SHORT COMMUNICATION

A simple PCR-RFLP test for direct identification of Melanocortin Receptor 1 (MC1R) alleles causing red coat colour in Holstein cattle

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ABSTRACT

A direct test to determine the presence of the recessive alleles causing red colour in Holstein cattle at DNA level is proposed. Digestions with two restriction enzymes were used to detect individuals carrying recessive alleles of Melanocortin Receptor 1 (MC1R) gene, responsible for coat coloration. Direct sequencing of the PCR products confirmed the identified genotypes. Compared to previously described methods this is an effective, relatively economic and quick method. This test could be employed not only to facilitate the detection of polymorphisms in populations but also to exclude animals carrying alleles resulting in an undesired coat colour from breeding schemes.

Key words: Coat colour, Cattle, Selection, PCR-RFLP

RIASSUNTO

UNA SEMPLICE METODICA PCR-RFLP PER L’IDENTIFICAZIONE DIRETTA DEGLI ALLELI DEL GENE MELANOCORTIN RECEPTOR 1 (MC1R) CHE CAUSANO LA COLORAZIONE ROSSA DEL MANTELLO NEI BOVINI DI RAZZA HOLSTEIN

In questo studio viene descritto un metodo per evidenziare gli alleli che determinano la colorazione rossa del mantello nei bovini di razza Holstein. Rispetto a metodi precedenti questo test è più rapido, efficace ed economico nell’individuare i portatori di alleli recessivi del gene Melanocortin Receptor 1 (MC1R), implicato nella colorazione del mantello, in quanto si basa su una singola reazione PCR e due digestioni del prodotto amplificato con enzimi di restrizione per identificare i diversi alleli. Per confermare la validità del metodo i frammenti di amplificazione usati per le digestioni sono stati sequenziati.

Il test proposto, oltre a facilitare l’individuazione di polimorfismi nelle popolazioni, può essere utilizzato per escludere dagli schemi di accoppiamento animali portatori di due alleli che determinano la pezzatura rossa.

Parole chiave: Colore del mantello, Bovini, Selezione, RFLP-PCR
**Introduction**

In mammals, primarily two loci, *extension* and *agouti*, control the distribution of the pigments eumelanin and phaeomelanin, which determine brown-black and yellow-red colorations, respectively. The *Extension* locus was found to be the gene encoding the MC1-R (melanocyte stimulating hormone receptor, Klungland *et al.*, 1995). Different alleles of MC1R have been implicated in colour determination by affecting eumelanin synthesis in mice (Robbins *et al.*, 1993) and horses (Johansson *et al.*, 1994).

In cattle, the black and red coat colours are determined, respectively, by dominant and recessive alleles (E<sup>d</sup>, and E<sup>+</sup> or e) at the MC1R gene (Klungland *et al.*, 1995).

The E<sup>+</sup> and e alleles differs from E<sup>d</sup> by a C (→) T transversion at nucleotide position 422 of the MC1R gene (GeneBank accession number U39469), eliminating an *Msp*<sub>AI</sub>I site. The e allele is characterised by presenting a G deletion at position 437 of the E<sup>+</sup> sequence (Klungland *et al.*, 2000), introducing a *Bsl*<sub>II</sub> site. Individuals that are not Ed homozygous may have progeny with coat colour unfit for selection schemes aimed at elimi-
nating animals with red coat color. Red colour is not linked to negative traits, but keeping the colours coded in the breed standard is considered a “brand” mark useful for the marketing. The aim of the present study is to determine the presence of the recessive alleles causing red colour in Holstein cattle at the DNA level using a direct test representing a valid alternative to previously described methods.

Material and methods

**PCR conditions**

DNA was extracted from blood of 7 unrelated Holstein animals bearing the different alleles using Wizard Genomic extraction kit (Promega) and was then amplified in a region including the mutation sites. The PCR reaction was performed using 50-100 ng of DNA template, 0.5 pmoles of each primer (MWG biotech), 1X PCR buffer, 1.5 mM MgCl2, 0.2mM each dNTPs and 1 U of Taq polymerase (Amersham Pharmacia) for a 30 µl final volume. The PCR profile included 3 min at 94 °C; 35 cycles of 30 s at 94 °C, 45 s at 63 °C, 1 min at 72 °C; and a final extension of 5 min at 72 °C.

The genotype was assessed by using the current method (Klungland et al., 2000).

**Primer sequences**

Primers described by Klungland et al. (1995) were used to amplify a 138 bp product:

- Forward: 5’-CAAGAACCGCAACCTGCACT-3’
- Reverse: 5’-GCCTGGGTGGCCAGGACA-3’

**Sequencing**

Direct sequencing of the PCR products of animals with different genotypes was performed using the Fmol sequencing kit (Promega) with near-infrared fluorescent dye end-labelled primers. The sequencing reactions were run on a Licor 4200 DNA automated sequencer, and true image data were analysed using Gene ImagiR software (LICOR).

**RFLP analysis**

The 138 base pair PCR-product, including the polymorphic sequence, was digested in two independent reactions, with: a) 1 U of MspAII (Promega) for 1h 30 min at 37 °C; and, b) 1 U of BsiI (New England Biolabs) for 1h 30 min at 55 °C. Fragments were resolved on a 3% metaphor agarose gel (Biospa) and visualised on UV light source after staining with ethidium bromide.

**Results and discussion**

Digestion with MspAII yielded two cut fragments of 106 and 32 bp for individuals which were homozygous at the dominant allele Ed, or the uncut fragment (138 bp) in the case of presence of the alleles E* and e (Figure 1a). Digestion of the same PCR product with BsiI resolved E* and e alleles, resulting in the cut fragments of 117 and 21 bp for individuals which were homozygous at the allele e, or the uncut fragment in the case of presence of E* and E' (Figure 1b). In both digestions, heterozygous individuals showed all three fragments. By considering the two digestion patterns it is possible to identify genotypes (Figure 2). Sequences of the PCR products confirmed the genotypes identified by digestion.

With respect to previously described methods for the identification of MC1R alleles (Joerg et al., 1996; Klungland et al., 2000), this test offers the advantages of being based on a single PCR reaction, therefore saving time and reagents. Also, the length of RFLP bands is suitable for detection on agarose gels. Moreover, to eliminate carriers of alleles involved in red coloration, it is sufficient to digest with MspAII, as Ed homozygotes are clearly distinguished from carriers of the other alleles, therefore saving time and money.

In previous studies, the sequence of the Holstein cattle alleles and detection methods were provided. In particular, a so called “AFLP assay” was proposed by Joerg et al. (1996) discriminating only two alleles. A PCR-RFLP was described by Klungland et al. (1995) by using two PCR reactions for discriminating the three alleles. The presented method shows the advantage of discriminating the three different alleles using a single PCR amplification.

**Conclusions**

The described PCR-RFLP is an effective, rela-
Polymorphisms at the MC1R locus. DNA of seven individuals has been amplified by PCR and the product, aliquoted in two batches, underwent (a) *Msp*AI digestion: samples 2, 3, 4 are homozygous for the $E^i$ allele; (b) *Bst*II digestion: samples 1, 5, 6, are heterozygous presenting $e$ allele. The 21 bp fragment is not visible on the agarose gel but it does not affect alleles identification; C: undigested PCR fragment; M: 100 bp ladders DNA fragments were resolved on a 3% agarose gel.

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