associated with the Chinchilla phenotype facilitate the breeding of rabbits with defined coat colours.

Key Words: *MLPH*, Rex rabbit, polymorphism, coat colour.

INTRODUCTION

Coat colour is the primary characteristic used to identify a rabbit breed. It is also an important quality trait of rabbit fur and rabbit skin, and a key factor in determining the quality and economic value of fur (Bennett and Lamoreux, 2003). Consequently, the breeding of certain rabbit coat colours and new colour varieties have always been highly valued by rabbit breeders. In the past, the number of coloured pedigree Rex rabbits kept in China was very small. We have previously observed that crossbreeding of Chinchilla rabbits can produce white Rex rabbits, black Rex rabbits and Chinchilla Rex rabbits (Figure 1). There is a strong correlation between human and animal hair and eye colour (Eiberg and Mohr, 1996; Mengel-From *et al.*, 2009), and most white rabbits have red eyes. The coat colour of ordinary white rabbits is related to the recessive mutation of the albinism gene in the C locus. The homozygous albino gene c causes the rabbit's coat colour and the eye's iris to produce no pigment. The eyeball iris lacks pigment to reflect the colour of blood vessels, so white rabbits are mostly red-eyed (Choudhury, 1987). As shown in Figure 1, the white Rex rabbit has red eyes. White Rex rabbit is an albino mutation type. For this reason, we chose black and Chinchilla Rex rabbits as experimental animals. After long-term collection and breeding of genetic resources, our team has obtained pure Chinchilla Rex rabbits, which provided research materials for this research.

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Figure 1: The coat colour of three Rex rabbits. (A) White Rex rabbit. (B) Black Rex rabbit. (C) Chinchilla Rex rabbit.

Melanophilin (MLPH) regulates the retention of melanosomes at the peripheral actin cytoskeleton of melanocytes, which is a process essential for normal mammalian pigmentation (Hume *et al.*, 2006). *MLPH* interacts with both Rab27a and Myo5a, functioning as a linker between Rab27a and Myo5a (Fukuda *et al.*, 2002; Nagashima *et al.*, 2002; Strom and M., 2002; Wu *et al.*, 2002). *MLPH* stimulates the ATPase activity of Myo5a (Li *et al.*, 2005) and increases the number of progressively moving Myo5a molecules (Sckolnick *et al.*, 2013). The *MLPH* and cargo adaptor proteins RILPL2 co-regulate myosin-5amotoractivity (Cao *et al.*, 2019). In mice, mutations in the myosin VA, Rab27a, and *MLPH* genes (Matesic *et al.*, 2001; Mercer *et al.*, 1991; Wilson *et al.*, 2000) encode proteins that constitute melanosome transport complexes (Barral and Seabra, 2004). The three-component complex formed by *MLPH*, Rab27a, and myosin VA is involved in the first process of melanosome transport. In humans, mutations in the *MLPH* gene have been shown to cause Griscelli syndrome type III, which is an autosomal recessive disorder characterised by pigment dilution of the skin and hair (OMIM 609227) (Ménasché *et al.*, 2003). Fontanesi *et al.* first investigated the rabbit homologous gene of the mouse dilute locus and excluded MYO5A as the determinant of the dilute locus in rabbit (Fontanesi *et al.*, 2012). After that, they found that a frameshift mutation in the *MLPH* gene (exon 5) causes the dilute coat colour in different rabbit (*Oryctolagus cuniculus*) breeds (Fontanesi *et al.*, 2014). However, the same results were not detected in our existing Rex rabbit population. This suggests that there may be other mechanisms for the function of *MLPH* in the formation of rabbit coat colour. Therefore, the *MLPH* gene seemed to be the most suitable candidate gene for coat colour formation in several Rex rabbits.

MATERIALS AND METHODS

Animals

All animal work was conducted in accordance with national and international guidelines for animal welfare. We obtained fresh ear tissue from 40 individual rabbits (Table 1) from two breeds (20 black Rex rabbits, and 20 Chinchilla Rex rabbits). All rabbits were from the Yuyao Xinnong Rabbit Industry.

