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Coat colour phenotype of Qingyu pig is associated with polymorphisms of melanocortin receptor 1 gene

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Objective: Qingyu pig, a Chinese indigenous pig breed, exhibits two types of coat colour phenotypes, including pure black and white with black spotting respectively. Melanocortin receptor 1 (*MC1R*) and agouti signaling protein (*ASIP*) are two widely reported pivotal genes that significantly affect the regulation of coat colour. The objectives of this study were to investigate whether the polymorphisms of these two genes are associated with coat colour and analyze the molecular mechanism of the coat colour separation in Qingyu pig.
Methods: We studied the phenotype segregation and used polymerase chain reaction amplification and Sanger sequencing to investigate the polymorphism of *MC1R* and *ASIP* in 121 Qingyu pigs, consisting of 115 black and 6 white with black spotted pigs.
Results: Coat colour of Qingyu pig is associated with the polymorphisms of *MC1R* but not *ASIP*. We only found 2 haplotypes, E^{QY} and E^{qy}, based on the 13 observed mutations from *MC1R* gene. Among which, E^{qy} presented a recessive inheritance mode in black spotted Qingyu pigs. Further analysis revealed a *g.462-463CC* insertion that caused a frameshift mutation and a premature stop codon, thus changed the first transmembrane domain completely and lost the remaining six transmembrane domains. Altogether, our results strongly support that the variety of Qingyu pig's coat colour is related to *MC1R*.
Conclusion: Our findings indicated that black coat colour in Qingyu pig was dominant to white with black spotted phenotype and *MC1R* gene polymorphism was associated with coat colour separation in Qingyu pig.

Keywords: Qingyu Pig; Coat Colour; Melanocortin Receptor 1 (*MC1R*); Agouti Signaling Protein (*ASIP*); Polymorphism

INTRODUCTION

Coat colour phenotype is one of the most obvious morphological traits in animals that is linked with concealment, communication, and regulation of physiological processes [1]. In pigs, there are several varieties of coat colours including solid black (Large Black and Laiwu), solid white (LargeWhite and Landrace), red (Duroc) and brown (Dahe), belted (Hampshire), and spotted (Pietrain and Rongchang), etc.

Hitherto, more than 350 genes are reported to be involved in pigmentation, such as tyrosinase-related protein 1 (*TYRP1*), KIT ligand (*KITLG*), and OCA2 melanosomal transmembrane protein (*OCA2*) that can directly or indirectly affect the production or regulation of two pigments pheomelanin (yellow to reddish brown) and eumelanin (black to brown) in mammals [2]. These two melanins are produced by melanocytes resident in skin and the proportions of which result in different coat colour [3]. Melanocortin receptor 1 (*MC1R*) and its peptide antagonist agouti signaling protein (*ASIP*) are two major genetic determinants of pigment phenotype in mammals [1,4,5]. When a-melanocyte-stimulating hormone (a-MSH) binds to MC1R, MC1R signaling

is activated, eumelanin synthesis is induced, and dark colours are produced; otherwise, pheomelanin is generated alone and lighter colours are produced in the absence of MC1R signaling [6]. To date, mutations in *MC1R* related to coat colour have been documented in a number of domestic mammals [7-9]. ASIP, a high-affinity antagonist of MC1R by nullifying the action of melanotropin alpha (a-MSH) [10], can influence MC1R signaling to determine coat colour in mammals, such as donkey [11], sheep [12], and dog [13].

Qingyu pig is a famous indigenous pig breed in China, which mainly lives in the mountainous areas of Sichuan province. This breed is characterized by its solid black coat colour, however, there are also some Qingyu pigs with white with black spotting. Up to now, many researches on coat colour variety of Chinese indigenous pigs have been performed [14-16], but there are no studies on the coat colour separation of Qingyu pig. To explore how the white with black spotted coat colour was formed in Qingyu pig, we documented and analyzed the phenotypes of this pig breed, investigated the variability of *MC1R*, *ASIP*, and analyzed their possible association with the white with black spotting coat colour.

