Indian J. Anim. Res., 53 (3) 2019: 288-293
Print ISSN:0367-6722 / Online ISSN:0976-0555

AGRICULTURAL RESEARCH COMMUNICATION CENTRE
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The mutations within *MC*1*R*, *TYRP*1, *ASIP* genes and their effects on phenotypes of coat color in wild pigs (*Sus scrofa ussuricus*)

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Received: 23-07-2017 Accepted: 27-05-2018 DOI: 10.18805/ijar.B-804

ABSTRACT

Animal coloration is a powerful model for studying the genetic mechanisms that determine animal phenotypes. But, there has not been comprehensive characterization of the molecular basis of the complex patterns of coat color phenotype variation in wild boars. This study results indicated that the wild-type allele E^+ of the *MC*1*R* gene was a dominant allele in wild boars and was not responsible for black, brown or other coat color phenotypes. A novel mutation c.695 T > C was identified in the 3′-UTR of the *ASIP* gene. The association analysis showed that the *C* mutation allele was highly significantly associated with wild-type coat colors between wild boars and Western pig breeds (*P*=1.35E-33). A non-synonymous g.2254 G > A substitution was found in exon 2 of the *TYRP*1 gene (p.143His>Arg). The association analysis demonstrated that the G mutation allele was also significantly associated with wild-type coat colors between wild boars and Western pig breeds (*P* = 5.09E-10). In short, a few mutation sites in *MC*1*R*, *ASIP*, and *TYRP*1 genes were identified and surveyed several polymorphisms molecular variations in Chinese wild boars. In our identified mutations have caused the morphological diversity in wild boars, but did not influence coat color phenotype variation in some domesticated pig breeds. The conclusion was obtained that some mutations in color-associated genes were associated with wild-type coat colors in wild boar population, and that similar coat colorations observed in domesticated pig and wild boars can be the product of underlying differences in the genetic basis of color variants.

Key words: *ASIP*, Coat color phenotype, *MC*1*R*, SNPs, *TYRP*1, Wild boar.

INTRODUCTION

Animal coloration is a powerful model for studying the genetic mechanisms that determine animal phenotypes (Hubbard *et al*., 2010). Analyzing phenotypic traits determined by ecological and evolutionary factors in natural populations is a fundamental goal of evolutionary genetics. Moreover, understanding the link between genotypes and phenotypes can elucidate mechanisms that shape phenotypic variation within populations and how these affect patterns of evolutionary change (Hubbard *et al*., 2010). Therefore, establishing such links can shed light on the origin and maintenance of phenotypic variation in wild and domesticated animals, and can provide insights into the history of domestication (Fang *et al*., 2009; Ludwig *et al*., 2009).

The wild boar (*Sus scrofa*) is an important resource. However, they have to face seasonal changes in temperature, food availability, and colors in their surroundings. During winter their fur is much denser. The color usually varies from dark grey to black or brown, but there are great regional differences in color. Domestication may have played a major role in numerous morphological, physiological and behavioral changes (Andersson *et al*., 2001). Among these, coat color seems to be an obvious change. Whether the genetic mechanisms of coat color phenotypes are shared or not between domestic pigs and their wild ancestors is still unknown. However, to date, there has not been comprehensive characterization of the molecular basis of the complex patterns of coat color phenotype variation in wild boars.

In a large number of mammalian species, the coat color diversity is mainly determined by the relative amount of two basic melanins; i.e., eumelanin (black/brown) and phaeomelanin (yellow/red) in which are genetically controlled by the *Extension* (*E*) and *Agouti* (*A*) loci, respectively (Searle., 1968). Dominant *Extension* alleles determine eumelanin production and black coat color, whereas recessive alleles determine red/yellow/pale pigmentation due to pheomelanin synthesis. The presence of wild-type *Extension* alleles is usually needed for the expression of the *Agouti* allele. Moreover, tyrosinase (*TYR*) is required for melanization in both types of melanosomes, whereas the tyrosinase-related protein 1 (*TYRP*1) is exclusive to the melanization of eumelanosome (Sturm *et al*., 1995). A notable fact is the conserved roles of the *MC*1*R*, *ASIP*, and *TYRP*1 genes in mammalian pigmentation. We therefore selected *MC*1*R*, *TYRP*1, and *ASIP* as candidate genes and revealed mutations in these coat color genes, the mutations

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were analyzed and considered their influence on complex phenotype patterns that occur in different coat color phenotypes in wild boar populations.

