PCR-RFLP identification of meat from red deer, sika deer, roe deer, fallow deer, mouflon, wild boar, hare and cattle

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Abstract

Meat authentication is currently a key topic in relation to the quality and safety of food of animal origin at all levels of production and the global distribution chain. New polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) based on digestion of PCR products with two restriction enzymes, MboII and AciI, have been developed for the specific identification of raw and heat-processed meat from red deer (*Cervus elaphus*), sika deer (*Cervus nippon*), roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*), mouflon (*Ovis musimon*), wild boar (*Sus scrofa*), hare (*Lepus europaeus*) and cattle (*Bos taurus*). The PCR primers were targeted in a well-conserved region of the cytochrome *b* (*CYTB*) gene to amplify a 378 bp region of all the analysed species. This simple, rapid and cost-effective method is suitable for identification of the meat of game species and their possible substitution by beef.

Authentication, game meat, cytochrome b, PCR, restriction enzymes

Identification of animal tissues is currently a key topic in relation to the quality and safety of food of animal origin at all levels of production and the distribution chain. Production of venison and venison meat products is a common object of intentional or unintentional fraud by substituting venison with cheaper and easily accessible meat of other animal species, as such substitution is often not easily recognised by ordinary people and results in a huge profit for the perpetrator. Methods of molecular biology, especially the polymerase chain reaction (PCR), have been recognised as highly applicable methods for tissue identification in selected animal species, particularly for its high specificity, sensitivity, rapidity, accurate interpretation and low cost.

Considering the high price of venison in comparison with the meat of ordinary livestock animals and the major price differences among species (roe deer, fallow deer, red deer and sika deer, wild boar, etc.) as well as its increasing popularity among consumers, this commodity may be the target of intentional substitution or falsification. Consumers who prioritise high-quality foodstuffs with high added value are increasingly looking for information about the composition, quality and origin of these foodstuffs. According to the Regulation (EC) No 172/2002, food laws must aim at the protection of the interests of consumers, including the prevention of food adulteration. The legislation of the European Union and the Czech Republic places great emphasis on the consumer's right to accurate information relating to the composition of foodstuffs, including content declaration of individual constituents, as provided by legislation in Regulation (EC) No. 1169/2011 on the provision of food information to consumers, Czech Act No. 110/1997 Coll. on foodstuffs and tobacco products, and Czech Act No. 166/1999 Coll. on veterinary care, as amended, which focus together with the implementing law on food labelling and protection of consumers.

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Various molecular methods developed over the past two decades are considered essential and reliable for meat species identification, and their use is becoming widespread for this purpose. The use of these approaches can overcome the disadvantages of many conventional methods based on the analysis of proteins. DNA-based methods present certain advantages such as the ubiquity of nucleic acids in every type of cells and greater DNA stability compared to proteins (Lockey and Bardsley 2000). A variety of different molecular techniques can be employed for the determination of species origin, including that of game meat. These methods include PCR using species-specific primers (Ha et al. 2006; Amaral et al. 2014; Amaral et al. 2015; Hossain et al. 2017), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Pfeiffer et al. 2004; Guan et al. 2018; Jiang et al. 2018), real-time PCR (Santos et al. 2012; Druml et al. 2015; Kaltenbrunner et al. 2018), nucleotide sequencing (Barbuto et al. 2010; Abdigini et al. 2015) and the random amplified polymorphic DNA (RAPD) technique (Martinez and Yman 1998; El-Jaafari et al. 2008).

The PCR-RFLP technique is one of the important molecular methods in meat species identification which employs restriction digestion of selected DNA fragments with appropriate restriction enzymes to generate fragments that are unique to the different animal species present in the sample. Both nuclear and mitochondrial genes have been used for the authentication of game meat species (Fajardo et al. 2010). Mitochondrial DNA (mtDNA) offers a number of advantages over nuclear DNA (nDNA). The copy number of mtDNA is several times more abundant than that of nDNA and presents more sequence diversity, thus allowing the discrimination of closely-related species and contributing towards improving test sensitivity (Partis et al. 2000). Among the mitochondrial genes, cytochrome *b* (*CYTB*), *12S rRNA*, *126S rRNA* and the D-loop are the most frequently used in molecular identification studies (Fajardo et al. 2010).

In this study, a PCR-RFLP technique involving the digestion of an amplified 378 bp region of the *CYTB* gene with two restriction enzymes to generate DNA profiles was developed for identification and verification of the authenticity of meat from game species (red deer, sika deer, roe deer, fallow deer, mouflon, wild boar and hare) due to their possible substitution by one another and by cheaper meat such as beef.

Materials and Methods

Samples and DNA extraction

The raw meat material of seven game species was obtained by hunting wild animals in 17 local hunting areas in the Czech Republic. The species included the red deer (*Cervus elaphus*) (n = 64), sika deer (*Cervus nippon*) (n = 82), roe deer (*Capreolus capreolus*) (n = 139), fallow deer (*Dama dama*) (n = 42), mouflon (*Ovis musimon*) (n = 37), wild boar (*Sus scrofa*) (n = 111), and European hare (*Lepus europaeus*) (n = 15). The carcasses were assessed by a trained person and identification of the individual game species was performed on the basis of anatomical and zoological characteristics. Beef samples (*Bos taurus*) (n = 28) were collected at slaughterhouses (Czech Republic). Meat samples (10 samples per each animal species) were also analysed after being subjected to experimental heat treatments at 120 °C, 100 °C, 70 °C, and 40 °C for 10 min.

