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CORRELATION OF SINGLE NUCLEOTIDE POLYMORPHISMS IN THE AGOUTI SIGNALING PROTEIN (ASIP) AND A TYPE III RECEPTOR PROTEIN-TYROSINE KINASE (KIT) LOCI WITH COLOUR IN THE BACTRIAN CAMEL (*Camelus bactrianus*)

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ABSTRACT

Coat colour is an important characteristic and economic trait in bactrian camels. The agouti signaling protein (ASIP) and a type III receptor protein-tyrosine kinase (KIT) that control melanogenesis are candidate coat colour genes in mammals. However, these genes are not well characterised in bactrian camels and their association with colour has not been established. Here, we sequenced the ASIP and KIT genes in a population of different coloured bactrian camels to gain an improved understanding of the effects of these genes on coat colour. Our analysis of the bactrian camel ASIP gene revealed one mutation that altered the amino acid sequence of the encoded protein. In the KIT gene, we detected nine mutations, of which six were silent mutations and three were single nucleotide polymorphisms (SNPs) that alter the amino acid sequence (V28A, H57R, T118A). Analysis of associations between phenotypic and genotypic characteristics revealed that none of the mutations in the ASIP and KIT loci correlated completely with coat colour in bactrian camels. Thus, our findings indicate that the coat colour phenotype of the bactrian camel is not related to the ASIP and KIT genes; however, further investigations using more advanced technology with larger numbers of animals are required to confirm this conclusion.

Key words: Agouti gene, bactrian camel, coat colour, KIT gene, polymorphism, SNP

The bactrian camel is an important livestock species in Asia and the surrounding areas with cooler climates. China has camel genetic resources consisting mainly of two humped camels (*Camelus bactrianus*), the domestic and wild bactrian camels. Five bactrian camel breeds (Alxa, Sunit, Qinghai, Tarim and Zhungeer) are derived from these resources (Ji *et al,* 2009). The bactrian camel provides milk, wool and meat and is an important mode of transportation in desert or semi-desert areas. Coat colour in animals is an obvious phenotypic trait that is relatively easy to assess and of broad public interest. During a long period of domestication and selective breeding in the bactrian population, a large number of different breeds have been established with different pigmentation phenotypes (Sponenberg, 1997).

The agouti gene encodes the agouti signaling protein (ASIP), which regulates pheomelanin (yellow) and eumelanin (black and brown) synthesis by the pigment-producing cells (melanocytes) within the hair follicle (Jackson, 1994). Melanocytes can switch between the production of these pigments during hair growth, resulting in hairs with different banding patterns. As an antagonistic ligand of the melanocortin-1 receptor (MC1R), the agouti protein contains a seven-transmembrane motif and is expressed on the surface of melanocytes (Bultman *et al,* 1992; Miller *et al,* 1993; Robbins *et al,* 1993). Functional mutation of the agouti gene can lead to variation in coat colours in some domestic animals, such as alpaca (Chandramohan *et al,* 2013), dog (Kerns *et al,* 2004), horse (Rieder *et al,* 2001; Ludwig *et al,* 2009), cat (Eizirik *et al,* 2003), sheep (Norris and Whan, 2008) and mouse (Bultman *et al,* 1994; Kuramoto *et al,* 2001; Miltenberger *et al,* 2002). KIT, which is another strong candidate gene for the control of coat colour variation in mammal, encodes is a type III receptor protein-tyrosine kinase. The KIT ligand (also called stem cell factor, SCF) binds to KIT via the second and third extracellular immunoglobulin

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domains (Haase *et al,* 2007). The KIT gene structure is complex, not only containing a coding region that plays an important role in the formation of melanin, but also a non-coding region that also has a great impact on hair traits. The KIT gene can determine the survival, differentiation, proliferation and migration of cells and is very important for survival and growth of melanocytes. KIT gene mutations have previously been shown to influence coat colour phenotypes in horses (Haase *et al,* 2007; Haase *et al,* 2009; Haase *et al,* 2013) and other mammals, including pigs, humans and mice (Giebel and Spritz, 1991; Guerif *et al,* 2002; Pielberg *et al,* 2002). To date, only a few studies have focused on genotype characterisation of candidate genes responsible for coat colour in Bactrian camels (Liang *et al,* 2016). In this study, we characterised the two coat colour candidate genes (agouti and KIT gene) in bactrian camels to investigate relationships between genotypes and the coat colour phenotypes.

