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Polymorphism of the Goat Agouti Signaling Protein Gene and Its Relationship with Coat Color in Italian and Spanish Breeds

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Abstract Agouti signaling protein (ASIP) is one of the key players in the modulation of hair pigmentation in mammals. Binding to the melanocortin 1 receptor, ASIP induces the synthesis of phaeomelanin, associated with reddish brown, red, tan, and yellow coats. We have sequenced 2.8 kb of the goat *ASIP* gene in 48 individuals and identified two missense (Cys126Gly and Val128Gly) and two intronic polymorphisms. *In silico* analysis revealed that the Cys126Gly substitution may cause a structural change by disrupting a highly conserved disulfide bond. We studied its segregation in 12 Spanish and Italian goat breeds ($N = 360$) with different pigmentation patterns and found striking differences in the frequency of the putative loss-of-function Gly_{126} allele (Italian 0.43, Spanish Peninsular 0.08), but we did not observe a clear association with coat color. This suggests that the frequency of this putative loss-of-function allele has evolved under the influence of

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demographic rather than selection factors in goats from these two geographical areas.

Keywords Goat · Agouti signaling protein · Coat color

Introduction

Binding of proopiomelanocortin to the melanocortin 1 receptor (MC1R) is the leading event that activates the synthesis of eumelanin via transcriptional and post-translational pathways (Hida et al. 2009). The agouti signaling protein (ASIP) is a soluble factor, secreted by dermal papilla cells in hair bulbs, that antagonizes the effects of proopiomelanocortin. In this way, ASIP promotes the synthesis of phaeomelanin, a yellow–red pigment (Hida et al. 2009). In cattle, the *ASIP* locus encompasses 5.33 kb and includes three exons (http://www.ensembl.org). Several alleles have been identified so far. There is evidence that they are associated with distinct phenotypes, including the modification of the wild-type pigmentation pattern (allele a^W, in breeds such as Brown Swiss), color lightening in the belly (a^i, Jersey and Limousine), and a dark recessive coat (Olson 1999, Seo et al. 2007). In sheep, there is compelling evidence that a duplication of the *ASIP* gene, with the second copy being ubiquitously expressed, is associated with a white coat (Norris and Whan 2008). In contrast, recessive black might be explained by promoter silencing in sheep harboring a single copy of the *ASIP* gene (Norris and Whan 2008).

The molecular basis of coat color inheritance is poorly understood in goats because many of the candidate genes potentially involved in pigmentation have not been sequenced or their levels of variability have not been assessed. Recently, several nonsense and missense amino acid substitutions at the goat *MC1R* gene have been reported (Fontanesi et al. 2009a). Genetic variability of the caprine *ASIP* gene has also been investigated by several authors (Tang et al. 2008; Fontanesi et al. 2009b; Li et al. 2010). None of the detected polymorphisms showed a complete association with coat color, suggesting that causal mutations remain to be identified (Fontanesi et al. 2009b). In fact, Fontanesi et al. (2009b) found evidence that duplication of the *ASIP* gene might be associated with the white coat of certain goat breeds. The main goal of our study is to characterize further the sequence variability of the goat *ASIP* gene and explore its relationship with coat color.

Materials and Methods

Nucleic Acid Isolation

Blood and hair samples were taken from 48 individuals belonging to the Malagueña, Cashmere, Saanen, Ajuy, Montefalcone, Sahelienne, Palmera, Majorera, Tinerfeña, Garganica, Jonica, Maltesa, Cilentana Nera, Grigia Molisana, Derivata di Siria, and Girgentana breeds. Genomic DNA extractions from the samples were made with the

DNeasy Blood and Tissue kit (Qiagen Iberia SL, Barcelona, Spain). A Nanodrop spectrophotometer ND-1000 (SG Servicios Hospitalarios, Barcelona, Spain) was employed to quantify the concentration of genomic DNA. We also sequenced complementary DNA obtained from skin samples corresponding to Palmera, Majorera, and Tinerfeña goats (tissue samples were not available for the remaining breeds). Skin samples were preserved in RNA-later (Applied Biosystems, Sant Andreu De Llavaneres, Spain) at a temperature of −20°C. RNA was isolated with the RiboPure kit (Applied Biosystems) and quantified with Agilent 2100 Bioanalyzer equipment (Agilent Technologies, Barcelona, Spain). Complementary DNA was synthesized with the Thermoscript RT-PCR System kit (Invitrogen, Barcelona, Spain).

