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Desmoglein 4 diversity and correlation analysis with coat color in goat

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ABSTRACT. Desmoglein 4 (DSG4) has an important role in the development of wool traits in domestic animals. The full-length *DSG4* gene, which contains 3918 bp, a complete open-reading-frame, and encodes a 1040-amino acid protein, was amplified from Liaoning cashmere goat. The sequence was compared with that of *DSG4* from other animals and the results show that the *DSG4* coding region is consistent with interspecies conservation. Thirteen single-nucleotide polymorphisms (SNPs) were identified in a highly variable region of *DSG4*, and one SNP (M-1, G>T) was significantly correlated with white and black coat color in goat. Haplotype distribution of the highly variable region of *DSG4* was assessed in 179

individuals from seven goat breeds to investigate its association with coat color and its differentiation among populations. However, the lack of a signature result indicates DGS4 haplotypes related with the color of goat coat.

Key words: Desmoglein 4; Diversity; Goat; Evolution; Correlation analysis

INTRODUCTION

To date, a series of candidate genes have been identified for wool or cashmere traits in domestic animals (Zhou et al., 2011; Geng et al., 2012; Wang et al., 2012). Desmoglein 4 (DSG4) is an example of a candidate gene, whose role in the biological function of coat and skin has been demonstrated in many studies. Green and Jones (1996) found that desmosomes, which are composed of several proteins including desmogleins and desmocollins, mediate cell-cell adhesion in hair follicles. Later, a deletion encompassing exons 5-8 of human *DSG4* in families with localized autosomal recessive hypotrichosis (LAH) was found, and a single nucleotide insertion in exon 7, along with a missense mutation in exon 6 in mice with lanceolate hair (lah) was identified (Kljuic et al., 2003). Subsequently, a number of mutations within *DSG4* that are associated with LAH (Messenger et al., 2005; Wajid et al., 2007) and monilethrix hairs in humans (Schaffer et al., 2006; Shimomura et al., 2006; Zlotogorski et al., 2006), and lah in rats (Jahoda et al., 2004; Meyer et al., 2004; Bazzi et al., 2006) were reported, which further indicated the importance of *DSG4*. In addition, some reports showed that members of the DSG family were associated with skin disease (Amagai, 2010; Amagai and Stanley, 2012). Recent studies have revealed that the *DSG4* genotype is strongly associated with wool traits in Chinese indigenous sheep (Zhang et al., 2011a; Ling et al., 2014). These studies strongly suggest that *DSG4* is a candidate gene that may contain polymorphic variations affecting wool traits in sheep. However, no study has addressed the complete coding sequence (CDS) region and the wool traits associated with DSG4 in goats.

Therefore, the aim of this investigation was to identify the complete CDS region in DSG4 in Chinese goats, as well as possible polymorphisms within this region and their correlation with coat color. Knowledge of the associations between *DSG4* and coat color and the differentiation of this gene among populations will be useful for animal breeding.

MATERIAL AND METHODS

Sample collection and RNA extraction

An ovary sample was collected from a Liaoning cashmere goat (Institute of Animal Science, Chinese Agriculture Academy Science, Beijing, China) following the method described by Zhao et al. (2015). The sample was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was isolated following the manufacturer protocol.

Primers, amplification, and sequencing of goat *DSG4* mRNA

As shown in Table 1, six pairs of continuous primers (from cDNA-P1 to cDNA-P6) were designed according to the sheep *DSG4* sequence (Zhang, 2011), and were synthesized by Shanghai Biological Engineering Technology Services Limited Co. First-strand cDNA was synthesized

according to the PrimeScript® RT Reagent Kit protocol. The RT-PCR product was stored at -20°C. The cDNA was then used as a template for subsequent PCR according to the High Fidelity Taq Enzyme protocol. PCR was performed by mixing 2 µL cDNA, 2 µL dNTPs (2.5 mM each), 1 µL (20 pM) each of forward and reverse primers, 0.25 µL (5 U/µL) High Fidelity Taq enzyme (TAKARA BIO, Inc., Dalian, China), 5 µL 10X PCR buffer (Mg^{2+} plus), and 36.75 µL sterilized and double-distilled water in a total 50-µL volume. PCR amplification of the *DSG4* gene was performed using an AB Applied Biosystems device (Life-Technologies™, USA) under the following conditions: 95°C for 5 min (initial denaturation), 30 cycles of at 95°C for 30 s, 60°C for 40 s, 72°C for 60 s, 72°C for 10 min, and 4°C for 30 min. One-fifth of each PCR product was electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

Table 1. Primer sequences, product size, and annealing temperature of RT-PCR.