Materials and Methods

Ethic Statement

No experiments with animals was performed for this study except the collection of blood from the jugular vein and the owner or researcher of the land gave permission to conduct the study on this site.

Animals and DNA extraction

Blood samples about 10mL were collected from 94 bactrian camels (55 Alxa bactrian camels, 14 Sunit bactrian camels, 13 Zhungeer bactrian camels, 12 Qinghai bactrian camels), with three coat colours: red (n = 14), brown (n = 60) and white (n = 20). Fibre colour charts for bactrian camels were unavailable; therefore, coat colour was determined according to the owner's assessment of the animal. Genomic DNA was extracted from 200 µl of EDTA anti-coagulated blood using the DNeasy tissue kit (Qiagen, Doncaster, Vic., Australia) according to the manufacturer's instructions. The quality and quantity of genomic DNA were determined with a NanoDrop spectrophotometer. Pre-tests was carried out on 9 animals (3 red, 3 white and 3 brown) and an additional 85 samples were subsequently analysed according to the results of the pre-test (exon1 mutations in the ASIP gene and exon2, exon4 and exon11 mutations in the KIT gene).

Amplification and sequencing ASIP and KIT genes

Sequences of the ASIP and KIT genes of bactrian camels were retrieved from the NCBI GenBank (http://www.ncbi.nlm.nih.gov/) and conserved regions were identified for the design of primers to amplify the coding region. PCR amplification of complete coding sequences was performed with the primers Table 1 in 50 µl reactions containing 5 µl 10× Taq Buffer (100 mM Tris-HCl, pH 8.8, at 25°C; 500 mM KCl, 0.8% (v/v) Nonidet), 1 µl dNTP (10 mM) (Sangon Biotech., Shanghai, China), 25 mM MgCl2 (Sangon Biotech.) 5 µl, 5 unit TaqDNA polymerase 0.5 µl, 1 µl each of forward and reverse primers and 1 µl genomic DNA. The cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles, each consisting of 94°C for 30 s, 55–60°C for 35 s and 72°C for 40–50s; with the final extension at 72°C for 5–8 min. Amplified DNA was electrophoresed in 1% (w/v) agarose gels in TAE buffer, stained with ethidium bromide and visualised by UV transillumination. The PCR products were purified using the Sangon PCR Cleanup Kit (Sangon). ASIP and KIT sequencing primers were designed for selected each exon (Table 1) using Big Dye Terminator Technology v3.1 (Applied Biosystems, Mulgrave, Victoria, Australia) and sequencing was performed on a 3730 DNA analyser (Applied Biosystems, USA).

Bioinformatics analyses

Sequences were analysed using Chromas, EditSeq and Seqman software to determine the genotypes of two randomly selected camels to verify the identities of the ASIP and KIT genes. Genotype and allele frequencies were counted directly and

Table 1. Primer pairs designed for amplification of ASIP and KIT gene exons from genomic DNA.

Gene	Primer	Sequence (5'-3')	Product size (bp)
ASIP	Ex1F Ex1R	TTGCTTCAGTCTCCCTCCC TTTCTCACAGCCTCTAACATGC	641 bp
KIT-2	Ex2F Ex2R	ACATCTTGGCCTGCATACC CTCATTAGGAAGAGTCGCACA	739 bp
KIT-4	Ex4F Ex4R	GGGGTAGAGTGCGTGCTTA AATCATTCAGAGAAACAGCATAAA	621 bp
KIT-11	Ex11F Ex11R	ACATAACAATGGCTTTAGGGAA GACCGATCACAAGAGCCAG	572 bp

the sequencing alignment was obtained using the DNAStar v5.2.2 program. The MegAlign program was used to align the ASIP and KIT amino acid sequences. The bactrian camel ASIP and KIT protein sequences were predicted from their respective exon sequences using the SpliceView program coupled with the known cattle coding sequence.

