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Review

A review of current PCR-based methodologies for the authentication of meats from game animal species

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The authenticity of food is currently a major issue for researchers, consumers, industries and policy markers at all levels of the production process. Particularly in the meat industry, products from game animals are susceptible targets for fraudulent labeling due to the economic profit that results from selling cheaper meat as meat from more profitable and desirable species. A part from meat species adulteration, illegal poaching of endangered game species may take place contributing to threat of wildlife populations. These reasons have encouraged the development of methods to ensure fair trade and labeling of game meats from production level to consumer use of end products. In the last years, full attention has been turning towards implementation of molecular genetic approaches for meat species identification because of their high sensitivity and specificity, as well as rapid processing time and low cost. This work presents an overview of the main PCR-based techniques applied to date to verify the authenticity of meat and meat products from game species.

Introduction

Over the last decades, meat industry has enforced strong measures towards establishment of effective traceability systems to preserve food safety and quality from farm to fork (Shackell, 2008). As a response to consumer demands for healthy food with distinctive qualities, high added-value meats from different game animals are becoming increasingly popular worldwide. The main motivations towards consumption of game meat products are: i) the particular texture and flavour of the meat, ii) the low fat and cholesterol content, iii) the lack of anabolic steroids or other drugs, and iv) the attraction of some people for the experience of eating new and exotic delicacies (Hoffman & Wiklund, 2006; La Neve, Civera, Mucci, & Bottero, 2008). However, as a consequence of the tremendous profit that results from selling cheaper meat as meat from more profitable and desirable species, fraudulent misdescription of game meat products is becoming a common practice among unscrupulous processors who apply deceptive practices on the products they are selling (Brodmann, Nicholas, Schaltenbrand, & Ilg, 2001).

Besides meat falsification, banned trade of certain endangered populations may also exist, threatening a multitude of species including primates, carnivores, ungulates and wild fowl. According to the latest annual survey compiled by the International Union for the Conservation of Nature (IUCN), which is the most complete global inventory of threatened species available, nearly 17,300 of the world's 47,677 assessed species are under threat of extinction. These comprise a fifth of the world's known mammals, a third of its amphibians, more than a quarter of its reptiles and 1223 species of birds (IUCN, 2009). Levels of exploitation of these species are high and the trade on them, together with other factors such as habitat loss, definitely contributes to severe depletion of biodiversity (Colombo, Cardia, Renon, & Cantón, 2004; Malisa, Gwakisa, Balthazary, Wasser, & Mutayoba, 2006).

Illegal wildlife commerce involves the illicit procurement, transport, and distribution of the live animals, but also of a vast array of products derived from them including

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food products, exotic leather or ivory goods, among others. The primary motivation to engage in such outlawed practices appears to be an economic gain. Driven by a demand for wildlife products that exceeds what the market can legally supply, the value of illegal wildlife products continues to increase as consumers are willing to pay greater amounts for the goods. However, due to its clandestine nature, illegal trade is difficult to quantify with any accuracy. Some analysts estimate that it is worth at least $5 billion, and may potentially exceed $20 billion annually, which would rank this trade as among the most lucrative illicit economies in the world, behind illegal drugs and possibly human trafficking and arms trafficking (Wyler & Sheikh, 2009). It is important to mention, however, that in many developing countries the income derived from illegal wildlife poaching and trading is often vital for sustaining the livelihoods, and even traditions and culture of impoverished peoples. This consideration may differentiate poor hunters and traders from international criminal syndicates who engage in the trade purely for profit. Adopted in 1973, the Convention on International Trade in Endangered Species of Fauna and Flora (CITES) is a conservation-minded agreement between 175 nations aiming to regulate, if not prohibit, the wildlife commerce (Abensperg-Traun, 2009). Although many wildlife species in trade are not endangered, the existence of an agreement to ensure the sustainability of the trade is important in order to safeguard these resources in the future.

Game meat authenticity not only relates to the industrial economic profit resulting from illegal trading, handling or substitution of species, but also to public health risks such as zoonoses or even allergies to a particular meat protein. In this context, although wild game meats may originate from farms having regulated hygienic standards and fair commercial practices (Hoffman & Wiklund, 2006), many industries worldwide export big amounts of wild game meats lacking of safety and traceability controls throughout the food and feed processing chain. To safeguard consumers, it is therefore essential to know the species origin of a product so that risks related to meat allergies, or zoonoses like tuberculosis, bovine spongiform encephalopathy (BSE), brucellosis, etc., affecting game mammals and birds may be prevented (Casoli, Duranti, Cambiotti, & Avellini, 2005; Stephenson, 2002). It should be noted that endangered gorillas, chimpanzees and other primates are also hunted for meat in many parts of the world, increasing the risk of infectious diseases "jumping" between apes and humans. Furthermore, researchers believe that the hunting and consumption of ape meat is the origin of human acquired immunodeficiency syndrome (HIV/AIDS), supporting the hypothesis that this fatal disease entered our species when Africans ate chimpanzee meat infected with Simian Immunodeficiency Virus (Chomel, Belotto, & Meslin, 2007).

Considering all the above mentioned aspects, enforcement of legislation on meat safety, traceability and authenticity is needed to achieve an active control on the commerce of game animals and their products. To fulfil this demand, the adoption of precise and efficient methodologies to assess meat sources and verify the authenticity of game meat products is of prime importance for the meat sector (Ballin, Vogensen, & Karlsson, 2009).

The analytical methods currently used for meat species authentication rely mainly on protein and DNA analysis. Protein-based techniques include electrophoretic (Montowska & Pospiech, 2007), chromatographic (Chou *et al*., 2007) and spectroscopic (Ellis, Broadhurst, Clarke, & Goodacre, 2005) approaches that have shown useful to identify the species origin of meats. However, they are limited when assaying heat-treated material due to denaturation of soluble proteins during food processing. Besides, analysis by immunoassays, which rely on the use of antibodies raised against a specific protein, are often hindered by cross-reactions occurring among closely related species (Ayaz, Ayaz, & Erol, 2006). The use of nucleic-acid-based analytical methods can overcome these difficulties because DNA is a very stable and long-live biological molecule present in all tissues of all organisms. Among DNA-based methods, polymerase chain reaction (PCR) is the most well developed molecular technique up to now and provides a simple, rapid, highly sensitive and specific tool for detecting constituents of animal origin in foods (Mafra, Ferreira, & Oliveira, 2008; Tobe & Linacre, 2008).

PCR amplification is based on the hybridisation of specific oligonucleotides to a target DNA and synthesis, *in vitro*, of millions of DNA copies flanked by these primers. The amplification of DNA fragments, followed by agarose gel electrophoresis for fragment size verification, is the simplest PCR strategy applied to evaluate the presence of a species in a meat product. Additional confirmation methods and/or examination of PCR products can be accomplished by: i) sequencing of DNA amplicons (PCR-sequencing) (Karlsson & Holmlund, 2007), ii) analysis of PCR-single strand conformation polymorphism (PCR-SSCP) (Ripoli, Corva, & Giovambattista, 2006), iii) simultaneous amplification of two or more fragments with different primer pairs (multiplex PCR) (Tobe & Linacre, 2008), iv) analysis of PCR-restriction fragment length polymorphism (PCR-RFLP) (Park, Shin, Shin, Chung, & Chung, 2007), v) analysis of random amplified polymorphic DNA (PCR-RAPD) (Arslan, Ilhak, Calicioglu, & Karahan, 2005) or vi) real-time fluorescence PCR assays (Jonker, Tilburg, Hägele, & De Boer, 2008). Other strategies focused on detection of genetic variability within closely related populations like short sequence repeat markers (microsatellites) or DNA chips (microarrays) can also be applied for meat speciation purposes (Felmer *et al*., 2008; Teletchea, Bernillon, Duffraisse, Laudet, & Hänni, 2008).

A primary aspect for successfully detecting a species by PCR is to choose adequate genetic markers to develop the assay. Both nuclear and mitochondrial genes have been broadly targeted for the identification of game and domestic

meat species (Fajardo *et al*., 2008a). The use of mitochondrial DNA (mtDNA) sequences offers a series of advantages over other genetic markers like 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. In addition, mitochondrial genes evolve much faster than nuclear ones and, thus, contain more sequence diversity facilitating the identification of phylogenetically related species (Girish *et al*., 2005). Among mitochondrial genes, the cytochrome b (Maede, 2006; Pfeiffer, Burger, & Brenig, 2004), the 12S and 16S ribosomal RNA subunits (Girish *et al*., 2007; Karlsson & Holmlund, 2007), and the displacement loop region (D-loop) (Krkoska, Nebola, Steinhauserová, Obroská, & Ernst, 2003; Montiel-Sosa *et al*., 2000) are the most commonly used markers in the development of DNA methods for meat authentication. Besides mitochondrial genes, nuclear markers are also described for meat species discrimination exploiting the existence of introns of different sizes which allow the amplification of species-specific DNA fragments. Some examples are the growth hormone gene (Brodmann & Moor, 2003), the actin gene (Hopwood, Fairbrother, Lockley, & Bardsley, 1999) or the melanocortin receptor 1 (*MC1R*) gene (Fajardo *et al*., 2008a).

The vast majority of PCR applications published to date for meat identification are focused on domestic animal species like cattle, sheep, goat, domestic pig, turkey or chicken (Girish *et al*., 2005; Stirtzel, Andree, Seuss-Baum, & Schwagele, 2007). In contrast, considerable lesser PCR-based approaches have been reported so far dealing with game meat authentication. The increasing importance and high commercial value of game and exotic meats in many parts of the world, together with the frequent fraudulent practices occurring at this level, is driving the development of appropriate tools for the authentication of a growing number of game species (La Neve *et al*., 2008; Brodmann *et al*., 2001). Within this context, this work presents an overview of the main PCR-based methodologies published so far in the literature about game meat identification. For the purpose of this review, the generic term *game meat* is adopted to refer to meats from a wide group of wild or farmed game and exotic animals. It should be also noted that although some of the techniques described here have not yet found a widespread application for verifying game meat authenticity, it is most likely that their use will be extended in the future.

