Analysis of melanocortin 1 receptor (*MC1R*) gene polymorphisms in some cattle breeds: their usefulness and application for breed traceability and authentication ofParmigiano Reggiano cheese

Vincenzo Russo, Luca Fontanesi, Emilio Scotti, Marco Tazzoli, Stefania Dall'Olio & Roberta Davoli

Analysis of melanocortin 1 receptor (MC1R) gene polymorphisms in some cattle breeds: their usefulness and application for breed traceability and authentication of Parmigiano Reggiano cheese

Vincenzo Russo, Luca Fontanesi, Emilio Scotti, Marco Tazzoli, Stefania Dall'Olio, Roberta Davoli

Dipartimento di Protezione e Valorizzazione Agroambientale. Università di Bologna, Italy

Corresponding author: Prof. Vincenzo Russo. DIPROVAL, Sezione di Allevamenti Zootecnici. Università di Bologna. Via F.lli Rosselli 107, 42100 Coviolo, Reggio Emilia, Italy - Tel. +39 0522 290522 - Fax: +39 0522 290523 - Email: vincenzo.russo@unibo.it

ABSTRACT

In cattle, the *MC1R* gene has been the subject of several studies with the aim to elucidate the biology of coat colour. Then, polymorphisms of this gene have been proposed as tools for breed identification and animal products authentication. As a first step to identify breed specific DNA markers that can be used for the traceability of mono-breed dairy cattle products we investigated, using PCR-RFLP and PCR-APLP protocols, the presence and distribution of some alleles at the *MC1R* locus in 18 cattle breeds for a total of 1360 animals. For each of seven breeds (Italian Holstein, Italian Brown, Italian Simmental, Rendena, Jersey, Reggiana and Modenese) a large number of animals (>70) was genotyped so the obtained results can be considered with more confidence. Allele E^D was identified only in black pied cattle (Italian Holstein and Black Pied Valdostana). Allele *E* (this nomenclature includes all alleles except E^D, *E1* and *e*) was observed in Italian Brown, Rendena, Jersey, Modenese, Italian Simmental, Grigio Alpina, Piedmontese, Chianina, Romagnola, Marchigiana, Swedish Red and White and Danish Red. Allele *E1* was identified in Italian Brown, Rendena, Grigio Alpina, Piedmontese, Swedish Red and White and Danish Red. The recessive allele *e*, known to cause red coat colour, was fixed in Reggiana and almost fixed in Italian Simmental. This allele was observed also in Italian Holstein, Italian Brown, Rendena, Jersey and Modenese albeit with low frequency. Moreover, this allele was detected in Valdostana, Pezzata Rossa d'Oropa, Piedmontese, Romagnola, Swedish Red and White, Danish Red, Charoleis and Salers. In the case of the Reggiana breed, which is fixed for allele *e*, the *MC1R* locus is highly informative with respect to breeds that carry other alleles or in which allele *e* is at very low frequency. In theory, using the *MC1R* locus it is possible to identify the presence of milk from some other breeds in Parmigiano Reggiano cheese labelled as exclusively from the Reggiana breed. This possibility was practically tested by setting up protocols to extract and analyse polymorphisms of the *MC1R* locus in several dairy products, including Parmigiano Reggiano cheese cured for 30 months. The lower detection limit was estimated to be 5% of non expected DNA. This test can represent a first deterrent against fraud and an important tool for the valorisation and authentication of Parmigiano Reggiano cheese obtained from only Reggiana milk.

Key words: Breed traceability, Dairy cattle products, Food authentication, *MC1R* polymorphisms, Parmigiano Reggiano cheese.

RIASSUNTO
ANALISI DI ALCUNE MUTAZIONI AL LOCUS MELANOCORTIN 1 RECEPTOR (*MC1R*) IN DIVERSE RAZZE BOVINE E LORO UTILIZZO PER LA TRACCIABILITÀ DI RAZZA DEL FORMAGGIO PARMIGIANO REGGIANO

Il legame tra un prodotto di origine animale e la razza da cui questo è originato rappresenta un aspetto importante per la valorizzazione di alcune produzioni. Il maggior prezzo che questi prodotti spuntano sul mercato fa emergere l'esigenza di poter autenticare o tracciare i prodotti mono-razza per smascherare e scoraggiare possibili frodi. A questo scopo sono stati proposti sistemi di analisi del DNA, alcuni dei quali utilizzano marcatori in geni che determinano il colore del mantello, che è uno dei principali caratteri che differenziano tra di loro le razze. Diverse mutazioni nel gene melanocortin 1 receptor (MC1R) sono già state associate a particolari effetti sul colore del mantello nella specie bovina. In questa ricerca abbiamo studiato la presenza dei principali alleli al locus MC1R, per valutare la possibilità di utilizzare questo gene per l'autenticazione e la tracciabilità di razza dei prodotti lattiero-caseari. Le mutazioni che permettono di distinguere questi alleli sono state analizzate utilizzando protocolli di PCR-RFLP e PCR-APLP su un totale di 1360 animali appartenenti a 18 razze bovine. Per ognuna delle seguenti razze, Frisona Italiana, Bruna Italiana, Pezzata Rossa Italiana, Jersey, Rendena, Reggiana e Modenese, è stato possibile analizzare più di 70 animali. L'allele E^D è stato identificato nella razza Frisona Italiana con una frequenza dello 0,886. L'allele E (nomenclatura che include tutti gli alleli tranne che e, E^D e E1) è stato identificato con alta frequenza nella Bruna Italiana (0,591), Rendena (0,738), Jersey (0,955) e Modenese (0,961) e con bassa frequenza nella Pezzata Rossa Italiana (0,029). Inoltre, questo allele è stato osservato nella Rossa Svedese, Rossa Danese, Grigio Alpina, Piemontese, Romagnola, Marchigiana e Chianina. In alcune di queste razze (Bruna Italiana, Rendena, Grigio Alpina, Piemontese, Rossa Svedese e Rossa Danese) è stato identificato anche l'allele E1. L'allele e è risultato fissato nella razza Reggiana e quasi fissato nella razza Pezzata Rossa Italiana. Inoltre, con bassa frequenza, è stato identificato in tutte le altre razze analizzate, tranne che nella Marchigiana. Le differenze osservate tra razze esaminate indicano che, almeno in alcuni casi, è possibile utilizzare i polimorfismi del gene MC1R per escludere o confermare l'impiego di latte di una determinata razza nella produzione di un prodotto lattiero-caseario. Il caso più interessante è quello del formaggio Parmigiano Reggiano prodotto con l'uso esclusivo di latte di bovine di razza Reggiana. Infatti, essendo presente in questa razza soltanto l'allele e il rilievo analitico di qualsiasi altro allele nel DNA estratto dal formaggio rivela l'uso di latte proveniente da altre razze. La messa a punto di un metodo PCR-RFLP per l'analisi del DNA estratto da prodotti lattiero caseari, incluso il Parmigiano Reggiano di oltre 24 mesi di stagionatura, rappresenta uno strumento importante per la difesa di questo prodotto mono-razza da eventuali frodi. I risultati ottenuti su 10 forme di formaggio prodotto esclusivamente con latte di bovine di razza Reggiana e su 15 forme di Parmigiano Reggiano commerciale ottenuto senza restrizione della razza di origine del latte hanno mostrato la validità del metodo del quale è stata valutata anche la sensibilità

Parole chiave: Tracciabilità di razza, Prodotti lattiero-caseari, Autenticazione alimentare, Mutazioni del gene MC1R, Formaggio Parmigiano Reggiano.