Results

The bactrian camel ASIP gene

The complete coding region of the bactrian camel ASIP gene encoded a protein of 132 amino acids with a predicted molecular mass of 14,543 Da. Amino acid alignment showed that there was high identity between the bactrian camel ASIP sequence and that of the counterparts in dromedary (98.1%), wild bactrian camel (98.1) and alpaca (96.2%). Furthermore, the bactrian camel protein exhibited the same identity with the pig, sheep, wild yak and cattle ASIP proteins (86.8%) (Fig 1).

The bactrian camel KIT gene

The complete coding region of the bactrian camel KIT gene encoded a protein of 982 amino acids with a predicted molecular mass of 109,662 Da. Amino acid alignment showed that there was high identity between the bactrian camel KIT sequence and that of the counterparts in dromedary (99.5%), wild bactrian camel (98.8%), alpaca (98.4%), pig (94.8%), wild yak (93.2). Furthermore, the bactrian camel protein exhibited the same identity with the sheep, goat and cattle KIT proteins (92.8%) (Fig 2).

Mutations in the bactrian camel ASIP gene

Sequencing of the ASIP gene coding region in 94 bactrian camels revealed three polymorphism in exon 1; while in the KIT gene, we identified 5 polymorphisms in exon 2, three in exon 4 and one in exon 11 (Table 2).

In exon 1 of the ASIP gene, we identified 2 synonymous single nucleotide polymorphisms (SNPs) and one nonsynonymous SNPs, c.A56C, which was predicted to cause an asparagine-to-threonine substitution at codon 19 (N19T). In the KIT gene, we found three nonsynonymous SNPs and six synonymous SNPs. In exon 2, two nonsynonymous SNPs were found: c.T82C was predicted to cause a valine-to-alanine substitution at codon 28 (V28A) and c.A169G predicted to cause a histidine-to-argnine substitution at codon 57 (H57R). Three synonymous mutations were also found: c.T26A (p.P9), c.A110C (p.T37) and c.T164A (p.N55). In exon 4, we identified one nonsynonymous mutation, c.A9G, which predicted to cause a threonine-to-alanine substitution at codon 118 (T118A). We also detected two nonsynonymous mutations, c.G7A (p.R187) and c.A90G (p.D215). In exon 11, one nonsynonymous mutation was found, c.G68T (p.S509) (Table 2 and Fig 3).

We hypothesised that SNP mutations associated with a change in the amino acid sequence may correlate with different coat colour in bactrian camels. Based on this hypothesis, we compared each genotype with coat colour to determine the relationship between SNP mutations and coat colour.

In exon 1 of the ASIP gene, 12 animals were heterozygous AC at N19T and anothor 12 were homozygous AA; the remaining 36 animals were homozygous for threonine. Among these animals, the majority were brown (n = 20), while nine were white and seven were red. In exon 2 of the KIT gene,

Table 2. Polymorphisms identified in the bactrian camel ASIP and KIT genes.

Polymorphism	Location	Amino acid change	Effect of amino acid change on protein
c.T23A	ASIP, exon 1	Synonymous	N/A
c.G25A	ASIP, exon1	Synonymous	N/A
c.A56C	ASIP, exon 1	N19T	Polar to polar
c.T26A	KIT, exon 2	Synonymous	N/A
c.T82C	KIT, exon 2	V28A	Nonpolar to nonpolar
c.A110C	KIT, exon 2	Synonymous	N/A
c.T164A	KIT, exon 2	Synonymous	N/A
c.A169G	KIT, exon 2	H57R	Polar to polar
c.A9G	KIT, exon 4	T188A	Polar to nonpolar
c.G7A	KIT, exon 4	Synonymous	N/A
c.A90G	KIT, exon 4	Synonymous	N/A
c.G68T	KIT, exon 11	Synonymous	N/A

