# Analysis of SNPs in the *KIT* Gene of Cattle with Different Coat Colour Patterns and Perspectives to Use These Markers for Breed Traceability and Authentication of Beef and Dairy Products

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## SHORT COMMUNICATION

# Analysis of SNPs in the *KIT* gene of cattle with different coat colour patterns and perspectives to use these markers for breed traceability and authentication of beef and dairy products

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## Abstract

The identification of the breed of origin of farm animals has recently assumed particular relevance, since increasing interests in marketing mono-breed labelled lines of beef and dairy products have in fact created the need to protect them from frauds. In order to develop DNA based breed traceability and authentication protocols, the first step is the identification of breed specific markers with high discriminatory power among breeds. We analysed two single nucleotide polymorphisms (SNP) identified in exon 2 (g.72779776C>T) and exon 3 (g.72783182A>G) of the *KIT* gene (a candidate gene for the *spotting locus*) in seven cattle breeds with different coat colour patterns (Italian Holstein-Friesian, no. = 61; Italian Brown, no. = 60; Italian Simmental, no. = 78; Jersey, no. = 60; Rendena, no. = 51; Reggiana, no. = 128; and Modenese, no. = 52). The two alleles of both SNPs were detected in all analysed breeds making their use unsuitable in breed traceabilty with a deterministic approach. Italian Simmental was almost fixed for the most common alleles (g.72779776C and g.72783182A). Haplotype analysis showed that spotted breeds (Italian Holstein-Friesian and Italian Simmental) had only two haplotypes, of which one ([C:A]) with high frequency (~90% and ~99%, respectively). Analysis of molecular variance (AMOVA) averaged over the two markers indicated that genetic variation between spotted and non-spotted groups of breeds amounted to 25.3% (P<0.05), supporting a possible involvement of the *KIT* gene in influencing the spotted phenotype, but probably not determining it, as we previously sug-gested. Pairwise $F_{st}$ values indicated signifi-cant differences among almost all pair of investigated breeds. The high discriminatory power of the analysed SNPs is an important characteristic for the inclusion of these mark-ers in SNP panels useful for breed allocation and traceability based on probabilistic approaches.

## Introduction

The identification of the breed of origin of farm animals has recently assumed particular relevance, since the increasing interest in marketing mono-breed labelled lines of beef and dairy products has created the need to pro-tect them from frauds (Fontanesi, 2009). At a molecular level, analysis of the DNA in animal products (including dairy products, as the milk contains somatic cells of the animal) can be used to trace back their origin to the individu-als and to infer their breed. However, in order to develop DNA based breed traceability and authentication protocols, the first step is the identification of breed specific markers with high discriminatory power among breeds. Colour genes are considered the most interest-ing candidates for this aim, as they affect a trait that was strongly selected during the con-stitution of the modern breeds, which, in turn, are usually recognised according to their coat colour phenotypes. Therefore, selection and fixation of coat colour at the phenotypic level should have indirectly caused a selection and fixation of causative alleles for genes involved in determining this trait.

A large number of genes affects coat colour and colour patterns through different mecha-nisms as evidenced in mice, considered the model species for these studies (Bennett and Lamoreux, 2003). For example, the production and relative amount of eumelanin and pheomelanin in hair (determining black/ brown and red/yellow pigmentation, respec-tively) are controlled mainly by the *Agouti* and *Extension loci* (Searle, 1968). These *loci* encode for the agouti signaling protein (*ASIP*; Bultman *et al.*, 1992) and for the melanocortin 1 receptor (*MC1R*; Robbins *et al.,* 1993) genes. Mutations in the cattle *MC1R* gene determine the dominant ($E^D$) or the recessive (*e*) alleles at the *Extension locus* causing black or red coat colour, respectively (Klungland *et al.,* 1995). These mutations have been already used for breed authentication purposes of dairy prod-ucts of French cattle breeds and of Parmigiano Reggiano obtained with Reggiana milk only (Maudet and Taberlet, 2002; Russo *et al.,*

2007). The use of these markers has been also proposed for breed authentication of beef prod-ucts (e.g.: Chung *et al.,* 2000; Rolando and Di Stasio, 2006). However, even if most of the possible frauds can be easily detected evidenc-ing the presence of the $E^D$ allele usually coming from the cosmopolitan Holstein-Friesian breed (almost fixed for this allele), in some cases it could be important to determine if white-spot-ted breeds contributed to produce the product under inspection (reviewed in Russo and Fontanesi, 2004).