Introduction

Traceability of farm animals to their source breed is becoming an important issue for the authentication of their products, as there is an increasing interest in marketing mono-breed labelled lines of meat as well as dairy products, which in some cases have obtained the protected denomination of origin (PDO). This interest derives from the fact that a marketing link between breed and their originated products can contribute to improve breed profitability and, in turn, sustainability of such farm animal production with significant impact on the rural economy of particular geographic areas and on breed conservation and biodiversity (i.e.: de Roest and Menghi, 2000; Gandini and Villa, 2003). A classical example regarding this issue is the recovery

of the Reggiana breed through the production and valorisation of Parmigiano Reggiano cheese obtained from this breed only (Russo and Mariani, 1975; Associazione Nazionale Allevatori Bovini Razza Reggiana, 2000). This mono-breed cheese is sold at about the double the market prize of undifferentiated Parmigiano Reggiano cheese.

Analysis of the DNA present in all animal products (including dairy products as the milk contains the somatic cells of the cow) can be used to trace back its origin to the individual animals and to infer their breed. Different approaches have been proposed for breed traceability using genetic markers: i) a probabilistic approach mainly based on the use of highly variable microsatellite or AFLP markers combined with different computational analyses that assign individuals to a particular breed with a certain probability (i.e.: Blott *et al.*, 1999; Ciampolini *et al.*, 2000; Maudet *et al.*, 2002; Negrini *et al.*, 2003); ii) a deterministic approach based on the use of few breed specific or exclusive markers whose informativeness is due only to their presence or absence in the analysed products (i.e.: Maudet and Taberlet, 2002; Carrión *et al.*, 2003). The former needs the constitution of databases of microsatellite or AFLP allele or genotype frequencies for each considered breed. It was proposed for breed traceability of individual meat cuts although some limits have been observed for the distinction of relatively genetically undifferentiated breeds (i.e.: Ciampolini *et al.*, 2000; Maudet *et al.*, 2002). Moreover, the main drawback of this approach is that it cannot be used to assign the breed of origin for products composed of mixtures of several/many animals, as is the case with most dairy products. The deterministic approach, which relies on the identification of markers that are present or absent in all

(or most) animals of a particular breed, can be applied to mixture of products obtained from more animals. Useful markers for this approach can be identified looking at mutations in genes affecting the main traits that differentiate the breeds.

Since the constitution of the first modern cattle breeds which dates back to the 1700's - 1800's, coat colour has been usually considered as an aid to breed identification. Therefore, registration of the animals to the herd-books required, and usually still requires, a specific coat colour and colour distribution pattern typical of that particular breed. These rules resulted more or less in the fixation of distinctive coat colours in most of the European cattle breeds. Classical genetic studies established that this trait is influenced by several genes that, in some cases, have been identified at the molecular level (reviewed in Olson, 1999). Thus, mainly as a consequence of human selection undergone during the constitution of the breed, mutations in some of these genes may result as diagnostic tools that could be used to attribute an animal to or exclude an animal from a particular cattle breed and then may also be useful to trace their dairy or meat products (Chung *et al.*, 2000; Maudet and Taberlet, 2002; Carrión *et al.*, 2003; Fernández *et al.*, 2004; Russo and Fontanesi, 2004).

Pigmentation in cattle, and in general in all mammals, is determined by the presence or absence of melanins in the hair (Searle, 1968). The melanins (eumelanin and phaeomelanin) are produced in specialized cells, the melanocytes, and are accumulated in the melanosomes that migrate by exocitosis to the hair during its growth. The relative amounts of black/brown (eumelanin) and red/yellow (phaeomelanin) pigments produced in melanocytes are controlled primarily by two loci, *Extension* and *Agouti*. The *Extension (E)* locus codes for the

melanocyte stimulating hormone receptor (MSHR) or melanocortin 1 receptor (MC1R) that is expressed in melanocytes (Robbins *et al.*, 1993). MC1R is a member of the superfamily of G-protein-coupled receptors consisting of seven transmembrane domains whose action on eumelanin synthesis is mediated, upon binding of the α-MSH and ACTH peptides, through the activation of adenylyl cyclase to elevate cAMP levels in melanocytes, that in turn affect tyrosynase activity (Mountjoy *et al.*, 1992). Classical genetic studies have indicated that dominant alleles at the *Extension* locus are associated with black coat colour while recessive alleles at this locus produce red/yellow coat colours (Searle, 1968). Several mutations of the *MC1R* gene have been described in mice (Robbins *et al.*, 1993), humans (Valverde *et al.*, 1995) and in different farm mammals, such as cattle (Klungland *et al.*, 1995), sheep (Våge *et al.*, 1999), pigs (Kijas *et al.*, 1998), horses (Marklund *et al.*, 1996) and rabbits (Fontanesi *et al.*, 2006). Some of them cause a constitutive activation of the MC1R protein dependent signalling pathway inducing eumelanin synthesis (black coat colour), while others cause a loss of function of the coded protein and induce phaeomelanin production (red/yellow coat colour). In cattle, the first molecular genetic studies identified three main alleles associated with coat colour at the *Extension* locus (Klungland *et al.*, 1995; Joerg *et al.*, 1996): E^+, the wild type allele that produces a variety of colours depending on the *Agouti* locus (Adalsteinsson *et al.*, 1995); E^D, the dominant allele, caused by a T>C missense mutation in the *MC1R* coding region determining an activation of the encoded receptor, which, in turn, gives black coat colour; *e*, the recessive allele caused by a single nucleotide deletion in the *MC1R* coding region that produces a non functional pre-

maturely terminated receptor and that, in homozygous animals, yields red/yellow coat colour. Then, another allele *(E1)*, determined by a duplication of 12 bp that subsequently causes a duplication of four amino acids in the third intracellular loop of the MC1R protein, was reported (Kriegesmann *et al.*, 2001; Rouzaud *et al.*, 2000; Maudet and Taberlet, 2002). Its effect on coat colour has not yet been completely clarified (Royo *et al.*, 2003). Two other alleles, indicated as E^{D1} and e^f, have been identified and their activity partially characterized *in vitro* (Graphodatskaya *et al.*, 2002; Maudet and Taberlet, 2002). Moreover, other mutations have been reported in different breeds (i.e.: Kriegesmann *et al.*, 2001; Maudet and Taberlet, 2002), whose specific effect on coat colour has not yet been investigated.

As a first step to identify breed specific DNA markers that can be used for the traceability of dairy cattle products, such as some typical and traditional Italian monobreed cheeses, we investigated the presence and distribution of some alleles at the *MC1R* locus in several cattle breeds that show different coat colours and patterns. Moreover, we set up a protocol to investigate admixture of milk of different breeds in dairy products focusing this procedure mainly on the traceability and authentication of Parmigiano Reggiano cheese which comes exclusively from the Reggiana breed.