Name	Primer sequence for PCR	Length (bp)	Annealing temperature (°C)
cDNA-P1	GACCAGGCTCAAATCAAATCTC	458	60
	CCTTTCTAAGTCTTCACCCCG		
cDNA-P2	CGACCGCCCTATGGAGTGTT	1058	60
	ATCTCGCCAGTCCTTGAATCT		
cDNA-P3	GTTTCACCACTCGGTTGCTT	814	60
	TGAAGCCTCAGTTTGGTCGT		
cDNA-P4	CCCCAGGGACAGCGGACA	741	60
	TGTCCAGGAAAGCCAGGTT		
cDNA-P5	GAGGAGGAACAGTTGAAGGG	1006	60
	TATGTTGGTGATTACAAGGTGC		
cDNA-P6	ACATCCCCAGTGACCTCTCG	1472	60
	GCAAGAAGCACTACAGTTATTT		

The PCR products were extracted using an Agarose Gel DNA Fragment Recovery Kit Ver. 2.0 Protocols (TaKaRa, Dalian, China) and then inserted into pMD18-T Simple Vectors (TaKaRa) according to the manufacturer protocols. Positive plasmids were named pMD18-T-DSG41 and sequenced using two-way sequencing by Tanyibiotech (Beijing, China).

Phylogenetic analysis of the DSG4 nucleotide and amino acid sequence

The nucleotide and amino acid sequences of Liaoning cashmere goat DSG4 were compared with those published for other species, including *Homo sapiens* (AAI32908.1), *Mus musculus* (AAP44999.1), *Rattus norvegicus* (AAQ88398.1), the DSG4 partial synthetic construct (AIC57789.1), *Bos taurus* (DAA16012.1; DAA16012.1), *H. sapiens* (NP 001127925.1; NP 817123.1), *Macaca mulatta* (XP 001102180.1), *Equus caballus* (XP 001496441.1), *B. taurus* (XP 002697734.1), *Oryctolagus cuniculus* (XP 002713485.1), *Callithrix jacchus* (XP 002757193.1), *Pongo abelii* (XP 002828187.1), *Ailuropoda melanoleuca* (XP 002926714.1), *Xenopus* (Silurana) *tropicalis* (XP 002934178.2), *Nomascus leucogenys* (XP 003262015.1), *Sus scrofa* (XP 003356443.2), *Cavia porcellus* (XP 003474064.1), *Sarcophilus harrisii* (XP 003759868.1), *Otolemur garnettii* (XP 003784813.1), *Pan paniscus* (XP 003830305.1; XP 003830306.1), *Papio anubis* (XP 003914318.1), *Saimiri boliviensis boliviensis* (XP 003924807.1), *Pan troglodytes* (XP