In cattle, the white-spotting phenotype (piebaldism), described as the presence of white patterns among coloured regions, is due to aberrant migration of melanocyte precur-sors from the neural crest to different skin areas. Classical genetic studies have indicated that cattle piebaldism is largely influenced by the *spotting (S) locus* for which a four-allele series has been reported (Olson, 1999): $S^H$ (Hereford pattern, characteristic of this breed), $S^P$ (Pinzgauer pattern or lineback, hav-ing as reference the Pinzgauer breed), $S^+$ (non-spotted, considered as the wild type and having as reference the Angus and other solid coloured breeds), and **s** (spotting pattern as presented in Holstein-Friesian and Simmental breeds). Alleles $S^H$ and $S^P$ seem codominant to each-other and incompletely dominant over S+. All these three alleles appear to be com-pletely dominant over the s allele (Olson, 1981; 1999).

The *spotting locus* (also known as *spotted locus*) was mapped to bovine chromosome 6 (BTA6), in a chromosome region including the

*KIT* gene (Grosz and MacNeil, 1999). In the same chromosome region, Reinsch *et al.* (1999) and Liu *et al.* (2009) localized a QTL for the degree of spotting in German Holstein-Friesian and Simmental cattle and in a Holstein-Friesian x Jersey cross. The *KIT* gene encodes the mast/stem cell growth factor receptor that is involved in melanogenesis driving the melanocyte migration from the neural crest along the dorsolateral pathway to colonize the final destination in the skin (Besmer *et al.*, 1993). Pigmentation defects in human and mouse are due to several *KIT* mutations (e.g.: Chabot *et al.*, 1988; Geissler *et al.*, 1988; Giebel and Spritz 1991; Spritz *et al.*, 1992). Mutations in the *KIT* gene cause different pigmentation patterns and colours in horses and pigs (e.g.: Marklund *et al.*, 1998; Pielberg *et al.*, 2002; Brooks and Bailey 2005; Haase *et al.*, 2007). Therefore, the bovine *KIT* gene is a major candidate for the *spotting locus* in cattle. For this reason we recently sequenced the 21 exons and intronic and flanking regions of the *KIT* gene in 18 cattle of three breeds (Angus, Hereford, and Holstein-Friesian), which were considered carriers of different putative alleles at the *spotting locus,* because of their coat colour patterns (Fontanesi *et al.*, 2010). The 111 identified polymorphisms were organized in 28 haplotypes with some evidences of selection signature presence in this region, that would be expected if selection pressure on the coat colour phenotypes tended to favour few *KIT* haplotypes (alleles) in the different breeds (Fontanesi *et al.*, 2010).

Here we selected two synonymous single nucleotide polymorphisms (SNPs) identified in exon 2 (g.72779776C>T) and exon 3 (g.72783182A>G) of the *KIT* gene, based on the fact that they could capture information of several *KIT* haplotypes, as indicated in Holstein-Friesian, Angus and Hereford (Fontanesi *et al.*, 2010). We analysed their frequency distribution in seven Italian cattle breeds and evaluated if these SNPs could be useful for breed allocation and consequently for breed traceability and authentication of dairy and beef products.

## Materials and methods

### Animals and samples

Milk, hair or semen were sampled from a total of 490 animals belonging to seven cattle breeds: Italian Holstein-Friesian, n=61 (17 sires and 44 cows); Italian Brown, n=60 (cows); Italian Simmental n=78 (cows); Jersey, n=60 (2 sires and 58 cows); Rendena, n=51 (cows); Reggiana, n=128 (sires); Modenese, n=52 (21 sires and 29 cows) (Table 1). Cows of Italian Holstein-Friesian, Italian Brown, Italian Simmental, Jersey, Rendena and Modenese were sampled in several farms in order to increase sire representation. Several artificial insemination centres provided sire semen. Almost all active Reggiana sires were analysed. DNA was extracted from the collected biological materials as previously reported (Russo *et al.*, 2007). Coat colour descriptions of the analysed breeds are reported as a note to Table 1.