PCR-sequencing

Sequencing of amplified fragments results in the highest amount of information without the need of using enzymes or post-analysis. By means of a universal primer pair, single-band amplification products from a wide range of animals can be obtained (Kocher *et al*., 1989). Further sequence analysis of the generated PCR amplicons can be used for interspecific and intraspecific identification of animal DNA in food products, allowing discrimination of even very closely related species. Due to their adequate level of mutation and great availability of sequences in the databases, mitochondrial cytochrome b, 12S and 16S rRNA genes are the most extended genetic markers for species discrimination by PCR-sequencing (Karlsson & Holmlund, 2007).

Among works focused on game meats, Chikuni, Tabata, Saito, and Monma (1994) developed a PCR-sequencing technique to identify different mammals including red deer species, as well as some birds like quail, song thrush and sparrow. A 646 base pair (bp) fragment of the mitochondrial cytochrome b gene was used to carry out the identification.

Brodmann *et al*. (2001) identified meats from red deer, fallow deer, roe deer and chamois by sequencing the PCR products achieved from a conserved 428 bp region of the mitochondrial cytochrome b gene. However, the method failed to differentiate between meats from wild boar and domestic pig due to the high homology of their mitochondrial sequences.

Wong, Wang, But, and Shaw (2004) used a 355 bp cytochrome b sequence for the authentication of snake meats to enforce wildlife conservation programs and restrain the illegal trading and consumption of endangered species.

Colombo *et al*. (2004) sequenced a 282 bp amplicon from the mitochondrial cytochrome b gene to identify meat samples suspected of containing chamois.

Li, Bai, Xu, Zhang, and Ma (2006) carried out the differentiation of cervid species by sequence analysis of 405 bp and 387 bp amplicons generated from the mitochondrial cytochrome b and 12S rRNA genes, respectively.

Kitano, Umetsu, Tian, and Osawa (2007) applied a PCR-sequencing method for the identification of a high number of vertebrates (mammals, birds, reptiles, amphibians and fish). Similarly, Karlsson and Holmlund (2007) identified a total of 28 different mammals including cervid species and wild boar. Both studies are based on conserved regions using primers designed to amplify small fragments (from 100 to 244 bp) on the mitochondrial 12S and 16S rRNA genes.

La Neve *et al*. (2008) developed an assay for the specific identification of meats from red deer, roe deer, pyrenean ibex and chamois by PCR-sequencing and capillary electrophoresis techniques targeting a 232 bp amplicon of the mitochondrial cytochrome b gene. The approach was also intended to enable the differentiation between these game meats and those from cattle, sheep and goat domestic species.

Girish *et al*. (2009) achieved the identification of quail, guinea fowl, ostrich and emu meats targeting a 456 bp fragment from the mitochondrial 12S rRNA gene to detect misrepresentation of poultry meat.

Other authors also use DNA sequence analysis for identifying the source of meats thought to be derived from

threatened species such as marine mammals (Palumbi & Cipriano, 1998).

Apart from meats, other profitable game by-products like elephant ivory (Lee *et al*., 2009) or horns from rhinoceros species (Hsieh *et al*., 2003) have been also identified by PCR-sequencing of the mitochondrial cytochrome b gene.

Although lesser works are available targeting nuclear markers, genes like 18S rRNA or the diglyceride acyltransferase 1 (*DGAT1*) have been sequenced for the discrimination of meats from species like kangaroo, crocodile or buffalo (Matsunaga, Shibata, Yamada, Shinmura, & Chikuni, 1998; Venkatachalapathy, Sharma, Sukla, & Hattacharya, 2008).

As a result of the big advance in nucleotide sequencing technology, PCR-sequencing analysis is progressing towards more robust, affordable and integrated tools for species identification (Kitano *et al*., 2007). Recently, the "DNA barcoding" technology developed by Hebert, Ratnasingham, and Dewaard (2003) has gained considerable support as a rapid, cost-effective and broadly applicable method for food authentication. DNA barcoding targets a small standardized fragment of 650 bp on the mitochondrial cytochrome oxidase I (COI) gene that is PCR amplified and sequenced to produce reference sequences or "DNA barcodes", which act as molecular identification tags for each species profiled. A number of studies carried out in a variety of taxa suggest that more than 95% of species in test assemblages of varied animal groups possess distinctive COI sequences (Dasmahapatra & Mallet, 2006; Ferri, Alu, Corradini, Licata, & Beduschi, 2009; Waugh, 2007). DNA barcoding employs standardized computational methodologies to supply a publicly accessible database for species identification, one that is explicitly derived from expert-authenticated reference DNA sequence data. The number of newly identified species is growing rapidly and, over the next 20 years, a barcode library for all eukaryotic life is expected. Nowadays, the Barcode of Life Data System (BOLD) provides an integrated bioinformatics platform that supports all phases of the analytical pathway, from specimen collection to tightly validated barcode library.

The introduction of DNA barcoding seems to be promising in different fields such as food authentication and biosecurity, forensic analysis, and wildlife enforcement to prevent poaching or illegal trade of threatened species (Ferri *et al*., 2009). Regarding food speciation, most of the studies reporting the use of this technique are focused on fishery products: Wong and Hanner (2008) identified the species origin of seafood products purchased from commercial markets and restaurants in North America. They achieved >97% sequence similarity for 90 of 91 samples tested when compared to DNA barcode database of reference specimens. Twenty-five percent of the samples were identified as mislabeled by this technique.

Holmes, Steinke, and Ward (2009) identified correctly shark and ray species by DNA barcode analysis. Many of the 27 classified species are listed on the World Conservation Union (IUCN) Red List and include one rated as critically endangered. Besides, Barbuto *et al*. (2010) employed the DNA barcoding approach to detect cases of species substitutions in shark slices sold in Italy. Results showed a high number of commercial frauds when comparing the analysed specimens with reference sequences from different databases.

In spite of its benefits, application of DNA barcoding for species identification may present constraints on samples in which thermal action or other processing effects degrade the DNA present in the tissues, since the amplification of the particularly large COI sequence can be prevented. Direct sequencing is also restricted in the analysis of mixed-species meats (sausages, pâtés, minced meat products, etc.) because the heterogeneous amalgam of sequences from the different species hinder result interpretation.

Future advances in DNA sequencing and computational technologies further promise the development of portable devices that will both gather barcode sequences in minutes and use an on-board barcode reference library to generate identifications. Such expedited access to biological identifications promises important benefits in a range of areas, from consumer protection and food safety, to disease prevention and better environmental monitoring (Waugh, 2007).

PCR-RAPD

The random amplified polymorphic DNA (RAPD) technique consists on the amplification of DNA fragments using a short arbitrary primer that ties multiple locations on the genomic DNA, followed by separation of amplified fragments based on their sizes using gel electrophoresis. Samples are identified by comparing the DNA bands of the fingerprints, which are expected to be consistent for the same primer, DNA and experimental conditions used (El-Jaafari, Panandam, Idris, & Siraj, 2008).

In the area of food analysis, PCR-RAPD method has been successfully used for species identification in meat, fish and vegetable foodstuffs (Arslan *et al*., 2005; Koveza, Kokaeva, Konovalov, & Gostimsky, 2005; Mohindra *et al*., 2007).

With respect to game meat authentication, the work of Comincini *et al*. (1996) describe the identification of phylogenetic relationships among different cervid species through the DNA profiles obtained by RAPD using eight primers with sizes ranging from 19 to 26 bp.

Chai, Huat, Thai, and Phang (1997) applied a RAPD method to generate discriminatory fingerprint patterns for ten bird species: pheasant, partridge, quail, guinea fowl, pigeon, emu, ostrich, chicken, local duck and mallard duck. Similarly, Koh, Lim, Chua, Chew, and Phang (1998) identified meats from wild boar, red deer, kangaroo and buffalo by RAPD technology.

Martínez and Yman (1998) carried out a RAPD study including game meats from elk, kangaroo, reindeer, buffalo and ostrich, as well as some domestic meat species. Species-specific profiles where obtained in fresh, frozen and canned samples.

Martínez and Danielsdottir (2000) correctly identified different seal and whale meat products (frozen, smoked, salted, dried, etc.) by RAPD and PCR-SSCP techniques using consensus primers designed on the mitochondrial cytochrome gene.

Yau, Wong, Shaw, But, and Wang (2002) authenticated by RAPD-PCR three snake species to avoid illegal trade of threatened populations.

Huang, Horng, Huang, Sin, and Chen (2003) performed the authentication of meats samples from ostrich, quail, dove, emu and pheasant using RAPD-PCR fingerprinting.

Arslan *et al*. (2005) differentiated by PCR-RAPD meats from wild boar, bear, camel and domestic species using a unique 10 bp oligonucleotide.

Wu, Liu, and Jiang (2006), and El-Jaafari *et al*. (2008) used this technique to identify different species belonging to the family *Cervidae* (sika deer, sambar deer, rusa deer, tufted deer, black muntjac and Reeve's muntjac).

Rastogi *et al*. (2007) applied successfully the RAPD-PCR technology to identify snake and buffalo, among other species, targeting the mitochondrial 16S rDNA and NADH dehydrogenase subunit 4 (ND4) genes and the nuclear actin gene.