Percent identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
1		98.1	98.1	96.2	86.8	86.8	86.8	86.8	81.1	80.8	80.8	79.2	79.2	79.2	**1**	bactrian camel
2	1.9		100.0	94.3	84.9	86.8	84.9	84.9	79.2	78.8	78.8	77.4	77.4	77.4	**2**	dromedary camel
3	1.9	0.0		94.3	84.9	86.8	84.9	84.9	79.2	78.8	78.8	77.4	77.4	77.4	**3**	wild bactrian camel
4	3.9	5.9	5.9		84.9	83.0	83.0	83.0	79.2	76.9	76.9	75.5	75.5	75.5	**4**	alpaca
5	14.6	16.9	16.9	16.9		81.1	84.9	84.9	84.9	80.8	80.8	77.4	77.4	75.5	**5**	pig
6	14.6	14.6	14.6	19.3	21.8		96.2	96.2	73.6	75.0	75.0	79.2	79.2	69.8	**6**	sheep
7	14.6	16.9	16.9	19.3	16.9	3.9		100.0	77.4	78.8	78.8	79.2	79.2	73.6	**7**	wild yak
8	14.6	16.9	16.9	19.3	16.9	3.9	0.0		77.4	78.8	78.8	79.2	79.2	73.6	**8**	cattle
9	21.8	24.4	24.4	24.4	16.9	32.6	27.0	27.0		80.8	80.8	75.5	75.5	75.5	**9**	human
10	19.7	22.3	22.3	24.9	19.7	27.6	22.3	22.3	22.3		100.0	80.8	80.8	78.8	**10**	goat
11	19.7	22.3	22.3	24.9	19.7	27.6	22.3	22.3	22.3	0.0		80.8	80.8	78.8	**11**	dog
12	24.4	27.0	27.0	29.7	27.0	24.4	24.4	24.4	29.7	22.3	22.3		100.0	69.8	**12**	donkey
13	24.4	27.0	27.0	29.7	27.0	24.4	24.4	24.4	29.7	22.3	22.3	0.0		69.8	**13**	horse
14	24.4	27.0	27.0	29.7	29.7	38.6	32.6	32.6	29.7	22.3	22.3	38.6	38.6		**14**	rabbit
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**		

Divergence

Fig 1. Amino acid sequence alignments of ASIP proteins from different species. 1. Bactrian camel (*Camelus bactrianus,* NW_011515153); 2. Dromedary camel (*Camelus dromedarius*, NW_011591043); 3. Wild bactrian camel (*Camelus ferus,* NW_006211580); 4. Alpaca (*Vicugna pacos*, NW-005882736); 5. Pig (*Sus scrofa*, NC_010459); 6. Sheep (*Ovis aries*, NC_019470); 7. Wild yak (*Bos mutus*, NW_005397034); 8. Cattle (B*os taurus,* AC_000170); 9. Human (*Homo sapiens*, NC_00002); 10. Goat (*Capra hircus*, EF587236); 11. Dog (*Canis lupus*, NC_006606); 12. Donkey (*Equus asinus*, NW_014638605); 13. Horse (*Equus caballuss*, NC_009165); 14. Rabbit (*Oryctolagus cuniculus*, NM_001082077).