### PCR, genotyping and sequencing

PCR was performed using a PTC-100 (MJ Research, Watertown, MA, USA) thermal cycler in a final 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 2.0 mM of MgCl$_2$. PCR was carried out using the following profile: 5 min at 95°C; 35 amplification cycles of 30 s at 95°C, 30 s at 59°C, 30 s at 72°C; 10 min at 72°C. Genotyping of the *KIT* exon 2 SNP (g.72779776C>T) was obtained by PCR-RFLP of a 234 bp fragment generated with primers (forward: 5'-TGTCGAGTACACAGAAGATGGAA-3'; reverse: 5'-AAGTCCACTTGACAAATCCTG-GA*CC*-3') designed on the bovine *KIT* gene sequence derived from the Btau_4.0 genome assembly (Ensembl ENSBTAG00000002699). When allele g.72779776C is present, a mismatched nucleotide in the reverse primer (underlined base in the primer sequence) inserts an artificial restriction site for *Msp*I restriction enzyme in the amplified products. Analysis of the exon 3 *KIT* SNP (g.72783182A>G) was carried out by PCR-RFLP after amplification of a 448 bp fragment (forward primer: 5'-CTGCAGTGGAAG-CATTTGAC-3'; reverse primer: 5'-ACAC-CCAGCAGAAAGCAAAC-3') and digestion with *Tai*I endonuclease. Enzyme digestion of the PCR fragments was obtained setting up two separated assays for the two SNPs using 5 μL of PCR products added with 5 U of *Msp*I (g.72779776C>T) or TaiI (g.72783182A>G) endonucleases (MBI Fermentas, Vilnius, Lithuania) in a total volume of 25 μL containing 1X reaction buffer at 37°C or 65°C overnight, respectively. All digested products were electrophoresed in 10% polyacryamide: bisacrylamide 29:1 TBE1X gels and DNA fragments were visualized with ethidium bromide. PCR-RFLP patterns for the g.72779776C>T were constituted by an undigested fragment for allele g.72779776T and by two fragments of 209 and 25 bp for allele g.72779776C, whereas the two alleles of SNP g.72783182A>G were

**Table 1. Alleles frequencies, genotypes, observed (Ho) and expected heterozygosity (He) of the g.72779776C>T and g.72783182A>G SNPs.**

| Breed* | No. of animals | g.72779776C>T | | | | | g.72783182A>G | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Allele frequency° | Genotypes (no.)# | | | Ho/He | Allele frequency° | Genotypes (no.)# | | | Ho/He |
| | | (C) | TT | CT | CC | | (A) | AA | AG | GG | |
| Italian Holstein-Friesian | 61 | 0.893 | 0 | 13 | 48 | 0.213/0.192 | 0.893 | 48 | 13 | 0 | 0.213/0.192 |
| Italian Brown | 60 | 0.192 | 38 | 21 | 1 | 0.350/0.312 | 0.817 | 38 | 22 | 0 | 0.367/0.302 |
| Italian Simmental | 78 | 0.987 | 0 | 2 | 76 | 0.026/0.026 | 0.987 | 76 | 2 | 0 | 0.026/0.026 |
| Jersey | 60 | 0.525 | 16 | 25 | 19 | 0.400/0.504 | 0.533 | 20 | 24 | 16 | 0.400/0.502 |
| Rendena | 51 | 0.343 | 19 | 29 | 3 | 0.569/0.455 | 0.882 | 40 | 10 | 1 | 0.196/0.210 |
| Reggiana | 128 | 0.562 | 20 | 72 | 36 | 0.570/0.493 | 0.695 | 59 | 60 | 9 | 0.477/0.428 |
| Modenese | 52 | 0.625 | 9 | 21 | 22 | 0.404/0.473 | 0.904 | 34 | 16 | 2 | 0.308/0.314 |

*Coat colour and patterns of the analysed breeds are the followings: Italian Holstein-Friesian, white with black spots, sometimes the spots are red; Italian Brown, solid brown with wide range of intensity (from light gray to dark fawn); Italian Simmental, white with red spots; Jersey, varying from a very light gray to a very dark fawn (sometimes broken); Rendena, solid dark brown (with different intensities); Reggiana, solid red; Modenese, solid white. °Allele frequency is reported for one of the two alleles. #The observed number of the three genotypes is reported.

evidenced by the presence of two fragments of 395 and 53 bp (allele g.72783182A) and three fragments of 230, 165, and 53 bp (allele g.72783182G).