Arbitrary primers have the advantage that no information on the gene fragments to be amplified is needed to generate species-specific patterns. Besides, PCR-RAPD is a fast and simple procedure, avoiding more complex analytical steps such as DNA restriction, sequencing or hybridisation (Wu *et al*., 2006). However, the main disadvantage of the method is the difficulty of obtaining reproducible results, since PCR amplifications have to be developed under strictly controlled and standardized conditions (temperature, number of cycles or reagents concentration). Also, because high quality starting DNA is particularly important to achieve reproducible RAPD profiles, the application of the technique is limited in highly processed meats with extensively degraded nucleic acids. Furthermore, due to the non-specific nature of the PCR reaction, RAPD technology is not appropriate for the identification of a target organism in admixed meats containing more than one species. Thus, PCR-RAPD for species identification is likely to be restricted to a rapid qualitative analysis, and known standards must be run together each time a sample is tested (Koh *et al*., 1998).

PCR-RFLP

PCR-restriction fragment length polymorphism (PCR-RFLP) has a special interest for meat species identification. The technique exploits the sequence variation that exists within defined DNA regions, allowing species differentiation of even closely related species by digestion of selected DNA fragments with appropriate restriction enzymes (Pascoal, Prado, Castro, Cepeda, & Barros-Velázquez, 2004).

PCR-RFLP has been thoroughly applied for species identification in meat and meat products (Girish *et al*., 2005; Maede, 2006), being one of the main genetic methods adopted by researchers aiming to identify game meat species. Table 1 summarizes the most relevant works published up to date about authentication of game meats by means of this technique. The table includes analysis of PCR-RFLP patterns from a wide array of game and domestic meat species using a single or a combination of different restriction endonucleases. The majority of the studies are based on mitochondrial markers like the cytochrome b and 12S rRNA genes due to their well suited features for meat speciation.

Conventional PCR-RFLP relies on the use of gel electrophoresis and staining for endpoint detection. These strategies are potentially hazardous and time-consuming and may produce variable results in certain instances. The miniaturization of biological and chemical analytical devices by micro-electro-mechanical-systems (MEMS) technology has posed a vital influence on the progress of PCR-based methodologies applied for species identification (Dooley, Sage, Clarke, Brown, & Garrett, 2005). In particular, microchip-based capillary electrophoresis technology represents a valuable recent advance for the analysis of complex restriction DNA banding patterns, in which the gel electrophoresis step is replaced by an automated LabChip electrophoretic system. The Agilent 2100 Bioanalyzer is the first commercially available device to utilize chip-based nucleic acid separation technology (Fig. 1a).

Among other applications, the PCR-RFLP lab-on-a-chip technology has emerged as a useful technique for the authentication of meat species like cattle, sheep, chicken, turkey or fish (Dooley & Garrett, 2001; Dooley *et al*., 2005). However, according to the reviewed literature, Fajardo, Gonzalez, Dooley, et al. (2009) is the only published study to date describing the identification of game meats by means of this technique (Fig. 1b). By adapting the novel LabChip support, these authors were able to integrate a high number of species in the assays, obtaining improved banding pattern resolution compared to conventional PCR-RFLP (Fajardo *et al*., 2006; Fajardo, González, López-Calleja, Martín, I. Rojas, *et al*., 2007). Miniaturization of analytical and biological instruments offers the following advantages over conventional techniques: better assay accuracy and precision, shorter time of analysis, higher versatility and reproducibility, minimal sample consumption and possibility of automation and storage of digital data (Dooley *et al*., 2005).

The PCR-RFLP technique presents the advantages of being simple, cheap and especially adaptable for routine large-scale studies such as those required in inspection programs (Pfeiffer *et al*., 2004). In contrast, the PCR-RFLP might not be applicable in the analysis of meats subjected

Table 1. Identification and differentiation of game meat species by PCR-restriction fragment length polymorphism (PCR-RFLP)

Species	Enzymes	Genetic marker (bp)	References
Red deer, roe deer, moose, antelope, chamois, mouflon, wild boar, kangaroo, buffalo, cattle, sheep, goat, domestic pig, horse, chicken and turkey	*Afl*III, *Alu*I, *Ase*I, *Cfo*I, *Dra*I, *Dra*III, *Eco*RI, *Hae*III, *Hind*I, *Hind*II, *Hin*fI, *Mbo*I, *Mbo*II, *Pst*I, *Rsa*I, *Sal*I, *Ssp*I, *Taq*I, *Tru9*I, *Xba*I	Cytochrome b (359 bp)	Meyer, Höfelen, Lüthy, and Candrian (1995)
Red deer, sika deer, cattle, sheep, goat and domestic pig	*BamH*I, *EcoR*I, *Sca*I	Cytochrome b (194 bp)	Matsunaga, Chikuni, *et al.*, 1998
Red deer, fallow deer, roe deer, bison and hare	*Alu*I, *Nco*I	Cytochrome b (981 bp)	Zimmermann, Zehner, and Mebs (1998)
Red deer, fallow deer, moose, antelope, gazelle, wildebeest, chamois, pyrenean ibex, kangaroo, buffalo, cattle, sheep, goat and hare	*Alu*I, *Ase*I, *Bam*HI, *Hae*III, *Hinc*II, *Hin*fI, *Mse*I, *Nla*III, *Rsa*I, *Ssp*I, *Taq*I	Cytochrome b (464 bp)	Wolf, Rentsch, and Hübner (1999)
Red deer, kangaroo, buffalo, horse, cattle, sheep, goat, domestic pig, emu, duck, chicken, turkey, rabbit, crocodile, barramundi, cat, dog, human, salmon, tuna, Nile perch and John dory	*Hae*III, *Hin*fI	Cytochrome b (359 bp)	Partis *et al.* (2000)
Wild boar and domestic pig	*Ava*II	D-loop region (531 bp)	Montiel-Sosa *et al.* (2000)
Ostrich	*Hae*III, *Hin*fI, *Rsa*I, *Tru9*I	Cytochrome b (359 bp)	Abdulmawjood and Buelte (2002)
Wild boar and domestic pig	*Tsp*509I	D-loop region (531 bp)	Krkoska *et al.* (2003)
Red deer, roe deer, wild boar, horse, cattle, goat, sheep, domestic pig, partridge, ostrich, duck, chicken, turkey and rabbit	*Alu*I, *Hin*fI, *Mbo*I, *Pal*I,	Cytochrome b (359 bp)	Pascoal *et al.* (2004)
Red deer, roe deer, cattle, sheep and goat	*Tsp*509I	Cytochrome b (195 bp)	Pfeiffer *et al.* (2004)
Buffalo, cattle, sheep and goat	*Alu*I, *Apo*I, *Bsp*TI, *Hha*I	12S rRNA (456 bp)	Girish *et al.* (2005)
Red deer, fallow deer, roe deer, cattle, sheep and goat	*Apo*I, *Bsl*I, *Mbo*II, *Mse*I	12S rRNA (720 bp)	Fajardo *et al.* (2006)
Cervids, bovines, porcines, equines and birds	*Alu*I, *Hae*III, *Hin*fI, *Mbo*I, *Pst*I, *Rsa*I, *Ta*I, *Xba*I	Cytochrome b (359–218bp)	Maede (2006)
Wildebeest, zebra, gazelle, impala, buffalo, reedbuck, kongoni, oryx, warthog and hippopotamus	*Rsa*I	D-loop region (664–246 bp)	Malisa *et al.* (2006)
Chamois, pyrenean ibex, mouflon, cattle, sheep and goat	*Apo*I, *Mse*I/*Mae*II	12S rRNA (720 bp) D-loop region (370 bp)	Fajardo *et al.* (2007a)
Guinea fowl, quail, chicken, duck and turkey	*Hin*fI, *Mph*1103I, *Mva*I, *Eco*47I	12S rRNA (456 bp)	Girish *et al.* (2007)
Peacock	*Alu*I, *Sau3A*I	12S rRNA (446 bp)	Saini *et al.* (2007)
Red deer, cattle, domestic pig, horse, chicken, duck and turkey	*Mbo*I, *Tsp*509I	12S rRNA (455 bp)	Park *et al.* (2007)
Wild boar and domestic pig	*BspH*I, *BstU*I	*MC1R* (795 bp)	Fajardo *et al.* (2008a)
Spotted deer, hog deer, barking deer, sika deer, musk deer and sambar deer	*Bsr*I, *Bst*SFI, *Dde*I, *Rsa*I,	12S rRNA (440 bp)	Gupta *et al.* (2008)
Red deer, sika deer, reindeer, elk and siberian maral deer	*Nla*IV, *Taq*I	Cytochrome b (466 bp) D-loop region (1175 bp)	Shin, Shin, Chung, and Chung (2008)
Quail, pheasant, red-legged partridge, chukar partridge, guinea fowl, capercaillie, Eurasian woodcock, woodpigeon, chicken, turkey muscovy duck and goose song thrus	*Alu*I, *Bfa*I/*Hin*fI, *Hpy188*III, *Mbo*II	12S rRNA (720 bp) D-loop region (310 bp)	Rojas *et al.* (2008; 2009a)
Red brocket deer, pygmy brocket deer and gray brocket deer	*Afl*III, *Bstn*I, *EcoR*II, *Ssp*I	Cytochrome b (224 bp)	González *et al.*, 2009
Indian crocodile species (mugger, saltwater and gharial)	*Hae*III, *Mbo*I, *Mwo*I	Cytochrome b (628 bp)	Meganathan, Dubey, and Haque (2009)
Buffalo, cattle, goat, domestic pig, quail, chicken and rabbit	*Alu*I, *Bso*fI, *BstU*I, *Mse*I, *Rsa*I	Cytochrome b (359 bp)	Murugaiah *et al.* (2009)

to DNA destructive processing, because amplification of the somewhat large DNA fragments that are commonly required for enzimatic restriction is impeded due to thermal DNA degradation. As happens with other techniques, PCR-RFLP analysis is neither the best choice for the analysis of admixed meats, since results may show