Material and methods

Animals

Hair, milk or semen samples were collected from a total of 1082 cows and 278 sires of 18 different breeds (Table 1). More than 70 animals were analysed from seven dairy or dual-purpose breeds (Italian Holstein-Friesian, Italian Brown, Italian Simmental, Jersey, Reggiana, Modenese and Rendena) for a total of 1272 analysed

Table 1. Breeds, number of genotyped animals, allele and genotype frequencies. The breeds of Group 1 are indicated in bold.

Breeds	N. of animals (sires + cows)	Allele frequencies§				Genotype frequencies (n. of animals)							
		E^D	E	E1	e	E^D/E^D	E^D/e	E/E	E/E1	E/e	E1/E1	E1/e	e/e
Italian Holstein-Friesian	261 (86+175)	0.890	-	-	0.110	0.805 (210)	0.157 (43)	-	-	-	-	-	0.038 (8)*
Italian Brown	248 (6+242)	-	0.591	0.377	0.032	-	-	0.343 (85)	0.472 (117)	0.024 (6)	0.121 (30)	0.040 (10)	-
Italian Simmental	208 (0+208)	-	0.029	-	0.971	-	-	0.005 (1)	-	0.048 (10)	-	-	0.947 (197)
Jersey	100 (2+98)	-	0.955	-	0.045	-	-	0.910 (91)	-	0.09 (9)	-	-	-
Rendena	82 (0+82)	-	0.738	0.250	0.012	-	-	0.512 (42)	0.427 (35)	0.024 (2)	0.037 (3)	-	-
Reggiana	297 (124+173)	-	-	-	1.00	-	-	-	-	-	-	-	1.00 (297)
Modenese	76 (21+55)	-	0.961	-	0.039	-	-	0.921 (70)	-	0.079 (6)	-	-	-
Grigio Alpina	6 (0+6)	-	0.750	0.250	-	-	-	0.500 (3)	0.500 (3)	-	-	-	-
Valdostana**	6 (0+6)	0.167	0.083	0.083	0.667	0.167 (1)	-	-	0.167 (1)	-	-	-	0.668 (4)
Pezzata Rossa d'Oropa	2 (0+2)	-	-	-	1.00	-	-	-	-	-	-	-	1.00 (2)
Piedmontese	6 (2+4)	-	0.834	0.083	0.083	-	-	0.833 (5)	-	-	-	0.167 (1)	-
Chianina	7 (4+3)	-	0.857	-	0.143	-	-	0.714 (5)	-	0.286 (2)	-	-	-
Romagnola	15 (13+2)	-	0.933	-	0.067	-	-	0.867 (13)	-	0.133 (2)	-	-	-
Marchigiana	4 (1+3)	-	1.00	-	-	-	-	1.00 (4)	-	-	-	-	-
Swedish Red and White	18 (1+17)	-	0.583	-	0.417	-	-	0.278 (5)	-	0.611 (11)	-	-	0.111 (2)
Danish Red	6 (0+6)	-	0.667	0.083	0.250	-	-	0.333 (2)	0.167 (1)	0.500 (3)	-	-	-
Charoleis	11 (11+0)	-	-	-	1.00	-	-	-	-	-	-	-	1.00 (11)
Salers	7 (7+0)	-	-	-	1.00	-	-	-	-	-	-	-	1.00 (7)
Total	1360 (278+1082)												

§ As the used genotyping protocol cannot distinguish allele E^+ from other less characterized alleles reported by several authors, we used E to consider all alleles except E^D, E1 and e.

* Eight red Italian Holstein-Friesian cows were purposely sampled.

** Of the 6 sampled Valdostana cows, 4 were red pied, one was black pied and 1 was solid brown.

samples. These seven breeds will be indicated henceforth as Group 1.

The remaining 88 animals were analysed to obtain a first preliminary evaluation or, in some cases, to confirm the variability at the *MC1R* gene in 11 other breeds, including some traditional beef breeds. These animals will be indicated henceforth as Group 2.

Cows of Italian Holstein Friesian, Italian Brown, Italian Simmental, Reggiana, Modenese, Rendena and Jersey were sampled in 17, 20, 7, 8, 4, 3 and 1 farms, respectively, located in Northern Italy. Grigio Alpina, Valdostana, Pezzata Rossa d'Oropa, Piedmontese, Romagnola, Marchigiana, Chianina, Swedish Red and White, Danish Red, Salers and Charoleis samples were collected during fair expositions in Northern Italy or in a few farms. Semen was provided by several artificial insemination centres.

Dairy products

Several products and cheese samples were collected. Some were only Reggiana milk derived products and were directly obtained from the producers. The mono-breed dairy products were the following: two milk pools exclusively from the Reggiana breed collected from Parmigiano Reggiano cheese vats, two whey samples collected from the same cheese vats, three samples of fresh Parmigiano Reggiano cheese (1-2 days old) obtained from only the Reggiana breed and 10 Parmigiano Reggiano cheese samples of 24 to 30 months obtained from only the Reggiana breed. Fifteen other Parmigiano Reggiano cheese samples (10-30 months old), not labelled as mono-breed products, purchased in retailer markets in Northern Italy, were considered as obtained by undefined animals without breed limitation.

DNA extraction

DNA extraction from hair roots was performed according to Healy *et al.* (1995). DNA was extracted from individual milk samples as described by Davoli *et al.* (1998) or using the Milk Extraction Kit (Nurex, Italy) and from semen using a Chelex 100 protocol (Walsh *et al.*, 1991).

For the listed dairy products DNA was extracted using commercial kits designed for blood (QIAamp DNA Blood Midi Kit, Qiagen) or food (Nucleo Spin Food, Macherey-Nagel, Düren, Germany). About 1-2 g of cheese was homogenized in a 15 ml tube. Then, 4 ml of bidistilled water and 200 µl of a 20 mg/ml proteinase K solution were added. This mix was vortexed and placed overnight in a rocking oven at 60°C. Then, about 2 ml (QIAamp DNA Blood Midi Kit) or 200 µl (Nucleo Spin Food) of this mix was used for DNA extraction according to the manufacturer's protocols.

The Macherey-Nagel kit was not used to extract DNA from cheese older than 20 months. For milk and whey pools, 2 ml were directly used for DNA extraction using the QIAamp DNA Blood Midi Kit, without previous proteinase-K overnight treatment.

PCR, analysis of mutations in individual samples and in dairy products and sequencing

PCR primers, designed on the bovine gene sequence (EMBL acc. no. S71017; Vanetti *et al.*, 1994), were used to amplify *MC1R* gene fragments (Table 2). PCR was performed using a PT-100 (MJ Research, Watertown, MA, USA) or a Perkin Elmer 9600 (Applied Biosystems) thermal cycler in a volume of 20 µl containing the DNA template (about 10-100 ng), 1 U DNA EuroTaq DNA polymerase (EuroClone Ltd., Paington, Devon, UK), 1X PCR Buffer, 2.5 mM dNTPs, 10 pmol of each primer and 1.0-2.0 mM of $MgCl_2$. PCR profile for the

Table 2. PCR analyses.