003953318.1), *Ovis aries* (XP 004020667.1), *Gorilla gorilla gorilla* (XP 004059331.1), *Odobenus rosmarus divergens* (XP 004412993.1), *Ceratotherium simum simum* (XP 004422718.1), *Dasypus novemcinctus* (XP 004484226.1), *Ochotona princeps* (XP 004579647.1), *Sorex araneus* (XP 004606182.1), *Octodon degus* (XP 004623757.1), *Jaculus jaculus* (XP 004654908.1), *Condylura cristata* (XP 004683867.1), *Mustela putorius furo* (XP 004803803.1), *Heterocephalus glaber* (XP 004905249.1), *Anas platyrhynchos* (XP 005009550.1), *Mesocricetus auratus* (XP 005065408.1), *Microtus ochrogaster* (XP 005355861.1), *Chinchilla lanigera* (XP 005373217.1), *Macaca fascicularis* (XP 005587029.1), *Canis lupus familiaris* (XP 005623044.1), *Capra hircus* (KM369171), *Myotis brandtii* (XP 005857122.1), *Bos mutus* (XP 005901112.1), *Pantholops hodgsonii* (XP 005960577.1), *Myotis lucifugus* (XP 006091735.1), *Tupaia chinensis* (XP 006150644.1), *Camelus ferus* (XP 006187223.1), *Vicugna pacos* (XP 006205143.1), *Tarsius syrichta* (XP 008071582.1), *Eptesicus fuscus* (XP 008146584.1), *Equus przewalskii* (XP 008505115.1), *Galeopterus variegatus* (XP 008580284.1), *Ursus maritimus* (XP 008691097.1), and *B. taurus* (XP 617938.3). The amino acid sequence of *DSG4* from Liaoning cashmere goat was aligned with that from other species using the software Clustal X (1.83). Bayesian inference (BI) and maximum likelihood frameworks were used to examine the phylogenetic position of the goat sequence. The best-fitting model (Jones-Taylor-Thornton + Gamma Distributed) of DNA substitution for BI was obtained using jModelTest V. 0.1.1. (Posada, 2008). The neighbor-joining (NJ) phylogenetic tree of *DSG4* sequences from these species was constructed using the MEGA (5.0) software (Tamura et al., 2011), and bootstrap values to support the nodes of the tree were based on 100 iterations of the heuristic search. Evolutionary relationships were clarified based on the results of this comparison.

Prediction and analysis of the antigenic domains of goat DSG4

The main antigenic domains (MADs) of goat DSG4 were predicted with the online tool http://imed.med.ucm.es/Tools/antigenic.pl, using the Jameson-Wolf method (Jameson and Wolf, 1988).

Polymorphism of the high variability region of *DSG4*

Blood samples were taken from 179 individuals of seven goat breeds over a large range in southern China, and from one commercial population. The geographic information of these individuals is presented in Table 2 and Figure 1. DNA was extracted using the phenol extraction method. The high variability region of the *DSG4* gene was amplified using primers DSG-HV-For (5'-AATGGGGACGTTTTTTGCTTA-3') and DRA-HV-Rev (5'-CTACAACACATAGAGTCGCAGA-3'), which have been previously used in sheep (Zhang, 2011b). PCR amplification was conducted in a PTC-100TM PCR instrument (MJ Research, Inc., USA) in a total reaction volume of 50 μL containing 150 ng DNA, 5 μL 10X PCR standard reaction buffer, 10 pM dNTPs, 50 mM $MgCl_2$, 20 pM each forward and reverse primer, and 2.5 U Taq DNA polymerase from Promega (China). Following an initial denaturation at 95°C for 3 min, 30 cycles were performed at 94°C for 45 s, 60.5°C for 45 s, and 72°C for 1 min. The final cycle was followed by extension at 72°C for 10 min. Tanyibiotech (Beijing, China) performed sequencing by DRA-HV-Rev.

The polymorphism information content (PIC) was estimated from allele frequencies with the Microsatellite toolkit. Screening for haplotypes, the Tajima test, and Fu and Li's F, D, F*, D* test were conducted by DnaSP5.10 (Rozas and Rozas, 1995). Phylogenetic network analyses based on NJ algorithms were performed to determine the evolutionary relationships and frequency distri-

bution of the haplotypes using the Networks software (Polzin and Daneshmand, 2003).

Associations between single nucleotide polymorphisms (SNPs) and hair color were assessed by the Fisher exact test (Statistica 8.0, Statsoft Software, Warsaw, Poland) using a generalized linear model and logistic regression analysis. A P value less than 0.05 was considered statistically significant.

Table 2. Information on animals sampled in this study.