MATERIALS AND METHODS

A total of 162 ear tissue samples (72 Wild boars, 30 Duroc, 30 Large White, and 30 Landrace) were collected from the wild boar population and three Western pig breeds. All experimental individuals were not in the same kinship. Coat color phenotype traits of all animals were recorded by direct visual inspection and photographs were taken outdoors. The samples were stored at -20°C untill isolation of genomic DNA, which was extracted from either tissue samples by a standard phenol/chloroform extraction method or a DNA extraction kit (Tiangen, China).

To detect the polymorphisms in the coding region of the *MC*1*R*, *TYRP*1, and *ASIP* genes, two primer pairs (Table 1) were designed to amplify two overlapping 835 and 878-bp fragments covering the entire *MC*1*R* coding region and partial flanking sequence (GenBank accession number: AF326520), six intron primer pairs (Table 1) in flanking of the *TYRP*1 gene exons 2–7 and one primer pair in the 3'-untranslated region (3' -UTR) from publicly available website sequences (ENSSSCT00000005725, NM_001025226, and NC_010443.1). Also, six primer pairs (Table 1) were used to amplify genomic regions covering the complete *ASIP* open reading frame (GenBank accession numbers: AJ427478). Mutation sites were identified by directly sequencing with DNA from 15 wild boar individuals with different coat colors as a template. Amplifications were performed on Eppendorf Mastercycler Thermal Cycler (Eppendorf, Germany) in a total volume of 25 µL, containing approximately 50 ng of genomic DNA, 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L Tris-HCl, 0.2 mmol/L of dNTP, 2.5 units of Taq DNA polymerase (Biocolor, China), 10 pmol of each primer. After pre-denaturation for 3 min at 95°C, 35 cycles of a denaturation step at 94°C for 30 s, an annealing step at optimal temperatures for 30 s (Table 1), and an elongation step at 72 °C for 45 s were followed with a final extension of 10 min at 72°C. The products were purified with a QIAquick PCR Purification Kit (Qiagen, Germany) and were subsequently bidirectionally sequenced with the respective PCR primers. The SNPs were identified by comparison of the obtained sequences using DNAstar (DNAStar, USA). Some identified SNPs were chosen for genotyping by a sequencing method in all sample of animals.

A *KpnI* PCR-RFLP assay was used to test the *TYRP*1 c.1484–1489del mutation (Ren *et al*., 2011). Genomic DNA was amplified with *TYRP*1-exon7 primer as described above. PCR products were digested with *KpnI* (NEB, USA) at 37°C for 4 h. The restriction fragments were separated on 2% agarose gels and genotypes were determined from the resulting band patterns. The association analysis between gene mutation sites and coat color phenotypes were performed using crosstabs with a Fisher's exact test implemented in the descriptive statistics procedure of SPSS version 19.0 (SPSS, USA).

RESULTS AND DISCUSSION

The whole coding region (CDS, 954 bp) and parts of the 5'- and 3'-untranslated regions (38 and 284 bp, respectively) of the *MC*1*R* gene were amplified and sequenced in 15 wild boar individuals. After analyzing and comparing the obtained sequence electropherograms, a total of nine SNPs were identified in the *MC*1*R* gene, of which two nucleotide substitutions, g.402 G > A and g.410 G > A, were found in the 5'-UTR. The two mutation SNPs in the 5' -UTR were not previously reported. The g.1110 G > A, 1197 G > A, 1318 C > T and 1554 G > A resulting in Val95 Met, Asp124Asn, Ala164Val, and Ala243Thr in the coding region of the wild boars were found after sequencing, respectively. Ala164Val and Ala243Thr corresponded with Ala160Val and Ala240Thr, which was reported by Kijas *et al*. (1998). Two silent mutations (g.1190 T > C, g.1556 G > A) were identified at 121(Asn) and 243(Ala) codon positions. Interestingly, one amino-acid replacement (g.1135 T > C, p.Leu102Pro) was

Table 1: Primer name, primer sequence, optimal annealing temperatures (Tm) and amplicon sizes.