Primer pair name	Forward (5'-3')	Reverse (5'-3')	Annealing temperature (°C)	PCR products (bp)	Use
Pair 1	CAAGAACCGCAACCTGCACT§	GCCTGGGTGGCCAGGACA§	65	137/138*	PCR-RFLP (*Msp*I and *Ssi*I)
Pair 2	AGTGCCTGGAGGTGTCCATCC	CAGCAGGATGGTGAGGTTG	63	243/255**	PCR-APLP and Sequencing
Pair 3	AGTGCCTGGAGGTGTCCATCC	GAAGTTCTTGAAGATGCAGCC	60	740***	Sequencing

§ *Primer sequences from Klungland et al. (1995).*
* *Allele* e *that is caused by 1 single nucleotide deletion results in a fragment of 137 bp.*
** *A fragment of 12 bp differs allele* E1 *from the other* MC1R *alleles.*
*** *The length of the wild type sequence was considered.*

analysis of individual samples was as follows: 5 min at 95°C; 35 amplification cycles of 30 sec at 95°C, 30 sec at the specific annealing temperature for each primer pair (Table 2), 30 sec at 72°C; 10 min at 72°C. PCR of the template DNA extracted from cheese samples was carried out with 40 amplification cycles.
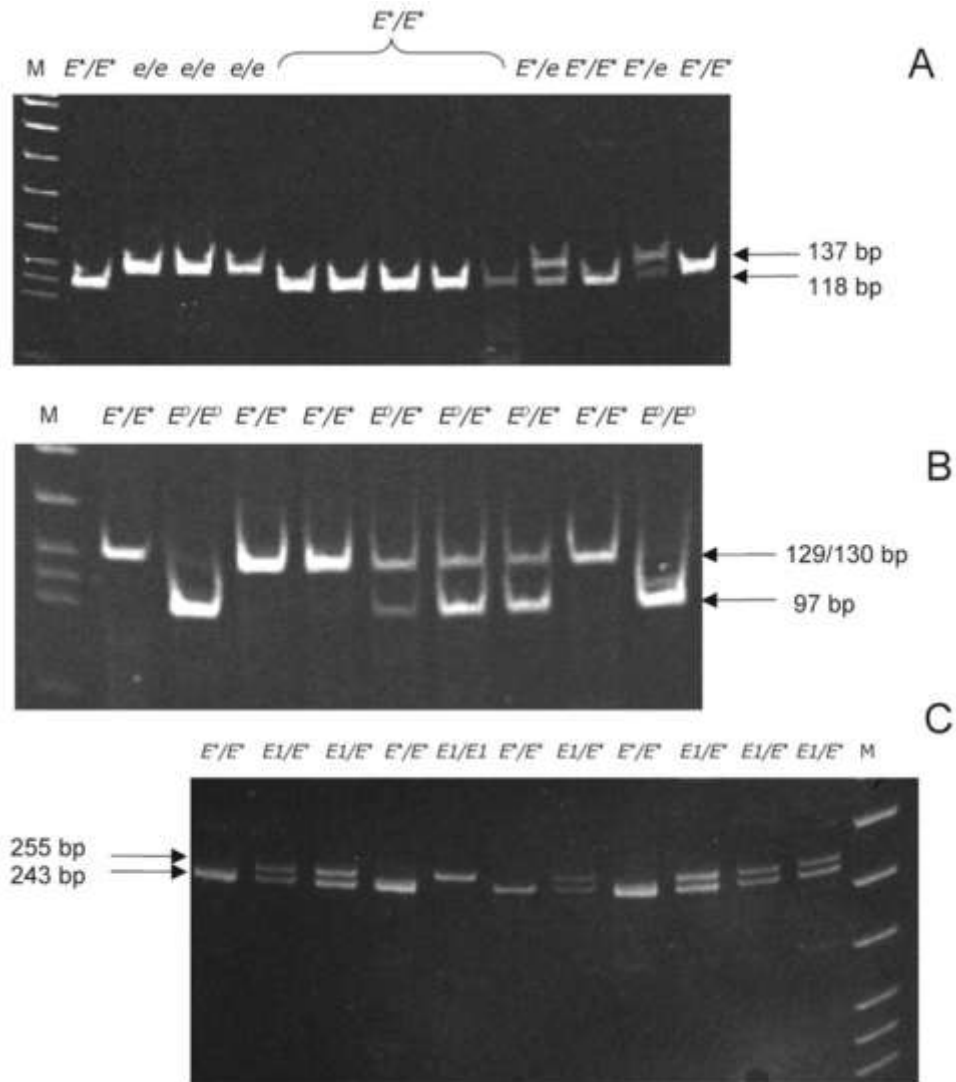
These PCR fragments obtained with primer pair 1 were analysed by means of a restriction fragment length polymorphism (RFLP) approach using *Msp*I (recognition sequence CCGG) and *Ssi*I (recognition sequence CCGC) to distinguish allele *e* from the other alleles and allele E^D from the other alleles, respectively (Figure 1 a and b). A PCR-amplified product length polymorphism protocol (PCR-APLP) was set up to analyse the *MC1R* region that contains the 12 bp insertion (primer pair 2) that differentiates allele *E1* from the other alleles (Figure 1). Sequencing was performed using the Big Dye v3.1 kit (Applied Biosystems, Foster City, CA, USA) on the PCR products obtained using primer pair 3 (Table 2) to amplify genomic DNA of two Italian Holstein Friesian and two Reggiana sires or using primer pair 2 to amplify genomic DNA of two Italian Brown cows.

The genotyping protocols used in the present study have been confirmed by sequencing parts of the single exon *MC1R* gene encompassing the investigated mutations in cattle with different genotypes.

Analysis of the sensitivity of the PCR-RFLP protocol

In order to have a first rough estimation of the sensitivity of the PCR-RFLP (*Msp*I digestion) allele detection method in dairy products we adapted and combined the methods described by Breem *et al.* (2000) and Maudet and Taberlet (2001). Several mixtures of DNA including different proportions of E^D/E^D Holstein DNA (1, 2, 5, 10, 25, 50 and 100%) in *e/e* Reggiana DNA were prepared. DNA concentration was evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). Restriction analysed fragments were electrophoresed and ethidium bromide stained bands were captured using a Kodak EDAS 290 instrument (Eastman Kodak Company, Scientific Imaging Systems, Rochester, NY, USA). Band peak intensity and band net intensity were obtained using the Kodak 1D image analysis software (Eastman Kodak

Figure 1.   A) PCR-RFLP analysis with *Msp*I and B) with *Ssi*I. C) PCR-APLP analysis.

The genotypes are indicated at the top of each lane. In A) as the analysis cannot distinguish alleles E^D, E and E1, the fragment of 118 bp was indicated with E* . The fragment of 20 bp, resulting from the digestion of the 138 bp fragment of the E^D, E or E1 alleles is not shown in the gel. In B) as the analysis cannot distinguish alleles E (130 bp), E1 (130 bp) and e (129 bp), the gel band of 129/130 bp was indicated with E*. Differences of 1 bp are undistinguishable in the gel. The fragment of 33 bp, resulting from the digestion of the 130 bp fragment of the E^D allele, as well as the fragment of 8 bp, due to the presence of a SsiI restriction site in the forward primer, are not shown in the gel. In C) as the analysis cannot distinguish alleles E^D, E and e, the fragment of 243 bp was indicated with E*. M = DNA molecular weight VIII (Roche Diagnostics).