DNA extraction and detection

A small piece of ear tissue from each rabbit was cut and placed in a centrifuge tube, which was stored in liquid nitrogen. DNA was extracted from fresh ear tissue using TIANamp Genomic DNA kits (Tiangen biotech, China, Beijing). Quality was tested using 1% agarose and NanoDrop 1000 (Thermo Scientific, USA) to detect concentration. Any unqualified DNA samples were re-sampled re-extracted or purified.

Sequencing and genotyping of MLPH

Primers were designed based on the genome sequence of *MLPH* from *Oryctolagus cuniculus* (NCBI Reference Sequence: NW_003159466.1 and Ensembl accession number ENSOCUG00000016496) using the Primer 5.0 (PREMIER Biosoft International, Canada) and the Oligo 6.0 (Molecular Biology Insights Inc., Cascade, CO, USA) software. The primers are listed in Table 2.

Polymerase chain reactions (PCRs) were performed using Taq Master Mix (Takara Biotech Co., Dalian, China). The reaction contained 1 µL genomic DNA and 1 µL of each primer in a total volume of 20 µL. Thermal cycling parameters were as follows: 94°C for 5 min; 35 cycles of 94°C for 45 s, 55-65°C (optimum annealing temperature) for 45 s, and 72°C for 1 min; and 72°C for 10 min. Completed reactions were stored at 4°C until sequencing and sent to the Huada Gene Company for sequencing (Shanghai, China).

Sequence analysis

Sequences were spliced and aligned using DNASTAR Lasergene 8.0, and the protein translation and calculation of protein molecular weight was also done with DNASTAR. Genotype and allele frequencies of polymorphisms were calculated using the direct counting method. The Hardy-Weinberg equilibrium was evaluated for each single nucleotid polymorfism (SNP) using the Chi-square test.

Data analysis

The test data was compiled using Excel 2013 software. The differences in the distribution of *MLPH* genotypes among coat colours were analysed using SPSS software and the χ^2 test. $P<0.05$ indicates a significant difference. Haploview 4.2 software was used for haplotype construction of 13 mutation loci in the *MLPH* gene. GC content and CpG islands were calculated with CpG plot (http://www.ebi.ac.uk/emboss/cpgplot/).

Table 1: Description of the Rex rabbit samples. All the animals were males.

	Black rex rabbit		Chinchilla rex rabbit	
	Ear number	Old (months)	Ear number	Old (months)
1	D40654	3.5	C31552	5.5
2	D40644	4	D40510	4
3	D40634	4.5	D40508	4
4	D40550	3.5	C31808	5
5	D40538	4.5	D40502	3.5
6	D40548	5	C31782	5
7	D40546	5	C31784	5
8	D40626	4	C50018	5.5
9	D40652	4	C31788	5
10	D40624	4	C31796	5
11	D40656	3.5	C50014	5.5
12	D40628	4	C31774	4.5
13	D40564	5	C31772	4.5
14	D40660	5.5	C31778	4
15	D40658	3.5	D40514	3.5
16	D40576	3	D40526	3.5
17	D40556	4	C30812	5.5
18	D40558	4	D40518	4
19	D40678	5.5	C50020	5
20	D40596	5	D40522	4

RESULTS

Structure of the rabbit MLPH gene

The genomic organisation of the rabbit *MLPH* gene was inferred by comparison of the genomic *Oryctolagus cuniculus* sequence with an experimentally derived rabbit cDNA sequence (Figure 2). We amplified the full-length sequence of *MLPH*, including the 1689 base pairs (bp) complete CDS region, encoding 562 amino acids, 15 exons, and 16 introns. The A+G content was 32.80%, and the G+C content was 67.2%. This shows that the *MLPH* gene has a very high GC content, which is significantly above the mammalian average of 41%. The *MLPH* mRNA contains five open reading frames, the longest of which has 1692 nt encoding a protein of 562 amino acids. The rabbit *MLPH* protein

Table 2: The 15 primer sequences and optimal temperature (T) for the *MLPH* gene.