Amplification and Resequencing of the Goat *ASIP* Locus

Five pairs of primers were used to characterize the goat *ASIP* gene (Table 1). Exons were numbered according to Fontanesi et al. (2009b). Most of the amplified regions have been previously sequenced in goats (GenBank accession no. EF587236), with the one exception of the PCR5 fragment, which encompasses the 3′ UTR region. The thermal profile of PCR1–2 and 4–5 consisted of 35 cycles of 94°C for 45 s, annealing temperature (Table 1) for 45 s, and 72°C for 1 min. The composition of these four amplification reactions was 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.2 μM each primer, 50-80 ng genomic DNA (PCR2 and PCR4-5) or 1.5 μL cDNA (PCR1), and 0.5 U *Taq* DNA polymerase (Ecogen, Barcelona, Spain) in a final volume of 20 μL. The Expand Long Template PCR System (Roche Diagnostics, Sant Cugat, Spain) was used to amplify a genomic region of 1.41 kb (PCR3). Amplification reactions contained 2.75 mM $MgCl_2$, 0.5 mM dNTPs, 0.3 μM each primer (ASIP-FW-EXON1, ASIP-REV-EXON1), 1.8 U *Taq* and *Tgo* DNA polymerase mix, and approximately 100 ng genomic DNA in a final volume of 50 μl. The thermal cycling profile was 94°C for 5 min; 30 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 90 s; with a final extension step of 72°C for 7 min. PCR purification used the ExoSAP-IT kit (GE Healthcare, Barcelona, Spain), and sequencing reactions (with the primers in Table 1) used the BigDye Terminator version 1.3 Cycle Sequencing Kit (Applied Biosystems). Genotyping was performed in a Sequenom MassArray iPlex platform at the Spanish National Genotyping Centre (CeGen, Santiago de Compostela, Spain).

Statistical Analysis

Population diversity parameters were estimated with the PopGen32 software (http://cc.oulu.fi/~jaspi/popgen/popgen.htm). We calculated observed heterozygosity (H_o), Shannon's index (Shannon and Weaver 1949), and the effective number of alleles (Hartl and Clark 1989). Deviations from Hardy–Weinberg equilibrium were tested using chi-square and likelihood ratio tests at each locus. Expected frequencies were calculated according to Levene (1949).

Table 1 Five primer pairs used in the characterization of the goat *ASIP* gene

PCR	Primer	Sequence[a]	Complementary region[a]	Annealing temp. (°C)
PCR1 (0.31 kb)	ASIP-FW-cDNA	CCTGGCTACCTTGCTGGTCT	561–580	62
	ASIP-REV-cDNA	GCACGGGTCGCAGCAG	5638–5653	
PCR2 (0.4 kb)	ASIP-FWGEXON1	GAAGAAAGCAGGAGGGCACA	459–478	60
	ASIP-REVGEXON1	GGGCACTTGATTCCTCCAGA	840–859	
PCR3 (1.41 kb)	ASIP-FW-EXON1	CCTGAGGAAAAGCCCAGAGA	616–635	60
	ASIP-REV-EXON1	CTTGTTCAGCGCTTCAAAGAGA	2006–2027	
PCR4 (1.12 kb)	ASIP-FW-EXON2	CTTCCAAGGTAGGCCTGGG	2074–2092	62
	ASIP-REV-EXON2	CTTTCAAATGGAAAGGCTCAGTTT	3175-3198	
PCR5 (0.61 kb)	ASIP-FW-EXON3	GGGACGTCTAGTCCGAGGAGT	5453–5473	60
	ASIP-REV-EXON3	CTGCCAGATCCAGAAAAGCG	–	

[a] Primer complementary regions, according to goat *ASIP* reference sequence EF587236, except ASIP-REV-EXON3, which is complementary to the 3′ downstream region of the bovine *ASIP* gene (Ensembl entry ENSBTAT00000048322)

