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Coat colours in the Massese sheep breed are associated with mutations in the agouti signalling protein (*ASIP*) and melanocortin 1 receptor (*MC1R*) genes

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Massese is an Italian dairy sheep breed characterized by animals with black skin and horns and black or apparent grey hairs. Owing to the presence of these two coat colour types, this breed can be considered an interesting model to evaluate the effects of coat colour gene polymorphisms on this phenotypic trait. Two main loci have been already shown to affect coat colour in sheep: Agouti *and* Extension *coding for the agouti signalling protein (*ASIP*) and melanocortin 1 receptor (*MC1R*) genes, respectively. The* Agouti *locus is affected by a large duplication including the* ASIP *gene that may determine the* Agouti *white and tan allele (*A^{Wt}*). Other disrupting or partially inactivating mutations have been identified in exon 2 (a deletion of 5 bp, D_5; and a deletion of 9 bp, D_9) and in exon 4 (g.5172T>A, p.C126S) of the* ASIP *gene. Three missense mutations in the sheep* MC1R *gene cause the dominant black* E^D *allele (p.M73K and p.D121N) and the putative recessive* e *allele (p.R67C). Here, we analysed these* ASIP *and* MC1R *mutations in 161 Massese sheep collected from four flocks. The presence of one duplicated copy allele including the* ASIP *gene was associated with grey coat colour (*$P = 9.4E\text{-}30$*). Almost all animals with a duplicated copy allele (37 out of 41) showed uniform apparent grey hair and almost all animals without a duplicated allele (117 out of 120) were completely black. Different forms of duplicated alleles were identified in Massese sheep including, in almost all cases, copies with exon 2 disrupting or partially inactivating mutations making these alleles different from the* A^{Wt} *allele. A few exceptions were observed in the association between* ASIP *polymorphisms and coat colour: three grey sheep did not carry any duplicated copy allele and four black animals carried a duplicated copy allele. Of the latter four sheep, two carried the* E^D *allele of the* MC1R *gene that may be the cause of their black coat colour. The coat colour of all other black animals may be determined by non-functional* ASIP *alleles (non-agouti alleles,* A^a*) and in a few cases by the* E^D Extension *allele. At least three frequent* ASIP *haplotypes ([D_5:g.5172T], [N:g.5172A] and [D_5:g.5172A]) were detected (organized into six different diplotypes). In conclusion, the results indicated that coat colours in the Massese sheep breed are mainly derived by combining* ASIP *and* MC1R *mutations.*

Keywords: coat colour, sheep, *ASIP*, *MC1R*, mutations

Implications

Two coat colour types segregate in Massese sheep: black and grey. This paper showed that mutations in the agouti signalling protein (*ASIP*) and melanocortin 1 receptor (*MC1R*) genes are associated with these two coat colour types in this breed, even if other genetic factors could be involved. In particular, the presence of different alleles containing a duplicated *ASIP* gene associated with the grey coat colour opens up other questions on its effects on reproduction and production traits that should be further investigated in comparison with other breeds.

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Introduction

Coat colour is one of the most important traits that can be used to distinguish farm animal breeds. This trait is influenced by a large number of genes (Bennett and Lamoreux, 2003) that are involved in determining the presence, distribution and biochemical activities of the melanocytes. These specialized cells synthesize eumelanins and pheomelanins, determining black/brown and red/yellow colours, respectively. Two main loci (*Agouti* and *Extension*) control and regulate the relative amount of these two melanin types in skin and hair (Searle, 1968). The *Agouti* locus encodes for the agouti signalling protein (ASIP; Bultman *et al.*, 1992). This small paracrine signalling molecule interacts with the

product of the *Extension* locus that encodes for the melanocortin 1 receptor (MC1R). MC1R is a seven transmembrane domains protein belonging to the G-protein coupled receptor present on the surface of the melanocyte membrane (Robbins *et al.*, 1993). Eumelanin synthesis is induced by the binding of MC1R with the α-melanocyte-stimulating hormone (αMSH). Alternative binding of MC1R with αMSH and ASIP causes a pigment-type switching from eumelanins to pheomelanins (Lu *et al.*, 1994; Ollmann *et al.*, 1998).

The *Agouti* and *Extension* loci show epistatic interactions with few exceptions. The presence of wild-type *Extension* alleles is usually needed for the expression of the *Agouti* allele. Dominant *Extension* alleles determine eumelanin production and black coat colour, whereas recessive alleles determine red/yellow/pale pigmentation due to pheomelanin synthesis. On the contrary, dominant *Agouti* alleles cause pheomelanin production, whereas recessive alleles determine eumelanin accumulation and black/dark coat colour (Searle, 1968).

Gain-of-function and loss-of-function mutations of the *MC1R* gene determining dominant or partially dominant black/dark and recessive or partially recessive red/yellow/pale coat colour phenotypes, respectively, have been described in a large number of mammals including, for example, mice (Robbins *et al.*, 1993), humans (Valverde *et al.*, 1995) and several farm animals like cattle (Klungland *et al.*, 1995; Joerg *et al.*, 1996; Rouzaud *et al.*, 2000), pigs (Kijas *et al.*, 1998 and 2001), horses (Marklund *et al.*, 1996), dogs (Everts *et al.*, 2000; Newton *et al.*, 2000), cats (Eizirik *et al.*, 2003; Peterschmitt *et al.*, 2009), rabbits (Fontanesi *et al.*, 2006) and goats (Fontanesi *et al.*, 2009a). In sheep, classical genetic studies have identified two alleles at the *Extension locus*: the *dominant black* (E^D) allele caused by two missense mutations in the *MC1R* gene (p.M73K and p.D121N) and present in the Norwegian Dala, Corriedale, Damara, Black Merino, Black Castellana and Karakul breeds (Våge *et al.*, 1999 and 2003; Royo *et al.*, 2008); and the *wild type* (E^+) allele widely distributed in most breeds (Searle, 1968; Sponenberg, 1997). We recently identified another *MC1R* missense mutation (p.R67C) in the Valle del Belice breed that we suggested to cause the recessive *e* allele at the ovine *Extension* series (Fontanesi *et al.*, 2010a).

