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A Simple Method for the Detection and Identification of Korean Brown Cattle (Hanwoo) using DHPLC

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Abstract

Denaturing high-performance liquid chromatography (DHPLC) is used in a wide variety of genetic applications and is an efficient method for detection of mutations involving one or a few nucleotides. We developed a high-throughput DHPLC method for identifying polymorphisms in the MC1R gene that are characteristic of Hanwoo cattle.

We compared 10 tissue samples from Hanwoo cattle, 10 samples from Holstein cattle and 10 samples from Hanwoo× Holstein crossbred cattle to determine whether DHPLC analysis can be used to distinguish between these genotypes. Samples obtained from Hanwoo cattle had a unique profile of peaks that could be used as a molecular fingerprint for this breed. We also analyzed two hundred samples in a trial in which we were blinded to the genotype of the samples and correctly identified the breed-of-origin of 594 out of 600 sequence variations (99%).

Introduction

Dominant mutations in the MC1R gene cause a general eumelanizing effect in mammalian species such as the mouse, red fox, sheep, and cattle[1)]. The MC1R gene determines the expression of a brown or a black colored pelage in these species. In cattle, two

polymorphisms of the MC1R locus have been observed: a T→C point-substitution that results in substitution of proline for leucine at position 99 of the amino acid sequence of the protein, and a G point-deletion that results in substitution of valine for glycine at position 104. When leucine and valine are present, cattle typically have a brown coat color such as that of Hanwoo cattle; when proline and glycine are present, the coat color is black such as that of Holstein cattle.

Denaturing high-performance liquid chromatography (DHPLC) is a rapid and sensitive method for detecting genetic mutations[2,4]. The technique consists of four steps: amplification by PCR, quantification, hybridization, and analysis of the hybridized product. During the hybridization step, heteroduplexes are formed between wild-type DNA and that of the unknown sample. Mismatches between wild-type and mutant DNA sequences disrupt the structure of the heteroduplex and are detected by DHPLC[2]. DHPLC has a wide range of applications, which include nucleotide polymorphism analysis[5], gene mapping[6], analysis of genes[7], screening for mutations[8], analysis of primer extensionproducts[9], and quantification of gene expression[10]. Here we describe a new application of this technique in which it is used to verify the presence of Hanwoo genes in cattle.

Compared to techniques currently used to verify that cattle are of the Hanwoo genotype, DHPLC is quick, economical, and capable of screening large numbers of samples in a relatively short time.

Thirty samples were tested to determine if DHPLC peak profile analysis could be used to distinguish samples from Hanwoo cattle from those from Holstein cattle. Samples from one Hanwoo and one Holstein, which were approved as being representative of their respective breeds, were used to distinguish between the genotypes of 600 other samples.

Materials and Methods

1. Materials

Guanidine-HCl and trizma base were from Sigma-Aldrich (USA). TEAA was from Transgenomic (USA) and acetonitrile was from Merck (USA). Genomic DNA Isolation Kits and Taq polymerase were from GeNet Bio (Korea).

2. DNA Extraction

Genomic DNA was extracted from cattle tissue samples. Briefly, a tissue lysis buffer was added to the tissue and incubated at 56 °C for 4~5 h. An equal volume of binding buffer and ethanol were added. The solution was added to a tube containing a binding column and centrifuged at 8,000 rpm for 1 min. The binding column was transferred to a microcentrifuge-tube, washed twice with washing buffer and dried for 1~2 min. The column was transferred to microcentrifuge-tube and eluted with elution buffer.

3. Polymerase Chain Reaction

The primers used for PCR of the MC1R gene were sense: 5'-CAAGAACCGCAACC TGCACTCC-3', antisense: 5'-AATGGCACCCAGGAAGCAGAG-3' (GenBank accession number Y19103) and yielded a product 223 bp in length. Each DNA extract was amplified in a volume of 20 μL containing 0.5 μM of each primer, 10X PCR Buffer, 0.5 U of Taq polymerase (GeNet Bio, Korea), 250 mM each of dATP, dGTP, dCTP and dTTP. A GeneAmp® PCR System 2700 (Applied Biosystems, USA) was used. The touchdown conditions for this PCR were an initial 5 min denaturation at 94 °C followed by 20 s at 94°C, 20 s at 65°C, and 30 s at 72 °C for 10 cycles and 25 cycles of 20 s at 94 °C, 20 s at 57 °C, 30 s at 72 °C followed by a 5 min extension at 72 °C. Four microliters of PCR product were separated in a 1.5% (w/v) agarose gel and stained with ethidium bromide.