Results

Identification of Polymorphism in the Goat ASIP Gene

We have amplified and sequenced 2.8 kb of the goat *ASIP* gene (Fig. 1; GenBank accession nos. GU224268 and GU076166). The alignment of sequences corresponding to 48 individuals allowed us to detect one *ASIP* variant, c.376 T>G, in the last exon (Fig. 2). This polymorphism is expected to be highly damaging because it involves the substitution of a highly conserved Cys_{126} residue, forming a disulfide bond (Yu and Millhauser 2007), by Gly. *In silico* analysis with the Panther software (Thomas and Kejariwal 2004) confirmed that this mutation might be highly deleterious (subPSEC = −5.82; probability of causing a deleterious effect: 0.94). Moreover, we found preliminary evidence of the existence of three additional SNPs. In this way, we identified a missense mutation at exon 4 (c.383 T>G, Val128Gly) and two intronic mutations (Supplementary Fig. 1). Although this exon 4 missense mutation is located in a conserved position of the cysteine-rich carboxy-terminal domain of the ASIP protein, *in silico* analysis with the Panther software (Thomas and Kejariwal 2004) predicted that this SNP does not have a major structural effect (probability of causing a deleterious effect: 0.46). Moreover, this mutation has been reported to have no association with coat color in several goat breeds (Fontanesi

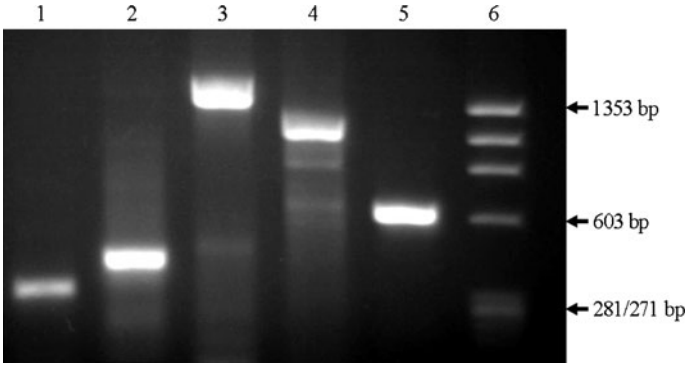

Fig. 1 Agarose gel electrophoresis of amplified products corresponding to the goat *ASIP* gene. *Lane 1* PCR1 (0.31 kb), *lane 2* PCR2 (0.4 kb), *lane 3* PCR3 (1.41 kb), *lane 4* PCR4 (1.12 kb), *lane 5* PCR5 (0.61 kb), *lane 6* lambda *Hin*dIII/phiX *Hae*III molecular weight DNA marker (1353, 1078, 872, 603, and 281/271 bp)

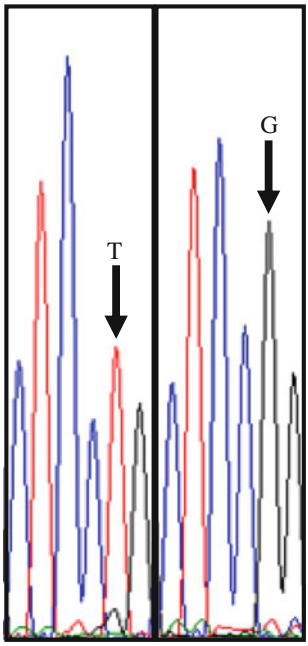

Fig. 2 Polymorphic site detected by sequencing of genomic DNA samples obtained from goats with TT (*left arrow*) and GG (*right arrow*) c.376 T>G *ASIP* genotypes

et al. 2009b). Because none of these three SNPs was expected to have functional effects, they were not validated by large-scale genotyping.

Segregation of the c.376 T>G Polymorphism in Goat Breeds with Different Coat Colors

We have analyzed the segregation of the c.376 T>G polymorphism in a panel of 360 goats belonging to 12 breeds (Fig. 3; Table 2). In sheep, a closely related