Breeds	Code	Sample size	Sampling location	North latitude	East longitude
Dazu black goat	DZ	27	Chongqing, China	29°39'26.25"	105°44'14.97"
Hechuan white goat	HW	17	Chongqing, China	29°58'29.98"	106°16'21.20"
Youzhou black-skin goat	YU	38	Chongqing, China	28°50'39.76"	108°45'48.46"
Inner Mongolia cashmere goat	NM	19	Alxa, China	47°52'6.67"	88°56'53.36"
Jianzhou big ear goat	JE	28	Chongqing, China	30°23'22.17"	104°31'38.75"
Jining grey goat	JG	21	Jining, China	35°23'48.59"	116°35'20.19"
Nubian goat	NB	29	Australia	Unknown	

Figure 1. Extrinsic features of seven goat. **A.** Inner Mongolia cashmere goat. **B.** Hechuan white goat. **C.** Dazu black goat. **D.** Jining grey goat. **E.** Youzhou black-skin goat. **F.** Jianzhou big ear goat. **G.** Nubian goat. **A** and **B**: pure white coat, **C** and **G**: pure black coat, **D**, **E**, and **F**: parti-color coat.

RESULTS AND DISCUSSION

Goat (*C. hircus*) is an important domestic animal worldwide. Coat fiber diameter, length, and color are key traits that contribute to the economic value of the goat; however, these traits are determined by genetic (Bunge et al., 1996; Lamoreux et al., 2001) and environmental (Kidson and Fabian, 1981) factors. To date, many common candidate genetic factors have been found to regulate coat color in other species, including the goat. For example, melanocortin 1 receptor (MC1R) and Agouti Signaling Protein (ASIP) are known to be major regulators of coat color in mice, and MC1R (Våge et al., 1999) and ASIP (Norris and Whan, 2008) are functionally linked to coat color phenotypes in sheep (Gratten et al., 2007; Yang et al., 2013) and yaks (Chen et al., 2009). In addition, tyrosinase-related protein 1 (TYRP1) is a strong candidate gene for coat color variation in Soay sheep (Gratten et al., 2007). Recently, researchers have focused on associations between candidate genes and coat color in goat, such as the red and black coat color phenotypes that are associated with MC1R (Fontanesi et al., 2009) and the brown coat color associated with TYRP1 variants (Becker et al., 2015; Dietrich et al., 2015). However, no single locus has been found to explain all of the divergence in coat color phenotypes. Therefore, there is a multi-locus response with respect to coat color. Recent studies have suggested that DSG4 is responsible for wool and cashmere traits in goat (Zhou et al., 2011; Wang et al., 2012).

In this study, the cDNA sequence of the *DSG4* gene from Liaoning cashmere goat including the full-length opening reading frame was obtained after splicing with six sequenced gene fragments of the target gene. The full gene sequence comprises 3120 bp and encodes 1040 amino acids. The molecular weight of the encoded receptor protein is 113.1236 kDa, with an isoelectric point of 4.51. We submitted the nucleotide and amino acid sequences of the protein to the GenBank Database (accession No. KM369171).

Evolutionary kinship in the phylogenetic tree (Figure 2) based on the sequence of the open-reading frame of 57 *DSG4* isoforms from different animal species, indicates that goat (*C. hircus*) is most closely related to antelope (*P. hodgsonii*) and sheep (*O. aries*). This relationship details the evolution of *DSG4* among different animal species and interspecies conservation, indicating that DSG4 displays an important and common biological function in different animal species.

The MADs of DSG4 were predicted by Jameson-Wolf methods using the online tool http://imed.med.ucm.es/Tools/antigenic.pl. The results indicated that 45 MADs lie from the 5th to the 1031th amino acid (Figure 3 and **Table S1**). These predictions may aid the selection of goat DSG4 antigenic epitopes to enable the preparation of antibodies for use in testing the tissue distribution of DSG4 *in vivo*.

The highly variable region of the *DSG4* gene was amplified and the total length of the aligned sequences was 557 bp, including 11 SNPs as follows; M-1: T>C at 86 bp, M-2: G>A at 117 bp, M-3: A>G at 180 bp, M-4: A>G at 205 bp, M-5: G>A at 219 bp, M-6: T>G at 268 bp, M-7: C>T at 319 bp, M-8: A>C at 359 bp, M-9:C>T at 361 bp, M-10: C>T at 455 bp, M-11: G>A at 503 bp, M-12: G>A at 523 bp, and M-13: C>T at 529 bp relative to the location of KT596879. Compared with the polymorphism from PIC, high diversity was observed in comparison with other locations among all populations, such as M-1 (0.359) and M-7 (0.261).

