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Identification of hare meat by a species-specific marker of mitochondrial origin

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ABSTRACT

Meat species identification in food has gained increasing interest in recent years due to public health, economic and legal concerns. Following the consumer trend towards high quality products, game meat has earned much attention. The aim of the present work was to develop a DNA-based technique able to identify hare meat. Mitochondrial cytochrome b gene was used to design species-specific primers for hare detection. The new primers proved to be highly specific to *Lepus* species, allowing the detection of 0.01% of hare meat in pork meat by polymerase chain reaction (PCR). A real-time PCR assay with the new intercalating EvaGreen dye was further proposed as a specific and fast tool for hare identification with increased sensitivity (1 pg) compared to end-point PCR (10 pg). It can be concluded that the proposed new primers can be used by both species-specific end-point PCR or real-time PCR to accurately authenticate hare meat.

1. Introduction

Food authenticity assessment is a very important issue in that it avoids unfair competition among producers and allows consumers to have accurate information about the products they purchase. Nowadays, in the meat industry, the substitution of higher commercial valued meats by low-priced ones and the fraudulent labelling of meat species is becoming a concern (Fajardo et al., 2010). In particular, products from game animals are considered to be susceptible targets for frauds as they generally command higher prices compared to other meats. Additionally, game meat consumption has increased in recent years due to several motivations, such as its particular taste and flavour, its healthier composition (with lower fat and cholesterol contents), the absence of antibiotics and anabolic steroids, and the attraction for some people of eating new and exotic delicacies (Fajardo et al., 2010).

In Central Europe, brown hare (*Lepus europaeus*) is one of the most abundant small game species (Fettinger, Smulders, Lazar, Omurtag, & Paulsen, 2010). In the Iberian Peninsula, the Iberian hare (*Lepus granatensis*) and the brown hare are two species that have been identified (Alves, Ferrand, Suchentrunk, & Harris, 2003). Cape hare (*Lepus capensis*) among all *Lepus* species has the largest distribution, ranging from South Africa across large parts of the continent to East, West, and North Africa, further to the Middle East, Central Asia, and the Pacific seaboard. Several other *Lepus* species have been identified, but phylogenetic relationships and systematics of hares are not well understood (Ben Slimen et al., 2008). Molecular studies based on mitochondrial DNA reveal quite complex evolutionary scenarios, such as the introgressive hybridisation between brown hares and mountain hares (*L. timidus*) (Thulin, Fang, & Averianov, 2006) and between Iberian hares and mountain hares (Alves et al., 2003). Recent reports support the hypothesis of conspecificity between cape and brown hares suggesting that differentiation patterns were not higher than within the taxa, being attributed to geographic distances rather than the occurrence of two species-specific gene pools (Ben Slimen et al., 2006, 2008).

The term "game" for culinary purposes is used to describe all birds and animals that are hunted for food (Cobos, Hoz, Cambero, & Ordofiez, 1995). Hare, as a game meat, has been used in many recipes, such as the typical Portuguese dishes "arroz de lebre" and "cabidela de lebre" having rice and hare as major ingredients. More recently, hare meat is used in some delicacies such as pâtés. Traditionally, hunted hares are bought whole, and are mainly sold entire, with hair, so the consumer can confirm its genuineness. Following the European Union food hygiene legislation, countries are allowed to issue national regulations for this particular sector of local supply of small quantities of food (European Commission, 2004). Consequently, in some European countries, hunters, under some provisions, able to put on the market not only eviscerated game carcasses, but also

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portioned and packed meat (Fettinger et al., 2010). Considering the above reasons and the accessibility of hares, restricted to hunting season and animal, hare meat is prone to be fraudulently substituted. Therefore, to be able to authenticate hare meat, analytical methodologies are required to specifically and unequivocally identify it.

Recently, DNA-based methods have been considered as essential tools for species identification in animal foods and feedstuffs. In particular, the polymerase chain reaction (PCR) technique using species-specific primers is extensively used because of its potential for simple, fast, specific and sensitive analysis, enabling the identification of species even in complex and processed foods (Bottero & Dalmasso, 2010; Mafra, Ferreira, & Oliveira, 2008). Most reported PCR applications for meat species identification have focused on domestic species like cattle, sheep, goat, domestic pig, turkey or chicken (Ballin, Vogensen, & Karlsson, 2009; Colgan et al., 2001; Girish et al., 2005; Mafra, Ferreira, Faria, & Oliveira, 2004; Mafra, Roxo, Ferreira, & Oliveira, 2007; Matsunaga et al., 1999; Soares, Amaral, Mafra, & Oliveira, 2010). In contrast, PCR assays dealing with game meat identification are less, but with an increasing trend in the past few years (Fajardo et al., 2010).