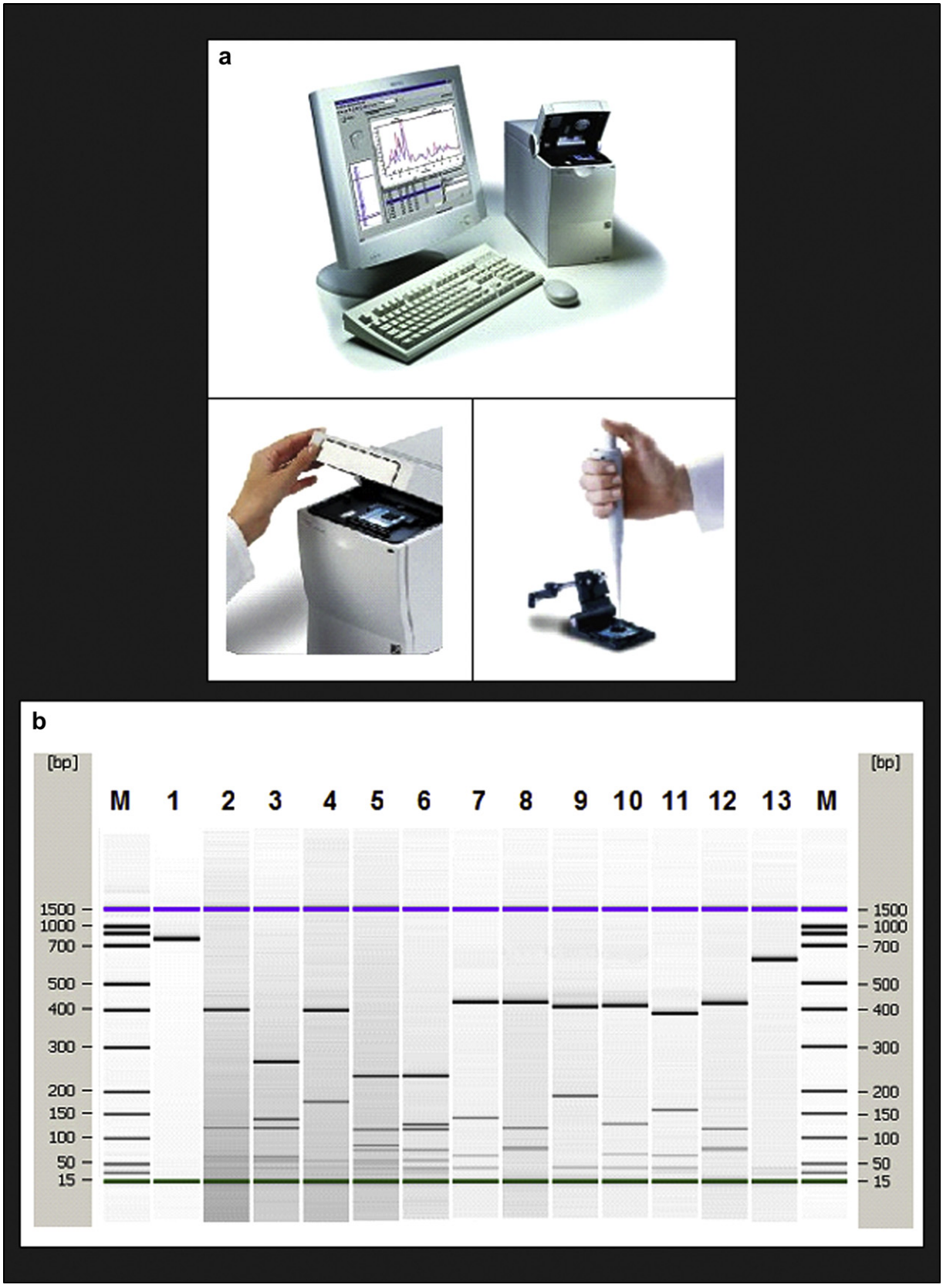

Fig. 1. PCR-RFLP lab-on-a-chip technology: (1a) Agilent 2100 Bioanalyzer lab-on-a-chip equipment. (1b) Computer-generated gel image using the 2100 Expert software including the 12S rRNA gene fingerprints generated by the *Mse*I restrictions. Meat samples are: undigested PCR product (1), red deer (2, 3), fallow deer (4), roe deer (5, 6), chamois (7), mouflon (8), pyrenean ibex (9), goat (10), cattle (11), sheep (12), and domestic pig (13). (M) Molecular weight marker 50-1000 bp Biomarker Low. Fajardo, Gonzalez, Dooley, *et al.* (2009).

a combination of miscellaneous restriction patterns representing all the possible species included in the sample (Girish *et al*., 2007). Accordingly, in the analysis of highly degraded or mixed-species food matrices, PCR using specific primers targeting short DNA fragments represents a suitable alternative for species identification (Stirtzel *et al*., 2007).

PCR using species-specific primers

The use of specifically designed oligonucleotides under restrictive PCR conditions has made possible the direct and specific identification of defined DNA fragments and its application to control food authenticity. By means of PCR with species-specific primers, a target sequence can be amplified very sensitively from a food matrix containing a pool of sequences, avoiding subsequent sequencing or restriction fragment length polymorphism (RFLP). PCR using species-specific primers has the preferences of being useful for routine analysis of large numbers of samples, even when aggressive processing treatments have been applied to the food (Rojas *et al*., 2009b; Mafra *et al*., 2008).

In contrast with the vast amount of PCR applications using specifically designed primers for domestic meat speciation (Haunshi *et al*., 2009; Nau *et al*., 2009), considerably fewer species-specific PCR-based studies have been reported so far referring to game meats. Table 2 compiles some published works about detection of game products by this methodology. For example, Fajardo *et al*. (2007a, 2007b) describe the use of specific oligonucleotides designed on the mitochondrial 12S rRNA and D-loop genes for the identification of various cervid and wild ruminant meats. Analysis of binary meat mixtures including each target species allowed the detection of 0.1% of all game species. The sensitivity of the PCR was not modified when raw, cured and heat-treated meat products were tested with the designed specific primer pairs.

Similarly, Rojas *et al*. (2009b), Rojas, González, Pavón, Pegels, Hernández, *et al*. (2010) developed PCR techniques with species-specific primers targeting the mitochondrial 12S rRNA and D-loop genes for the identification of various game bird species. The detection level of the PCR assays was set on 0.1% either on raw and sterilized muscular binary mixtures for each of the targeted species.

Ha, Jung, Nam, and Moon (2006) achieved the unequivocal identification of deer and other ruminant species in animal feedstuffs by using species-specific primers targeting the mitochondrial 12S and 16 rRNA genes. The detection limit of the assay was set on 0.05% for the four developed primer pairs.

PCR using species-specific primers directed to short DNA fragments offers simplicity, specificity and high sensitivity for meat authentication studies. On one hand, the specific identification of a target species in matrices containing a pool of heterogeneous genomic DNA sequences

Table 2. Identification and differentiation of game meat species by PCR using species-specific primers

Species	Genetic marker	Specific PCR product (s)	References
Ostrich and emu	Cytochrome b	543 and 229 bp, respectively (rp)	Colombo, Viacava, and Giaretti (2000)
Cervid species (Ceylon spotted deer, Ceylon hog deer, Ceylon sambhur and barking deer)	Cytochrome b	450 bp	Rajapaksha, Thilakaratne, Chandrasiri, and Niroshan (2002)
Buffalo	Cytochrome b	242 bp	Rajapaksha, Thilakaratne, Chandrasiri, and Niroshan (2003)
Tiger	Cytochrome b	408 bp	Wan and Fang (2003)
Camel	Cytochrome b	208 bp	Chen, Wu, Xu, Wan, and Qian (2005)
Chinese alligator	Cytochrome b	180 bp	Yan *et al*. (2005)
Deer, cattle, sheep, goat and ruminants	12S and 16S rRNA	104, 99, 108, 105, and 191 bp (rp)	Ha *et al*. (2006)
Red deer, roe deer and fallow deer	12S rRNA	175, 169 and 175 bp (rp)	Fajardo *et al*. (2007a)
Chamois, *Pyrenean ibex* and mouflon	D-loop region	178, 88 and 155 bp (rp)	Fajardo *et al*. (2007b)
Pheasant, quail, guinea fowl, chicken, turkey, duck and goose	Cytochrome b	164, 187, 192, 133, 71, 95 and 237 bp (rp)	Stirtzel *et al*. (2007)
Red deer, cattle, sheep, goat, domestic pig, horse, donkey, cat, dog, fox, guinea pig, hedgehog, badger, harvest mouse, house mouse, rat, rabbit and human	Cytochrome b	From 89 to 362 bp	Tobe and Linacre (2008)
Guinea fowl, chicken, duck, and turkey	Cytochrome b	186, 188, 189 and 186 bp (rp)	Nau *et al*. (2009)
Pigeon, chicken, duck, and domestic pig	Cytochrome b and D-loop region	401, 256, 292 and 835 bp (rp)	Haunshi *et al*. (2009)
Snake species (Indian rock, rat snake and Indian cobra)	16S rRNA	380, 265 and 165 bp (rp)	Dubey et al. (2009)
Cetacean species	12S rRNA	172 and 49 bp	Shinoda *et al*. (2009)
Quail, pheasant, partridge and guinea fowl	12S rRNA	129, 113, 141 and 130 bp (rp)	Rojas *et al*. (2009b)
Quail, pheasant, partridge, guinea fowl, pigeon, Eurasian woodcock and song thrush	D-loop	96, 100, 104, 106, 147, 127, and 154 bp (rp)	Rojas, González, Pavón, Pegels, Hernández, *et al*., (2010a)

is possible and, on the other, it is a well adapted technique for the analysis of thermally or otherwise processed products with highly damaged DNA. The main drawback, however, relies on the need of accurate data on the species target sequences in order to design the corresponding specific primers (Rojas *et al*., 2009b).

