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Authentication of meat from game and domestic species by SNaPshot minisequencing analysis

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Abstract

The aim of the present study is to develop an assay for the specific identification of meat from *Capreolus capreolus*, *Cervus elaphus*, *Capra ibex*, *Rupicapra rupicapra*, targeting sequences of the cytochrome b (cyt b) gene of mitochondrial DNA. The assay is also intended to enable differentiation between meat from these wild species as well as *Ovis aries*, *Capra hircus*, *Bubalus bubalis*, *Bos taurus* and *Sus scrofa* domestic species.

The primers used in the preliminary PCR were designed in well conserved regions upstream and downstream of the diagnosis sites. They successfully amplified a conserved 232 bp region from the cyt b gene of all the species taken into consideration. The sites of diagnosis have been interrogated using a minisequencing reaction and capillary electrophoresis. All the results of the multiplex PER (primer extension reaction) test were confirmed by fragment sequencing. The assay offers the possibility of discriminating nine species at the same time.
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1. Introduction

In the last few years, the consumption of game meat has gained increasing favour among consumers, who appreciate its texture and flavour as well as the low fat and cholesterol content and its lack of anabolic steroids or other drugs. The high commercial value of game meat has sometimes induced fraud, such as mislabelling or selling less valuable meat as meat from more appreciated species (Fajardo et al., 2006). Therefore, it is important to establish the animal species in order to detect possible falsifications.

Nowadays a number of different assays have been developed in this field. Those based on protein analysis such as electrophoresis and immunoenzymatic tests (Hsieh, Sheu, & Brifgman, 1998) have been abandoned because of their low specificity (Berger, Mageau, Schwab, & Johnston, 1988). Game species are difficult to discriminate, as they are closely related to each other and are phylogenetically similar to some domestic species. Hence, the assays based on DNA analysis are preferable for differentiation and identification. In particular, due to the high mutation rate of mitochondrial DNA (mtDNA), 10 times greater than nuclear DNA, point mutations accumulate very quickly allowing the discrimination of closely related species (Jorde, Bamshad, & Rogers, 1998). Cytochrome b (cyt b) contains species-specific information and it has been widely used in a considerable number of studies on phylogenesis and in studies dealing with forensic science and food inspection. In these fields, the application of polymerase chain reaction (PCR) seems to give the most satisfactory results (Teletchea, Maudet, & Hänni, 2005). However, species-specific primers cannot be designed when species are very closely related. In these cases it is advisable to apply

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techniques such as PCR-RFLP (restriction fragment length polymorphism) which is based on the analysis of the single species-specific mutations (sites of diagnosis). This method allows the amplification of DNA by PCR and the detection of genetic variation between species by digestion of the amplified fragment with restriction enzymes (Kelly, Carter, & Cole, 2003).

PCR-RFLP analysis of a conserved fragment from the mitochondrial 12S rRNA has been applied to identify several closely related domestic animal and game species (Fajardo et al., 2006; Pfeiffer, Burger, & Brenig, 2004; Wolf, Rentsch, & Hubner, 1999).

However, the possible individual mutations make the selection of endonucleases in conserved restriction sites difficult, and several enzymes are often needed to discriminate between phylogenetically related species and when several species have to be differentiated simultaneously (Wolf et al., 1999).

In addition the use of many restriction enzymes and analysis of more than one diagnosis site make PCR-RFLP difficult to automate.

Other method such as RAPD (random amplified polymorphic DNA) was successfully associated with PCR-RFLP for game species differentiation. The main advantage of RAPD is that the technique usually generates products which can be seen as DNA fingerprints on gel electrophoresis. However, the patterns are not always reproducible due to factors such as cycling conditions or intra-species polymorphism (Koh, Lim, Chua, Chew, & Phang, 1998). So, other techniques have been suggested. Among these, the sequencing is particularly suitable when a specific attributions needed and it is not sufficient to confirm or exclude the presence of a species (Bartlett & Davidson, 1992).

Recently Bottero, Dalmasso, Cappelletti, Secchi, and Civera (2007) successfully applied a method based on minisequencing reaction to differentiate closely related species of tuna fish in canned products. This technique consists in the analysis of the diagnosis sites present in a fragment previously amplified and is based on the dideoxy (ddNTP) single base extension of an unlabelled oligonucleotide (sequencing primer) at the 3′ end of the base immediately adjacent to the diagnosis site. Each ddNTP is labelled with different fluorescent dyes and a fifth color is used to label the internal size marker. In particular the fluorescent dyes are assigned to the individual ddNTPs as follows: A(dR6G, green), C(dTAMRA, black), G(dR110, blue) and T(dROX, red). The extended SNaPshot primers used to interrogate different diagnosis site differ in color and size (the length of a primer being modified by the addition of poly-T tails at the 5′-end) (Quintàns et al., 2004).

Meat from venison is becoming increasingly popular in European markets. The main species consumed in Europe are roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*) and chamois (*Rupicapra rupicapra*).

Moreover the hunt of steinbock (*Capra ibex*) in some country is selective or prohibited.

In the present study we describe a rapid and reliable method for the specific identification of game meats from roe deer, red deer, steinbock and chamois based on minisequencing reaction and a capillary electrophoresis of a conserved fragment from the mtDNA cyt b gene. The assay is also intended to enable the differentiation between these wild ungulate meats and those from buffalo (*Bubalus bubalis*) sheep (*Ovis aries*), goat (*Capra hircus*), cattle (*Bos taurus*) and swine (*Sus scrofa*) domestic specie, which can be sold in place of more expensive venison meat.

2. Materials and methods

2.1. Samples and DNA extraction

Muscle samples of roe deer, red deer, steinbock and chamois, were obtained from the Department of Animal Production (Faculty of Veterinary Medicine, University of Torino, Italy) and from hunted killed animals in different Italian regions (Italian Wildlife Institute (INFS), Ozzano Emilia, Bologna, Italy). Goat, sheep, buffalo, cattle and swine meat samples were obtained from local abattoirs. Thirty specimens of each species were morphologically identified before sampling muscles for analysis.

The investigation was carried out on raw meat and on autoclave-treated meat (121 °C for 15 min.) of each species object of this study.

Genomic DNA was extracted from meat using Dneasy Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions with slight modification (400 mg of sample and 100 μl the final elution volume).

The DNA was quantified by spectrophotometry (Biophotometer 6131, Eppendorf AG, Hamburg, Germany).

In order to evaluate the sensitivity of the preliminary PCR, dilutions of roe deer DNA (25, 5, 2.5, 1, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, 0.005, 0.0025 ng) were prepared.

In addition, to evaluate the "dilution" effect on patterns reproducibility a series of dilutions of the purified PCR product has been prepared (0.4, 0.2, 0.1, 0.05, 0.01, 0.005, 0.001 pmol).

2.2. Primers design

The cyt b sequences downloaded from the GenBank database, corresponding to *Rupicapra rupicapra* (GenBank accession no. AF034725), *Capreolus capreolus* (GenBank accession no. AJ000024), *Cervus elaphus* (GenBank accession no. AJ000021), *Capra ibex* (GenBank accession no. AF034735), *Bubalus bubalis* (GenBanK accession no. AY488491), *Capra hircus* (GenBank accession no. X56289), *Ovis aries* (GenBank accession no. DQ097429), *Bos taurus* (GenBank accession no. AB074968) and *Sus scrofa* (GenBank accession no. NC_000845) were aligned with the Clustal X program (Higgins, Bleasby, & Fuchs, 1992) in order to detect polymorphic sites to be used as diagnosis position (Tables 1 and 2).

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