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PCR identification of meats from chamois (*Rupicapra rupicapra*), pyrenean ibex (*Capra pyrenaica*), and mouflon (*Ovis ammon*) targeting specific sequences from the mitochondrial D-loop region

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Abstract

A polymerase chain reaction (PCR) assay was developed for the identification of meats from chamois (*Rupicapra rupicapra*), pyrenean ibex (*Capra pyrenaica*), and mouflon (*Ovis ammon*) by using oligonucleotides targeting mitochondrial D-loop sequences. A D-loop region (~700–1000 bp) was firstly amplified and sequenced from various game and domestic meat DNAs, and three primer sets were then designed on the basis of nucleotide multialignment of the generated D-loop sequences. As expected from sequence analysis, PCR amplification of the targeted D-loop fragments was successfully achieved from chamois (88 bp), pyrenean ibex (178 bp), and mouflon (155 bp) meats, showing adequate specificity and reproducibility against a number of game and domestic meats. Mouflon and sheep meats were amplified together in accordance to the high nucleotide identity of their mt D-loop sequences. In this work, satisfactory amplification was also accomplished in the analysis of experimentally pasteurized (72 °C for 30 min) and sterilized (121 °C for 20 min) meats, with a detection limit of ~0.1% for each of the targeted species. The proposed PCR assay represents a rapid and straightforward method for the detection of possible adulterations in game meat products.
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1. Introduction

To enforce control measures on the authentication of meat, legislative authority establishes that meat products must be accurately labelled regarding species content (Pascal & Mahé, 2001). In this framework, game meat is a susceptible target for fraudulent labelling due to the economic profit that results from selling cheaper meat as meat from more profitable and desirable species. Moreover, illegal trade of certain wild game species may contribute, not only to meat adulteration but also to severe depletion of biodiversity (Brodmann, Nicholas, Schaltenbrand, & Ilg, 2001; Colombo, Cardia, Renon, & Cantoni, 2004). For these reasons, development of accurate methods for determining the animal species in raw and processed meats are highly needed to protect both consumers and producers from frauds, and also to avoid over-exploitation and illegal market trafficking of game meat products (Lenstra, Buntjer, & Janssen, 2001; Teletchea, Maudet, & Hänni, 2005).

A number of classic analytical methods have been developed that allow the determination of the authenticity of meat through the study of its components, either by capillary electrophoresis (Vallejo, González, Mazorra, & Rodríguez, 2005), gas chromatography (Toorop, Murch, & Ball, 1997), or immunological techniques (Giovannacci et al., 2004), among other approaches. Although most of these methods are of considerable value in certain instances, they are not convenient for routine sample analysis because they

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are relatively costly, time consuming, and complex to perform. Moreover, they have the limitation of relying on the analysis of proteins, many of which are heat labile (Chen & Hsieh, 2000). As an alternative to protein analysis, DNA-based methods applied to meat speciation offer several advantages: (i) DNA is a relatively stable molecule, allowing analysis of processed and heat-treated food products, (ii) the information content of DNA is greater than that of proteins due to the degeneracy of genetic code as one goes from DNA to protein, and (iii) DNA is present in all kinds of tissue due to its ubiquity in every type of cell (Meyer & Candrian, 1996).

Among DNA-based methods, PCR technology provides the food analyst with a simple, specific, and highly sensitive tool for the authentication of species in food products (Fei et al., 1996). In particular, the use of mitochondrial DNA (mtDNA) sequences for species identification by PCR may offer a series of advantages over other genetic markers, such as cell nucleus DNA. Whereas detection of nuclear DNA might be limited as a result of the generally low copy number of sequences, utilization of mtDNA increases PCR amplification sensitivity because there are several copies of mtDNA per cell. Besides, the large variability of mtDNA allows reliable identification and differentiation of precise species in food mixtures (Girish et al., 2004). Among mitochondrial genes, the cytochrome *b* (Matsunaga et al., 1999; Verkaar, Nijman, Boutaga, & Lenstra, 2002; Wolf, Rentsch, & Hübner, 1999), the 12S and 16S ribosomal RNA subunits (Bottero et al., 2003; Fajardo et al., 2006; Rodríguez et al., 2003, 2004), and the displacement loop region (D-loop) (Fei et al., 1996; Gao, Liang, Zhang, & Zhu, 2004; Montiel-Sosa et al., 2000) are the most widely used markers in the development of DNA methods for species identification.