Compared to single-species PCR systems, multiplex PCR, in which many primers are used together for the amplification of more than one target region, is a hopeful technique to save costs and enhance the speed, efficiency and reliability of analysis for the simultaneous identification various meat species (Tobe & Linacre, 2008).

Real-time PCR

Real-time PCR approaches allow the detection of even minute traces of different animal species in products of complex composition and are considered one of the most promising molecular tools for meat authentication (Koppel, Zimmerli, & Breitenmoser, 2009). Particularly, real-time PCR refers to the process where the production of amplification products is directly monitored during each amplification cycle and can be measured when the PCR reaction is still in the exponential phase and none of the reaction components is limited. The assay allows quantifying at an early stage in the PCR process, which is inherently more accurate than the end point analysis typically associated with gel agarose or polyacrilamide electrophoresis. Real-time data collection is achieved using fluorescent molecules that provide a strong correlation between fluorescence intensity and PCR product abundance (López-Andreo, Lugo, Garrido-Pertierra, Prieto, & Puyet, 2005).

The real-time PCR technique has been largely used for gene expression analysis, identification of microorganisms and detection and quantification of genetically modified organisms, whereas only recently suggested for food species identification (Hanna, Connor, & Wang, 2005). In the last years, real-time PCR assays have been described for the detection of a number of meat species like beef, pork, lamb, horse, chicken, turkey and duck, among others (Jonker *et al*., 2008; Laube, Zagon, & Broll, 2007). Besides, to meet current legal measures against the spread of BSE, this technology is being increasingly applied for the detection of ruminant, avian, porcine and fish DNA in feedstuffs (Cawthraw *et al*., 2009). However, as occurs with other DNA-based approaches reviewed in this paper, very few works are available in the literature concerning the application of real-time PCR for the identification of less commonly consumed meats such as those from game species.

There are various fluorescence based chemistries adapted to real-time PCR detection, which can be classified into four types: hydrolysis probes such as TaqMan®, hairpin probes like molecular beacons, fluorescent-labeled hybridisation (FRET) probes, and DNA intercalating dyes. In particular, probe-based chemistries like TaqMan® real-time PCR assays permit the use of very small amplicons and internal probes that will only bind to the desired specific sequence within the amplicon. These assays can therefore amplify specifically and efficiently, while improve detection mainly when enhanced sensitivity is required. However, a possible limitation is the design and availability of primers and probes, which must be selected according to very rigid conditions that cannot always be easily met (Jonker *et al*., 2008; Laube *et al*., 2007). Besides probe-based approaches, the simplest, least expensive and most direct fluorescent system adapted to real-time PCR detection involves the incorporation of the SYBR Green I dye, whose fluorescence under UV greatly increases when bound to the minor groove of the double helical DNA. SYBR Green I dye real-time PCR protocols are deficient in the specificity conferred by fluorescent DNA probes, but have the advantage of allowing the interpretation of DNA melting curves to discard the specific amplicon after the PCR from false positive signals due to non specific amplification or primer-dimers (López-Andreo, Garrido-Pertierra, & Puyet, 2006; Sawyer, Wood, Shanahan, Gout, & McDowell, 2003).

As examples of the application of real-time PCR technology to game speciation, the authors Wetton, Tsang, Roney, and Spriggs (2002) identified DNA from tiger using a species-specific oligonucleotide pair targeting the mitochondrial cytochrome b gene and the SYBR Green fluorescent intercalator.

Hird *et al*. (2004) achieved the identification of deer and some domestic species by the optimization of real-time TaqMan® technology with truncated primers located on the mitochondrial cytochrome b gene.

López-Andreo *et al*. (2006) developed TaqMan® real-time PCR systems on the mitochondrial cytochrome b gene for the detection and quantification of DNA from ostrich and other meat species. This research group also evaluated the usefulness of post-PCR melting temperature analysis for the identification of kangaroo, horse, bovine and porcine species in mixed samples using mitochondrial cytochrome b sequences and the SYBR Green fluorescent molecule (López-Andreo *et al*., 2006).

Chisholm, Sánchez, Brown, and Hird (2008) identified DNA from pheasant and quail in commercial food products using species-specific primers and TaqMan® probes designed on the mitochondrial cytochrome b gene.

Fajardo *et al*. (2008b, 2008c) accomplished a SYBR Green real-time PCR assay to detect red deer, fallow deer, roe deer, chamois and pyrenean ibex in meat mixtures using species-specific primers targeting the mitochondrial 12S rRNA and D-loop genes. In a further study, these authors reported an improvement of the assay in terms of specificity, sensitivity, efficiency and accuracy by using specific TaqMan® probes instead of the SYBR Green fluorescent intercalator (Fajardo, González, Martín, *et al*., 2009).

Rojas, González, Pavón, Pegels, Lago, *et al*., 2010b developed real-time PCR assays using TaqMan® probes for verifying the authenticity of meat and commercial meat

products from game birds including quail, pheasant, partridge, guinea fowl, pigeon, Eurasian woodcock and song thrush. The assay is based on specific primers and probes designed for each target species on the mitochondrial 12S rRNA gene.

The work of Chen *et al*. (2009) is among the exiguous number of studies in which detection of DNA from game species in animal feedstuffs is reported. The fluorescent molecule EvaGreen® and specific cytochrome b primers were used for the identification of species belonging to the family *Cervidae* (red deer, sika deer, sambar deer, white-lipped-deer, fallow deer, reindeer and milu).

Some of the advantages of real-time PCR-based fluorescence technology are: a) the potential for quantitative measurements at an early stage in the PCR process which is more precise than the end point analysis, b) the discrimination of the origin of DNA without the need for any additional time-consuming and laborious steps such as sequencing, enzyme digestion or conformational analysis, c) fluorescence data can be collected directly from a real-time PCR instrument or a fluorescence spectrophotometer, avoiding the need for electrophoresis, d) the rapidity of assays, allowing the routine high-throughput screening of multiple samples, and e) the great reduction of the potential contamination of the PCR mixture with target DNA because the reaction tubes remain closed throughout the assay (Brodmann & Moor, 2003; Chisholm *et al*., 2008).

In quantitative real-time PCR assays, analyses are performed by comparing the amplification (C_p) of a target (generally a mtDNA sequence) in an unknown (blind) sample with a calibration curve prepared with known (reference) concentrations of the same target in the sample. In this context, the accuracy and quantification potential of the method can be influenced by factors affecting to DNA yield such as the extent of DNA degradation due to processing treatments and the presence of different tissues in the samples potentially containing different type and amounts of DNA yielding cells. As consequence, although the technique has a proven quantitative potential, quantitative estimation of a target species content in commercial meat products would be only be useable if identical terms of composition and processing treatment are available for the preparation of suitable calibration standards, which is not feasible from a practical point of view (Prado *et al*., 2009). Moreover, the application of the technique may be restrained by the high cost derived from the specific fluorescent probes when used (Fajardo, González, Martín, *et al*., 2009).

Conclusions

The increasing demands imposed by EU legislation in relation to food and feed labeling and traceability have induced a great deal of research into the development of molecular tools for food authentication. This has been due in no small part to public health concerns associated with the BSE crisis and other risks like food allergies to certain food components. However, food authenticity is substantially addressed to avoid of unfair competition practices of producers, processors and sellers who would gain an economic advantage from food misrepresentation, as well as to prevent over-exploitation and illegal trafficking and consumption of endangered species through international trade.

DNA-based techniques, and particularly those based on the PCR, have special value for use in meat speciation. Although much of the recent applications that have occurred within this area have been primarily directed to common domestic species, differential consumer preferences and globalization of the meat industry have pushed the emergence of molecular identification tools specifically designed for alternative meats like those from game animals. The PCR-based approaches discussed herein show sufficient advantages in terms of reliability, speed, sensitivity and specificity to find application on game meat authenticity testing. However, the feasibility of their use in the food industry is still today dependent on the lowering of instrument and running costs. Considering the increasing amount of research in recent years, it is likely that the potential of PCR-based methodologies for meat authentication will continue to grow as species coverage in the databases increases and, in time, perhaps these techniques can be adopted as complementary regulatory tools to allow farm to fork traceability and discourage game meat substitution in the marketplace.

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References

Abdulmawjood, A., & Buelte, M. (2002). Identification of ostrich meat by restriction fragment length polymorphism (RFLP) analysis of cytochrome b gene. *Journal of Food Science*, *67*(5), 1688–1691.

Abensperg-Traun, M. (2009). CITES, sustainable use of wild species and incentive-driven conservation in developing countries, with an emphasis on southern Africa. *Biological Conservation*, *142*(5), 948–963.

Arslan, A., Ilhak, I., Calicioglu, M., & Karahan, M. (2005). Identification of meats using random amplified polymorphic DNA (RAPD) technique. *Journal of Muscle Foods*, *16*(1), 37–45.

Ayaz, Y., Ayaz, N. D., & Erol, I. (2006). Detection of species in meat and meat products using enzyme-linked immunosorbent assay. *Journal of Muscle Foods*, *17*(2), 214–220.