Across all individuals, nine haplotypes were constructed by 13 SNPs (Table 3), and their phylogenetic relationship and distribution are shown in Figure 4. All sequences were submitted to GenBank (KT596879-KT596887). The Jianzhou big ear goat (JE) carried more haplotypes (H_2, H_3, H_4, H_8, H_9) than any other breed, which is consistent with its domestic history. According to Animal Genetic Resources in China: Sheep and Goats (China National Commission of Animal Genetic Resources, 2011), JE is a neutral hybrid between the Nubian goat and indigenous breed

rounding in Southwest China from the Second World War. Two decades ago, these ecotype individuals were consternated, and a specific breed was constructed by artificial breeding for meat production. The genetic material revealed that JE not only shares certain haplotypes (H_1, H_2) with the Nubian goat but also shares a private haplotype (H_4), which is represented in southwest breeds (Dazu black goat and Youzhou black-skin goat). These results suggest that genetic variants of *DSG4* may provide molecular evidence and tools for tracing the breeding history of domestic animals.

Figure 2. Phylogenetic tree of the Desmoglein 4 (*DSG4*) gene. Neighbor-joining phylogenetic tree of *DSG4* from different species constructed using the MEGA5 software. Bootstrap values to support the nodes of the tree were based on 100 interactions of the heuristic search.

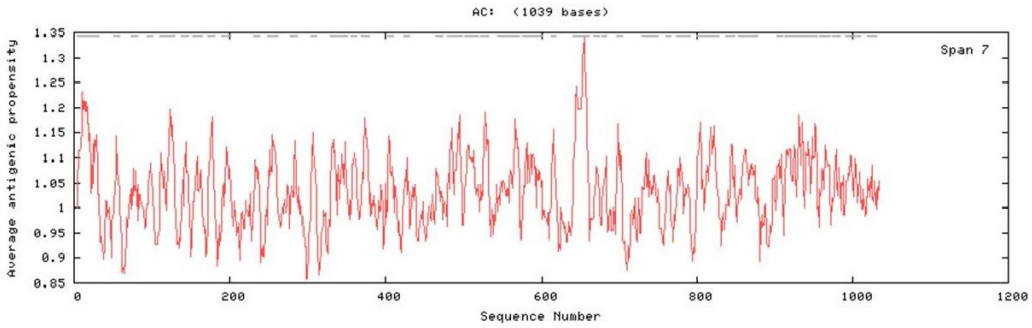

Figure 3. Antigenic domains of DSG4 from Liaoning cashmere goat.

Table 3. Distribution of single-nucleotide polymorphisms (SNPs) in each haplotype.

Hap	M-1	M-2	M-3	M-4	M-5	M-6	M-7	M-V8	M-9	M-10	M-11	M-12	M-13	FEQ	GN
H_1	T	G	A	A	G	T	C	A	C	C	G	G	C	186	KT596879
H_2	C	G	A	A	G	T	C	A	C	C	G	G	C	86	KT596880
H_3	C	G	A	A	G	T	T	A	C	C	G	G	C	72	KT596881
H_4	T	G	A	A	G	T	T	A	C	C	G	G	C	5	KT596882
H_5	T	G	A	A	A	T	C	A	C	C	G	G	C	2	KT596883
H_6	C	G	A	A	G	T	C	A	C	C	A	G	C	1	KT596884
H_7	C	G	A	A	G	T	C	A	C	T	G	G	C	2	KT596885
H_8	C	A	G	G	G	G	C	C	T	C	G	A	C	3	KT596886
H_9	C	A	G	G	G	G	C	C	T	C	G	A	T	1	KT596887

Hap = haplotype; FEQ = frequency of each haplotype among all individuals; BN = GenBank accession No.

