ABSTRACT

The duplex-PCR technique, proposed to identify and differentiate cattle and water buffalo DNA using primers described by Rea et al. (2001) for identification of cattle and buffalo DNA in Italian cheese, was tested on mitochondrial DNA extracted from meat muscle samples.

The optimized PCR amplified 113 bp and 152 bp products for cattle and buffalo respectively. Meat processing technology (salting, drying, smoking, and cooking) affects the integrity of the extractable DNA. Also many times meat samples are brought to the laboratory for speciation one or two days after slaughter under unpreserved conditions. So different levels of autolysis were experimentally produced, and PCR successfully amplified cyt b gene from meat samples that were putrefied even after 48 h and cooked at various conditions.

The technique was successful in detecting up to 1 pg adulteration in a cattle-buffalo meat mixture. The test is a valuable tool for meat authentication and screening of cooked, putrefied and mixed samples of cattle and buffalo flesh.

Keywords: meat speciation, polymerase chain reaction, cattle, buffalo, cyt b

INTRODUCTION

The determination of food authenticity and the detection of adulteration are major issues in the food industry and are attracting an increasing amount of attention. Therefore, reliable techniques to identify the species of origin of components in a food product derived from animals are necessary for food authentication purposes. Identification of the species of origin of meat samples is relevant to consumers for the possible economic loss from fraudulent adulterations, medical requirements of individuals who might have specific allergies, and religious reasons (Miguel et al., 2004).

The conventional methodology used for the determination of species origin in meat products are predominantly based on immunochemical and electrophoretic analysis of protein. Additionally, through the acquisition of sequence data, DNA can potentially provide more information than protein, due to the degeneracy of the genetic code and the presence of many non-coding regions. Polymerase chain reaction (PCR) is the most widely used molecular biology technique. Following PCR, the amplified DNA fragments normally require further analysis to identify the species of origin. The methods used include Single Strand Conformation Polymorphism (SSCP), Restriction Fragment...
Length Polymorphism (RFLP), Denaturing Gradient Gel Electrophoresis (DGGE), Thermal Gradient Gel Electrophoresis (TGGE) and Sequencing.

A particular type of PCR, based on the multiplex-PCR principles described by Dieffenbach and Dveksler (1995) and Innis et al. (1990), was used for species identification in meat and meat products in two different ways by Fei et al. (1996) and Matsunaga et al. (1999). The advantage of this technique is that the species is directly identified by PCR, without requiring further analyses.

A number of studies have addressed meat species identification in the recent past and attempts were made to differentiate closely related meat species (Rajapaksha, 2002; Rodriguez, 2004; Maccabiani, 2005; Martina et al., 2006).

Fewer studies have been published so far reporting the application of DNA-based techniques for differentiation of water buffalo meat from cattle meat, but with limited utility in the case of mixed and processed meat (Girish et al., 2005; Rastogi et al., 2004; Jain, et al., 2007). In this context, development of highly sensitive and specific method for identification and differentiation of cattle and water buffalo meat is necessary for law enforcement.

In the present paper, a variation of multiplex-PCR, the duplex-PCR, is proposed to identify and differentiate cattle and water buffalo meat. A common primer is used along with two specific primers that allow two different DNA fragments to be amplified, one specific to cattle and the other to water buffalo. These are used to identify meat and meat products from the two species. This work presents a specific, sensitive, effective and inexpensive alternative to the existing methods.

MATERIALS AND METHODS

DNA isolation from meat samples
Mitochondrial DNA, along with genomic DNA, was extracted from meat samples of each species by using the method described by Ausubel et al. (1987) with some modifications. The quality and purity of DNA were checked on agarose gel electrophoresis and DNA was quantified using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies).

Polymerase chain reaction
A small fragment of the cyt b gene of mitochondrial DNA extracted from fresh, cooked and putrefied samples was amplified. For this purpose species specific primers described by Rea et al. (2001) for identification of cattle and buffalo DNA in Italian cheese were tested on DNA extracted from meat samples. A common forward primer (5'-CTT CTT ATT CGC ATA CGC AAT CTT ACG ATC- 3') and species specific reverse primers, cattle specific (5'-TGC TCT AAT CCC CTC A TA CAC ACC TCC A- 3') and water buffalo specific (5'-TAT GAT GTT CCG GCC ATT CTA CTA CAC ACC TCC A- 3') were used, as described by Rea et al. (2001).