Company). The relative intensity (RI) was calculated as the averaged ratio of the band peak intensity and band net intensity obtained for the 118 bp and 137 bp fragments.

Results and discussion

Analysis of the MC1R *mutations in several cattle breeds*

The combination of the PCR-RFLP and PCR-APLP analyses (Figure 1) made it possible to distinguish the four main alleles at the bovine *MC1R* locus: i) allele E^D causing black coat colour; ii) allele *e*, causing red coat colour; iii) allele *E1* caused by a 12 bp insertion; iv) the wild type allele E^+, that actually is not possible, using these genotyping methods, to distinguish from other less characterized alleles reported by several authors, thus henceforth it will be indicated as allele *E*.

Allele and genotype frequencies obtained in the analysed animals are reported in Table 1. Among the breeds of Group 1, allele E^D was identified only in the Italian Holstein Friesian (0.886) while in Group 2 it was observed only in the sampled Black Pied Valdostana cow. Considering Group 1, allele *E* was observed with high frequency in the Italian Brown (0.591), Rendena (0.738), Jersey (0.955) and Modenese (0.961), while with low frequency in Italian Simmental (0.029). In Group 2, this allele was identified in the Red Swedish, Red Danish, Grigio Alpina, Piedmontese, Romagnola, Marchigiana and Chianina breeds. Allele *E1* was observed in Italian Brown (0.377) and Rendena (0.250) and, among the breeds of Group 2, in Grigio Alpina, Piedmontese, Swedish Red and White and Danish Red. Allele *e* was fixed in Reggiana (breed characterized by solid red coat colour) and almost fixed in Italian Simmental (0.971). This allele was also observed in all other investigated breeds of Group 1, even with low frequency.

In breeds of Group 2, the recessive *e* allele was identified in Valdostana, Pezzata Rossa d'Oropa, Piedmontese, Romagnola, Swedish Red and White, Danish Red, Charoleis and Salers.

Other investigators have studied this locus in some of the breeds included in the present work thus it is possible to compare their results with what is reported in Table 1. For some of these breeds (Italian Holstein-Friesian, Italian Brown, Italian Simmental, Rendena and Jersey) our report included a much larger number of animals compared to the previous studies. For other breeds (Reggiana and Modenese, among Group 1, and Grigio Alpina, Swedish Red and White and Danish Red, among Group 2), the present study is the first report.

The presence of allele E^D only in black breeds (Italian Holstein-Friesian and Black Pied Valdostana) was expected and confirms the results of previous studies (Joerg *et al.*, 1996; Rouzaud *et al.*, 2000; Maudet and Taberlet, 2002; Crepaldi *et al.*, 2003; Rolando and Di Stasio, 2006). All black Holstein animals carried at least one copy of E^D. Moreover, it is also well known that allele *e* is present in the Italian Holstein-Friesian. Our study, which to our knowledge is the largest investigation at this locus in this breed, obtained, considering only black animals (the red Italian Holstein-Friesians were purposely sampled, thus they were excluded from the allele frequency estimation), a frequency of the recessive *e* allele of about 8.5%. If we assume Hardy-Weinberg equilibrium at this locus, homozygous *e/e* Italian Holstein-Friesian animals should be about 0.7% in the Italian population.

A few Italian Brown and Rendena animals were analysed by Crepaldi *et al.*

(2005) who observed in these breeds only the presence of the *E* and *E1* alleles. However, our study, which included a larger number of animals, reported the presence of allele *e* in both these breeds, although with low frequency and only in heterozygous state. In the Italian Simmental Crepaldi *et al.* (2005) observed only allele *e* while in the present study we identified, unexpectedly, also few *E/e* and *E/E* cows (10 and 1, respectively). These animals were sampled in two different farms and for four of them it was possible to identify a common ancestor that unfortunately was not possible to sample. Thus, it could be supposed that the Italian Simmental may not be completely fixed at the *MC1R* locus due to more or less remote crossing with other breeds. However, according to the used genotyping protocols it is not possible to exclude in the Italian Simmental breed the presence of allele *e^f*, which is indistinguishable from other alleles apart from the three (*E^D*, *E1* and *e*) directly genotyped. This allele should confer a similar red phenotype to the animals (Graphodatskaya *et al.*, 2002). In Jersey, despite what was reported by Berryere *et al.* (2003), we also identified for the first time, although with low frequency (0.045), allele *e*. Among the breeds of Group 2, it is worth reporting that we identified alleles e and *E1* in the Piedmontese breed that was suggested to be only *E$^+$/E$^+$* by Rolando and Di Stasio (2006), and we confirmed the presence of allele *e* in the Romagnola and Chianina breeds according to what was already reported by others (Maudet and Taberlet, 2002; Crepaldi *et al.*, 2003). Miming the allelic structure at the *MC1R* locus in these two Italian white beef breeds, allele *e* was also observed in another Italian white breed, Modenese, a local dairy breed. This presence may produce red animals if heterozygous sires are actively used thereby causing problems for the registration to the herd book of animals without the characteristic coat colour (AIA, 2006). In the Romagnola breed this problem was solved by starting a program aimed to eradicate allele *e* from the breed (Marilli *et al.*, 2005). According to what we observed, a similar program may be useful in the Modenese breed also considering the potential applications for breed traceability when a breed is fixed for a particular allele.

The Reggiana breed was thoroughly investigated at this locus. All active used sires were genotyped together with a large number of cows. According to our results, it is possible to say that Reggiana breed is fixed for allele *e*. Other two typical red breeds, Danish Red and Swedish Red and White, were not fixed for the e allele as also alleles *E* and *E1* were presented. On one hand, this was unexpected considering that these breeds are recognised by their characteristic red coat colour and that a phenotypic survey suggested that all animals of these two breeds are *e/e* (Kantanen *et al.*, 2000). On the other hand, the heterogeneity at the *MC1R* locus could be predicted considering the fact that, during the 1970's, Brown Swiss breeding was introduced into the Danish Red bloodline and that, in the 1960's, Ayrshire blood was introgressed into Swedish Red and White lines (Mason, 1996).

The few animals that we have analysed for the Valdostana, Pezzata Rossa d'Oropa, Marchigiana, Charoleis and Salers confirm the results obtained for these breeds by other Authors (Rouzaud *et al.*, 2000; Maudet and Taberlet, 2002; Crepaldi *et al.*, 2003).

Potential and limits of the MC1R *locus for breed traceability*
Considering the allele and genotype frequencies obtained for the analysed breeds

it is possible to note some differences that can be used, in theory, to identify or exclude, at least in some cases, the breed of origin of dairy products. For this purpose we have focused our attention mainly on the seven breeds included in Group 1. For the other breeds only few animals have been genotyped, thus in depth investigations are necessary to assess the power of the *MC1R* gene for breed traceability in their cases.