4. Heteroduplex Formation

Following quantification of the PCR products, DNA heteroduplexes were formed by mixing equal amounts of Hanwoo control DNA and sample DNA. Hybridization reactions were performed using a GeneAmp® PCR System 2700. A ten-minute preincubation at 94 °C followed by cooling at a rate of 1.5 °C per min to 25 °C was applied.

5. DHPLC Analysis

To determine if the primer set would produce discriminating peak profiles, 20 samples from cattle were analyzed by DHPLC and reverse-phase HPLC using WAVE® software and a DNA fragment Analysis System® (both from Transgenomic, Omaha, NE, USA). Injections of hybridized sample (8 μL, 20~30 ng DNA) were exposed to 63 °C, 64 °C, 65 °CC, 66 °C, and 67 °C in the presence of an analytical gradient (0.9 mL/min) generated by the WaveMaker software for elution of the MC1R amplicons. The 200 blinded samples were analyzed at 65 °C using the analytical gradient. Amplicons from Hanwoo cattle eluted as single peaks, whereas heterozygous amplicons from Holstein cattle or Hanwoo× Holstein crossbred cattle eluted as double peaks. The mobile phase was composed of buffer A, which contained 0.1 M triethylammonium acetate (Transgenomic), pH 7.0 and buffer B, which contained 0.1 M triethylammonium acetate, pH 7.0, and 25% acetonitrile. The columns were 50 mm long and had an internal diameter of 4.4 mm (DNASep®cartridges) and were packed with nonporous polystyrene-di-vinylbenzene copolymer particles 2.1± 0.12 μm in diameter.

6. Sequencing of PCR Products

The PCR Product was purified using an PCR Purification Kit (GeNet Bio). All samples analyzed by DHPLC were subject to DNA sequencing with a 3730XL DNA Analyzer (Applied Biosystems).

Results and Discussion

Each sample was mixed with a Hanwoo control sample of verified genotype to form a homoduplex and/or a heteroduplex. If the genotype of the sample is Hanwoo, a single peak would be expected; if the genotype of the sample is Holstein or Hanwoo × Holstein, double peaks would be expected. We used DHPLC to analyze two hundred samples whose origins were blinded to us and correctly identified sequence variations in 594 out of 600 (99%) samples.

Individual identification is widely used in the stud industry. In the case of breeding stock, verification of the genotype of individual sires and dams is essential because traits that are passed on to their offspring affect the gene pool of the breed. The purpose of this study was to develop an efficient method for screening for the presence of Hanwoo genes using DHPLC.

To evaluate the ability of DHPLC to identify Hanwoo genes, we compared elution peaks from two control samples (one from a Hanwoo and one from a Holstein) with those of 600 samples whose origins were blinded to us. Of the unknown samples, 594 were identified correctly. DHPLC has a 99% detection rate and is easy to perform. We identified the detection of the 99 T−to−C substitution that alters a leucine to a proline or the 104 G deletion that alters a glycine to a valine, which determines whether the sample is from a Hanwoo or a Holstein. Furthermore, in our mixed HanwooHolstein samples, we detected less than 80:20 mixture in the Hanwoo control background. Elution profiles seem to be highly characteristic of genotypes.

DHPLC is an efficient method for detecting genetic variants such as single nucleotide polymorphisms, insertions, and deletions, and has been used successfully for detection of mutations[11]. The advantages of this method include automation and speed of analysis[4]. The experimental conditions described herein are particularly cost−effective because very high sensitivity and specificity were obtained with one run temperature.

Because of the capability of DHPLC to detect variations in DNA sequences, its high throughput, and the utility of the MC1R gene in identifying Hanwoo cattle, it may be possible to refine this method for the rapid screening of large numbers of samples. It may also be possible to analyze regions of variability in species−specific genes, which could be exploited for strain typing. DHPLC appears to be a promising technique for identifying and characterizing the Hanwoo genotype without having to sequence DNA.

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