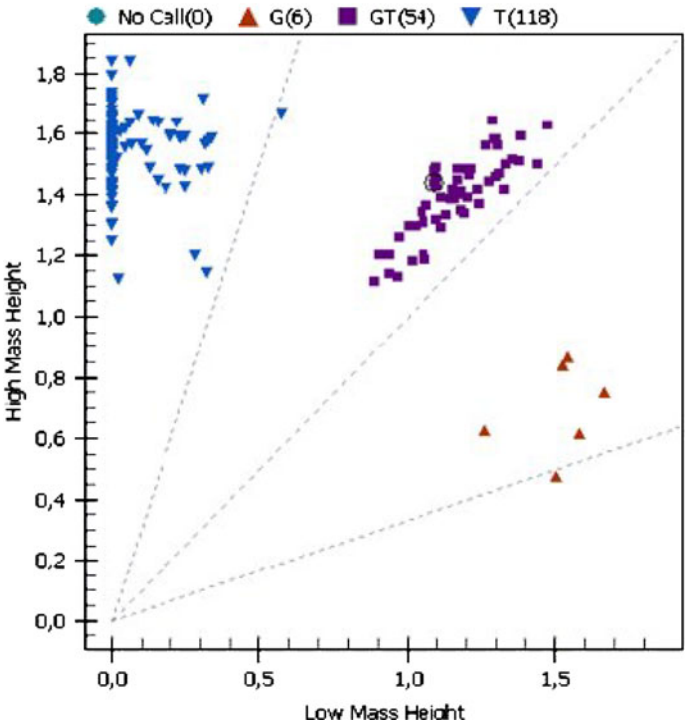

Fig. 3 Cluster plot of *ASIP* genotypes obtained with the Sequenom MassArray iPlex technology

species, the assignment of *ASIP* alleles to specific loci is very complex because there are two highly similar duplicated copies of *ASIP* (Norris and Whan 2008). In the absence of opposing evidence, we have assumed that the Cys_{126} and Gly_{126} are two alleles of a single-copy goat *ASIP* locus. This assumption is consistent with the pattern of segregation we have observed (GG, GT, and TT genotypes are present) and with data reported by Fontanesi et al. (2009b). Population diversity indices (Table 3) happened to be higher in Italian and Canarian breeds than in their Spanish Peninsular counterparts. Hardy–Weinberg analysis showed that most of the populations did not depart significantly from this model (Table 3), except for the Girgentana, Jonica, and Murciano-Granadina breeds. The populations of Girgentana and especially Jonica are relatively small, so these results should be interpreted with caution. The number of genotyped Murciano-Granadina goats, however, was quite large, and the evidence of a departure from Hardy–Weinberg happened to be highly significant.

Discussion

Sequencing the goat *ASIP* gene in several individuals revealed four variable sites, of which two were missense and coincided with those reported by Fontanesi et al.

Table 2 Segregation of the c.376 T>G polymorphism in 12 goat breeds

Breed	Individuals sampled	Coat color	Genotype			Location
			TT	TG	GG	
Cilentana Nera	22	Black	0.09	0.68	0.23	South Western Italy
Garganica	26	Black, sometimes with reddish areas	0.12	0.61	0.27	South Eastern Italy
Derivata di Siria	11	Red	0.10	0.30	0.60	Southern Italy
Girgentana	19	White, with brownish or brown areas in the front and cheeks	0.85	0.10	0.05	Southern Italy
Jonica	10	White, sometimes slightly reddish and/or with brownish areas in the head and neck	0.80	0.10	0.10	Southern Italy
Malagueña	52	Cream to dark red	1.00	0.00	0.00	Southern Spain
Murciano-Granadina	81	Black or dark brown	0.85	0.12	0.03	Southern Spain
Saanen	58	White	1.00	0.00	0.00	Switzerland
Rasquera	10	White, very often with black spots and more rarely with reddish/cream spots	0.70	0.30	0.00	North Eastern Spain (Catalonia)
Palmera	37	Red	0.57	0.38	0.05	Spain (Canary Islands)
Tinerfeña	18	Black or dark brown	0.67	0.22	0.11	Spain (Canary Islands)
Majorera	16	Wide variety	0.37	0.5	0.13	Spain (Canary Islands)

(2009b). We decided to focus our study on the c.376 T>G polymorphism because it involves an amino acid change predicted to have structural consequences. In principle, this SNP is not expected to have effects on *ASIP* mRNA levels because it does not map to a regulatory region. Moreover, nonsense-mediated mRNA decay just targets mRNAs containing premature translation termination codons (Conti and Izaurralde 2005), which is not the case. In a similar study performed in the Xalda sheep breed, Royo et al. (2008) analyzed a putative loss-of-function mutation in the coding region of the ovine *ASIP* gene and did not find any relationship with mRNA levels. In summary, it is reasonable to assume that the c.376 T>G polymorphism might alter ASIP function by disrupting a highly conserved disulfide bridge rather than by affecting its transcriptional rate.