Allele E^D makes it possible to distinguish black and white Holstein-Friesian milk from all other breeds as this allele is not present in non black animals confirming what was suggested by Maudet and Taberlet (2002). Allele *E1*, which is present in Italian Brown and Rendena in which it is not fixed and even is not the most frequent, is only partially informative for breeds that do not have this allele. Namely, if this allele is evidenced it is possible to deduce that milk of cows of these breeds is present while if this allele is not detected it does not exclude the presence of Italian Brown or Rendena milk in the analysed dairy products. The Reggiana breed, for which there is a very high interest in the possibility to trace its mono-breed Parmigiano Reggiano cheese, resulted fixed for allele *e*. This situation makes it possible to say that the *MC1R* locus is highly informative for this breed with respect to breeds that carry other alleles or in which allele *e* is at very low frequency. Namely, using the *MC1R* locus it is possible to differentiate Parmigiano Reggiano cheese from the Reggiana breed from cheese produced using milk of Italian Holstein-Friesian (black and white), Italian Brown, Rendena, Modenese and Jersey, breeds which all, except Rendena, are present in the geographic area of production of the Parmigiano Reggiano cheese. However, it should be pointed out that Italian Simmental and Reggiana products cannot be distinguished as the former breed has a very high frequency of allele *e*. Moreover, the low expected and actual frequency of red *e/e* Italian Holstein-Friesian cows in this breed makes it highly improbable, but still possible, to produce Parmigiano Reggiano cheese from milk of only cows with this genotype. Thus, only in these two cases it is not possible to exclude the use of milk of other breeds/type of animals (Italian Simmental and red Italian Holstein-Holtesin cows) for the production of Parmigiano Reggiano cheese from only Reggiana cows. On the other hand, as some mono-breed cheeses in the north-eastern part of Italy are also produced only with Italian Simmental milk, similar considerations may be applied or deduced for this breed. In this case it is worth considering that Reggiana cows are not present in the geographic region in which mono-breed cheeses are manufactured using only Italian Simmental milk.

However, as Reggiana (solid red) and Italian Simmental and red Italian Holstein-Friesian (red and white) differ in their coat colour pattern distribution, the identification of (a) genetic marker(s) that may distinguish nonspotted from spotted animals could solve the limit of the *MC1R* locus for traceability of Parmigiano Reggiano cheese exclusively from Reggiana cows. The possibility of distinguishing Italian Simmental from red Italian Holstein-Friesian dairy products remains to be resolved.

From crossbreeding studies it was deduced that in spotted animals a recessive allele, *s*, is fixed at the *Spotted* locus and that other alleles at this locus (S^H, S^P and S^+ of the Hereford colour pattern, Pinzgauer colour-sided pattern and nonspotted wild type, respectively) act as dominant suppressors of the spotted phenotype

(Olson, 1999), although other modifier genes may be involved in this phenotype. The *Spotted* locus was then mapped to bovine chromosome 6 in the region of the v-KIT Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog *(KIT)* gene (Grosz and MacNeil, 1999) whose mutations, in other species (i.e.: Jackson, 1997), have been indicated to affect reduced or localized pigmentation. Moreover, a major QTL for the degree of spotting in Simmental and Holstein cattle was localized in the same position of chromosome 6, suggesting the presence of more than one recessive *s* allele at the *Spotted* locus (Reinsch *et al.*, 1999). Thus, the *KIT* gene seems an obvious candidate to identify genetic markers that may be useful in distinguishing spotted from nonspotted cattle. Nevertheless, a single nucleotide polymorphism (SNP) identified in intron 3 of this gene (Olsen *et al.*, 2000) resulted not informative for this purpose (Maudet and Taberlet, 2002; our unpublished result). Further studies on the bovine *KIT* gene may provide more useful DNA markers.

Application of MC1R *gene polymorphisms for breed traceability and authentication of Parmigiano Reggiano cheese*
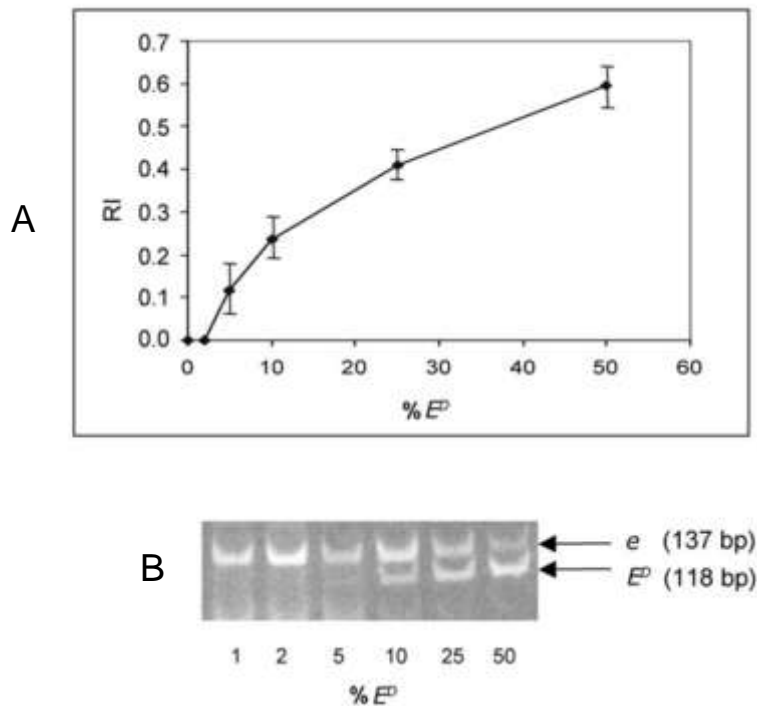
As milk contains a large number of somatic cells that, in turn, are included as components in the cheese and in other processed dairy products, the DNA that is present in these cells represents the trace of the milk producer animals (Lipkin *et al.*, 1993). Thus, to use this animal trace, the first step is the extraction of the DNA. Several studies have already reported the possibility of extracting and analysing DNA from cheese (i.e.: Branciari *et al.*, 2000; Maudet and Taberlet, 2001, 2002).

In the present work we set up two differ-ent extraction protocols that, although they were based on a kit originally designed for blood or general food, made it possible to obtain amplifiable DNA from all collected Parmigiano Reggiano cheese samples, as well as from the milk and milk whey pools. Thus it was possible to isolate and amplify DNA from cheese cured for up to 30 months indicating that during such a long curing period the animal DNA is not completely destroyed.