MATERIALS AND METHODS

Animals and sampling

All experimental procedures involving animals were approved by the Animal Care and Use Committee of College of Animal Science and Technology, Sichuan Agricultural University, and were carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Qingyu pigs we studied were from a Qingyu pig nucleus farm in Bazhong County, Sichuan Province, China. The phenotypes of 121 piglets were collected. These piglets were from mating of 7 black boars with 10 black sows. Ear tissues of all these piglets were sampled in this study. The 121 ear samples were prepared and preserved in 75% ethanol at –20°C. Genomic DNA was extracted from ear samples with the Tiangen Genomic DNA Kit (Beijing, China).

Polymerase chain reaction amplification and Sanger sequencing

To detect the potential mutations in the exons along with partial region sequences, primer pairs were designed by the program Primer 5.0 based on the published sequences of Sus scrofa *MC1R* (AF326520) and *ASIP* (AJ427478), respectively (Table 1, Figure 2A, 2B). The 25 μL polymerase chain reaction (PCR) reaction system contained 2 μL DNA (approximately 25 ng/μL), 1 μL 10 pmol/L forward primer, 1 μL 10 pmol/L reverse primer, 12.5 μL 2×Taq PCR Mastermix (TIANGEN BIOTECH, Beijing, China) and 8.5 μL ddH_2O (double-distilled water). PCR amplifications were performed on a PTC-200 Programmable Thermal Controller (MJ Research Inc., Waltham, MA, USA) by the following steps: 95°C for 3 min, 35 cycles of amplification including 30 s at 95°C, annealing at the Tm (Table 1) for 30 s and 1 min at 72°C, and finally 72°C for 5 min. The products were detected by electrophoresis on 1.5% agarose gel, ethidium bromide stained and then purified. The qualified PCR products were directly sequenced by an ABI 3730XL DNA analyzer (Applied Biosystems, Carlsbad, CA, USA) using the BigDye Direct Cycle Sequencing Kit.

Table 1. PCR primers and conditions used for detecting mutations of *MC1R* and *ASIP*

Primer name	Primer sequence	Primer binding region	Size (bp)	T_m (°C)
MC1R-1F	GCTGAGCACAGGCGAGGTTG	5'UTR	885	62
MC1R-1R	AGGAAGCAGAGGCTGGACAC	Exon1		
MC1R-2F	CGCCAAGAACCGCAACCTG	Exon1	901	62
MC1R-2R	GTCCAGCGTCCATACCTTCAG	3'UTR		
ASIP-1F	GTAGCAGTCGGAGCTGAAATC	Intron1	409	58
ASIP-1R	CGAATGTTCTTCTCACCTTGC	Intron2		
ASIP-2F	CGCCTTCTTAGATTTCCCCTTTG	Intron2	437	58
ASIP-2R	CGCCAGGAAGTTTTTTGGTAGC	Intron3		
ASIP-3F	ACAGGCAGAGGTGAGGAAGAGTG	Intron3	702	57
ASIP-3R	GGAAAGGGTGAAAGGCTGAATG	3'UTR		

PCR, polymerase chain reaction; *MC1R*, melanocortin receptor 1; *ASIP*, agouti signaling protein.

Data analysis

Nucleic acid and protein database searching were performed using BLAST at NCBI. DNA sequence data were analyzed by DNASTAR 7.1 software. Haplotype combinations of *MC1R* were constructed with PHASE 2.1.1 software [17]. TMpred was utilized to predict the protein domains of MC1R. Phenotypes separation analysis, the frequencies of *MC1R* allele and genotype, and chi square test of the association between phenotypes and genotypes were performed using SAS Software (version 9.2, USA).

RESULTS AND DISCUSSION

Phenotypes separation of Qingyu pig

Phenotypes separation analysis can define the inheritance of the main coat colour phenotypes [18]. In the nucleus farm of Qingyu pig, all boars and sows are solid black coat colour, while there are black and white with black spotting pigs in the descendants (Figure 1). The same coat colour polymorphism had been documented in Banna Mini-pig [19]. Among all the pigs in the farm, we found the phenotype of white with black spotted just existed in pigs from two litters (Table 2), and offspring from other litters were all black phenotype. According to the phenotypes distribution of Qingyu pig, we can determine that the black phenotype was completely dominant over white with black spotted coat colour and infer that the inheritance of Qingyu pigs' coat colour may result from a single gene segregation.