Primer name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Size/bp	Tm/°C
MC1R-Exon-1	AGGCAGGGGTGTCTCTGTGTC	GCCAGGCAGCAGACGAAGTAG	835	65
MC1R-Exon-2	TCGCCCATGTACTACTTCG	GTCCAGCGTCCATACCTTC	878	57.1
TYRP1-Exon1	CCTGCTAATGAAGGCTTTTGA	TAGTGGGAAATGTCTTTGGGG	1175	55
TYRP1-Exon2	GGCATTACAAGACCATTAGGC	GTTCTATCCCTTGCCTTGCTC	1135	63
TYRP1-Exon3	GGTGGAAATGCCGAGTAGAAG	TTAGGTTGCCCAGACTATGCC	983	60
TYRP1-Exon4	TAGTCTTTCAGGGCTCTTTGC	TTCACATCAACTGACAGGCTC	1087	63
TYRP1-Exon5	AGCCTTTTCCTGCTCTTCCTT	AAGTGCTTAGCCTCAACGGAT	1190	63
TYRP1-Exon6	GATGAGAGGCAATAGGAAACA	ATCTACTCTCTCTGTCCCCCA	1167	61.2
TYRP1-Exon7	TGGGCTCCAAAACCAACAGTA	TATCTCTCACCCCTTCCCCAC	962	63
ASIP-Exon1	GAACACCACCATACACTTTGC	CAGATAACTTAGATGCCACCC	809	55
ASIP-Exon2	TCAACGGCTGCTTCTGACTTC	CTGCTCCTCCACAAAAGTTCC	1075	54.8
ASIP-Exon3	TGGGTGTCAGAATACAGCATA	GTCATTCTCAAATCTCAACCC	1088	54.8
ASIP-Exon4	CTCACATAGGGCACACAGTTC	ACAGCAAGCCTCAGATTTAGA	1242	56.1
ASIP-Exon5	GCCAGAGAACTTCAGAGACCA	CTTGTTTTTTCGGCGATAGGA	1173	62.2
ASIP-3'-UTR	CCGTGGAGCTGAGTGGGA	CAGTCTCAGGGCCGGTAA	691	59.4

absent from all wild boars, making it easy to distinguish between domestic pigs and wild boars. According to the nomenclature for these *MC*1*R* alleles proposed by Fang *et al*. (2009) and Kijas *et al*. (1998, 2001), seven allelic variants corresponded to five different *E* alleles (Table 2). Almost all tested wild boars carried the E^+ allele (70/72); whereas E^p allele was not present. Fang *et al*. (2009) investigated genetic variation in the *MC*1*R* gene among 15 wild and 68 domestic pigs from both Europe and Asia and they found that all mutations were silent in the wild animals, suggesting purifying selection, but nine of ten mutations found in domestic pigs resulted in an altered protein sequence, suggesting that early farmers intentionally selected for novel coat color. This addresses why coat color is much more variable in domestic animals than in their wild ancestors. Interestingly, in the current study, one amino-acid replacement (g.1135 T > C) that cause changes at Leu102Pro was absent from all wild boars, making it easy to distinguish between domestic pigs and wild boars. In the Duroc breed, no inactivation mutations in *MC*1*R*, which are responsible for red-colored *e* alleles, were observed in wild boars. Twelve wild boars were heterozygous (E^+/e) and three wild boars were homozygous (*e*/*e*) for the *e* allele, but this may be the result of gene flow from domestic pigs. The *e* allele is of European origin and differs from native Chinese wild boar alleles by at least two synonymous and two non-synonymous substitutions (Fang *et al*., 2009).