The coding region of the *ASIP* gene is determined by three exons (2, 3 and 4, according to the nomenclature in mice). The ASIP protein sequence contains a secretion signal, a basic amino-terminal domain, a proline stretch and a cysteine-rich carboxy-terminal domain that folds in a characteristic knot structure. The cysteine-rich domain is responsible for melanocortin binding activity *in vitro* (Kiefer *et al.*, 1997; McNulty *et al.*, 2005). A large number of alleles have been described at the *Agouti* locus in mice, with most having been characterized at the DNA level (e.g. Bultman *et al.*, 1992; Miltenberger *et al.*, 2002). Mutations at this locus affecting or associated with coat colours or skin pigmentation have been also identified in other species. These mutations can affect both coding (rat, Kuramoto *et al.*, 2001; horse, Rieder *et al.*, 2001; dog, Kerns *et al.*, 2004; Berryere *et al.*, 2005; cat, Eizirik *et al.*, 2003; fox, Våge *et al.*, 1997; rabbit, Fontanesi *et al.*, 2010b) and non-coding/regulatory regions (human: Kanetsky *et al.*, 2002; cattle: Girardot *et al.*, 2006). In sheep, at least three mutations were suggested to cause the non-agouti recessive black coat colour (Norris and Whan, 2008; Royo *et al.*, 2008; Gratten *et al.*, 2010): a 5 bp deletion in exon 2 (g.100-105delAGGAA, denoted D_5), a missense mutation in exon 4 (g.5172T>A or p.C126S) and a regulatory mutation only indirectly deduced but not yet characterized. Another deletion of 9 bp in exon 2 (g.10-19delAGCCGCCTC, denoted D_9), positioned just 10 bp after the ATG start codon, would result in the loss of a tripeptide that might partially impair the ASIP function (Norris and Whan, 2008). In addition, a copy number variation (CNV) of a large genomic region including the ovine *ASIP* gene has been shown to be the genetic cause of the dominant white/tan (A^{Wt}) *Agouti* allele in sheep, determining white hair colour (Norris and Whan, 2008).

Massese is an Italian local sheep breed that accounts for about 5000 animals registered to the herd book. It is considered a dairy breed that was originated in the province of Massa and that nowadays is mainly reared in the Tuscany and Emilia Romagna regions. Massese is also considered one of the few black Italian sheep breed. However, sheep have black skin and horns and black or grey hairs (Figure 1). It is also worth mentioning that the apparent grey coat colour in some of these animals could be due to the presence of pale hairs in a black background. Owing to the presence of these two coat colour types, this breed is an interesting model to evaluate the effects of coat colour gene polymorphisms on this phenotypic trait.

Here, we analysed the *ASIP* and *MC1R* genes in Massese sheep and showed that mutations at these two loci are associated, even if not completely, with the two coat colour types of this breed: black and grey.

Material and methods

Animals and samples

Hair root or milk samples were collected from 161 adult (1 to 7 years old) Massese sheep of four different flocks (14, 5, 69 and 73 sheep from flocks A, B, C and D, respectively). The hair colour of these sheep was recorded during sample collection based on visual classification by two independent persons (L.F. and S.D.) using a classification into two classes: black ($n = 121$) or grey ($n = 40$; Figure 1). The grey class included animals with different greyness, from pale to dark grey. Hair root samples were also collected from 10 Appenninica (from two flocks), 10 Bergamasca (from one flock) and 10 Sarda (from two flocks) sheep with white hair and unpigmented skin.

DNA extraction and genotyping

DNA was extracted from milk using the Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA) and from hair roots using rapid extraction methods (Russo *et al.*, 2007).

PCR-RFLP was used to genotype the animals for four missense mutations: three missense mutations have been

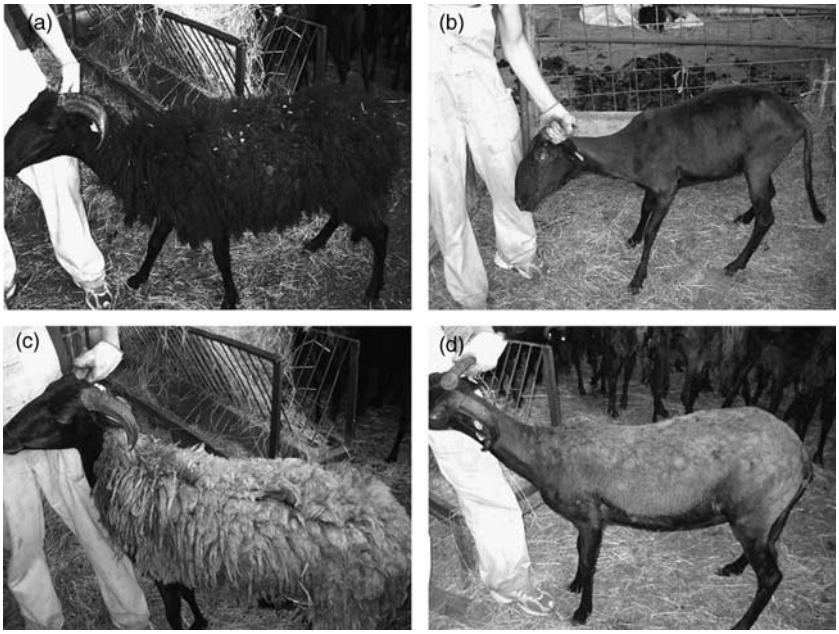

Figure 1 Black Massese sheep before (a) and after (b) shaving. Grey Massese sheep before (c) and after (d) shaving.