Mutations of *ASIP* in Qingyu pig

Figure 1. The phenotypes of white with black spotted (right) and solid black (left) Qingyu pigs.

ASIP was documented as an important gene associated with coat colour [20]. Sus scrofa *ASIP*, including three exons, two introns and parts of the 5'UTR and 3'UTR, was established (Figure 2A). Primers were set to amplify these three agouti exons, as well as parts of the intronic flanking regions to identify the mutations of *ASIP*. Six SNPs, two in introns (*g.379G>A* and *g.5250G>A*) and four in exons (*g.133A>G*, *g.257G>A*, *g.5003G>A*, and *g.5049C>T*), were detected in Qingyu pigs (Figure 2A). Among all these mutations, only one nucleotide substitution *g.257G>A* (p.V53M) in coding sequence altered the encoded amino acid. Herein, the Met53 allele was found in both solid black and white with black spotted Qingyu pigs, which had also been documented in brownish red, blond and black Chinese indigenous breeds [16,21]. Apparently, we can exclude the *g.257G>A* mutation as a causative mutation for the coat colour variety of Qingyu pig. Though no mutations changed the function of *ASIP* protein, it is worthwhile to further analyse the regulatory region of *ASIP* gene, since a regulatory mutation in *ASIP* has been documented to affect the phenotype in mammals [22,23].

Table 2. Phenotypes separation in Qingyu pig

Boar[1)]	Sow[1)]	n	Offspring			
			Black		White with black spotting	
			♀	♂	♀	♂
114	170	10	3	5	2	0
87	192	13	6	3	1	3
Totel		23	9	8	3	3

[1)] Number of the boars and sows indicated the ID of the pigs.

The correlation between polymorphisms of *MC1R* and coat colour separation in Qingyu pig

MC1R is a critical gene known to be a major determinant of pig coat colour and is known for its role in regulating the change between eumelanin and pheomelanin biosynthesis pathways in mammals [24,25]. It has been documented that polymorphisms of *MC1R* determined different coat colour in numerous studies [26,27]. Because *MC1R* variant has been associated with the