Unexpectedly, we were unable to find a clear relationship between *ASIP* c.376 T>G genotypes and coat color (Table 2). In the Italian breeds, the putative loss-of-function Gly_{126} allele had very different allelic frequencies in goats with black coats and those with white coats. In this way, this allele was relatively abundant in the Garganica (0.58) and Cilentana Nera (0.57) black breeds, while its frequency was very low in Girgentana (0.10) and Jonica (0.15) white goats. The Gly_{126} allele, however, was also abundant in the red-coated Derivata di Siria goats. This finding is surprising because non-functional alleles of the *ASIP* gene promote

Table 3 Population diversity for the c.376 T>G polymorphism in 12 goat breeds

Breed	Population diversity[a]			Hardy–Weinberg test	
	H_o	I	N_e	χ^2	P
Cilentana Nera	0.681	0.683	1.963	2.957	0.085
Garganica	0.615	0.681	1.953	1.513	0.218
Derivata de Siria	0.272	0.536	1.541	0.889	0.345
Girgentana	0.100	0.325	1.219	5.473	0.019*
Jonica	0.100	0.422	1.342	5.647	0.017*
Malagueña	0.000	0.000	1.000	–	–
Murciano-Granadina	0.123	0.294	1.187	4.312	0.037*
Saanen	0.000	0.000	1.000	–	–
Rasquera	0.300	0.422	1.342	0.198	0.655
Tinerfeña	0.222	0.529	1.528	2.833	0.092
Majorera	0.500	0.661	1.882	0.019	0.889
Palmera	0.378	0.554	1.582	0.007	0.931

[a] H_o Observed heterozygosity, I Shannon's index, N_e effective number of alleles

* Significant deviation from Hardy–Weinberg equilibrium

the synthesis of eumelanin rather than of phaeomelanin. Similarly, we did not find a clear relationship between the c.376 T>G polymorphism and coat color in Spanish breeds. The Cys_{126} allele was fixed in the Malagueña breed that displays a variety of colors from cream to dark red. The putative loss-of-function allele was nearly absent from the Murciano-Granadina population (0.09) that has a solid black coat. This unexpected result could be due to genetic heterogeneity (i.e., the existence of additional nonfunctional alleles in the *ASIP* gene that remain to be identified) or to the occurrence of epistatic interactions with other loci. Finally, Canarian breeds (Palmera, Majorera, and Tinerfeña) displayed the Gly_{126} allele at frequencies of 0.22 to 0.38. This finding agrees well with previous results indicating that Canarian goats have a genetic background and a phylogeographic structure remarkably different from that of Iberian breeds (Amills et al. 2004).

In concordance with our population diversity measurements, Tang et al. (2008) analyzed a G → T polymorphism, mapping to exon 4 of the goat *ASIP* gene, in 12 Chinese breeds and found that most of them were at equilibrium; the exceptions were Nanjiang Brown (a fast growth strain) and Guizhou populations. They interpreted the deviations as evidence of selective pressure acting on the goat *ASIP* gene. Similarly, Fontanesi et al. (2009b) studied the segregation of three ASIP polymorphisms (Ala96Gly, Cys126Gly, and Val128Gly) in six goat breeds and found that Ala96Gly and Cys126Gly were in equilibrium, but Val128Gly was in disequilibrium in the Girgentana and Saanen goat populations. The lack of Hardy–Weinberg equilibrium in certain goat populations might be explained by multiple technical (sample size) and biological (genetic drift, selection, and inbreeding) factors. Inbreeding is not expected to have a major effect on *ASIP* genotype frequencies because, in general, local goat breeds are managed in an extensive way,

and the number of half-sibs derived from a given buck is relatively small. In Holstein dairy cattle, where artificial insemination is very common and bulls can have thousands of daughters, inbreeding coefficients are around 0.04 (Thompson et al. 2000). It is reasonable to expect that inbreeding coefficients of Mediterranean goat breeds are well below this value, meaning that inbreeding has a negligible effect on their genotype frequencies. Although directional selection might act on the goat *ASIP* gene in certain scenarios, in the absence of clear effects of c.376 T>G on pigmentation it is difficult to justify it from a biological point of view. Conceivably, the Gly_{126} mutation might be linked to another one with causal effects, such as the CNV reported by Fontanesi et al. 2009b, favoring the occurrence of genetic hitchhiking. Genetic drift, founder effects (associated with breed creation), and bottlenecks (in endangered breeds such as Rasquera) might have also modified *ASIP* allele frequencies in a substantial way, producing different patterns in Iberian, Italian, and Canarian breeds. In-depth sequencing of the goat *ASIP* gene and haplotype reconstruction would allow neutrality tests, such as Tajima's *D* (Tajima 1989), with the aim of detecting significant departures from mutation-drift equilibrium. Even in this case, we anticipate that it would be difficult to disentangle the effects of demographic and selective forces.