Various combinations of primers and DNA of cattle and buffalo origin were tested in a final volume of 25 μl containing 1x PCR master mix (MBI Fermentas, Canada) 10 pmole of each primer and 90-100 ng of DNA template (cattle and/or buffalo). Amplification was performed in Master Cycler gradient thermocycler (Eppendorf, Germany) with the following cycling conditions: after an initial heat denaturation at 95°C for 5 minutes, 35 cycles were programmed as follows: 95°C for 30 seconds, 65°C for 30 seconds, 72°C for 30 seconds and final extension at 72°C for 5
minutes. This optimized PCR amplified a 113 bp product for cattle and a 152 bp product for buffalo which were confirmed by using Genesnap and Genetool programmes (Syngene, UK) and running the products parallel to a 100 bp MW marker.

**RESULTS AND DISCUSSION**

Rea et al. (2001) successfully used an annealing temperature of 55°C for differentiation of cattle and buffalo DNA from cheese samples using the same set of primers, but in the present study mild cross reactions were observed when 55°C annealing was used (Figure 1) so the annealing temperature was increased by 2°C each time, until there were no cross reactions at 65°C (Figure 2 - lane 3 and 4). The specificity and sensitivity of the test greatly depends on the annealing temperature of the primers. These primers yielded PCR products only from the DNA extracted from species that they were designed for and showed no cross-reactivity with the DNA from sheep and goat. PCR products were not obtained for the samples of negative controls with any of the species-specific primer sets.

**PCR profiles of cooked and putrefied meat samples**

Species identification of cooked meat is often warranted. The processing technology (salting, drying, smoking, and cooking) applied during the manufacture of meat products affect the integrity of the extractable DNA, causing its degradation into small size fragments (Dias et al., 1994; Martinez and Man, 1998). For this reason, in the present study, meat samples were cooked at 100°C and 120°C in dry (hot air oven) and moist heat (water bath and autoclave) for 45 minutes to simulate cooking. Proper cooking was evident from discolored meat. Many times meat samples are brought to the laboratory for speciation one or two days after slaughter under unprocessed conditions. Looking to the reality of the situation that exists, different levels of autolysis was produced by allowing the meat samples to putrefy for variable periods (48 h or more) of time at room temperature in unprocessed conditions to stimulate the autolysis in meat. PCR successfully amplified small fragment of the cyt b gene from cooked and putrefied meat samples, indicating that partial degradation of DNA because of cooking or putrefaction of meat does not inhibit amplification of the cyt b gene region.

Mitochondrial DNA was used in the study as it offers two main advantages: first that mt DNA is present in thousands of copies per cell (as many as 2,500 copies), especially in the case of post –mitotic tissues such as skeletal muscle (Greenwood and Paboo, 1999). This increases the probability of achieving a positive result even in the case of samples suffering severe DNA fragmentation due to intense processing conditions (Bellagamba et al., 2001) and second that the large variability of mt DNA targets as compared with nuclear sequences facilitates the discrimination of closely related animal species even in the case of mixture of species (Prado et al., 2002).

Detection of adulteration from degraded DNA obtained from cooked and putrefied samples is one of the very important merits of this technique as a tool for meat species detection.

**Sensitivity of the assay**

To measure the detection limit of the duplex-polymerase chain reaction, DNA samples (cooked and putrefied) in 10 fold dilution i.e. 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 ng each were tested and positive signals up to 0.001 ng (i.e 1 pg)
Figures 1 and 2 showing gel electrophoresis pattern on 2% agarose.
Lane 1: Cattle DNA with common forward primers and cattle specific reverse primers
Lane 2: Buffalo DNA with common forward primers and buffalo specific reverse primers
Lane 3: Cattle DNA with common forward primers and buffalo specific reverse primers
Lane 4: Buffalo DNA with common forward primers and cattle specific reverse primers
Lane 5: Cattle and buffalo mixed DNA with common forward primers and cattle specific reverse primers
Lane 6: Cattle and buffalo mixed DNA with common forward primers and buffalo specific reverse primers
Lane 7: Cattle and buffalo mixed DNA with common forward primers and cattle specific and buffalo specific reverse primers
Lane 8: Cattle DNA with common forward primers and cattle specific and buffalo specific reverse primers
Lane 9: Buffalo DNA with common forward primers and cattle specific and buffalo specific reverse primers
Lane 10: 100bp DNA molecular weight marker.
of template DNA were observed.

It can be concluded that cattle and buffalo meat could be reliably identified and differentiated using duplex PCR at optimized conditions, detecting up to 1 pg adulteration in cattle-buffalo meat mixture.

Also this method can be applied with equal efficiency to fresh, cooked and putrefied meat.

REFERENCES


Miguel, A. R., G. Teresa, G. Isabel, A. Luis, E.H.