Primer name	Primer sequences 5'- 3'	T	Primer name	Primer sequences 5'- 3'	T
MLPH1-F	CCTGGCCCTGCTGTTTCAG	62°C	MLPH9-F	GTTCTCCCGCTCTCTCAGC	62°C
MLPH1-R	GCAGGACGTCGGAGGACC		MLPH9-R	TAGTGGCAGTACCTGTGCTG	
MLPH2-F	CTGGCCGTGTGTTCCCTTTC	61°C	MLPH10-F	AGCCGCTGTCCCTCTCTTT	62°C
MLPH2-R	CGAGAGAGCCCATCTTCACC		MLPH10-R	CCACAGGCCGACTGTCAC	
MLPH3-F	CAGGGCTGGCTCTGTGAC	64°C	MLPH11-F	AGACTCGCGAGTGGAGTACA	63°C
MLPH3-R	AGAGACAGGCATGCACTCAC		MLPH11-R	AAACACAGGCGGGAGAGATC	
MLPH4-F	GATGGACACTCCGCTGTCAG	62°C	MLPH12-F	CCTCTCCAGCAGGCGTCTAA	61°C
MLPH4-R	GAAGTCAGAGCCTGGCAGTG		MLPH12-R	CCTGATGTCAGAGGCTCACT	
MLPH5-F	GTCTGGTCCTGTCCTTCGAG	61°C	MLPH13-F	GTCTCTGGGTCTGCAAGGAC	63°C
MLPH5-R	TGTCCCTGTCCTGAGAGCC		MLPH13-R	GGTAATGAGCTGCTCACGCT	
MLPH6-F	CAAGAACCAGGACCAGCGTC	60°C	MLPH14-F	GCCAGGTCCCCTTTAATGCT	58°C
MLPH6-R	TGTCCCTACCACGCACACT		MLPH14-R	GCGAGCACGTTTTGCTGTAA	
MLPH7-F	CCAGGTCCGTCTGAGGTACT	60°C	MLPH15-F	CGTGTGTCTCTGTGCCTCAT	63°C
MLPH7-R	GGAAGCACTGTCCCTACCAC		MLPH15-R	GAGAGCCAGGGCAGGGAA	
MLPH8-F	GAGCCGTGGTTCTCATGTCC	60°C			
MLPH8-R	GTCTCTGTACACTACGCAGCA				

was predicted to have a molecular weight of 60.7 kDa, a pI of 5.55, and shows 64.87% identity to the orthologous human protein (lack of homologous amino acids encoded by human *MLPH* exon 9).

Estimation of allele frequencies using the Hardy-Weinberg equilibrium test

The genotype frequencies and alleles frequencies of different alleles of the aforementioned mutation sites in different coat colour groups were calculated (Table 3). Thirteen SNPs were detected in two strains of Rex rabbits. Among them, the following had a Chi-square greater than 5.99 ($P< 0.05$): g.606C>A, g.610T>C, g.642C>G alleles of exon 5, g.1067>G, g.1095C>T of exon 9 and g.1462G>A alleles of exon 12. This indicates that different genotypes were significantly different between two Rex rabbit strains, and these alleles were associated with the coat colour of Rex rabbits.

The genetic structure of the loci of the 7 missense mutation SNPs was analysed, and we found that they presented high heterozygosity, observed in the two strains of Rex rabbits (Table 4). This indicates that the 13 loci were variable in the population. The Hardy-Weinberg test of two Rex rabbits revealed that there was no significant difference between the two groups of data, according to the df= 2 (Degree of freedom) and Chi-square test (HW<5.99, $P>0.05$). Except for the g.606C>A SNP in the Chinchilla Rex rabbit, the rest of the SNPs achieved a Hardy-Weinberg equilibrium.

Amino acid changes and mutation types

There are seven missense mutations and six silent mutations in the 13 mutation loci (Table 5). There were two mutations that were mutated to arginine (p.Trp204Arg and p.Lys356Arg) and two mutations that were mutated to alanine (p.Gly314Ala and p.Val318Ala). The remaining mutations are p.Leu302Pro and p.Asp488Ser. Compared with