The genomic regions containing the two selected SNPs (g.72779776C>T and g.72783182A>G) were resequenced in a panel of 9 cattle (3 each Italian Holstein-Freisian, Italian Brown and Reggiana) with different PCR-RFLP genotypes in order to verify the results of the genotyping protocols. Amplification of the sequenced products was obtained as reported above except for the fragment containing exon 2 for which a different forward primer (5'-TGTCGAGTACACAGAAGATGGAA-3') placed in intron 1 of the *KIT* gene was used. Briefly, 3-5 μL of PCR product was 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 v3.1 kit (Applied Biosystems, Foster City, CA, USA) and sequencing reactions, after a few purification steps using EDTA 0.125M, ethanol 100% and ethanol 70%, were loaded on an ABI3100 Avant sequencer (Applied Biosystems). All sequences were visually inspected and aligned with the help of the CodonCode Aligner software (*http://www. codoncode.com/aligner*).

## Data analysis

Haplotypes including the two SNPs were inferred using the PHASE program v. 2.1 with default settings (Stephens *et al.*, 2001). ARLEQUIN software v. 3.1 (*http://cmpg.unibe.ch/software/arlequin3*) was used to calculate $F_{is}$ values and population pairwise $F_{st}$ genetic distance on haplotype frequencies (with 1000 permutations for significance), observed heterozygosity (Ho), expected heterozygosity (He), and departure from Hardy-Weinberg equilibrium for each *locus*. This software was also used for the analysis of molecular variance (AMOVA) with 1000 permutations, including single markers or haplotypes, testing the effect of the coat colour pattern in evaluating population differentiation with a model including spotted (Italian Holstein-Friesian and Italian Simmental) vs non spotted breeds, spotted phenotype/breeds, individuals/breeds and individuals.

## Results and discussion

Allele frequencies and genotypes for the g.72779776C>T and g.72783182A>G SNPs identified in seven cattle breeds are reported in Table 1. For the exon 2 polymorphism, allele g.72779776C is the most frequent in the two spotted breeds, Italian Holstein-Friesian (~90%) and Italian Simmental (almost fixed). This allele is present in all other breeds, even if at lower frequency (ranging from ~60% in Modenese to ~20% in Italian Brown). On the contrary, the alternative allele (g.72779776T) is the most frequent in Italian Brown and Rendena, two solid coloured breeds. For the exon 3 polymorphism, allele g.72783182A is the most frequent in all breeds (ranging from ~99% in Italian Simmental to ~53% in Jersey). The two SNPs did not deviate from Hardy Weinberg equilibrium (P>0.05) in all breeds.

Sequencing carried out to confirm the genotyping results for the two amplified fragments containing exon 2 and exon 3, respectively, did not reveal any other polymorphisms. This confirm that another synonymous SNP we previously detected in exon 3 (g.72783146G>T) in only one Holstein-Friesian sire is quite rare (Fontanesi *et al.*, 2010).

Deterministic approaches for breed traceability and authentication of dairy and beef products are based on the possibility to exclude the presence of frauds derived from admixture or substitution of milk or meat obtained from not allowed breeds, using one or few DNA markers with high resolution power (Fontanesi, 2009). The optimal situation would be the case of an allele fixed in one breed and not present in others. In the two SNPs analysed for the *KIT* gene, two alleles were observed in all breeds for both polymorphic sites. For this reason, theoretically it is not possible to apply a precise authentication system based on these DNA markers. However, since in the Italian Simmental breed allele g.72779776C is almost 100%, in some situations this polymorphic site could be useful to evaluate if the tested products derive from this breed only. It is worth to point out that the detection of this allele only cannot be considered a direct proof of authenticity, since it has a high frequency in other breeds (Table 1).