identified in the ovine *MC1R* gene and determine the putative recessive *e* allele (c.199C>T or p.R67C) and the black dominant E^D allele (c.218T>A or p.M73K and c.361G>A or p.D121N) at the *Extension* locus (Våge *et al*., 1999; Fontanesi *et al*., 2010a); another missense mutation has been identified in exon 4 of the ovine *ASIP* gene (g.5172T>A or p.C126S; Norris and Whan, 2008) and is associated with black non-agouti coat colour.

PCR analyses were carried out in a volume of 20 μl containing a 10 to 100 ng DNA template, 1 U DNA EuroTaq DNA polymerase (EuroClone Ltd, Paington, Devon, UK), 1X PCR Buffer, 2.5 mM dNTPs, 10 pmol of each primer and optimized $MgCl_2$ concentrations (from 1.0 to 2.0 mM). PCR profile was as follows: 5 min at 95°C; 35 amplification cycles of 30 s at 95°C, 30s at 58°C to 64°C, 30 s at 72°C; 5 min at 72°C (Table 1).

Restriction enzyme digestion was obtained with 5 μl of PCR product in a total of 25 μl of reaction volume including 5 U of restriction enzyme and 1X reaction buffer. The resulting DNA fragments were electrophoresed in 10% polyacrylamide : bisacrylamide 29 : 1 TBE 1X gels. DNA bands were visualized with 1X GelRed Nucleid Acid Gel Stain (Biotium Inc., Hayward, CA, USA). Details of the PCR-RFLP protocols used for the analysis of the three *MC1R* missense mutations have been already reported in Fontanesi *et al*. (2010a; see also Table 1). The g.5172T>A *ASIP* polymorphism was genotyped inserting an artificial restriction site for *Pst*I during amplification with a mismatched reverse primer. Digestion of a 273 bp amplified fragment with *Pst*I endonuclease (Fermentas; recognition sequence CTGCAG) at 37°C overnight produced two fragments (251 and 22 bp) when allele g.5172T was present, whereas only one fragment (undigested product) was obtained when allele g.5172A was present.

Fragment analysis was used to genotype the sampled sheep for the D_5 and D_9 deletions already reported in exon 2 of the *ASIP* gene (Smit *et al*., 2002; Norris and Whan, 2008). PCR amplification was carried out as described above but in a 10 μl final volume including a 5′ 6-FAM-labelled primer (Table 1). Amplified products were loaded on an ABI3100 Avant capillary sequencer (Applied Biosystems, Foster City, CA, USA). For this analysis, 1 to 2 μl of reaction product was diluted in 10 μl of Hi-Di formamide (Applied Biosystems) and added to 0.1 μl of Rox-labelled DNA ladders (500HD Rox, Applied Biosystems). Labelled DNA fragments were sized using GeneScan version 3.7 and Genotyper version 3.7 software (Applied Biosystems).

Fragment analysis was also used to identify if the sampled animals carried a duplicated DNA copy including the *ASIP* gene (Norris and Whan, 2008). For this analysis, a three-primer multiplex PCR was used to amplify both the junction between the duplicated DNA copies and the 5′-breakpoint sequences as reported by Norris and Whan (2008). Primers Agt16 and Agt18 spanning the junction between the duplicated copies produced a unique PCR product of 242 bp, whereas Agt16 and Agt17 spanning the 5′-breakpoint sequence produced a fragment of 238 bp. The amplification was carried out by asymmetric competitive PCR as described by Norris and Whan (2008) with few modifications (10 μl of final reaction volume and 25 cycles), with the Agt16 primer labelled in 5′ with 6-FAM. If the amplification resulted in these two fragments, the animal carried at least a copy of the duplicated chromosome region, whereas when only the fragment of 238 bp was obtained the animal did not carry any duplicated DNA region. Obtained fragments were analysed by capillary electrophoresis on an ABI3100 Avant sequencer (Applied Biosystems) as reported above. The relative ratio between the peak height of the two fragments (when a duplicated DNA copy was present) was calculated and compared between animals.