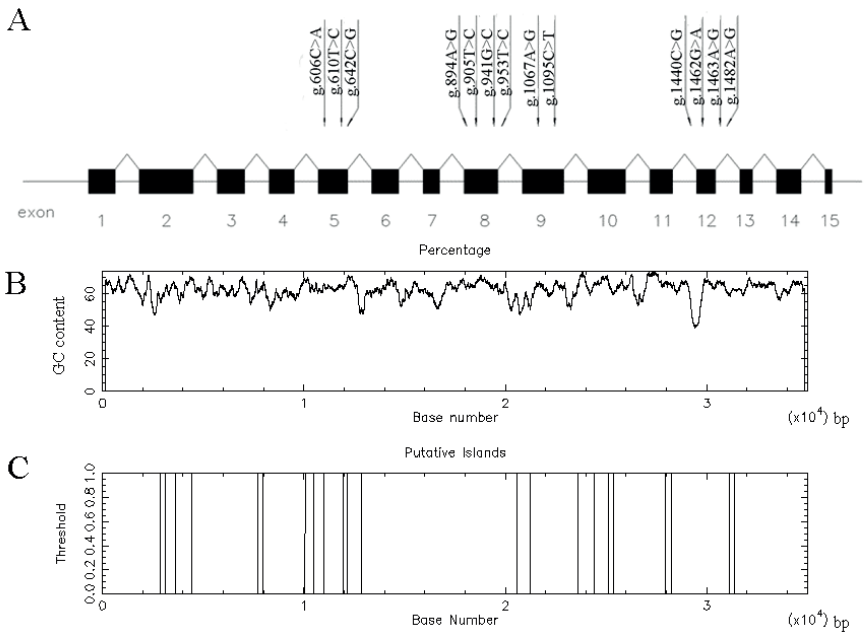

Figure 2: Structure of the *MLPH* gene. (A) The 13 polymorphisms that were identified in two strains of Rex rabbit are indicated. Coding regions of exons are indicated in black. The 5' is on the left, and the 3' non-coding regions are on the right. The positions of 13 single nucleotid polymorphisms chosen for further analyses are indicated by arrows. (B) Illustration of the unusually high GC content of the *MLPH* gene. The GC content was calculated using a 300 base pairs (bp) window. (C) CpG island of *MLPH* gene. CpG island criteria were: GC>0.5, CpGobs/CpGexp>0.6, and length >200 bp.

human and mouse *MLPH* proteins, the g.953T>C locus led to p.Val318Ala, Alanine is consistent with humans. And the mutated arginine (p.Lys356Arg) is identical to both human and mouse proteins (Figure 3).

Correlation analysis between haplotype and phenotypic traits

To evaluate the potential association between haplotypes and coat colour, the Haploview 4.2 software was used to construct the haplotypes of the seven missense mutation alleles of *MLPH*. Ten haplotypes were found for the seven mutant SNPs in the population of two Rex rabbit strains (Table 6). In theory, if there is no linkage between the loci, 128 (2^7) haplotypes should be produced at seven mutation alleles. It indicates that these loci may be in tight linkage disequilibrium. The H1 and H2 haplotype were the main haplotypes found in all Rex rabbits. The major

Table 3: Allele frequency and genotype frequency in rabbits with different coat colors.