Reggiana is a further cattle breed for which there is interest in tracing cheese obtained from its own milk only. Parmigiano Reggiano cheese produced with Reggiana milk only is sold at about twice the price of the common Parmigiano Reggiano cheese. An authentication system based on *MC1R* gene markers has been already developed and routinely applied (Russo *et al.*, 2007; Fontanesi, 2009). This system would be improved if markers able to distinguish solid coloured from spotted breeds were identified. As a matter of fact, Reggiana breed is fixed for the recessive *e* allele of the *MC1R/ Extension locus*, like the Italian Simmental breed (in which this allele is ~99%) (Russo *et al.*, 2007). Another problem could derive from spotted red Italian Holstein-Friesian. As Reggiana cows are solid red and Italian Simmental (and a few Italian Holstein-Friesian cows as well) animals are spotted red, the products of the two (three) breeds could be distinguished if a DNA marker associated to the two different phenotypes could be identified. Unfortunately, this is not the case, at least for the two genotyped SNPs of the *KIT* gene (Table 1).

Results of haplotype analysis including the g.72779776C>T and g.72783182A>G polymorphisms are reported in Table 2. Only two haplotypes [T:G] and [C:A] were identified in Italian Holstein-Friesian and Italian Simmental, with one of them ([C:A]) having very high frequency in both breeds. In Jersey, only one animal carried a different haplotype ([T:A]) from those of the two spotted breeds. This haplotype was the most frequent in Italian Brown and Rendena (62.5% and 53.9%, respectively). Reggiana and Modenese had 55.5% and 61.5% of the [C:A] haplotype. These results confirmed the high frequency of the [C:A] haplotype despite the coat colour pattern of the animals, as already reported by Fontanesi *et al.* (2010), who analysed the haplotype structure of the *KIT* gene in Holstein-Friesian, Hereford and Angus based on 111 polymorphic sites. In our previous study we derived the presence of 28 *KIT* gene haplotypes in which the combination of the g.72779776C>T and g.72783182A>G SNPs resulted in 23 of them, constituted by [C:A] with a frequency across the analysed breeds of ~90%. Other 4 haplotypes carried the combination [T:G] (identified in Angus, Hereford, and Holstein-Friesian), whereas only one was [T:A] (identified in Angus) (Fontanesi *et al.*, 2010). In addition to these haplotypes, in Reggiana and Modenese we identified a rare haplotype ([C:G]; considering only the two analysed SNPs), which was not reported in our previous study. Therefore, even if in the present investigation we analysed only two polymorphisms, we can confirm the complex heterogeneity of the *KIT* gene in breeds having different coat colour patterns, making unlikely this gene to determine the spotted phenotype in Holstein-Friesian and Simmental breeds. On the other hand, it is interesting to note that only the [C:A] and [T:G] haplotypes were identified in the spotted breeds (even if here we analysed markers in a small portion of the *KIT* gene). This might be in agreement to the QTL experiments that located a multi-allele QTL for the degree of spotting on BTA6 in the region containing the *KIT* gene (Reinsch *et al.*, 1999; Liu

*et al.,* 2009), suggesting that the *KIT* gene might affect the extension of the spotted areas in spotted breeds. AMOVA based on single markers (averaged) or haplotypes showed that genetic variation between spotted and non-spotted groups of breeds amounted to 25.3% (16.8% for the g.72779776C>T SNP and 9.5% for the g.72783182A>G SNP) or 21.2% of total variation (P<0.05 and P<0.10, respectively), supporting a possible involvement of the *KIT* gene in influencing the spotted phenotype, but probably not determining it (at least in all animals), as already suggested (Fontanesi *et al.,* 2010).