Moreover, the use of *MC1R* gene polymorphisms to trace and evaluate the authenticity of mono-breed dairy products relies, other than on the possibility to extract and PCR amplify DNA from the mentioned dairy products, on the sensitivity of the mutation detection methods used to identify the presence of different alleles in the amplified DNA. The evaluation of the sensitivity of the genotyping protocols was intended as the identification of the lower detection limit of possible contaminating DNA, namely DNA of not allowed breeds, in the analysed products. This issue was investigated using as PCR template mixtures of DNA including different proportions of DNA of animals with diverse *MC1R* genotype. The use of already extracted DNA instead of mixture of milk or purposely manufactured cheese containing known proportions of milk of animals with different *MC1R* genotype overcomes the problem due to the large variation on somatic cell count of different milk samples. The PCR-RFLP test was applied considering the possibility to identify the presence of alleles different from the allele *e* that is fixed in the Reggiana breed as, in this case, there is a direct interest in avoiding fraud in the production of Parmigiano Reggiano from milk of only this breed. Figure 2 reports the plotted RI as well as the captured gel picture of the two main DNA fragments (118 and 137

Figure 2.   A) RI curve obtained for the PCR-RFLP *(MspI)* analysis of artificial constructed reference DNA samples containing different concentrations of contaminating DNA of E^D/E^D animals. B) A PCR-RFLP *(MspI)* gel analysis of artificial constructed reference DNA samples used for the construction of the "reference curve".

bp) for the analysed artificial constructed reference samples. The lower limit at which it was possible to identify the presence of different alleles other that the *e* allele was 5%. For the 1 and 2% reference samples it was not possible to detect the 118 bp fragment. Although the point of interest was the identification of the lower limit of detection that shows if a dairy product derives from an admixture of milk of different breeds, the obtained "reference curve" can represent a rough semi-quantitative estimation of the level of mixture of DNA with different genotypes. Thus it could be used, with caution, to evaluate the level of admix-ture of milk in a dairy product. The parameters that might interfere with the accuracy of the methods may be i) the somatic cell count of individual milk samples, as already mentioned, that are affected by several factors, and that alters the contribution of the individual cow DNA to the milk pool from which the dairy products are obtained, and ii) the sensitivity of the mutation detection methods that in general give distorted estimations for alleles at lower concentration (Breem *et al.*, 2000). The sensitivity of this method could be improved using alternative genotyping approaches. For example, Maudet and Taberlet (2002) detected the

presence of 1% of Holstein's milk in a milk curd using a competitive oligonucleotide priming PCR method together with the genotyping of fluorescently labelled reaction products in a sequencer.

The PCR-RFLP test that was applied on the 10 samples of long cured Parmigiano Reggiano cheese, the three samples of fresh Parmigiano Reggiano cheese and the samples of milk and milk whey pools from only Reggiana cows revealed, as expected, only the fragment corresponding to allele *e*. When this test was applied on Parmigiano Reggiano cheese of unknown breeds, only the fragment of 118 bp (alleles *E*, *E^D* or *E1*), or two fragments of 118 (alleles *E*, *E^D* or *E1*) and 138 bp (allele *e*), were identified showing that these cheeses where not produced using only Reggiana milk.

Conclusions

The present study investigated for the first time polymorphisms of the *MC1R* gene in some cattle breeds and added new information on the population genetic structure at this locus for several other breeds. This information has been evaluated to implement a breed traceability strategy for some mono-breed products. Even if the use of the *MC1R* polymorphisms alone cannot give a complete answer to the question that arises in a field application of cattle breed traceability of dairy products it was shown that this gene, considering the four analysed alleles and the genotype frequencies in the investigated breeds, is useful to exclude or assign, at least in some cases, the breed of origin of a product. The most favourable situation is for the Reggiana breed that presents only allele *e* at the *MC1R* locus. The genetic test that was used to analyse the animal trace in cheeses cured up to 30 months can represent a first deterrent against frauds and an important tool for

the valorisation and authentication of Parmigiano Reggiano cheese obtained from only Reggiana milk.

REFERENCES

Adalsteinsson, S., Bjarnadottir, S., Vage, D.I., Jonmundsson, J.V., 1995. Brown coat color in Icelandic cattle produced by the loci Extension and Agouti. J. Hered. 86:395-398.

AIA (Italian Breeders Association), 2006. Registro Anagrafico delle popolazioni bovine autoctone e gruppi etnici a limitata diffusione. Disciplinare del Registro Anagrafico. Home page address: http://www.aia.it/tecnico/bovini/Pubblicazione RA_file/page0001.htm.

Associazione Nazionale Allevatori Bovini Razza Reggiana, 2000. La Razza Reggiana. ANABoRaRe, Reggio Emilia, Italy.

Berryere, T.G., Schmutz, S.M., Schimpf, R.J., Cowan, C.M., Potter, J., 2003. TYRP1 is associated with dun coat colour in Dexter cattle or how now brown cow?. Anim. Genet. 34:169-175.

Blott, S.C., Williams, J.L., Haley, C.S., 1999. Discriminating among cattle breeds using genetic markers. Heredity 82:613-619.

Branciari, R., Nijman, I.J., Plas, M.E., Di Antonio, E., Lenstra, J.A., 2000. Species origin of milk in Italian mozzarella and Greek feta cheese. J. Food Prot. 63:408-411.

Breem, G., Harold, D., Ralston, S., Shaw, D., St. Clair, D., 2000. Determining SNP allele frequencies in DNA pools. Biotechniques 28:464-470.

Carrión, D., Day, A., Evans, G., Mitsuhashi, T., Archibald, A., Haley, C., Andersson, L., Plastow, G., 2003. The use of *MC1R* and *KIT* genotypes for breed characterisation. Arch. Zootec. 52:237-244.

Chung, E.R., Kim, W.T., Kim, Y.S., Han, S.K., 2000. Identification of Hanwoo meat using PCR-RFLP marker of MC1R gene associated with bovine coat color. Korean J. Anim. Sci. 42:379-390.

Ciampolini, R., Leveziel, H., Mozzanti, E., Grohs, C., Cianci, D., 2000. Genomic identification of the

breed of an individual or its tissue. Meat Sci. 54:34-40.

Crepaldi, P., Marilli, M., Gorni, C., Meggiolaro, D., Cicogna, M., Renieri, C., 2003. Preliminary study on MC1R polymorphism in some cattle breeds raised in Italy. Ital. J. Anim. Sci. 2 (Suppl. 1):13-15.

Crepaldi, P., Fornarelli, F., Marilli M., 2005. MC1R gene: comparison between different farm animal species. Ital. J. Anim. Sci. 4 (Suppl. 2):43-45.

Davoli, R., Dall'Olio, S., Milc, J., Russo, V., 1998. Diffusione dell'allele G di α_{S1}-caseina in razze bovine allevate in Italia. Proc. 41th Nat. Congr. SISVet, Silvi Marina (TE), Italy, 41: 493-494.

de Roest, K., Menghi, A., 2000. Reconsidering 'traditional' food: the case of Parmigiano Reggiano cheese. Sociol. Ruralis 40:439-451.

Fernández, A., Fabuel, E., Alves, E., Rodriguez, C., Silió, L., Óvilo, C., 2004. DNA tests based on coat colour genes for authentication of the raw material of meat products from Iberian pigs. J. Sci. Food Agric. 84:1855-1860.

Fontanesi, L., Tazzoli, M., Beretti, F., Russo, V., 2006. Mutations in the melanocortin 1 receptor *(MC1R)* gene are associated to coat colours in the domestic rabbit *(Oryctolagus cuniculus)*. Anim. Genet. 37:489-493.

Gandini, G.C., Villa, E., 2003. Analysis of the cultural value of local livestock breeds: a methodology. J. Anim. Breed. Genet. 120:1-11.

Graphodatskaya, D., Joerg, H., Stranzinger, G., 2002. Molecular and pharmacological characterization of the MSH-R alleles in Swiss cattle breeds. J. Recept. Sig. Transd. 22:421-430.