Table 1 *Primer sequences and PCR conditions for MC1R and ASIP sequencing, PCR-RFLP and fragment analyses*

Gene	Primer pair name	Forward and *reverse* primers (5′-3′)	Gene region (amplified fragment length)	PCR conditions[†]	Use
ASIP	ex2	cacatgcatttgccagacc	Part of intron 1, exon 2 and part of intron 2 (309 bp)	57/2.0/35/PT	Sequencing
		atcggcttggagagtgtttg			
ASIP	ex3	cagaagctgctggcctaagt	Part of intron 2, exon 3 and part of intron 3 (369 bp)	58/2.0/35/PT	Sequencing
		gctcgaggtcaggaaggttt			
ASIP	ex4	gcaggtggggacatctagtc	Part of intron 3, exon 4, CDS and 3′-UTR (391 bp)	55/2.0/35/PT	Sequencing
		gccccaacgtcaatagcc			
ASIP	ex4_5172	gcaggtggggacatctagtc cagcaggtggggttgagcacgctgc[‡]	Exon 4 (273 bp)	56/2.0/35/PT	PCR–RFLP analysis with *Pst*I (g.5172T>A; p.C126S)
ASIP	ex2_del	6FAM-ctacctgactgccttctctg	Exon 2 (174 bp)	59/2.0/25/PT	Fragment analysis (genotyping of the exon 2 deletions)
		aacaggttcatggaagaattg			
ASIP	Agt17[‖]	gtttctgctggacctcttgttc	Duplication breakpoint at the *Agouti* locus (238/242 bp)	58/2.0/23/PT	Fragment analysis (duplication breakpoint analysis, asymmetric competitive PCR)
	Agt18[‖]	gtgccttgtgaggtagagatggtgtt			
	Agt16[‖]	*6FAM-cagcaatgaggacgtgagttt*			
MC1R[§]	1_MC1R[¶]	agtgcctggaggtgtccatcc	5′-flanking region, 5′-UTR, part of CDS (169 bp)	62/2.0/35/PT	PCR–RFLP analysis with *Ssi*I (c.199C>T; p.R67C) and *Hin1*II (c.218T>A; p.M73K)
		ctgacgctcaccagcaagt			
MC1R	2_MC1R[¶]	agccatgagttgagcaggac	Part of CDS (376 bp)	64/1.0/35/PT	Sequencing
		caggacaccagcctccag			
MC1R	3_MC1R[¶]	gtgagcgtcagcaacgtg	Part of CDS (366 bp)	61/2.0/35/TG	Sequencing and PCR–RFLP analysis with *Tru1*I (c.361G>A; p.D121N)
		acatagaggacggccatcag			
MC1R	4_MC1R[¶]	gcctggttggcttcttcata *tggtctagcgatcctctttg*	Part of CDS and part of 3′-UTR (456 bp)	58/2.0/35/TG	Sequencing

[†]Annealing temperature (°C)/$MgCl_2$ concentration (mM)/no. of cycles/thermal cycler (TG = TGradient thermal cycler; PT = PT-100 thermal cycler).
[‡]The underlined base in primer reverse inserts an artificial restriction site for *Pst*I when allele g.5172T is present in the amplified product.
[§]The *MC1R* gene is constituted by only one exon.
[‖]Primers reported by Norris and Whan (2008).
[¶]Primers reported by Fontanesi *et al.* (2010a).

Sequencing

Sequencing of the three coding exons (exons 2, 3 and 4) with partial intronic regions of the *ASIP* gene and of the single exon *MC1R* gene was carried out for nine Massese sheep. PCR primers and conditions for amplification of these *ASIP* and *MC1R* gene fragments are reported in Table 1. Before sequencing, 3 to 5 μl of these PCR products were treated with 2 μl of ExoSAP-IT® (USB Corporation, Cleveland, Ohio, USA) following the manufacturer's protocol. Cycle sequencing of the PCR products was obtained with the Big Dye version 3.1 kit (Applied Biosystems) and sequencing reactions, after a few purification steps using EDTA 0.125 M, Ethanol 100% and Ethanol 70%, were loaded on an ABI3100 Avant sequencer (Applied Biosystems). All sequences were visually inspected and aligned using the BioEdit version 7.0.5.2 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and the CodonCode Aligner (http://www.codoncode.com/aligner) software.

Data analysis

Association between DNA markers and coat colours was tested using 2×2 contingency tables with Fisher's exact test implemented in the procedure FREQ of SAS version 8.02 (SAS Institute Inc., Cary, NC, USA). Haplotypes including the exon 2 deletions and the g.5172T > A single nucleotide polymorphism (SNP) were inferred using the PHASE program version 2.1 (Stephens *et al.*, 2001) in Massese sheep that did not carry any duplicated DNA region. Evaluation of the Hardy–Weinberg equilibrium was obtained with χ^2 analysis from the HWE software program (Linkage Utility Programs, Rockefeller University, New York, NY, USA).

Results

ASIP and MC1R genotypes

Analysing 161 Massese sheep with the assay designed to test the presence of a duplicated copy allele including the *ASIP* gene, one fragment of 238 bp was amplified in 120 animals, indicating that they did not carry any duplication at this locus. However, two fragments of 238 and 242 bp were amplified in 41 Massese sheep (7, 14 and 20 in flocks A, C and D, respectively), meaning that at least a duplicated copy allele was present in these animals (Table 2). The same assay was applied to the Appenninica, Bergamasca and Sarda sheep and all showed both DNA fragments (Table 2), as expected from their coat colour, according to what was indicated by Norris and Whan (2008). The relative ratio between the two fragment peaks in the 41 Massese sheep was 2.57 ± 0.26 on average (ranging from 3.26 to 1.94). The relative ratio in the Appenninica, Bergamasca and Sarda sheep was around 1 (1.07 ± 0.01). Figure 2 shows the