Ballin, N. Z., Vogensen, F. K., & Karlsson, A. H. (2009). Species determination – can we detect and quantify meat adulteration? *Meat Science*, *83*(2), 165–174.

Barbuto, M., Galimberti, A., Ferri, E., Labra, M., Malandra, R., Galli, P., et al. (2010). DNA barcoding reveals fraudulent substitutions in shark seafood products: the Italian case of "palombo" (*Mustelus* spp.). *Food Research International*, *43*(1), 376–381.

Brodmann, P. D., & Moor, D. (2003). Sensitive and semi-quantitative TaqMan® real-time polymerase chain reaction systems for the

detection of beef (*Bos taurus*) and the detection of the family *Mammalia* in food and feed. *Meat Science*, *65*(1), 599–607.

Brodmann, P. D., Nicholas, G., Schaltenbrand, P., & Ilg, E. C. (2001). Identifying unknown game species: experience with nucleotide sequencing of the mitochondrial cytochrome b gene and a subsequent basic local alignment search tool search. *European Food Research Technology*, *212*(4), 491–496.

Casoli, C., Duranti, E., Cambiotti, F., & Avellini, P. (2005). Wild ungulate slaughtering and meat inspection. *Veterinary Research Communications*, *29*(2), 89–95.

Cawthraw, S., Saunders, G. C., Martin, T. C., Sawyer, J., Windl, O., & Reaney, S. D. (2009). Real-time PCR detection and identification of prohibited mammalian and avian material in animal feeds. *Journal of Food Protection*, *72*(5), 1055–1062.

Chai, K. M., Huat, L. C., Thai, C. S., & Phang, S. T. W. (1997). Random amplified polymorphic DNA (RAPD) fingerprint profiling of domestic and game birds. *Asia-Pacific Journal of Molecular Biology and Biotechnology*, *5*(3), 173–182.

Chen, Y., Wu, Y. J., Wang, J., Xu, B. L., Zhong, Z. Y., & Xia, J. S. (2009). Identification of *Cervidae* DNA in feedstuff using a real-time polymerase chain reaction method with the new fluorescence intercalating dye EvaGreen. *Journal of AOAC International*, *92*(1), 175–180.

Chen, Y., Wu, Y. J., Xu, B. L., Wan, J., & Qian, Z. M. (2005). Species-specific polymerase chain reaction amplification of camel (*Camelus*) DNA extracts. *Journal of AOAC International*, *88*(5), 1394–1398.

Chikuni, K., Tabata, T., Saito, M., & Monma, M. (1994). Sequencing of mitochondrial cytochrome b genes for the identification of meat species. *Animal Science and Technology*, *65*, 571–579.

Chisholm, J., Sánchez, A., Brown, J., & Hird, H. (2008). The development of species-specific real-time PCR assays for the detection of pheasant and quail in food. *Food Analytical Methods*, *1*(3), 190–194.

Chomel, B. B., Belotto, A., & Meslin, F. X. (2007). Wildlife, exotic pets, and emerging zoonoses. *Emerging Infectious Diseases*, *13*(1), 6–11.

Chou, C. C., Lin, S. P., Lee, K. M., Hsu, C. T., Vickroy, T. W., & Zen, J. M. (2007). Fast differentiation of meats from fifteen animal species by liquid chromatography with electrochemical detection using copper nanoparticle plated electrodes. *Journal of Chromatography B–Analytical Technologies in the Biomedical and Life Sciences*, *846*(1–2), 230–239.

Colombo, F., Cardia, A., Renon, P., & Cantón, C. (2004). A note on the identification of *Rupicapra rupicapra* species by polymerase chain reaction product sequencing. *Meat Science*, *66*(3), 753–755.

Colombo, F., Viacava, R., & Giaretti, M. (2000). Differentiation of the species ostrich (*Struthio camelus*) and emu (*Dromaius novaehollandiae*) by polymerase chain reaction using an ostrich-specific primer pair. *Meat Science*, *56*(1), 15–17.

Comincini, S., Sironi, M., Bandi, C., Giunta, C., Rubini, M., & Fontana, F. (1996). RAPD analysis of systematic relationships among the Cervidae. *Heredity*, *76*(3), 215–221.

Dasmahapatra, K. K., & Mallet, J. (2006). DNA barcodes: recent successes and future prospects. *Heredity*, *97*(4), 254–255.

Dooley, J. J., & Garrett, S. D. (2001). *Development of meat speciation assays using the Agilent 2100 bioanalyzer*. Agilent Technologies Application. Note no 5988–4069EN.

Dooley, J. J., Sage, H. D., Clarke, M. L., Brown, H. H., & Garrett, S. D. (2005). Fish species identification using PCR-RFLP analysis and lab-on-a-chip capillary electrophoresis: application to detect white fish species in food products and an interlaboratory study. *Journal of Agricultural and Food Chemistry*, *53*(9), 3348–3357.

Dubey, B., Meganathan, P. R., & Haque, I. (2009). Multiplex PCR assay for rapid identification of three endangered snake species of India. *Conservation Genetics*, *10*(6), 1861–1864.

El-Jaafari, H. A. A., Panandam, J. M., Idris, I., & Siraj, S. S. (2008). RAPD analysis of three deer species in Malaysia. *Asian-Australasian Journal of Animal Sciences*, *21*(9), 1233–1237.

Ellis, D. I., Broadhurst, D., Clarke, S. J., & Goodacre, R. (2005). Rapid identification of closely related muscle foods by vibrational spectroscopy and machine learning. *Analyst*, *130*(12), 1648–1654.

Fajardo, V., González, I., Dooley, J., Garret, S., Brown, H. M., García, T., et al. (2009). Application of PCR-RFLP analysis and lab-on-a-chip capillary electrophoresis for the specific identification of game and domestic meats. *Journal of the Science of Food and Agriculture*, *89*(5), 843–847.

Fajardo, V., González, I., López-Calleja, I., Martín, I., Hernández, P. E., García, T., et al. (2006). PCR-RFLP authentication of meats from red deer (*Cervus elaphus*), fallow deer (*Dama dama*), roe deer (*Capreolus capreolus*), cattle (*Bos taurus*), sheep (*Ovis aries*) and goat (*Capra hircus*). *Journal of Agricultural and Food Chemistry*, *54*(4), 1144–1150.

Fajardo, V., González, I., López-Calleja, I., Martín, I., Rojas, I., Pavón, M. A., et al. (2007). Analysis of mitochondrial DNA for authentication of meats from chamois (*Rupicapra rupicapra*), pyrenean ibex (*Capra pyrenaica*) and mouflon (*Ovis ammon*) by polymerase chain reaction-restriction fragment length polymorphism. *Journal of AOAC International*, *90*(1), 179–186.

Fajardo, V., González, I., López-Calleja, I., Martín, I., Rojas, M., Hernández, P. E., et al. (2007a). Identification of meats from red deer (*Cervus elaphus*), fallow deer (*Dama dama*) and roe deer (*Capreolus capreolus*) using polymerase chain reaction targeting specific sequences from the mitochondrial 12S rRNA gene. *Meat Science*, *76*(2), 234–240.

Fajardo, V., González, I., López-Calleja, I., Martín, I., Rojas, M., Hernández, P. E., et al. (2007b). PCR identification of meats from chamois (*Rupicapra rupicapra*), pyrenean ibex (*Capra pyrenaica*) and mouflon (*Ovis ammon*) targeting specific sequences from the mitochondrial D-loop. *Meat Science*, *76*(4), 644–652.

Fajardo, V., González, I., Martín, I., Rojas, M., Hernández, P. E., García, T., et al. (2008a). Differentiation of European wild boar (*Sus scrofa scrofa*) and domestic swine (*Sus scrofa domestica*) meats by PCR analysis targeting the mitochondrial D-loop and the nuclear melanocortin receptor 1 (*MC1R*) genes. *Meat Science*, *78*(3), 314–322.

Fajardo, V., González, I., Martín, I., Rojas, M., Hernández, P. E., García, T., et al. (2008b). Real-time PCR for detection and quantification of red deer (*Cervus elaphus*), fallow deer (*Dama dama*), and roe deer (*Capreolus capreolus*) in meat mixtures. *Meat Science*, *79*(2), 289–298.

Fajardo, V., González, I., Martín, I., Rojas, M., Hernández, P. E., García, T., et al. (2008c). Real-time PCR for quantitative detection of chamois (*Rupicapra rupicapra*) and pyrenean ibex (*Capra pyrenaica*) in meat mixtures. *Journal of AOAC International*, *91*(1), 103–111.

Fajardo, V., González, I., Martín, I., Rojas, M., Hernández, P. E., García, T., et al. (2009). A LigthCycler TaqMan® PCR assay for quantitative detection of chamois (*Rupicapra rupicapra*) and pyrenean ibex (*Capra pyrenaica*) in meat mixtures. *International Journal Food Science and Technology*, *44*(10), 1997–2004.

Felmer, R., Sagredo, B., Chávez, R., Iraira, S., Folch, C., Parra, L., et al. (2008). Implementation of a molecular system for traceability of beef based on microsatellite markers. *Chilean Journal of Agricultural Research*, *68*(4), 342–351.

Ferri, G., Alu, M., Corradini, B., Licata, M., & Beduschi, G. (2009). Species identification through DNA "Barcodes". *Genetic Testing and Molecular Biomarkers*, *13*(3), 421–426.

Girish, P. S., Anjaneyulu, A. S. R., Viswas, K. N., Haunsh, S., Bhilegaonkar, K. N., Agarwal, R. K., et al. (2009). Poultry meat speciation by sequence analysis of mitochondrial 12S rRNA gene. *Indian Journal of Animal Sciences*, *79*(2), 217–220.