In this study, we have analyzed eight additional goat populations in previous studies characterizing ASIP variation (Tang et al. 2008; Fontanesi et al. 2009b). Our conclusion match those of Fontanesi et al. (2009b) in the sense that the Gly_{126} allele does not seem to be clearly associated with darker pigmentation. The recent identification of copy number variations in the caprine *ASIP* locus illustrates the complex inheritance of coat color in this ruminant species (Fontanesi et al. 2009b). In the future, the structural characterization of additional genes and the assessment of their levels of polymorphism in diverse goat populations will be necessary to elucidate the genetic factors that modulate pigmentation as well as to unveil the intricate network of interactions that they establish in order to express this trait.

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References

Amills M, Capote J, Tomàs A, Kelly L, Obexer-Ruff G, Angiolillo A, Sanchez A (2004) Strong phylogeographic relationships among three goat breeds from the Canary Islands. J Dairy Res 71:257–262

Conti E, Izaurralde E (2005) Nonsense-mediated mRNA decay: molecular insights and mechanistic variations across species. Curr Opin Cell Biol 17:316–325

Fontanesi L, Beretti F, Riggio V, Dall'Olio S, Gómez González E, Finocchiaro R, Davoli R, Russo V, Portolano B (2009a) Missense and nonsense mutations in melanocortin 1 receptor (*MC1R*) gene of different goat breeds: association with red and black coat color phenotypes but with unexpected evidences. BMC Genet 10:47

Fontanesi L, Beretti F, Riggio V, Gómez González E, Dall'Olio S, Davoli R, Russo V, Portolano B (2009b) Copy number variation and missense mutations of the agouti signaling protein (*ASIP*) gene in goat breeds with different coat colors. Cytogenet Genome Res 126:333–347

Hartl DL, Clark AG (1989) Principles of Population Genetics, 2nd edn. Sinauer Associates, Sunderland, pp 123–132

Hida T, Wakamatsu K, Sviderskaya EV, Donkin AJ, Montoliu L, Lynn Lamoreux M, Yu B, Millhauser GL, Ito S, Barsh GS, Jimbow K, Bennett DC (2009) Agouti protein, mahogunin, and attractin in pheomelanogenesis and melanoblast-like alteration of melanocytes: a cAMP-independent pathway. Pigment Cell Melanoma Res 22:623–634

Levene H (1949) On a matching problem in genetics. Ann Math Stat 20:91–94

Li XL, Zhao JW, Tang CJ, Zhou RY, Zheng G, Li LH, Guo XL (2010) Sequencing of part of the goat agouti gene and SNP identification. Biochem Genet 48:152–156

Norris BJ, Whan VA (2008) A gene duplication affecting expression of the ovine *ASIP* gene is responsible for white and black sheep. Genome Res 18:1282–1293

Olson TA (1999) Genetics of colour variation. In: Fries R, Ruvinsky A (eds) The genetics of cattle. CABI Publishing, Wallingford

Royo LJ, Alvarez I, Arranz JJ, Fernández I, Rodríguez A, Pérez-Pardal L, Goyache F (2008) Differences in the expression of the *ASIP* gene are involved in the recessive black coat colour pattern in sheep: evidence from the rare Xalda sheep breed. Anim Genet 39:290–293

Seo K, Mohanty TR, Choi T, Hwang I (2007) Biology of epidermal and hair pigmentation in cattle: a mini-review. Vet Dermatol 18:392–400

Shannon CE, Weaver W (1949) A mathematical model of communication. University of Illinois Press, Urbana

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595

Tang CJ, Zhou RY, Li XL, Zhao JW, Li LH, Feng FJ, Li DF, Wang JT, Guo XL, Keng JF (2008) Variation of 423G > T in the Agouti gene exon 4 in indigenous Chinese goat breeds. Biochem Genet 46:770–780

Thomas PD, Kejariwal A (2004) Coding single-nucleotide polymorphisms associated with complex vs. Mendelian disease: evolutionary evidence for differences in molecular effects. Proc Natl Acad Sci USA 101:15398–15403

Thompson JR, Everett RW, Hammerschmidt NL (2000) Effects of inbreeding on production and survival in Holsteins. J Dairy Sci 83:1856–1864

Yu B, Millhauser GL (2007) Chemical disulfide mapping identifies an inhibitor cystine knot in the agouti signaling protein. FEBS Lett 581:5561–5565