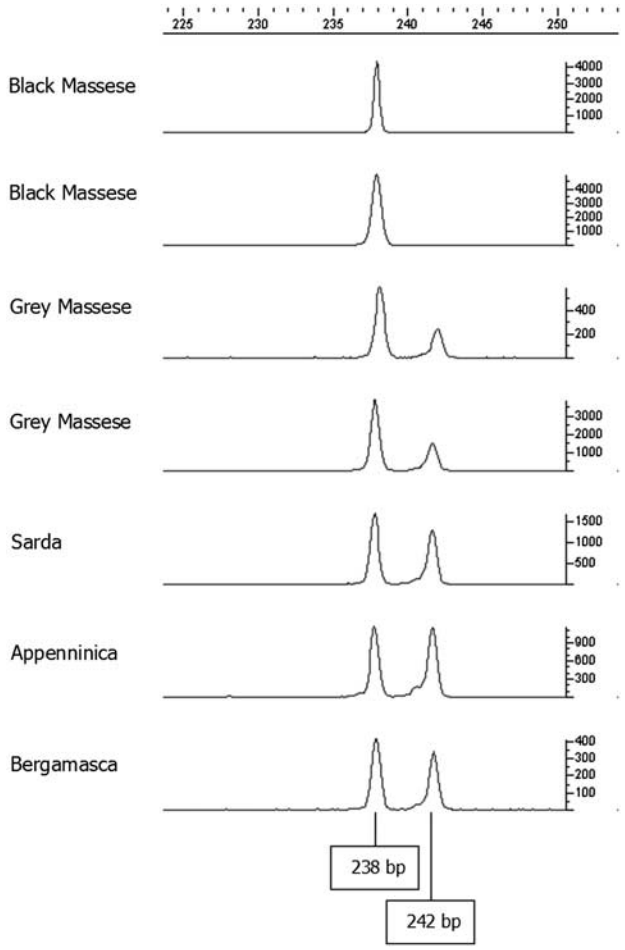

Figure 2 Electropherograms showing the results of the asymmetric competitive PCR used to evaluate the absence (only one fragment of 238 bp) or presence (two fragments of 238 and 242 bp) of a duplicated copy allele including the *ASIP* gene.

Table 2 *Results of the duplication breakpoint analyses at the* Agouti *locus (*ASIP *gene) in the investigated sheep*

			No. of sheep with different hair colour		
Breed	Duplicated DNA copy including *ASIP*[†]	No. of analysed sheep	Black	Grey	White
Massese	Absence	120 (4)[‡]	117 (4)[‡]	3	–
	Presence	41 (2)[‡]	4 (2)[‡]	37	–
Appenninica	Presence	10	–	–	10
Bergamasca	Presence	10	–	–	10
Sarda	Presence	10	–	–	10

Association with different hair colours.
[†]Absence = amplification of the 238 bp fragment only. Presence = amplification of the 238 and 242 bp fragments (Norris and Whan, 2008).
[‡]Numbers in parenthesis indicate the sheep carrying the E^D allele at the *Extension* locus.

Table 3 *Association between Massese hair colour and genotypes of the* ASIP *exons 2 and 4 mutations and the absence/presence of a duplicated DNA copy including the* ASIP *gene*

Duplicated DNA copy including *ASIP*[†]	*ASIP* polymorphism[‡]	Genotype[§]	No. of massese sheep with different hair colour		
			Black	Grey	Total
Absence	Exon 2 deletions (D_5/D_9/N)	D_5D_5	89 (2)[‖]	3	92 (2)[‖]
		D_5N	25 (2)[‖]	–	25 (2)[‖]
		NN	2	–	2
		D_5D_9	1	–	1
		Total	117 (4)[‖]	3	120 (4)[‖]
	Exon 4 (g.5172T>A)	TT	29 (1)[‖]	–	29 (1)[‖]
		TA	59 (1)[‖]	3	62 (1)[‖]
		AA	29 (2)[‖]	–	29 (2)[‖]
		Total	117 (4)[‖]	3	120 (4)[‖]
Presence	Exon 2 deletions (D_5/D_9/N)	D_5D_9N	3 (2)[‖]	17	20 (2)[‖]
		D_5D_9	1	17	18
		D_9N	–	3	3
		Total	4 (2)[‖]	37	41 (2)[‖]
	Exon 4 (g.5172T>A)	TT	1	10	11
		TA	3 (2)[‖]	27	30 (2)[‖]
		AA	–	–	–
		Total	4 (2)[‖]	37	41 (2)[‖]

[†]Absence = amplification of the 238 bp fragment only. Presence = amplification of the 238 and 242 bp fragments (Norris and Whan, 2008).
[‡]D_5 = g.100-105delAGGAA; D_9 = g.10-19delAGCCGCCTC; N = normal exon 2 allele without deletions.
[§]In the sheep with duplicated DNA copy including *ASIP*, the genotypes are reported as genotyping results.
[‖]Numbers in parenthesis indicate the sheep carrying the E^D allele at the *Extension* locus.

different electrophoretic patterns observed in Massese sheep and in the other ovine breeds.