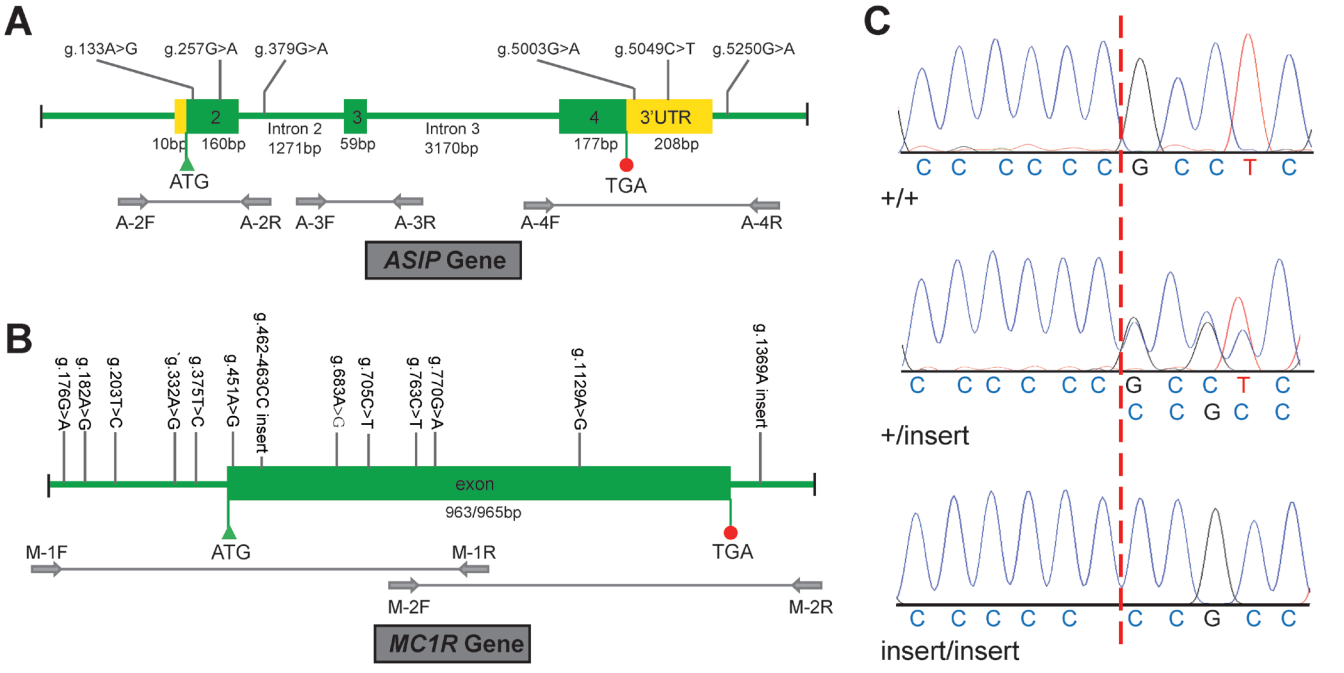

Figure 2. Mutations of *MC1R* and *ASIP* in Qingyu pig. (A) The structure and mutations of *MC1R* in Qingyu pig. (B) The structure and polymorphisms of *ASIP* in Qingyu pig. (C) Sequencing results of each genotype at *g.462-463CC* insert locus of *MC1R* gene. Green boxes indicate protein coding sequences, while yellow boxes stand for 5'UTR or 3'UTR. The start codon (ATG) and the stop codon (TGA) are indicated by the green triangle (▲) and red circle (●) symbols, respectively. Numbers below the gene structure indicate the length of exons and introns in bp. *MC1R*, melanocortin receptor 1; *ASIP*, agouti signaling protein.

Table 3. Haplotypes and mutations in Qingyu pig

Haplotype	Alleles												
	176	182	203	332	375	451	462-463	683/685	705/707	763/765	770/772	1129/1131	1369/1371
E^{QY}	G	A	T	A	T	A	-	A	C	C	G	A	-
E^{qy}	A	G	C	G	C	G	CC	G	T	T	A	G	A

Table 4. The distribution of *MC1R* haplotypes and genotypes in Qingyu pig

Phenotype	n	Haplotype frequency[1)]		Genotype (No./freq)			x^2
		E^{QY} (%)	E^{qy} (%)	$E^{QY}E^{QY}$ (%)	$E^{QY}E^{qy}$ (%)	$E^{qy}E^{qy}$ (%)	
White with black spotting	6	0	100	0/0	0/0	6/100	121**
Black	115	83.48	16.52	77/66.96	38/33.04	0/0	

MC1R, melanocortin receptor 1.
x^2: Chi square value, df = 2, x^2 = 5.99, p = 0.05, x^2 = 9.21, p = 0.01. ** Meant the extremely significant level.
[1)] The haplotype E^{qy} owned *g.462-463CC* insert but E^{QY} did not.

Japanese brindling coat colour in rabbits [28], we sequenced the exon along with flanking regions of *MC1R* gene of these two litters to elucidate the genetic mechanisms behind coat colour separation of Qingyu pig. A total of 13 mutations have been detected (Figure 2B). We observed 7 nucleotide substitutions (*g.451A>G*, *g.462-463CC* insertion, *g.683A>G*, *g.705C>T*, *g.763C>T*, *g.770G>A*, *g.1129A>G*) occurred in the coding region and 6 polymorphisms (*g.176G>A*, *g.182A>G*, *g.203T>C*, *g.332A>G*, *g.375T>C*, *g.1369A* insertion) were distributed in the flanking regions. We analyzed the haplotypes of *MC1R* utilizing the polymorphisms both in exon and introns, and found two longer haplotypes, E^{QY} and E^{qy}, from these 13 closely linked mutations (Table 3). To further determine the haplotypes, we sequenced *MC1R* of 98 solid black Qingyu pigs from another 8 litters and the same mutations and haplotypes were detected. Then, we analyzed the *MC1R* genotypes of all these 121 Qingyu pigs. The distribution of *MC1R* genotypes was significantly different between black and spotted Qingyu pigs (p<0.01). All the 6 white with black spotted Qingyu pigs were $E^{qy}E^{qy}$ genotypes with no E^{QY} haplotype, while E^{QY} occurred in all the 115 black pigs (Table 4). Hence, we inferred that the phenotype separation of Qingyu pig was associated with *MC1R* gene and E^{qy} presented a recessive inheritance mode in black spotted Qingyu pigs. Interestingly, we found that the *g.451A>G* mutation was a synonymous mutation whereas *g.462-463CC* insertion caused a frameshift mutation which both changed the amino acids after 23rd codon and generated a premature stop codon at position 56 (Figure 2C, Figure 3). MC1R, a known major determinant of pigment phenotype, is