Girish, P. S., Anjaneyulu, A. S. R., Viswas, K. N., Santhosh, F. H., Bhilegaonkar, K. N., Agarwal, R. K., et al. (2007). Polymerase chain reaction-restriction fragment length polymorphism of mitochondrial 12S rRNA gene: a simple method for identification of poultry meat species. *Veterinary Research Communications*, *31*(4), 447–455.

Girish, P. S., Anjaneyulu, A. S. R., Viswas, K. N., Shivakumar, B. M., Anand, M., Patel, M., et al. (2005). Meat species identification by

polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of mitochondrial 12S rRNA gene. *Meat Science*, *70*(1), 107–112.

González, S., Maldonado, J. E., Ortega, J., Talarico, A. C., Bidegaray-Batista, L., García, J. E., et al. (2009). Identification of the endangered small red brocket deer (*Mazama bororo*) using noninvasive genetic techniques (*Mammalia; Cervidae*). *Molecular Ecology Resources*, *9*(3), 754–758.

Gupta, A. R., Patra, R. C., Das, D. K., Gupta, P. K., Swarup, D., & Saini, M. (2008). Sequence characterization and polymerase chain reaction-restriction fragment length polymorphism of the mitochondrial DNA 12S rRNA gene provides a method for species identification of Indian deer. *Mitochondrial DNA*, *19*(4), 394–400.

Ha, J. C., Jung, W. T., Nam, Y. S., & Moon, T. W. (2006). PCR identification of ruminant tissue in raw and heat-treated meat meals. *Journal of Food Protection*, *69*(9), 2241–2247.

Hanna, S. E., Connor, C. J., & Wang, H. H. (2005). Real-time polymerase chain reaction for the food microbiologist: technologies, applications, and limitations. *Journal of Food Science*, *70*(3), 49–53.

Haunshi, S., Basumatary, R., Girish, P. S., Doley, S., Bardoloi, R. K., & Kumar, A. (2009). Identification of chicken, duck, pigeon and pig meat by species-specific markers of mitochondrial origin. *Meat Science*, *83*(2), 454–459.

Hebert, P. D. N., Ratnasingham, S., & Dewaard, J. R. (2003). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London Series B*, *270*(Suppl. 1), S96–S99.

Hird, H., Goodier, R., Schneede, K., Boltz, C., Chisholm, J., Lloyd, J., et al. (2004). Truncation of oligonucleotide primers confers specificity on real-time polymerase chain reaction assays for food authentication. *Food Additives and Contaminants*, *21*(11), 1035–1040.

Hoffman, L. C., & Wiklund, E. (2006). Game and venison – meat for the modern consumer. *Meat Science*, *74*(1), 197–208.

Holmes, B. H., Steinke, D., & Ward, R. D. (2009). Identification of shark and ray fins using DNA barcoding. *Fisheries Research*, *95* (2–3), 280–288.

Hopwood, A. J., Fairbrother, K. S., Lockley, A. K., & Bardsley, R. G. (1999). An actin gene-related polymerase chain reaction (PCR) test for identification of chicken in meat mixtures. *Meat Science*, *53*, 227–231.

Hsieh, H. M., Huang, L. H., Tsai, L. C., Kuo, Y. C., Meng, H. H., Linacre, A., et al. (2003). Species identification of rhinoceros horns using the cytochrome b gene. *Forensic Science International*, *136* (1–3), 1–11.

Huang, M. C., Horng, Y. M., Huang, H. L., Sin, Y. L., & Chen, M. J. (2003). RAPD fingerprinting for the species identification of animals. *Asian-Australasian Journal of Animal Sciences*, *16*(10), 1406–1410.

IUCN (2009). Numbers of threatened species by major groups of organisms (1996–2009). http://www.iucnredlist.org/documents/summarystatistics/2009RL_Stats_Table_1.pdf

Jonker, K. M., Tilburg, J. J. H. C., Hägele, G. H., & De Boer, E. (2008). Species identification in meat products using real-time PCR. *Food Additives and Contaminants*, *25*(5), 527–533.

Karlsson, A. O., & Holmlund, G. (2007). Identification of mammal species using species-specific DNA pyrosequencing. *Forensic Science International*, *173*(1), 16–20.

Kitano, T., Umetsu, K., Tian, W., & Osawa, M. (2007). Two universal primer sets for species identification among vertebrates. *International Journal of Legal Medicine*, *121*(5), 423–427.

Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Pääbo, S., Villablanca, F. X., et al. (1989). Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences*, *86*(16), 6196–6200.

Koh, M. C., Lim, C. H., Chua, S. B., Chew, S. T., & Phang, S. T. W. (1998). Random amplified polymorphic DNA (RAPD) fingerprints for identification of red meat animal species. *Meat Science*, *48*(3–4), 275–285.

Koppel, R., Zimmerli, F., & Breitenmoser, A. (2009). Heptaplex real-time PCR for the identification and quantification of DNA from beef, pork, chicken, turkey, horse meat, sheep (mutton) and goat. *European Food Research and Technology*, *230*(1), 125–133.

Koveza, O. V., Kokaeva, Z. G., Konovalov, F. A., & Gostimsky, S. A. (2005). Identification and mapping of polymorphic RAPD markers of pea (*Pisum sativum L.*) genome. *Genetika*, *41*(3), 341–348.

Krkoska, L., Nebola, M., Steinhauserová, I., Obroská, I., & Ernst, M. (2003). Using the PCR-RFLP method. *Fleischwirtschaft International*, *2*, 39–42.

La Neve, F., Civera, T., Mucci, N., & Bottero, M. T. (2008). Authentication of meat from game and domestic species by SNaPshot minisequencing analysis. *Meat Science*, *80*(2), 216–224.

Laube, I., Zagon, J., & Broll, H. (2007). Quantitative determination of commercially relevant species in foods by real-time PCR. *International Journal of Food Science and Technology*, *42*(3), 336–341.

Lee, J., Hsieh, H. M., Huang, L. H., Kuo, Y. C., Wu, J. H., Chin, S. C., et al. (2009). Ivory identification by DNA profiling of cytochrome b gene. *International Journal of Legal Medicine*, *123*(2), 117–121.

Li, B., Bai, S. Y., Xu, Y. C., Zhang, W., & Ma, J. Z. (2006). Identification of sika deer and red deer using partial cytochrome b and 12S ribosomal RNA genes. *Journal of Forestry Research*, *17*(2), 160–162.

López-Andreo, M., Lugo, L., Garrido-Pertierra, A., Prieto, M. I., & Puyet, A. (2005). Identification and quantitation of species in complex DNA mixtures by real-time polymerase chain reaction. *Analytical Biochemistry*, *339*(1), 73–82.

López-Andreo, M., Garrido-Pertierra, A., & Puyet, A. (2006). Evaluation of post-polymerase chain reaction melting temperature analysis for meat species identification in mixed DNA samples. *Journal of Agricultural and Food Chemistry*, *54*(21), 7973–7978.

Maede, D. (2006). A strategy for molecular species detection in meat and meat products by PCR-RFLP and DNA sequencing using mitochondrial and chromosomal genetic sequences. *European and Food Research Technology*, *224*(2), 209–217.

Mafra, I., Ferreira, I. M. P. L. V. O., & Oliveira, M. B. P. P. (2008). Food authentication by PCR-based methods. *European Food Research and Technology*, *227*(3), 649–665.

Malisa, A. L., Gwakisa, P., Balthazary, S., Wasser, S. K., & Mutayoba, B. M. (2006). The potential of mitochondrial DNA markers and polymerase chain reaction-restriction fragment length polymorphism for domestic and wild species identification. *African Journal of Biotechnology*, *5*(18), 1588–1593.

Martínez, I., & Danielsdottir, A. K. (2000). Identification of marine mammal species in food products. *Journal of the Science of Food and Agriculture*, *80*(4), 527–533.

Martínez, I., & Yman, I. M. (1998). Species identification in meat products by RAPD analysis. *Food Research International*, *31*(6–7), 459–466.

Matsunaga, T., Chikuni, K., Tanabe, R., Muroya, S., Nakai, K., Shibata, K., et al. (1998). Determination of mitochondrial cytochrome b gene sequence for red deer (*Cervus elaphus*) and the differentiation of closely related red deer meats. *Meat Science*, *49*(4), 379–385.

Matsunaga, T., Shibata, K., Yamada, J., Shinmura, Y., & Chikuni, K. (1998). Identification of meat species based on the difference of 18S ribosomal RNA genes. *Journal of the Japanese Society for Food Science and Technology*, *45*(12), 719–723.

Meganathan, P. R., Dubey, B., & Haque, I. (2009). Molecular identification of Indian crocodile species: PCR-RFLP method for forensic authentication. *Journal of Forensic Sciences*, *54*(5), 1042–1045.

Meyer, R., Höfelen, C., Lüthy, J., & Candrian, U. (1995). Polymerase chain reaction-restriction fragment polymorphism analysis:

a simple method for species identification in food. *Journal of AOAC International*, *78*(6), 1542–1551.

Mohindra, V., Khare, P., Lal, K. K., Punia, P., Singh, R. K., Barman, A. S., et al. (2007). Molecular discrimination of five Mahseer species from Indian peninsula using RAPD analysis. *Acta Zoologica Sinica*, *53*(4), 725–732.

Montiel-Sosa, J. F., Ruiz-Pesini, E., Montoya, J., Rocalés, P., López-Pérez, M. J., & Pérez-Martos, A. (2000). Direct and highly species-specific detection of pork meat and fat in meat products by PCR amplification of mitochondrial DNA. *Journal of Agricultural and Food Chemistry*, *48*(7), 2829–2832.