Results of the genotyping for the exon 2 deletions and for the g.5172T>A polymorphism in exon 4 of the *ASIP* gene in Massese sheep are reported in Table 3, separately for the animals without a duplicated copy allele and for the animals with a duplicated copy allele. For the first group, three alleles were identified. The most frequent one was D_5 (87.5%), followed by an allele without any deletion (allele N; 12.1%), whereas allele D_9 was observed in one sheep only of flock A (0.4%). No allele with both the D_9 and D_5 deletions was identified. The three identified exon 2 alleles were observed in four genotypes with D_5D_5 (76.7%) that was the most frequent one (Table 3). In these sheep, the two alleles of the g.5172T>A SNP showed exactly the same frequency (50%). The g.5172TA genotype was the most frequent (51.7%). Both polymorphic regions (exon 2 deletions and exon 4 SNP) were in Hardy–Weinberg equilibrium considering the whole Massese sampled population ($P = 0.96$ and $P = 0.71$, respectively) or analysing the data independently for the two flocks with a larger number of samples ($P = 0.63$ and $P = 0.32$ for flock C; $P = 0.81$ and $P = 0.37$ for flock D). Haplotypes between the exons 2 and 4 polymorphic regions/sites were inferred with $P \geqslant 0.99$ for all animals, except for the only sheep that carried the D_9 allele (that had genotypes D_5D_9 and g.5172TA). Three haplotypes were deduced in the remaining 119 sheep. All these haplotypes were putatively non-functional as they carried one (haplotype 1: [D_5:g.5172T]) or the other (haplotype 2: [N:g.5172A]) or both (haplotype 3: [D_5:g.5172A]) non-functional sites. Their observed frequencies were 49.6%, 12.2% and 38.2%, respectively. The six possible haplotype combinations (diplotypes) are reported in Table 4. Considering all sampled Massese sheep that did not carry any duplicated copy *ASIP* allele, the diplotypes were in Hardy–Weinberg equilibrium ($P = 0.48$). The Hardy–Weinberg equilibrium of the diplotypes was also observed in flock D ($P = 0.74$), whereas significant departure was evidenced in flock C ($P = 0.04$), probably due to population stratification.

Table 4 ASIP *diplotypes (haplotype combinations) in Massese sheep without any duplicated DNA copy including the* ASIP *gene*

ASIP diplotype[†]	No. of Massese sheep with different hair colour		
	Black	Grey	Total[‡]
D_5:g.5172T – D_5:g.5172T	28	–	28
D_5:g.5172T – D_5:g.5172A	47	3	50
D_5:g.5172T – N:g.5172A	12	–	12
D_5:g.5172A – D_5:g.5172A	14	–	14
D_5:g.5172A – N:g.5172A	13	–	13
N:g.5172A – N:g.5172A	2	–	2
Total	116	3	119

[†]Haplotypes have been inferred between *ASIP* the exons 2 and 4 mutations. D_5 = g.100-105delAGGAA; N = normal exon 2 allele without deletions.
[‡]One sheep carrying the D_9 deletion was not included as it was not possible to infer with high confidence its haplotypes. Therefore, the total number of considered animals was 119 instead of 120.

Different results were obtained in the 41 Massese sheep that carried a duplicated copy *ASIP* allele. However, owing to the reason that a duplicated copy allele was present, it was not possible to distinguish the different single copy alleles

from the multiple *ASIP* copies present in one allele. Therefore, genotypes are reported as genotyping results (Table 3). Almost half of the animals (48.8%) carried all three exon 2 alleles (D_5D_9N), whereas 43.9% resulted with genotype D_5D_9 and the remaining 7.3% had genotype D_9N. Another indication of the presence of CNV was the excess of heterozygous sheep at the g.5172T > A SNP (73.2%; Table 3). Departure from the Hardy–Weinberg equilibrium was highly significant ($P = 0.000018$), but this result is clearly biased by the presence of duplicated copies in which one copy (or more copies) could carry the g.5172T allele and the other(s) could carry the g.5172A allele. Similar results are common in loci with CNV (Fontanesi *et al.*, 2009b).

The genotyping results obtained from the sheep of the other breeds, all of whose animals carried duplicated copy alleles, were, in general, different from those obtained in the 41 Massese sheep. Eight Sarda sheep had genotype D_5N and two showed only the N allele. Six Appenninica sheep had genotype D_5N, two had only allele N, one had all three alleles and one was with genotype D_5D_9. Among the 10 analysed Bergamasca sheep, 5 were D_5N, 3 were D_5D_9N and 2 had only allele N.

Three missense *MC1R* mutations were genotyped in all Massese sheep, as in part reported by Fontanesi *et al.* (2010a). Six out of 161 animals carried the dominant black E^D allele. They had genotype E^DE^+, whereas all other sheep were E^+E^+. Therefore, the frequency of the E^D allele we observed in this breed was about 2%. Of these six animals, two carried a duplicated copy *ASIP* allele (Tables 2 and 3). The E^D allele was not identified in the Sarda, Appenninica and Bergamasca genotyped animals.

Association between ASIP and MC1R polymorphisms and coat colour in Massese sheep

Of the 161 Massese sheep we collected, 121 were classified as black (75%) and 40 as grey (25%). This is a good approximation of the frequency of these two coat colours in this breed as the animals were sampled randomly in the four flocks considered. The analysis of the genotyping data (Table 3) clearly indicated that polymorphisms in the *ASIP* and *MC1R* genes affect coat colour in Massese sheep. First of all, the presence of a duplicated copy allele including the *ASIP* gene was highly significantly associated with the grey coat colour ($P = 9.4E\text{-}30$, Fisher's exact test). Almost all animals with a duplicated copy allele (37 out of 41) showed uniform apparent grey hair (even if with different intensities of grey) and almost all animals without a duplicated allele (117 out of 120) were completely black. Only a few animals did not follow these rules: three grey sheep did not carry any duplicated copy allele and four black animals carried a duplicated copy allele. Of the latter four sheep, two were heterozygous at the *MC1R* locus; namely, they carried a dominant black allele (E^D). This is the reason because, despite the *ASIP* duplication that may determine grey colour, these two animals were black, according to the epistatic action of the *Extension* locus over the *Agouti* locus. Therefore, the inconsistencies between genotypes in the *ASIP* and *MC1R* genes and coat colour were for only 5 out of 161 Massese sheep, all belonging to flock D. The animals for which there was no correspondence between *ASIP* and *MC1R* genotypes and coat colour, as well as the other four animals with *ASIP* D_5D_5 genotype, were sequenced for both genes. However, no other mutations were identified in addition to those that have been already reported (Norris and Whan, 2008; Fontanesi *et al.*, 2010a).