The E^+ allele was significantly associated with wild-type coat color in the wild boar population ($P = 0.027$, Chi-square test). However, they are not probably due to one factor. In particular, the mutation alleles were not completely associated with black or brown coat color in wild boars. A unique *MC*1*R* allele (E^+) has also been identified in European wild boars, associated with the wild-type coat color that is not found in any of the domestic breeds (Koutsogiannouli *et al*., 2010). Thus, *MC*1*R* was a less likely candidate for coat color phenotype in wild boars, although we cannot rule out the possibility of regulatory mutation(s) of *MC*1*R* determining the black, brown or silver brown coat color phenotypes. It may be that mutations of the *MC*1*R* gene, in natural populations, have been implicated in the vast majority of cases, possibly due to the minimal pleiotropic effects.

In the *ASIP* gene, only one SNP (c.695 T > C) was identified in the 3'-UTR by screening six PCR products, which resulted from a T to C single point mutation, other any mutations did not find in the coding regions of the *ASIP* gene. The c.695 T > C polymorphism was genotyped in 72 wild boars belonging to seven coat color phenotypes and three Western pig breeds. The results showed that all wild pigs were *CC* homozygotes or *CT* heterozygotes (Table 3). It was never identified in three Western pig breeds (*TT*). Mao *et al*. (2010) reported that a novel missense mutation c.157G > A was identified in exon 2 of the *ASIP* gene, their results showed that all pigs were *GG* homozygote, except for six heterozygotes in brownish red Tibetan pigs and four heterozygotes in solid black Tibetan pigs, apparently excluding it as a causative mutation for the brownish red coat color in Tibetan pigs. In the present study, the c.157 G > A mutation was not found in the *ASIP* gene. However, for the c.695 T > C mutation site, the genotyping results

Table 2: *MC*1*R* and *Extension* genotypes and allele frequencies among all tested wild boars.

Breed	Coat color phenotypes	Total	*Extension* genotypes						*Extension*/*MC*1*R* allele frequencies				
			E^+/E^+	E^+/E^{D1}	E^+/E^{D2}	E^+/e	e/e	E^p/E^p	E^+	E^{D1}	E^{D2}	e	E^p
Wild boar	Black	5	1	3	0	0	1	0	0.5	0.3	0	0.2	0
	Brown	8	1	4	1	2	0	0	0.56	0.25	0.06	0.13	0
	Reddish brown	3	2	0	0	1	0	0	0.83	0	0	0.17	0
	Black brown	22	14	2	0	6	0	0	0.82	0.04	0	0.14	0
	Dark brown	15	8	2	0	3	2	0	0.7	0.07	0	0.23	0
	Silver brown	11	10	1	0	0	0	0	0.95	0.05	0	0	0
	Pale brown	8	5	2	1	0	0	0	0.81	0.13	0.06	0	0

Table 3: Genotypes and allele frequencies at the *ASIP* c 695 T>C mutation site in wild boars and Western pig breeds.

Breeds	Coat color phenotypes	No	*ASIP* c.696 C > T genotypes			Allele frequencies	
			CC	CT	TT	C	T
Wild boar	Black	5	3	2	0	0.8	0.2
	Brown	8	2	6	0	0.625	0.375
	Reddish brown	3	3	0	0	1	0
	Black brown	22	9	13	0	0.705	0.295
	Dark brown	15	5	10	0	0.667	0.333
	Silve brown	11	9	2	0	0.909	0.091
	Pale brown	8	7	1	0	0.937	0.063
Duroc	Red	30	0	0	30	0	1
Landrace	White	30	0	0	30	0	1
Large White	White	30	0	0	30	0	1

demonstrated that it is difficult to exclude it as the causative mutation for the wild-type coat color in wild boars. The association analysis provided evidence that the *C* mutation allele was highly significantly associated with wild-type coat colors (P = 1.35E-33). Furthermore, Kingsley *et al*. (2009) found a 125-kb deletion, which included the upstream regulatory region and exons 1 and 2 of *Agouti*, results in a loss of *Agouti* expression and is perfectly associated with melanic color. Comparative sequencing of all *ASIP* exons and *ASIP* cDNAs between Mangalitza and Piétrain pigs did not reveal any differences associated with the coat color phenotype (Dröemüller *et al*., 2006). Although functional variation was not detected in the coding region of the *ASIP* gene in wild boar populations, further analysis in the *ASIP* regulatory region was worthwhile. These results suggested that the mutation site *C* allele identified in the *ASIP* gene was associated with wild-type coat color phenotypes in wild boar populations, other coat color function genes maybe determine the coat color phenotype of the wild boars.