			genotype frequency			Allele frequency					
Exon	SNPs	color	CC	CA	AA	C	A	He	χ^2	PIC	HW
5	g.606C>A	Ch	0.20	0.80	0.00	0.60	0.40	0.48	7.200	0.365	8.889
		Bl	0.90	0.10	0.00	0.95	0.05	0.095	12.800	0.090	0.055
			TT	TC	CC	T	C				
	g.610T>C	Ch	0.10	0.55	0.35	0.375	0.625	0.469	6.100	0.359	0.600
		Bl	0.20	0.35	0.45	0.375	0.625	0.469	1.900	0.359	1.284
			CC	CG	GG	C	G				
	g.642C>G	Ch	0.10	0.55	0.35	0.375	0.625	0.469	6.100	0.359	0.600
		Bl	0.20	0.50	0.30	0.45	0.55	0.495	2.800	0.372	0.002
8			AA	AG	GG	A	G				
	g.894A>G	Ch	0.00	0.5	0.5	0.25	0.75	0.375	0.000	0.305	2.222
		Bl	0.20	0.45	0.35	0.425	0.575	0.489	1.900	0.369	0.126
			TT	TC	CC	T	C				
	g.905T>C	Ch	0.00	0.5	0.5	0.25	0.75	0.375	0.000	0.305	2.222
		Bl	0.20	0.50	0.30	0.45	0.55	0.495	2.800	0.372	0.002
			GG	GC	CC	G	C				
	g.941G>C	Ch	0.00	0.6	0.4	0.3	0.7	0.42	0.800	0.332	3.673
		Bl	0.25	0.55	0.20	0.525	0.475	0.499	4.300	0.374	0.211
			AA	AG	GG	A	G				
	g.953T>C	Ch	0.00	0.6	0.4	0.3	0.7	0.42	0.800	0.332	3.673
		Bl	0.25	0.55	0.20	0.525	0.475	0.499	4.300	0.374	0.211
9			AA	AG	GG	A	G				
	g.1067A>G	Ch	0.20	0.75	0.05	0.575	0.425	0.489	16.300	0.369	5.714
		Bl	0.40	0.40	0.20	0.600	0.400	0.480	1.600	0.365	0.556
			CC	CT	TT	C	T				
	g.1095C>T	Ch	0.30	0.65	0.05	0.625	0.375	0.469	10.900	0.359	2.990
		Bl	0.60	0.25	0.15	0.725	0.275	0.399	6.700	0.319	2.783
12			CC	CG	GG	C	G				
	g.1440G>A	Ch	0.10	0.55	0.35	0.375	0.625	0.469	6.100	0.341	0.600
		Bl	0.20	0.55	0.25	0.475	0.525	0.499	4.300	0.373	0.211
			GG	GA	AA	G	A				
	g.1462G>A	Ch	0.15	0.50	0.35	0.40	0.60	0.480	3.700	0.365	0.035
		Bl	0.15	0.55	0.30	0.425	0.575	0.489	4.900	0.369	0.314
			AA	AG	GG	A	G				
	g.1463A>G	Ch	0.15	0.50	0.35	0.40	0.60	0.480	3.700	0.365	0.035
		Bl	0.20	0.55	0.25	0.475	0.525	0.499	4.300	0.373	0.211
			AA	AG	GG	A	G				
	g.1482A>G	Ch	0.15	0.50	0.35	0.40	0.60	0.480	3.700	0.365	0.035
		Bl	0.20	0.55	0.25	0.475	0.525	0.499	4.300	0.373	0.211

He: expected heterozygosity. X^2: chi-square value. PIC: polymorphism information content, a measure of the amount of information that a genetic marker polymorphism can provide in linkage analysis. HW: Hardy-Weinberg equilibrium. Ch: Chinchilla Rex rabbit. Bl: Black Rex rabbit.

Table 4: Correlation analysis of 7 missense mutations in each Rex rabbit.

SNP locus	Ho	He	HW (*P*)	Minimum allele frequency	Allele	r^2
g.610T>C	0.417	0.483	0.3849	0.408	C:T	1.0
g.905T>C	0.483	0.477	1.0	0.392	C:T	0.842
g.941G>C	0.533	0.495	0.7842	0.45	C:G	0.935
g.953T>C	0.533	0.491	0.7357	0.433	C:T	1.0
g.1067>G	0.533	0.495	0.7842	0.45	A:G	1.0
g.1462G>A	0.483	0.497	0.9908	0.458	A:G	0.935
g.1482A>G	0.483	0.499	0.9633	0.475	G:A	1.0

haplotypes of the Chinchilla and black Rex rabbit were H1/H2/H3/H4/H5 and H1/H2/H3/H6/H8, respectively. The special haplotypes of Chinchilla Rex rabbit (H4, H5, H7) were consistently associated with the Chinchilla phenotype.

As shown in Figure 4, there were 8 SNP loci where r^2=1, which indicates complete linkage. The r^2 of other loci were all greater than 0.8. This further suggests that these seven loci are in a tight linkage disequilibrium state.