In addition to the role of the duplicated DNA copy including the *ASIP* gene in determining the grey colour in Massese sheep, the black colour of a few Massese animals was due to the *MC1R* E^D allele (that was heterozygous with a wild-type allele in six sheep), as indicated above. However, almost all other black sheep (excluding the few exceptions indicated above) carried the non-functional *ASIP* alleles (haplotypes 1 to 3; Tables 3 and 4) that can be considered the black recessive non-agouti alleles at the *Agouti* locus (Sponenberg, 1997; Norris and Whan, 2008; Royo *et al.*, 2008).

In the 41 Massese sheep with a duplicated *ASIP* allele, the relative ratio between the peaks of two amplified fragments obtained in the assay, compared to that of other white breeds, clearly indicated a non-equal content of the two amplified regions, which can be interpreted with the fact that these Massese animals were heterozygous for an allele harbouring a duplicated DNA region including the *ASIP* gene with the other allele without any duplication. As a consequence, the duplicated copy allele(s), which was (were) associated with the grey coat colour, can be considered dominant (or partially dominant) over the non-duplicated copy alleles, which are associated with black coat colour. Following this assumption, the frequency of the duplicated copy allele(s) in the analysed Massese population was 12.7% (10.1% in flock C and 13.7% in flock D). The departure from the Hardy–Weinberg equilibrium was significant considering all sampled Massese sheep ($P = 0.014$) but not in flock C ($P = 0.208$) or in flock D ($P = 0.074$). It is also interesting to note that of these 41 Massese sheep, 18 had genotype D_5D_9 (17 of them were grey and one was black) with g.5172TT or g.5172TA. Considering that these animals were heterozygous with a non-duplicated allele, it is likely that this single copy allele harbours D_5 (according to its allele frequency in the Massese population) included in haplotypes 1 or 2 (with g.5172T or g.5172A). Therefore, the duplicated copy allele(s) (in sheep showing the D_5D_9 genotype) may carry two (or more) copies of the D_9 allele or both the D_5 and D_9 alleles. Both these *ASIP* variants are predicted to be non-functional or partially functional (Norris and Whan, 2008), but their effects on coat colour in Massese sheep seem evident and apparently indistinguishable from the effects of other duplicated alleles, coding for at least a functional ASIP, that are associated with grey coat colour. As a matter of fact, even if the grey of the animals classified in this coat colour category varied in intensity (from pale to dark), there was no association between the duplicated copy genotype and the degree of greyness (data not shown). The grey colour could be derived by the presence of pale hairs in a black skin background, but this issue should be further investigated as

we did not analytically determine the melanin contents in the different Massese sheep. The classification of the Massese animals into the two coat colour categories (black and grey) cannot discriminate between differences in distribution/ratio of eumelanic and pheomelanic fibres among sheep.

Discussion

As recognized early by classical genetic experiments, black coat colour in sheep can be due to homozygosity of a recessive non-agouti allele (A^a) at the *Agouti* locus or to the presence of a dominant *Extension* allele (E^D). Black determined in these two ways is not phenotypically distinguishable (Searle, 1968; Sponenberg, 1997). More recently, a few other studies have investigated polymorphisms in the ovine *ASIP* and *MC1R* genes and evaluated their effects on hair colour in several sheep breeds/populations (Våge *et al.*, 1999 and 2003; Norris and Whan, 2008; Royo *et al.*, 2008; Fontanesi *et al.*, 2010a; Gratten *et al.*, 2010).

We evaluated the effects of *ASIP* and *MC1R* gene variants on Massese sheep coat colour. In black, solid-coloured animals, at least three frequent *ASIP* haplotypes ([D_5:g.5172T], [N:g.5172A] and [D_5:g.5172A]) were detected with an additional rare haplotype including the D_9 allele (with g.5172T or g.5172A). These haplotypes may correspond to different A^a *Agouti* alleles. Six different diplotypes, which were originated combining the three major haplotypes, were almost completely associated with black coat colour (Table 4). The presence of more than one putative A^a *Agouti* allele (characterized by the combination of D_5 and the exon 4 SNP) was also observed in a feral population of Soay sheep in which different morphs segregate (Gratten *et al.*, 2010). In addition, another putative A^a allele present in this wild population as well as in black Merino sheep was suggested to be caused by a *cis*-regulatory mutation that might control *ASIP* gene expression (Norris and Whan, 2008; Royo *et al.*, 2008; Gratten *et al.*, 2010). A regulatory mutation (probably the same) was also supposed to be the most important cause of the recessive black coat colour phenotype in the Spanish Xalda breed, together with the less frequent D_5 allele (Royo *et al.*, 2008). According to the data obtained, we could not deduce the presence of an *ASIP* regulatory mutation in the analysed Massese sheep as the observed genotypes could all explain the black coat colour in their animals (Table 4). If this additional regulatory mutation is present in the Massese breed, it would have a very low frequency.