Lastly, PCR primers were designed to amplify seven exons and exon–intron boundary sites of the *TYRP*1 gene (Table 1). Eleven SNPs, including one cSNP and 10 intronic polymorphisms, were detected in the *TYRP*1 gene. *TYRP*1 g.2254 G>A (*c*.428 G > A, p.143His>Arg) is located in exon 2 and caused a non-conservative amino acid change from histidine to arginine at the 143 codon position (p.143His>Arg), and five SNPs (*TYRP*1.*g* 2603 G > C, *TYRP*1.*g* 2896 A > G, *TYRP*1.*g* 2921 A > G, *TYRP*1.*g* 5074 A > C, and *TYRP*1.*g* 5397 G>A) were located in intron 2, two SNPs (*TYRP*1.*g* 8836 C > G and *TYRP*1.*g* 8929 A > C) were located in intron 3, one SNPs (*TYRP*1.*g* 11615 T > A) was located in intron 4, and two SNPs (*TYRP*1.*g* 16548 C > T, *TYRP*1.*g* 16551 C > T) were located in intron 5. These SNPs were not further analyzed in our study, except for *TYRP*1 g.2254 G > A. Moreover, a 6-bp deletion mutation was not found in exon 8 (c.1484-1489del) of the *TYRP*1 gene. However, the non-synonymous (c.428G > A, p.143His>Arg) and deletion (c.1484-1489del) mutations were used for further investigation. The g.2254 G>A polymorphism was analyzed by directly sequencing. The deletion (c.1484-1489del) mutation was identified using *KpnI* PCR–RFLP by exon 7 primer amplification (Table 1). The wild-type allele (+) was represented by two fragments of 390 bp and 572 bp and the del allele by an uncut amplicon of 956 bp (Fig. 1). Genotypes and allele frequencies of the g.2254G > A and c.1484-1489del mutations are shown in Tables 4 and 5.

Ren *et al*. (2011) provided the compelling evidence that brown colors in Chinese indigenous pigs are caused by the same ancestral mutation (c.1484-1489del) in *TYRP*1. Although we investigated the distribution of genotypes at the g.2254 G > A and c.1484-1489del sites in wild boars with different coat color phenotypes and three Western pig breeds, the analysis results demonstrated that a majority of the non-brown and brown-coated individuals segregated for the g.2254 G > A polymorphism (Table 4). The results

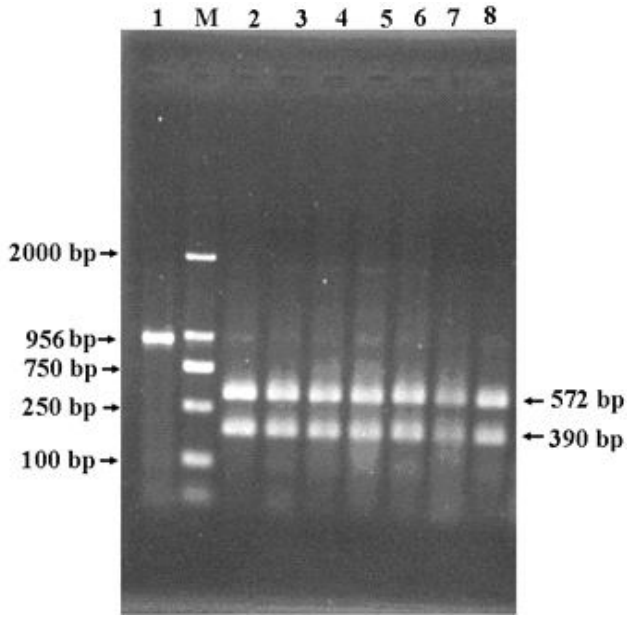

Fig 1: The electrophoresis patterns of the *TYRP1* c.1484-1489del mutation by a *KpnI* PCR-RFLP assay. Line 1: del/del. Lines 2-8: +/+.

Table 4: Genotypes and allele frequencies at the *TYRP*1 g.2254 G > A polymorphic site (p.143His>Arg) in wild boars and Western pig breeds.