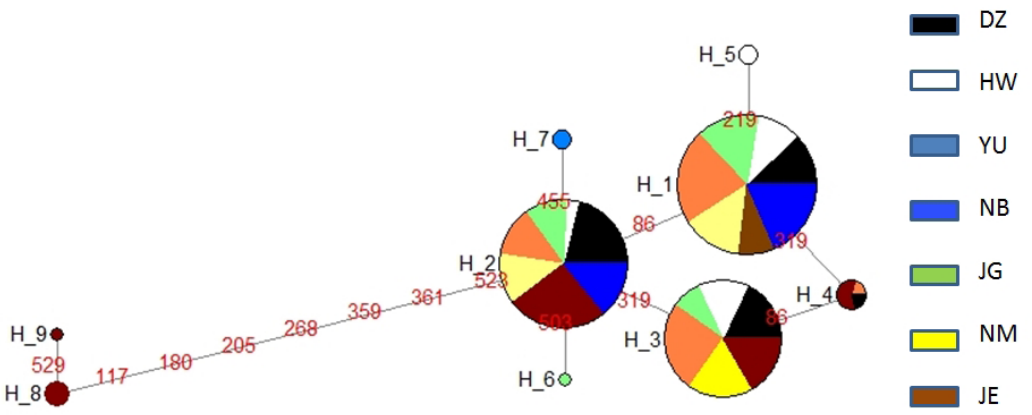

Figure 4. Distribution of haplotype frequencies of the *DSG4* highly variable region in each breed.

In addition, the results of the Tajima test and the Fu test are listed in Table 4. Nucleotide diversity, defined as the average number of pairwise nucleotide sequence differences, ranges from 0.00101 (Inner Mongolia cashmere goat) to 0.00328 (Jining grey goat) in this study, indicating a positive signature because of the sweep caused by genetic hitchhiking. In addition, a series of tests, such as Tajima's D and Fu and Li's F, D, F*, D* were performed to assess the deviation from neutrality in each breed. However, the lack of a signature result indicates a pattern of DGS4 haplotypes that deviates from that expected under neutrality.

Table 4. Summary statistics of the *DSG4* highly variable region in goat breeds.

Population	Nucleotide diversity (π)	Tajima's D	Fu and Li's D	Fu and Li's F	Fu and Li's D*	Fu and Li's F*
DZ	0.00160	1.79859	0.73633	1.22511	0.01370	-0.51761
HW	0.00197	0.97478	0.97343	1.14037	0.96844	1.12121
JG	0.00171	-0.12836	-0.00734	-0.04553	0.08537	0.03561
YU	0.00138	1.034844	0.72650	1.06394	0.72938	1.06371
NM	0.00101	0.35678	0.73633	1.22511	0.77710	0.75923
NB	0.00158	0.71408	0.87876	0.97048	0.87667	0.96470
JZ	0.00328	-0.45347	0.78341	0.43129	0.76583	0.42548

Eleven SNPs with a low frequency variant were not included in the correlation analysis between genotype and coat color. According to the correlation analysis between coat color [black color, white color, hybrid color (parti-color); Figure 1] and M-1, M-7 indicated that there is no significant correlation using the generalized linear model. However, the M-1 location had a highly significant (P = 0.0481) correlation with the coat color phenotype. Detailed information regarding the results among white and black individuals using logistic regression analysis is presented in Table 5. Previous reports have shown that the coat color phenotype and its regulating factors are not as simple as expected. Therefore, the identification of a genetic mutation (M-1) in *DSG4* may be useful as a potential biomarker for elucidating the genetic mechanism responsible for the development of white and black coats. However, whether the *DSG4* variability is responsible for coat color requires further study.

Table 5. Correlation analysis between *DSG4* SNPs and coat color phenotype.

	Genotype frequency			Generalized linear model				Logistic regression			
Loci	CC	TT	CT	Estimate	Std. Error	t-value	Pr>| t |	Estimate	Std. Error	Z-value	Pr>| z |
M-1	55	71	53	0.0528	0.0792	0.6669	$5.0567e^{-01}$	-0.5301	0.2682	-1.976	0.0481*
M-7	130	28	21	-0.0390	0.0886	-0.4403	$6.6024e^{-01}$	0.2886	0.2973	0.971	0.3316

Conflicts of interest

The authors report no conflicts of interest.

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Supplementary material

Table S1. The main antigenic domains (MADs) of goat DSG4).

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