DISCUSSION

There are different types and amounts of melanin pigmentation in animal fur, which affects the formation of differing skin and coat colours. There are numerous genes that regulate the deposition of melanin, including *TYR*, *MITF*, *KIT*, and *MC1R*. Among them, the *MLPH* gene is a candidate gene that plays an important role in the formation of mammalian coat colour and has been studied in cats, dogs, and mink. Ishida *et al.* (Ishida *et al.*, 2006) found that sequence analysis in dilute cats identified a single base pair deletion in exon 2 of *MLPH* transcripts that introduces a stop codon 11 amino acids downstream. This resulted in the truncation of the bulk of the *MLPH* protein. Philipp *et al.* (2005) found that a set of SNPs near exon 2 were identified that were highly significantly associated to the dilute phenotype. Bauer *et al.* (2018) identified *MLPH*:c.705G>C as a variant explaining a second canine dilution allele. In a study of minks of violet and silver-blue colour, Cirera *et al.* (2013) found that a phenotypic deletion in *MLPH* on exon 8 of the silver-blue mink resulted in the deletion of myosin VA (*MYO5A*) binding domain. This affected the transport of melanosomes and melanoma and resulted in a silver-blue phenotype.

In the present study, we sequenced 1689 bp of the entire CDS region of the Rex rabbit *MLPH* gene, and 13 SNPs were found in 15 exons. Lehner *et al.* (2013) found that, in both colour diluted rabbits (Netherland Dwarf and Loh), the skipping of exons 3 and 4 was present. This resulted in altered amino acids at p.QGL(Reed, 1989; Krawczak *et al.*, 1992; Roscigno *et al.*, 1993) QWA and a premature stop codon at p.K40*. Additionally, Fontanesi *et al.* (2014) sequenced 6357 bp of the *MLPH* gene in 18 rabbit breeds. There were seven missense mutations and six

Table 5: Mutation types of 13 SNPs.

Exon	SNPs	Mutation type	Representation
5	g.606C>A	silent	p.Ser202=
	g.610T>C	missense	p.Trp204Arg(W204R)
	g.642C>G	silent	p.Ser214=
8	g.894A>G	silent	p.Gly298=
	g.905T>C	missense	p.Leu302Pro(L302P)
	g.941G>C	missense	p.Gly314Ala(G314A)
	g.953T>C	missense	p.Val318Ala(V318A)
9	g.1067A>G	missense	p.Lys356Arg(K356R)
	g.1095C>T	silent	p.Val365=
12	g.1440C>G	silent	p.Val480=
	g.1462G>A	missense	p.Asp488Ser(Codon 1)(D488S)
	g.1463A>G	missense	p.Asp488Ser(Codon 2)
	g.1482A>G	silent	p.Pro494=

Figure 3: Alignment of *MLPH* proteins from different species. The *MLPH* protein sequences were translated from nucleotide database accessions [NP_001284414.1 GI: 661902992] (rabbit), [AKI70657.1 GI: 823670892] (human), and [NP_443748.2 GI: 87080831] (mouse), respectively. The three major predicted protein domains of *MLPH* are indicated in accordance with EBI (https://www.ebi.ac.uk/Tools/msa/clustalo/). The big difference between the sequences is caused by the fact that rabbit is lacking a homologous exon to human exon 9. In human this exon is not used constitutively and for the alignment a protein isoform without the amino acids encoded by this alternative exon was used. Polymorphisms that affect the amino acid sequence of the rabbit *MLPH* protein are indicated with arrows.

Table 6: Haplotypes and haplotype frequencies in each Rex rabbit for 7 missense mutation loci.

Colour	Name	Haplotype	Haplotype frequency	Colour	Name	Haplotype	Haplotype frequency
CH	H1	CCCCGAG	0.450	BL	H1	CCCCGAG	0.400
	H2	TTGTAGA	0.250		H2	TTGTAGA	0.350
	H3	CCCCAAG	0.150		H3	CCCCAAG	0.075
	H4	TCCCAGA	0.075		H6	CTGTAGA	0.075
	H5	TCGTAGA	0.050		H8	CCGTAAG	0.050
	H7	CCCCGGA	0.025		H9	CCGTAAA	0.025
					H10	TTGTAAA	0.025

CH: Chinchilla Rex rabbit; BL: Black Rex rabbit.