Breeds	Coat color phenotypes	No	*TYRP1* g.2254 G > A genotypes			Allele frequencies	
			GG	GA	AA	G	A
Wild boar	Black	5	4	1	0	0.900	0.100
	Brown	8	4	2	2	0.625	0.375
	Reddish brown	3	2	1	0	0.8333	0.1667
	Black brown	22	17	3	2	0.7727	0.2273
	Dark brown	15	11	4	0	0.8667	0.1333
	Silver brown and gray	11	4	2	5	0.455	0.545
	Pale brown	8	3	2	3	0.500	0.500
Duroc	Red	30	0	3	27	0.050	0.950
Landrace	White	30	0	0	30	0.000	1.000
Large White	White	30	10	8	12	0.467	0.533

Table 5: Genotypes and allele frequencies of the *TYRP*1 c.14841489del mutation in wild boars and Western pig breeds.

Breeds	Coat color phenotypes	No	Genotypes			The *del* frequencies
			+/+	*+/del*	*del/del*	
Wild boar	Black	5	5	0	0	0
	Brown	8	8	0	0	0
	Reddish brown	3	3	0	0	0
	Black brown	22	22	0	0	0
	Dark brown	15	15	0	0	0
	Silver brown and gray	11	11	0	0	0
	Pale brown	8	8	0	0	0
Duroc	Red	30	30	0	0	0
Landrace	White	30	30	0	0	0
Large White	White	30	30	0	0	0

excluded it as the cause of the brown coat color phenotype in wild boar populations. However, the association analysis indicated that the A mutation allele was significantly associated with wild-type coat colors (P = 5.09E-10). Therefore, these results could not exclude it as a causative mutation for the wild-type coat color in wild pigs. Meanwhile, these results can also exclude the c.1484-1489del mutation as the cause of the brown coat color phenotype in wild boar populations, because all of the wild boars were homozygous for the wild-type allele (+) (Table 5).

It should be noted that the determination of coat coloration is a complex process in which several genes interact. Many color-associated genes and their alleles have epistatic effects. So far, studies of coat-color-associated genes in wild animals have been focused on *ASIP* and *MC1R*. There is no detailed insight into the underlying molecular mechanism of patterning in wild species. This is mainly because of a lack of investigation because most studies have been carried out on domesticated animals. However, a large number of mutations have been described in domesticated animals and therefore some limited conclusions can be drawn (Cieslak *et al*., 2011). Hence, it is worthwhile to evaluate other coat color genes as candidates for different coat color phenotypes in wild boars. The whole genome-wide association approach has been successfully used to map and identify causal genes responsible for several traits, especially for monogenic traits including white and spotted coat color in dogs (Karlsson *et al*., 2007; Salmon Hillbertz *et al*., 2007). Hence, we expect that a high-density SNP chip tool would be useful to perform a whole-genome association study to locate the gene(s) affecting coat color in wild boar populations.

CONCLUSION

The molecular variation within *MC*1*R*, *ASIP*, and *TYRP*1 genes were surveyed in Chinese wild pigs. These mutations have caused the morphological diversity of their wild relatives, but did not influence coat color phenotype variation in some domesticated pig breeds. Although most mutations in color-associated genes were probably present in wild populations of ancestor species, artificial selection caused their fixation and an increase in frequency. In most animal coat color phenotypes, several genes can cause similar phenotypes in different species. In contrast, some similar coat color phenotypes can also be caused by different genes. The conclusion was obtained that some mutations in color-associated genes were associated with wild-type coat colors in wild boar population, and that similar coat colorations observed in domesticated pig and wild boars can be the product of underlying differences in the genetic basis of color variants. The genome-wide association study need to locate the gene(s) affecting coat color in wild boar populations by a high-density SNP chip tool.

ACKNOWLEDGEMENT

This study was supported by He'nan Research Program of Foundation and Advanced Technology of China (092102110088), Foundation of He'nan Educational Committee of China (2010B230007, 14B230017), Cooperation Project of Henan Science and Technology Department of China (182107000041), and Innovation Scientists and Technicians Troop Construction Projects of Henan Province of China.

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