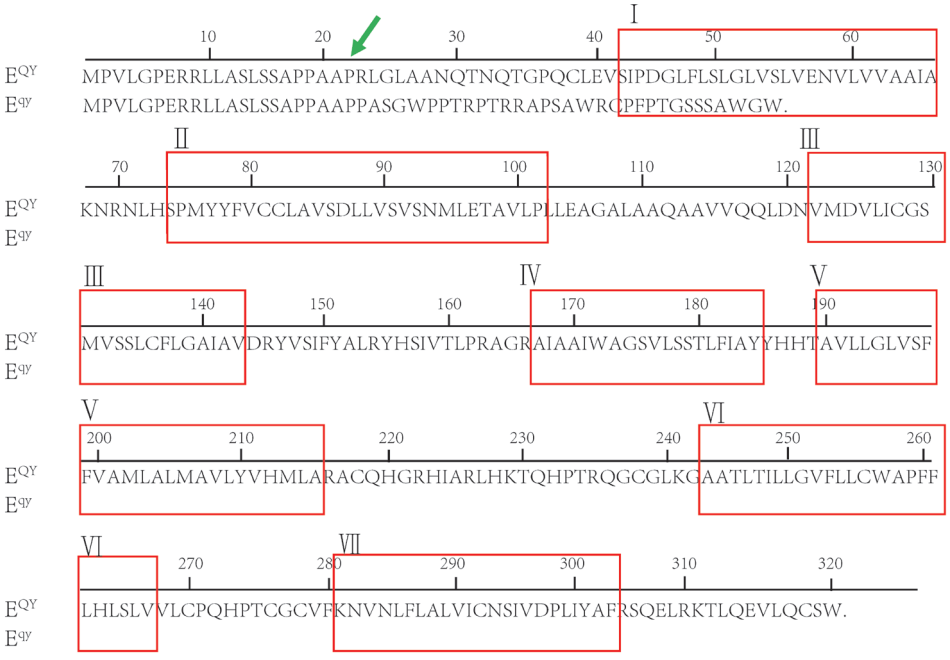

Figure 3. Amino acid sequence alignment of *MC1R* variants in Qingyu pig. The seven transmembrane domains are indicated in boxes and denoted with roman numerals. The arrow is used to sign the beginning of amino acid changes. *MC1R*, melanocortin receptor 1.

a seven-transmembrane G-protein-coupled protein [29]. Further analysis revealed that the insertion mutation caused a complete change of the first transmembrane domain, and deletion of the remaining six transmembrane domains (Figure 3). The *CC* insertion was supposed to produce a red pigmentation caused by the complete loss of MC1R signaling. However, the observed phenotypes of the *CC* insertion mutation were highly diverse and the coat colour can range from white, red with black spotted, white with black spotted to almost solid black [25,30,31]. It has been documented that the *CC* insertion was unstable in organisms and the somatic reversion events restored the frame and function of MC1R, resulted in black spotted [32]. Since there was no evidence to explain the formation of the white background of coat colour, we can just infer that it may be produced by the absence of melanocytes or modified by other genes [33]. In general, the coat colour segregation of Qingyu pigs is related to *MC1R*.

CONCLUSION

MC1R gene played a significant role in regulation of coat colour variety. Meanwhile, not only gene verification but also phenotype separation suggested that the white with black spotted phenotype was recessive to black coat colour in Qingyu pig. Mutations were detected both in exon and introns of *MC1R* gene, and we found 2 haplotypes, E^{QY} and E^{qy}, from 13 closely linked mutations of *MC1R*. Moreover, the mutations in *MC1R* can be used as diagnostic makers for Qingyu pig breeding to clear the coat colour of white with black spotting. In summary, the study advanced our understanding of the molecular basis of coat colour variation in Qingyu pig.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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