synonymous mutations found in this experiment. These results were not consistent with those previously reported. It is speculated that differences in variety and breeding quality may lead to the divergence in SNP loci. Interestingly, we found that the g.1462G>A and g.1463 A>G SNPs are the first and second nucleotides of the same codon (Table 2). There is a strong possibility that their transposition led to an amino acid change from Asp to Ser. Except for the g.606C>A SNP in Chinchilla Rex rabbits, all other loci were in Hardy-Weinberg equilibrium. This indicates that this population of two Rex rabbit strains was not susceptible to external disturbances in the selection. After a long period of evolution, selection reached equilibrium, and the seven missense mutations could provide reasonable genetic information. Under the dual action of artificial and natural selection, some loci of Rex rabbits have been selected and mutated. The genetic structure of the Rex rabbit genome has been mutated to a certain extent, which leads to increased homogeneity of the group. These unbalanced loci can be used as marker genes. When this is combined with the different production traits of Rex rabbits and screening for exceptional individuals, it could provide a basis for the breeding of Rex rabbits.

The genomic structure of the *MLPH* gene is similar but not identical in rabbit, human and mouse. Differences were observed with respect to the rabbit exon 8 and exon 9, which is lacking from other species and the human/mouse exon 9 that could not be identified within the genomic rabbit sequence by sequence comparisons. The dog *MLPH* protein is also lacking a homologous exon to human exon 9. All the experimental rabbit cDNA sequences obtained in this study lacked a corresponding sequence. This substitute exon may not be conserved in the rabbit gene due to the presence of a known splice variant in human lacking exon 9. An alternative explanation would be that the homology between the human and rabbit exon 9 is very low, so that it cannot be identified by cross-species sequence comparison.

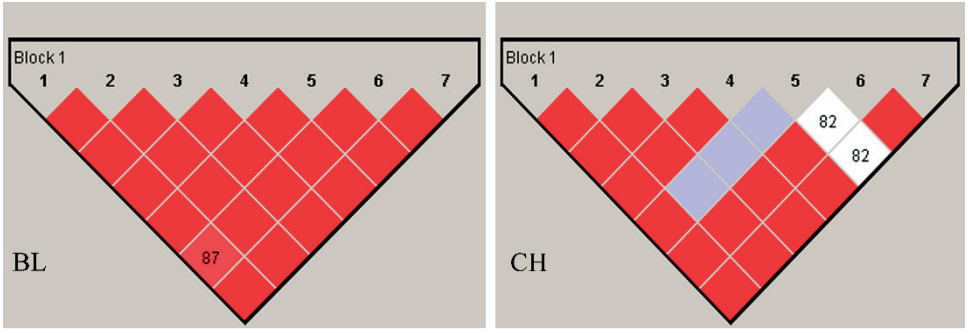

Figure 4: Haploview map in linkage disequilibrium (LD) mode of seven missense mutations. The colour of the LD map ranges from light to dark (white to red) to indicate a degree of linkage from low to high, respectively, and deep red indicates complete linkage (r^2=1). The value of r^2 indicates if one locus can reflect the degree of information of another locus, and r^2=1 is considered a perfect LD. At this time, only one mark is observed to provide all of the information of another mark. BL: Black Rex rabbit; CH: Chinchilla Rex rabbit.

Construction of haplotypes requires a linkage disequilibrium analysis. If two SNPs are not in linkage disequilibrium, then they are independent of each other and do not affect each other, and thus, constructing a haplotype is meaningless. Linkage disequilibrium is commonly used for r^2 or D', and the magnitude of r^2 is related to the efficacy of association analysis. Studies have shown that if $r^2>0.33$, then the two markers of the constructed haplotype can be considered to be closely linked and can be inherited as a whole (Ardlie *et al.*, 2002; Shifman *et al.*, 2003) .

The current study indicates that the above seven SNPs were consistent with an $r^2>0.33$, and they were in linkage disequilibrium. It is speculated that the seven missense mutations and seven major haplotypes are important functions to distinguish the Chinchilla, black Rex rabbits, and provide valuable molecular markers for directional breeding of Rex rabbits.

CONCLUSION

We characterised the rabbit *MLPH* gene and identified 13 polymorphisms of this gene that occur in two strains of Rex rabbits. We obtained 10 haplotypes, and the main haplotypes of the two Rex rabbit populations were different. The special haplotype of Chinchilla Rex rabbit (H4, H5, H7) was consistently associated with the Chinchilla phenotype. The coat colour formation may be caused by one or more mutations within or near the *MLPH* gene in several Rex rabbit strains.

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