Grosz, M.D., MacNeil, M.D., 1999. The "Spotted" locus maps to bovine chromosome 6 in a Hereford-cross population. J. Hered. 90:233-236.

Healy, P.J., Dennis, J.A., Moule, J.F., 1995. Use of hair root as a source of DNA for the detection of heterozygotes for recessive defects in cattle. Aust. Vet. J. 72:392.

Jackson, I.J., 1997. Homologous pigmentation mutations in human, mouse and other model organisms. Hum. Mol. Genet. 6:1613-1624.

Joerg, H., Fries, H.R., Meijerink, E., Stranzinger, G.F., 1996. Red coat color in Holstein cattle is associated with a deletion in the MSHR gene. Mamm. Genome 7:317-318.

Kantanen, J., Olsaker, I., Brusgaard, K., Eythorsdottir, E., Holm, L.E., Lien, S., Danell, B., Adalsteinsson, S., 2000. Frequencies of genes for coat colour and horns in Nordic cattle breeds. Genet. Select. Evol. 32:561-576.

Kijas, J.M.H., Wales, R., Törnsten, A., Chardon, P.,

Moller, M., Andersson, L., 1998. Melanocortin Receptor 1 *(MC1R)* mutations and coat color in pigs. Genetics 150:1177-1785.

Klungland, H., Våge, D.I., Gomez-Raya, L., Adalsteinsson, S., Lien, E., 1995. The role of melanocyte-stimulating hormone (MSH) receptor in bovine coat color determination. Mamm. Genome 6:636-639.

Kriegesmann, B., Dierkes, B., Leeb, T., Jansen, S., Brenig, B., 2001. Two breed-specific bovine MC1-R alleles in Brown Swiss and Saler breeds. J. Dairy Sci. 84:1768-1771.

Lipkin, E., Shalom, A., Khatib, H., Soller, M., Friedmann, A., 1993. Milk as a source of deoxyribonucleic acid and as a substrate for the polymerase chain reaction. J. Dairy Sci. 76:2025-2032.

Marilli, M., Fornarelli, F., Casaleggi, M., Milanesi, E., Filippini, F., Crepaldi, P., 2005. MC1R gene and coat color of Romagnola breed. pp 241-246 in Proc. 4th World Congr. Italian Beef Cattle, Perugia, Italy.

Marklund, L., Johansson Moller, M., Sandberg, K., Andersson, L., 1996. A missense mutation in the gene for melanocyte-stimulating hormone receptor *(MC1R)* is associated with the chestnut coat color in horses. Mamm. Genome 7:895-899.

Mason, I.L., 1996. A world dictionary of livestock breeds, types and varieties. 4th ed., CABI publisher, Wallingford, UK.

Maudet, C., Luikart, G., Taberlet, P., 2002. Genetic diversity and assignment tests among seven French cattle breeds based on microsatellite DNA analysis. J. Anim. Sci. 80:942-950.

Maudet, C., Taberlet, P., 2001. Detection of cows' milk in goats's cheeses inferred from mitochondrial DNA polymorphism. J. Dairy Res. 68:229-235.

Maudet, C., Taberlet, P., 2002. Holstein's milk detection in cheeses inferred from melanocortin receptor 1 *(MC1R)* gene polymorphism. J. Dairy Sci. 85:707-715.

Mountjoy, K.G., Robbins, L.S., Mortrud, M.T., Cone, R.D., 1992. The cloning of a family of genes that encode the melanocortin receptors. Science 257:1248-1251.

Negrini, R., Milanesi, E., Moazami-Goudarzi, K., Laloé, D., Chegdani, F., Bernardi, J., Lenstra, J.A., Ajmone-Marsan, P., 2003. Preliminary comparison between microsatellite and AFLP multilocus genotypes for bovine breed assignment. Ital. J. Anim. Sci. 2 (Suppl.1):22-24.

Olsen, H.G., Våge, D.I., Lien, S., Klungland, H., 2000. A DNA polymorphism in the bovine c-kit

gene. Anim. Genet. 31:71.

Olson, T.A., 1999. Genetics of colour variation. In: R. Fries, A. Ruvinsky (eds.) The genetics of the cattle. CABI Publishing, Wallingford, UK, pp. 33-53.

Reinsch, N., Thomsen, H., Xu, N., Brink, M., Kalm, E., Brockmann, G.A., Gruppe, S., Kühn, C., Schwerin, M., Leyhe, B., Hiendleder, S., Erhardt, G., Medjugorac, I., Russ, I., Förster, M., Reents, R., Averdunk, G., 1999. A QTL for the degree of spotting in cattle shows synteny with the *KIT* locus on chromosome 6. J. Hered. 90:629-634.

Robbins, L.S., Nadeau, J.H., Johnson, K.R., Kelly, M.A., Roselli-Rehfuss, L., Baack, E., Mountjoy, K.G., Cone, R.D., 1993. Pigmentation phenotypes of variant Extension locus alleles result from point mutations that alter MSH receptor function. Cell 72:827-834.

Rolando, A., Di Stasio, L., 2006. MC1R gene analysis applied to breed traceability of beef. Ital. J. Anim. Sci. 5:87-91.

Rouzaud, F., Martin, J., Gallet, P.F., Delourme, D., Goulemont-Leger, V., Amigues, Y., Ménissier, F., Levéziel, H., Julien, R., Oulmouden, A., 2000. A first genotyping assay of French cattle breeds based on a new allele of the extension gene encoding the melanocortin-1 receptor (Mc1r). Genet. Sel. Evol. 32:511-520.

Royo, L.J., Álvarez, I., Luque, A.J., Fernández, I., de Argüello, S., Molina, A., Robles, M., Goyache, F., 2003. Genotipado del locus Extension (Mc1r) en cinco razas bovinas españolas de capa castagña.

Proc. 10th Meet. on Animal Production, Interprofessional Association for the Development in Agriculture, Zaragoza, Spain. www.dcam.upv.es/acteon/CONGRESOS/AIDA2003/royo.pdf

Russo, V., Mariani, P., 1975. Modenese e Reggiana. Stato attuale e prospettive di due razze bovine che vanno scomparendo. L'Italia Agricola 113(3):56-72.

Russo, V., Fontanesi, L., 2004. Coat colour gene analysis and breed traceability. pp 95-100 in Proc. 7th World Brown Swiss Conf., Verona, Italy.

Searle, A.G., 1968. Comparative Genetics of Coat Colour in Mammals. Logos Press, London, UK.

Våge, D.I, Klungland, H., Lu, D., Cone, R.D., 1999. Molecular and pharmacological characterization of dominant black coat color in sheep. Mamm. Genome 10:39-43.

Valverde, P., Healy, E., Jackson, I., Rees, J.L., Thody, A.J., 1995. Variants of the melanocyte-stimulating hormone receptor gene are associated with red hair and fair skin in humans. Nature Genet. 11:328-330.

Vanetti, M., Schonrock, C., Meyerhof, W., Hollt, V., 1994. Molecular cloning of a bovine MSH receptor which is highly expressed in the testis. FEBS Lett. 348:268-272.

Walsh, P.S., Metzger, D.A., Higuchi, R., 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10:506-513.