For the analysed breeds $F_{is}$ (obtained using haplotype frequencies), which measures the inbreeding coefficient of the individuals relative to the considered subpopulations, ranged from -0.167 (Italian Brown) to +0.231 (Jersey) (Table 2), but deviated significantly from zero in the Jersey breed only (P<0.05), that means there was a slight deficiency of heterozygotes than expected only in this group of animals. Pairwise $F_{st}$ measures (Table 3) indicated that all breed comparisons were significant, except for Reggiana vs. Modenese. These local breeds are traditionally reared in a very close geographic area in the North of Italy (provinces of Reggio Emilia and Modena, from which their names come from), suggesting that the population distribution of the analysed *KIT* markers might be derived, at least partially, from the geographic closeness of these two breeds, together with their similar solid coat pattern (even if of different colour). On the other hand, for all other breeds, the reproductive barriers might be the reason of the significant differences obtained with the $F_{st}$ measures, together with a possible indirect selection pressure on the gene due to direct selection over some different coat colour patterns. This result might indicate that the two *KIT* markers could be useful in defining SNP panels for breed allocation using probabilistic approaches.

## Conclusions

The identification of polymorphisms in genes affecting one of the most important phenotypic traits for breed identity (coat colour) could result in DNA markers useful for authentication and traceability of mono-breed products. We analysed two polymorphisms in the *KIT* gene, a candidate gene for the *spotting locus*, in several Italian cattle breeds with different coat colour patterns. These markers were not fixed in any considered breeds. However, AMOVA indicated that these *KIT*

**Table 2. Haplotypes and $F_{is}$ for the two *KIT* SNPs in seven cattle breeds.**

| Breeds | No. of animals | Haplotypes | | | | $F_{is}$ |
|---|---|---|---|---|---|---|
| | | [T:A] | [T:G] | [C:A] | [C:G] | |
| Italian Holstein-Friesian | 61 | - | 13 | 109 | - | -0.111 |
| Italian Brown | 60 | 75 | 22 | 23 | - | -0.167 |
| Italian Simmental | 78 | - | 2 | 154 | - | -0.006 |
| Jersey | 60 | 1 | 56 | 63 | - | +0.231 |
| Rendena | 51 | 55 | 12 | 35 | - | -0.144 |
| Reggiana | 128 | 36 | 76 | 142 | 2 | -0.141 |
| Modenese | 52 | 20 | 19 | 64 | 1 | +0.067 |

**Table 3. Pairwise $F_{st}$ values between breeds from haplotype frequencies.**

| Breeds | Italian Holstein-Friesian | Italian Brown | Italian Simmental | Jersey | Rendena | Reggiana | Modenese |
|---|---|---|---|---|---|---|---|
| Italian Holstein-Friesian | - | | | | | | |
| Italian Brown | 0.547 | - | | | | | |
| Italian Simmental | 0.076 | 0.677 | - | | | | |
| Jersey | 0.273 | 0.340 | 0.469 | - | | | |
| Rendena | 0.442 | 0.021 | 0.592 | 0.274 | - | | |
| Reggiana | 0.151 | 0.252 | 0.274 | 0.031 | 0.169 | - | |
| Modenese | 0.137 | 0.245 | 0.297 | 0.094 | 0.141 | 0.011 | - |

polymorphisms could capture a quote of genetic variation distinguishing spotted breeds (Italian Holstein-Friesian and Italian Simmental) from solid coloured breeds (or almost solid, as some Jersey animals have a broken coat colour phenotype). This indication might indirectly suggest that the *KIT* gene is involved in influencing the degree of spotting in these breeds, but it does not seem to be the causative factor (or the only causative factor) of the spotted phenotype. Other genes might be involved in causing piebaldism in Holstein-Friesian and Simmental cattle. Further studies are needed to clarify this issue.

The fact that none of the two analysed SNPs are fixed in any considered breeds, indicates that these markers are not useful for breed authentication of dairy products usually derived from the contribution of several animals. Only the Italian Simmental was almost fixed for one of the two alleles for both polymorphisms (g.72779776C and g.72783182A, haplotype [C:A]). However, these alleles are the most frequent in most of the analysed breeds except in Italian Brown and Rendena; it could therefore be possible, at least in some cases, monitoring the Italian Simmental dairy products to detect any milk addings from the latter two different breeds. On the other hand, the discriminatory power of these markers

could point out on the inclusions of the g.72779776C>T and g.72783182A>G polymorphic sites in SNP panels developed for breed allocation and traceability of beef products relying on probabilistic approaches.

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