New developments of quantitative analysis based on real-time PCR offer the potential for rapid results, high throughput, high sensitivity and opportunities for automation (López-Calleja et al., 2007a, b). In real-time PCR, the amplification of products is directly monitored along each amplification cycle, allowing the measurement of the PCR process at an early stage of the exponential phase and, therefore, providing a quantitative result. Data collection is achieved using fluorescent molecules able to provide a strong correlation and to measure minute amounts of different animal species (Fajardo et al., 2008; Mafra, Ferreira, & Oliveira, 2008). Real-time PCR is based on the use of fluorescent compounds, which can be either a general dye, such as SYBR Green, that intercalates in DNA molecules, or different probe-based systems, which confer an added specificity to the reaction. In both cases, fluorescence is proportional to the amount of DNA present (Dooley, Paine, Garrett, & Brown, 2004; Fajardo et al., 2008; Mafra, Ferreira, & Oliveira, 2008). The use of DNA binding dyes offers a suitable and less expensive alternative to real-time PCR since it is a more flexible method without requiring an individual probe design and optimisation steps (Fajardo et al., 2008). The use of SYBR Green real-time PCR has been successfully applied to the identification and quantification of game meats, namely red deer, fallow deer and roe deer, in meat mixtures (Fajardo et al., 2008). More recently, EvaGreen has been reported as a novel DNA intercalating dye, more stable and sensitive than SYBR Green (Wang, Chen, & Xu, 2006). The enhanced fluorescent EvaGreen dye can be used at higher concentrations than SYBR Green I, generating greater fluorescent signals, increased sensitivity and excellent stability without causing PCR inhibition. The application of real-time PCR with EvaGreen fluorescent dye has been reported for the identification of cervidae species (Chen et al., 2009).

The aim of the present work was to develop a highly specific and sensitive PCR technique to identify and authenticate hare meat. For this purpose, hare specific primers were designed targeting the cytochrome b (cyt*b*) mitochondrial gene to amplify a 127 bp fragment. The new primers were used to develop a simple species-specific PCR approach to identify hare meat. By the use of real-time PCR with the new generation dye EvaGreen, a highly sensitive technique for hare detection was proposed.

2. Materials and methods

2.1. Samples

Samples of authentic hare meat were acquired from local hunters and retail market. Each sample consisted on an entire animal, not eviscerated, with hair, to confirm its authenticity. For each animal, fresh muscle portions were selected and minced in a blender. Reference binary mixtures containing known proportions of hare meat in pork meat (0.01%, 0.1%, 0.5%, 1%, 2.5%, 5%, 10% and 20% (w/w)) were prepared by homogenising both minced meats with a blender to a final weight of 100 g. To avoid contamination, each mixture was processed separately using different material and different blender containers previously treated with DNA decontaminator solution.

A wide range of non-target animal species was also included in the study for specificity assays, namely rabbit (*Oryctolagus cuniculus*), partridge (*Alectoris* spp.), red legged partridge (*Alectoris rufa*), pheasant (*Phasianus colchinus*), quail (*Coturnix coturnix*), turkey (*Meleagris gallopavo*), chicken (*Gallus gallus*), duck (*Anas platyrhynchos*), ostrich (*Struthio camelus*), beef (*Bos taurus*), sheep (*Ovis aries*), goat (*Capra hircus*), pig (*Sus scrofa domestica*), wild boar (*Sus scrofa scrofa*) and red deer (*Cervus elaphus*).

All samples were extracted immediately or stored at −20 °C to prevent enzymatic DNA degradation.

2.2. DNA extraction

DNA was extracted using the Wizard method as described by Mafra, Silva, Moreira, Ferreira da Silva, and Oliveira (2008) with minor modifications. Briefly, 100 mg of ground and homogenised samples were transferred to a 2 mL sterile reaction tube followed by the addition of 860 μL of TNE extraction buffer (10 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% SDS), 100 μL of 5 M guanidine hydrochloride solution and 40 μL proteinase K solution (20 mg/mL). After the incubation at 60 °C for 3 h, with occasional stirring, the suspension was centrifuged (15 min, 18,514 *g*) and 500 μL of the supernatant was mixed with 1 mL of Wizard DNA purification resin (Promega, Madison, WI, USA). The mixture was eluted through a column and the resin washed with 2 mL of isopropanol solution (80%, v/v). After drying the column, the DNA was eluted by centrifugation (1 min 10,000 *g*) with 100 μL of Tris–EDTA buffer (10 mM Tris, 1 mM EDTA) at 70 °C to a new reaction tube. The extractions were performed in duplicate assays for each binary mixture.

The quality of extracted DNA was analysed by electrophoresis in a 1.0% agarose gel containing Gel Red 1x (Biotium, Hayward, CA, USA) for staining and carried out in TAE buffer (40 mM Tris–acetate, 1 mM EDTA) for 40 min at 120 V. The agarose gel was visualised under UV light and a digital image was obtained using a Kodak Digital ScienceTM DC120 (Rochester, NY, USA).

2.3. DNA quantification and purity

The DNA was quantified by spectrophotometry using a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) as described by Somma (2006). The DNA concentration was determined by UV absorbance at 260 nm (1 absorbance unit corresponds to 50 μg/mL of dsDNA). The purity of the extracted DNA was determined by the ratio of the absorbances at 260 and 280 nm.

2.4. Target gene selection and oligonucleotide primers

Oligonucleotide primers to identify hare meat were designed on the basis of the mitochondrial cyt*b* gene sequence from various animal and plant species available in the NCBI (National Center for Biotechnology Information) Genbank database. The specific primers were designed using the software Primer3 Output designing tool (http://frodo.wi.mit.edu/primer3/). The nucleotide sequence was submitted to the basic local alignment search tool BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), which identifies regions of local similarity among homologue sequences of different species and calculates the statistical significance of the matches (Altschul, Gish, Miller, Myers, & Lipman, 1990).