Percent identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
1		99.5	98.5	98.4	94.8	93.2	92.8	92.8	92.8	91.6	91.1	89.9	88.5	80.0	**1**	bactrian camel
2	0.3		99.5	99.3	95.2	93.7	93.2	93.6	93.7	91.9	91.8	90.4	89.3	80.4	**2**	dromedary camel
3	0.7	0.4		99.0	94.6	93.2	92.8	93.2	93.4	91.7	91.5	90.0	89.0	80.0	**3**	wild bactrian camel
4	0.8	0.7	0.7		94.9	93.4	93.0	93.4	93.6	91.9	91.7	90.2	89.1	80.4	**4**	alpaca
5	5.1	5.0	5.2	5.1		94.6	94.1	94.5	94.6	93.1	92.9	91.5	89.9	81.5	**5**	pig
6	6.7	6.6	6.6	6.6	5.4		99.0	99.4	99.7	92.3	92.0	91.4	89.8	80.9	**6**	sheep
7	7.4	7.2	7.3	7.2	6.0	1.0		99.3	99.9	91.8	91.6	91.2	89.6	80.5	**7**	wild yak
8	6.9	6.8	6.9	6.9	5.7	0.5	0.7		99.3	92.2	92.1	91.4	89.9	80.7	**8**	cattle
9	6.7	6.6	6.5	6.5	5.4	0.1	1.1	0.7		92.3	92.1	91.3	89.9	80.9	**9**	human
10	8.7	8.6	8.5	8.5	7.1	8.2	8.8	8.4	8.2		99.7	90.9	89.3	80.8	**10**	goat
11	8.9	8.7	8.7	8.7	7.2	8.3	9.0	8.5	8.3	0.3		90.6	89.2	80.6	**11**	dog
12	10.7	10.3	10.3	10.3	9.0	9.2	9.6	9.3	9.3	10.1	10.4		88.6	80.0	**12**	donkey
13	11.6	11.5	11.6	11.5	10.5	10.6	11.1	10.8	10.7	11.5	11.6	12.4		80.1	**13**	horse
14	22.6	22.4	22.7	22.4	21.4	21.9	22.6	22.3	21.9	22.1	22.3	22.8	22.9		**14**	rabbit
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**		

Divergence

Fig 2. Amino acid sequence alignments of KIT proteins from different species. 1. Bactrian camel (*Camelus bactrianus,* NW_011515153); 2. Dromedary camel (*Camelus dromedarius*, NW_011591043); 3. Wild bactrian camel (*Camelus ferus*, NW_006211580); 4. Alpaca (*Vicugna pacos*, NW-005882736); 5. Pig (*Sus scrofa*, NC_010459); 6. Sheep (*Ovis aries,* NC_019470); 7. Wild yak (*Bos mutus*, NW_005397034); 8. Cattle (*Bos taurus*, AC_000170); 9. Human (*Homo sapiens*, NC_00002); 10. Goat (*Capra hircus,* EF587236); 11. Dog (*Canis lupus*, NC_006606); 12. Donkey (*Equus asinus*, NW_014638605); 13. Horse (*Equus caballuss*, NC_009165); 14. Rabbit (*Oryctolagus cuniculus*, NM_001082077).

three animals (2 brown and 1 red) were homozygous for the alanine allele at V28A. The heterozygous genotype was present in 21 animals, while the remaining 67 were homozygous for valine, with three varying phenotypes. At H57R of the KIT gene, four animals (3 brown and 1 red) were homozygous for the arginine allele and no white colour homozygotes were detected in our population. Thirty animals were heterozygous for the histidine allele and the remaining 68 were homozygous. Two red animals red and two brown animals were homozygous for the alanine allele at T118A. There were no white