Besides the selection of adequate genetic markers, a crucial feature of any PCR-based method is the design of the oligonucleotides. The use of specifically designed primers under restrictive conditions of PCR amplification have made possible the direct and specific identification of amplified mtDNA fragments, avoiding subsequent sequencing or restriction fragment length polymorphism (RFLP) identification (Kusama, Nomuta, & Kadowaki, 2004; Rodríguez et al., 2004).

The demand for game meat is growing because consumers are increasingly becoming concerned about healthy and safe products. Game meat is low in fat and cholesterol, it has high levels of polyunsaturated fatty acids, the animals are not injured with hormones or steroids, and the meat has a fine texture and delicate flavour when prepared properly (Hoffman & Wiklund, 2006). Among ruminant bovids, the subfamily *Caprinae* includes the closely related genera *Rupicapra*, *Capra* and *Ovis* (Groves & Shields, 1996). The three European game meat species comprising these genera are *Rupicapra rupicapra* (chamois), *Capra pyrenaica* (pyrenean ibex), and *Ovis ammon* (mouflon) (Wolf et al., 1999). Considering all the above-mentioned aspects, we describe in the present study a PCR method using specific primers for the identification of meats from these three *caprinae* species. The assay is based on the selective amplification of mt D-loop sequences from chamois, pyrenean ibex, and mouflon.

2. Materials and methods

2.1. Selection and preparation of meat samples

Authentic muscle samples of chamois (*Rupicapra rupicapra*), mouflon (*Ovis ammon*), pyrenean ibex (*Capra pyrenaica*), red deer (*Cervus elaphus*), fallow deer (*Dama dama*), and roe deer (*Capreolus capreolus*), were obtained from the Department of Animal Pathology (Facultad de Veterinaria, Universidad Autónoma de Barcelona, Spain) and from several meat-cutting installations of the Comunidad de Madrid (Parque Natural "El Pardo"), Andalucía (Parque Natural Sierra de Cazorla, Segura y Las Villas), Castilla-La Mancha (Ciudad Real and Toledo), and Extremadura (Badajoz).

Cattle (*Bos taurus*), sheep (*Ovis aries*), goat (*Capra hircus*), and swine (*Sus scrofa domestica*) muscle samples were obtained from several local abattoirs and retail markets (Madrid, Spain). Each species was morphologically identified before the samples were obtained.

Fresh muscle portions from all selected specimens were processed immediately or stored frozen at −20 °C until use. Chamois, pyrenean ibex, and mouflon meats were also analyzed after being subjected to experimental pasteurization (72 °C for 30 min) and sterilization (121 °C for 20 min) treatments.

Binary mixtures of chamois, pyrenean ibex or mouflon meat with swine meat were prepared using raw muscles from the selected species. For each meat mixture, five different percentages containing 0.1%, 1%, 5%, 10%, and 25% (wt/wt) of the target species were prepared with a blender (Sunbeam Oster, FL) to a final weight of 50 g. The 100% from chamois, pyrenean ibex, mouflon, and swine meats was also used as control in the analysis of the binary mixtures. Fifteen grams of each mixture was pasteurized at 72 °C for 30 min, and another 15 g was sterilized at 121 °C for 20 min.

2.2. DNA extraction

Genomic DNA was obtained from meat using a Wizard® DNA Clean-up System kit (Promega Corp., Madison, WI) as described in a previous work (Fajardo et al., 2006).

The DNA extracted from raw and processed tissues was electrophoresed in a 1% D1 (Hispanlab S.A., Torrejón, Spain) agarose gel containing 1 μg/ml ethidium bromide in Tris–acetate buffer (0.04 M Tris–acetate and 0.001 M EDTA, pH 8.0) for 30 min at 110 V. Genomic DNA was visualized by UV transillumination using a Geldoc 1000 UV Fluorescent Gel Documentation System-PC (Bio-Rad Laboratories, Hercules, CA).

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