Montowska, M., & Pospiech, E. (2007). Species identification of meat by electrophoretic methods. *Acta Scientarium Polonorum, Technologia Alimentaria*, *6*(1), 5–16.

Murugaiah, C., Noor, Z. M., Mastakim, M., Bilung, L. M., Selamat, J., & Radu, S. (2009). Meat species identification and Halal authentication analysis using mitochondrial DNA. *Meat Science*, *83*(1), 57–61.

Nau, F., Desert, C., Cochet, M. F., Pasco, M., Jan, S., Baron, F., et al. (2009). Detection of turkey, duck, and guinea fowl egg in hen egg products by species-specific PCR. *Food Analytical Methods*, *2*(3), 231–238.

Palumbi, S. R., & Cipriano, F. (1998). Species identification using genetic tools: the value of nuclear and mitochondrial gene sequences in whale conservation. *Journal of Heredity*, *89*(5), 459–464.

Park, J. K., Shin, K. H., Shin, S. C., Chung, K. Y., & Chung, E. R. (2007). Identification of meat species using species-specific PCR-RFLP fingerprint of mitochondrial 12S rRNA gene. *Korean Journal for Food Science of Animal Resources*, *27*(2), 209–215.

Partis, L., Croan, D., Guo, Z., Clark, R., Coldham, T., & Murby, J. (2000). Evaluation of DNA fingerprinting method for determining the species origin of meat. *Meat Science*, *54*(4), 369–376.

Pascoal, A., Prado, M., Castro, J., Cepeda, A., & Barros-Velázquez, J. (2004). Survey of authenticity of meat species in food products subjected to different technological processes, by means of PCR-RFLP analysis. *European Food Research and Technology*, *218*(3), 306–312.

Pfeiffer, I., Burger, J., & Brenig, B. (2004). Diagnostic polymorphisms in the mitochondrial cytochrome b gene allow discrimination between cattle, sheep, goat, roe buck and red deer by PCR-RFLP. *Genetics*, *5*, 30–35.

Prado, M., Fumière, O., Boix, A., Marien, A., Berben, G., & von Holst, C. (2009). Novel approach for interlaboratory transfer of real-time PCR methods: detecting bovine meat and bone meal in feed. *Anaytical and Bioanalytical Chemistry*, *394*(5), 1423–1431.

Rajapaksha, W. R. A. K. J., Thilakaratne, I. D. S. I. P., Chandrasiri, A. D. N., & Niroshan, T. D. (2002). Development of PCR assay for differentiation of some important wild animal meal of Sri Lanka. *Journal of Veterinary Medicine Series B*, *49*(7), 322–324.

Rajapaksha, W. R. A. K. J., Thilakaratne, I. D. S. I. P., Chandrasiri, A. D. N., & Niroshan, T. D. (2003). Development of PCR assay for identification of buffalo meat. *Asian-Australasian Journal of Animal Sciences*, *16*(7), 1046–1048.

Rastogi, G., Dharne, M. S., Walujkar, S., Kumar, A., Patole, M. S., & Shouche, Y. S. (2007). Species identification and authentication of tissues of animal origin using mitochondrial and nuclear markers. *Meat Science*, *76*(4), 666–674.

Ripoli, M. V., Corva, P., & Giovambattista, G. (2006). Analysis of a polymorphism in the DGAT1 gene in 14 cattle breeds through PCR-SSCP methods. *Research in Veterinary Science*, *80*(3), 287–290.

Rojas, M., González, I., Fajardo, V., Martín, I., Hernández, P. E., García, T., et al. (2008). Polymerase chain reaction-restriction fragment length polymorphism authentication of raw meats from game birds. *Journal of AOAC International*, *91*(6), 1416–1422.

Rojas, M., González, I., Fajardo, V., Martín, I., Hernández, P. E., García, T., et al. (2009a). Identification of raw and heat-processed meats from game bird species by polymerase chain reaction-restriction fragment length polymorphism of the mitochondrial D-loop region. *Poultry Science*, *88*(3), 669–679.

Rojas, M., González, I., Fajardo, V., Martín, I., Hernández, P. E., García, T., et al. (2009b). Authentication of meats from quail (*Coturnix coturnix*), pheasant (*Phasianus colchicus*), partridge (*Alectoris spp*), and guinea fowl (*Numida meleagris*) using polymerase chain reaction targeting specific sequences from the mitochondrial 12S rRNA gene. *Food Control*, *20*(10), 896–902.

Rojas, M., González, I., Pavón, M. A., Pegels, N., Hernández, P. E., García, T., et al. (2010a). Polymerase chain reaction assay for verifying the labeling of meat and commercial meat products from game birds targeting specific sequences from the mitochondrial D-loop region. *Poultry Science*, *89*(5), 1021–1032.

Rojas, M., González, I., Pavón, M. A., Pegels, N., Lago, A., Hernández, P. E., García, T., et al. (2010b). Novel TaqMan real-time polymerase chain reaction assay for verifying the authenticity of meat and commercial meat products from game birds. *Food Additives and Contaminants*, *27*(6), 749–763.

Saini, M., Das, D. K., Dhara, A., Swarup, D., Yadav, M. P., & Gupta, P. K. (2007). Characterisation of peacock (*Pavo cristatus*) mitochondrial 12S rRNA sequence and its use in differentiation from closely related poultry species. *British Poultry Science*, *48*(2), 162–166.

Sawyer, J., Wood, C., Shanahan, D., Gout, S., & McDowell, D. (2003). Real-time PCR for quantitative meat species testing. *Food Control*, *14*(8), 579–583.

Shackell, G. H. (2008). Traceability in the meat industry – the farm to plate continuum. *International Journal of Food Science and Technology*, *43*(12), 2134–2142.

Shin, K. H., Shin, S. C., Chung, K. Y., & Chung, E. R. (2008). Identification of deer antler species using sequence analysis and PCR-RFLP of mitochondrial DNA. *Korean Journal for Food Science of Animal Resources*, *28*(3), 276–282.

Shinoda, N., Yoshida, T., Kusama, T., Takagi, M., Onodera, T., & Sugiura, K. (2009). Development of primers for detection of heat-treated cetacean materials in porcine meat and bone meal. *Journal of Food Protection*, *72*(7), 1496–1499.

Stephenson, J. (2002). "Bush meat" threat. *Journal of the American Medical Association*, *287*(18), 2353.

Stirtzel, S., Andree, S., Seuss-Baum, I., & Schwagele, F. (2007). Authentification of the most common poultry species by means of PCR. *Fleischwirtschaft*, *87*(6), 86–89.

Teletchea, F., Bernillon, J., Duffraisse, M., Laudet, V., & Hänni, C. (2008). Molecular identification of vertebrate species by oligonucleotide microarray in food and forensic samples. *Journal of Applied Ecology*, *45*(3), 967–975.

Tobe, S. S., & Linacre, A. M. T. (2008). A multiplex assay to identify 18 European mammal species from mixtures using the mitochondrial cytochrome b gene. *Electrophoresis*, *29*(2), 340–347.

Venkatachalapathy, R. T., Sharma, A., Sukla, S., & Hattacharya, T. K. (2008). Cloning and characterization of DGAT1 gene of Riverine buffalo. *DNA Sequence*, *19*(3), 177–184.

Wan, Q. H., & Fang, S. G. (2003). Application of species-specific polymerase chain reaction in the forensic identification of tiger species. *Forensic Science International*, *131*(1), 75–78.

Waugh, J. (2007). DNA barcoding in animal species: progress, potential and pitfalls. *BioEssays*, *29*(2), 188–197.

Wetton, J. H., Tsang, C. S. F., Roney, C. A., & Spriggs, A. C. (2002). An extremely sensitive species-specific ARMs PCR test for the presence of tiger bone DNA. *Forensic Science International*, *126*(2), 137–144.

Wolf, C., Rentsch, J., & Hübner, P. (1999). PCR-RFLP analysis of mitochondrial DNA: a reliable method for species identification. *Journal of Agricultural and Food Chemistry*, *47*(4), 1350–1355.

Wong, E. H. K., & Hanner, R. H. (2008). DNA barcoding detects market substitution in North American seafood. *Food Research International*, *41*(8), 828–837.

Wong, K. L., Wang, J., But, P. P. H., & Shaw, P. C. (2004). Application of cytochrome b DNA sequences for the authentication of endangered snake species. *Forensic Science International, 139*(1), 49–55.

Wu, X. B., Liu, H., & Jiang, Z. G. (2006). Identification primers for sika deer (*Cervus nippon*) from a sequence-characterised amplified region (SCAR). *New Zealand Journal of Zoology, 33*(1), 65–71.

Wyler, L. S., & Sheikh, P. A. (2009). International illegal trade in wildlife: Threats and U.S. policy. *Congressional Research Reports*. Order Code RL34395.

Yan, P., Wu, X. B., Shi, Y., Gu, C. M., Wang, R. P., & Wang, C. L. (2005). Identification of Chinese alligators (*Alligator sinensis*) meat by diagnostic PCR of the mitochondrial cytochrome b gene. *Biological Conservation, 121*(1), 45–51.

Yau, F. C. F., Wong, K. L., Shaw, P. C., But, P. P. H., & Wang, J. (2002). Authentication of snakes used in Chinese medicine by sequence characterized amplified region (SCAR). *Biodiversity and Conservation, 11*(9), 1653–1662.

Zimmermann, S., Zehner, R., & Mebs, D. (1998). Identification of animal species in meat samples by DNA-analysis. *Fleischwirtschaft, 78*(5), 530–533.