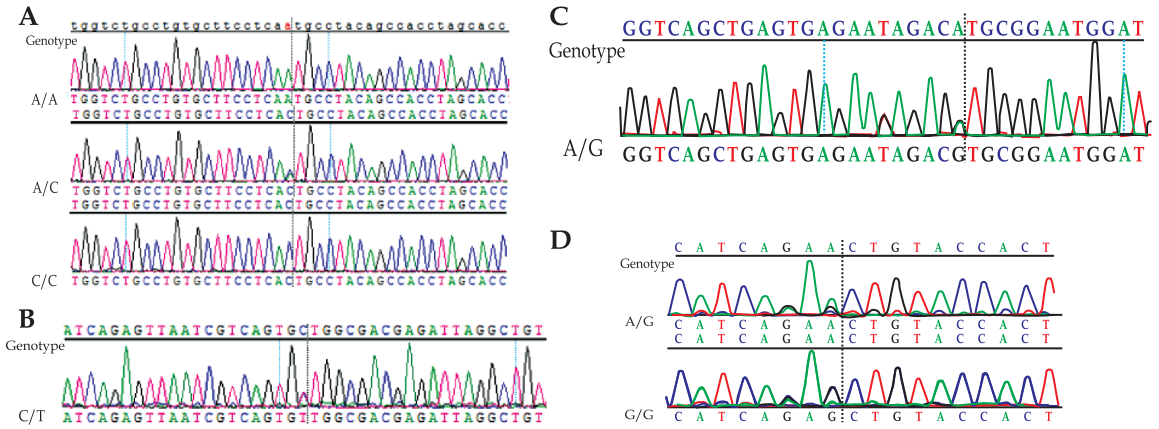

Fig 3. Mutations in the bactrian camel ASIP and KIT gene. (A) Exon 1 (ASIP), c.A56C; (B) Exon 2 (KIT), c.T82C; (C) Exon 2 (KIT), c.A169G; (D) Exon 4 (KIT), c.A9G.

animals with this genotype, 19 were heterozygous for the threonine allele and the remaining 48 were homozygous, with three varying phenotypes.

Discussion

In some domesticated species, coat colour is regulated by two main types of melanin: eumelanin and pheomelanin. The agouti protein is a paracrine-signaling molecule that is normally expressed in the skin and exhibits high homology among mammals. Loss-of-function mutations in the ASIP gene could perturb the ASIP signaling pathway and interfere with melanogenesis. Very few studies on functional genes in bactrian camels have been reported compared with those of other mammals, such as cattle and sheep. In particular, there are few studies on the funtional genes related to coat colour (Girardot *et al,* 2005; Royo *et al,* 2005; Li *et al,* 2014; Zhang *et al,* 2017), probably because of the genetic complexity of this characteristic. Assuming that colour is a complex phenotype, we investigated the ASIP and KIT genes, which have been identified as candidate genes. In this study, we detected a possible association between genotype (c.A56C, c.T82C, c.A169G, c.A9G) and coat colour phynotype in bactrian camels. All the SNPs in the ASIP and KIT gene can not bright out colour variation. Fiber colour charts for bactrian camels were unavailable; therefore, fibre colour was classified according to the owner's assessment, which may lead to in our analysis of the effects of SNPs on bactrian

Table 3. Genotype vs. phenotype.

Exon	Polymorphism	White individual	Red individual	Brown individual	Total
ASIP (exon1)	N19T				
	AA	4	3	5	12
	AC	1	1	10	12
	CC	9	7	20	36
KIT (exon2)	V28A				
	TT	14	8	45	67
	CT	6	4	11	21
	CC	0	1	2	3
KIT (exon2)	H57R				
	AA	14	8	46	68
	AG	6	3	21	30
	GG	0	1	3	4
KIT (exon4)	T118A				
	AA	9	3	36	48
	AG	6	4	9	19
	GG	0	2	2	4

camel fleece colour. Moreover, due to the complexity of the interactions among genes, studies of multiple candidate genes, including key regulatory regions and intronic regions of the ASIP and KIT genes and with larger sample numbers are required to fully elucidate the these association of these genes with coat colour in bactrian camels. In addition, the MC1R gene is a plausible choice for investigation because of its highly conserved nature among eutherian mammals.

Conclusion

Exon 1 of the ASIP gene and the exons 2, 4 and 11 of the KIT gene were sequenced to determine the existence of any correlation between ASIP and KIT gene polymorphisms and different coat colours in Bactrian camels. In total, 12 polymorphisms were identified in 94 bactrian camels. Three polymorphisms were detected in exon 1 of the ASIP gene, while the KIT gene contained five polymorphisms in exon 2, three polymorphisms in exon 4 and one polymorphism in exon 11. However, no association was identified between the genotypes of the polymorphisms in the ASIP and KIT genes and coat colour trait, possibly due to the complexity of coat colour gene interactions. Further investigations using more advanced technology with larger numbers of animals are required to confirm this conclusion.

Conflict of interest

The authors declare that they have no conflicts of interest.

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