The duplicated *ASIP* copy allele(s) observed in Massese sheep could correspond to the A^{Wt} allele and/or to the other intermediate *Agouti*-locus alleles (e.g. grey and tan, A^{gt}; light grey, A^{kg}; grey, A^g) that may produce uniform mixtures of eumelanic and pheomelanic fibres resulting in a grey colour (Sponenberg, 1997). We tended to believe that more than one of these alleles (or additional forms of the A^{Wt} allele) are present in Massese sheep, and probably in several other breeds. The duplication detected by a specific designed PCR assay is not actually represented by only one sequence. Heterogeneity of the duplicated *ASIP* sequence was clearly evidenced by the analysis of the exon 2 deletions. Striking differences are evident comparing the exon 2 genotyping results of Massese sheep carrying a duplicated allele with those of other white sheep breeds. For example, the putative partially functional D_9 allele was always present in the grey Massese sheep (with a duplicated copy allele) but was not detected in the Sarda breed and was rare in the Appenninica and Bergamasca breeds. It was predicted that at least a fully functional copy of the *ASIP* gene should be present in a duplicated allele (A^{Wt} allele) to cause the white phenotype (Norris and Whan, 2008). The grey coat colour could be determined by the presence in duplicated *ASIP* alleles of the supposed partially functional D_9 variant together with the putative non-functional *ASIP* haplotypes carrying D_5 and/or A or, in the alternative, with putative fully functional *ASIP* haplotypes. Another possible explanation of the grey coat colour could be due to the fact that Massese sheep with this phenotype may be heterozygous with one of the three frequent non-agouti haplotypes (1, 2 and 3), and therefore the duplicated copy allele(s) might be partially dominant. Segregation of dominant white (or partially dominant) and recessive black coat colours, similar to that observed in Massese sheep, occurs in the Spanish Xalda breed (Royo *et al.*, 2008). The duplicated copy allele(s) (A^{Wt} or others) might have been introduced by crossing with other white breeds or it could be part of the original genetic pool from which the current Massese breed was originated. As a matter of fact, a duplicated copy allele including the *ASIP* gene could have been originated earlier after the domestication process of the sheep and single copy non-functional alleles (determining the non-agouti black phenotype) might be derived by non-homologous recombination (Norris and Whan, 2008; Gratten *et al.*, 2010). CNV are unstable genomic regions and non-homologous recombination could also create heterogeneity in the number of copies and in their organization (Cooper *et al.*, 2007). This could also be the reason for the differences in the duplicated *ASIP* variants that we observed in the Massese and other sheep breeds.

Association between Massese coat colour and polymorphisms in the *ASIP* gene was highly significant. However, a few animals did not follow the expected rule. We also excluded the presence of the missense mutation in the tyrosinase-related protein 1 (*TYRP1*) gene responsible for coat colour variation in a feral population of Soay sheep (Gratten *et al.*, 2007; data not shown). All of these few animals belonged to the same flock in which other genetic factors could segregate and modify the effects of the *ASIP* alleles. We tended to exclude the possibility of sampling errors or of misclassification of these animals. However, different levels of greyness could be due to environmental factors or to the age of the animals. No additional *MC1R* polymorphisms were detected apart from the few animals carrying the E^D allele that contributed to explain the coat colour in two sheep that otherwise would have been grey as they carried a duplicated *ASIP* allele. The other four animals with this allele were not different from the other black sheep, as a result of which their coat colour was determined by the recessive non-agouti alleles.

Furthermore, it is interesting to note that a mild selection pressure (as deduced by the departure from the Hardy–Weinberg equilibrium) could have been operated against the duplicated copy allele(s). It could be possible that sheep homozygous for a duplicated copy allele would be much more white than heterozygous animals and for this reason they might not be registered in the Massese breed herd book. Homozygous putative white animals could be culled whereas heterozygous sheep would not be eliminated, preserving the grey morph in the Massese populations. As a matter of fact, if strong selection pressure, both against heterozygous and homozygous grey/white animals would be applied, the duplicated copy allele(s) could be quite easily eliminated. A few breeders consider the grey Massese sheep more productive in terms of milk yield in a single lactation, but this information has not been experimentally verified as yet. Nevertheless, the duplicated alleles might have negative pleiotropic effects represented by reduced fecundity and greater seasonality in reproduction as evidenced in Icelandic sheep for the A^{Wt} allele or by decreased fertilization rate as reported for a *grey* allele (Adalsteinsson, 1970 and 1975). Massese sheep are characterized by a partial seasonality of oestrus, which can occur at any time during the year (Franci *et al*., 1999), and this trait could be due to the usual black coat colour that might exclude or maintain in heterozygosity the duplicated copy allele(s) with putative negative effects when homozygous. Further studies are needed to evaluate the effects of *ASIP* alleles on production and reproduction traits in the Massese breed.

Another issue of particular interest is the possible association between *ASIP* alleles and prion protein (*PrP*) alleles. The *ASIP* and *PrP* genes are both located on sheep chromosome 13, about 15 cM apart (Sawalha *et al*., 2009). Polymorphisms at the ovine *PrP* gene are associated with susceptibility to scrapie. Selection against some *PrP* alleles is the basis of current breeding programmes implemented to control this disease (Vascellari *et al*., 2005; Hunter, 2007; Dawson *et al*., 2008). Sawalha *et al*. (2009) showed a strong significant association of *PrP* genotypes with coat colour in Badgerfaced Welsh Mountain and Shetland sheep, probably due to *Agouti* locus alleles. The implication of these associations is that selection to increase resistance to scrapie based on *PrP* genotypes could modify *ASIP* allele frequencies in Massese and, in turn, change the ratio between black and grey animals with possible effects on other traits.

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