DNA was extracted from raw and heat-processed meat and raw and heat-processed meat juice using the NucleoSpinFood kit (Macherey-Nagel GmbH & Co. KG, Duren, Germany) according to the manufacturer's instructions. Approximately 200 mg of meat were used for DNA extraction. The DNA solution was stored at 4 °C until used as PCR templates.

DNA sequencing

Ten samples from each species, i.e. red deer, sika deer, roe deer, fallow deer, mouflon, wild boar, hare and cattle, were amplified, sequenced and compared. A polymerase chain reaction was performed in 25 µl volumes containing 50 ng of genomic DNA, PPP Master Mix without $MgCl_2$ (Top-Bio, Vestec, Czech Republic), 0.2 µM of forward (5′-CAAATATCATTCTGAGGAGCAAC-3′) and reverse (5′-TRGCTYCTTCTTTGAGTCTTAG-3′) primers each, and 2.0 mM $MgCl_2$. The PCR thermal profile consisted of pre-denaturation at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 53 °C for 15 s, elongation at 72 °C for 50 s, and final extension at 72 °C for 7 min. The PCR products after purification were sequenced using the ABI 3500 genetic analysis sequencer (Applied Biosystems, Foster City, CA, USA) for subsequent RFLP studies. The

program CLUSTAL Omega (https://www.ebi.ac.uk/Tools/msa/clustalo) was used for sequence alignment of the DNA sequences obtained from each sample, and the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for comparison of sequence similarities in the NCBI database.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

Polymerase chain reaction amplification was carried out at a final volume of 25 µl. Each reaction contained 1 × Master Mix – FastStar PCR Master (Roche, Basel, Switzerland), 0.2 µM of forward (5′-TGAGGACAAATATCAT TCTGAGGA-3′) and reverse (5′-GTTTGCTGGGGTGTAGTTATCTG-3′) primers each, and 50 ng of template DNA. The PCR thermal profile consisted of an initial denaturation step at 95 °C for 4 min followed by 30 cycles of denaturation step at 95 °C for 30 s, annealing at 50 °C for 30 s, and an extension step of 72 °C for 45 s, with a final extension step of 72 °C for 7 min. The specificity of amplified products was verified by agarose gel electrophoresis (3% agarose gel with ethidium bromide staining).

Polymerase chain reaction amplicons (378 bp) of the mitochondrial *CYTB* gene were digested with restriction enzymes MboII (TaKaRa Biotechnology, Dalian Co. Ltd., China) and AciI (New England Biolabs, UK). The digestion reactions were performed at a total volume of 15 µl containing 2 µl of 10X enzyme buffer, 5 µl of PCR products, and 2 units of endonuclease. The reaction mixture was incubated overnight at 37 °C (16 h). The digested products were visualised by electrophoresis in 3% agarose gel by ethidium bromide staining.

Results

DNA sequence analysis of the *CYTB* gene

The present study generated results of the technique for specific identification of meats from seven game species and one domestic species based on RFLP analysis of sequences from the mitochondrial *CYTB* gene. Fragment of the *CYTB* gene of a size of 819 bp was amplified and analysed from samples of all 8 species. The sequences were submitted to the European Molecular Biology Laboratory (EMBL) database and assigned with accession numbers KX550266 *Cervus elephus* (red deer), KX550267 *Cervus nippon* (sika deer), KX550268 *Capreolus capreolus* (roe deer), KX550269 *Dama dama* (fallow deer), KX550270 *Ovis aries musimon* (mouflon), KX550271 *Sus scrofa* (wild boar), and KX550272 *Bos taurus* (cattle). The BLAST search results revealed sequence similarities of the analysed species to the sequence in the database within the range of 99.4–100%. Sequences of the mitochondrial *CYTB* gene revealed variation between the analysed species enabling the application of PCR-RFLP to distinguish between them. Based on the obtained *CYTB* fragment sequences for all the tested species, a search was undertaken for restriction endonuclease sites (Fig. 1). The enzymes MboII and AciI were chosen on the basis of the predictable specific pattern that they would produce (Table 1).

Table 1. Restriction pattern of mitochondrial *CYTB* gene for different animal species after digestion with restriction endonucleases MboII and AciI.

	Red deer	Sika deer	Roe deer	Fallow deer	Mouflon	Wild boar	European hare	Cattle
MboII	195	154	378	113	195	243	243	243
	117	117		89	183	135	135	135
	66	66		66				
		41		64				
				46				
AciI						208	378	280
						170		98

Identification of game species by PCR-RFLP

Our proposed universal primer pair was able to successfully amplify the region of the mitochondrial *CYTB* gene in all analysed samples (n = 490), providing a single PCR product of 378 bp. The identification of meat from all seven game species (red deer, sika

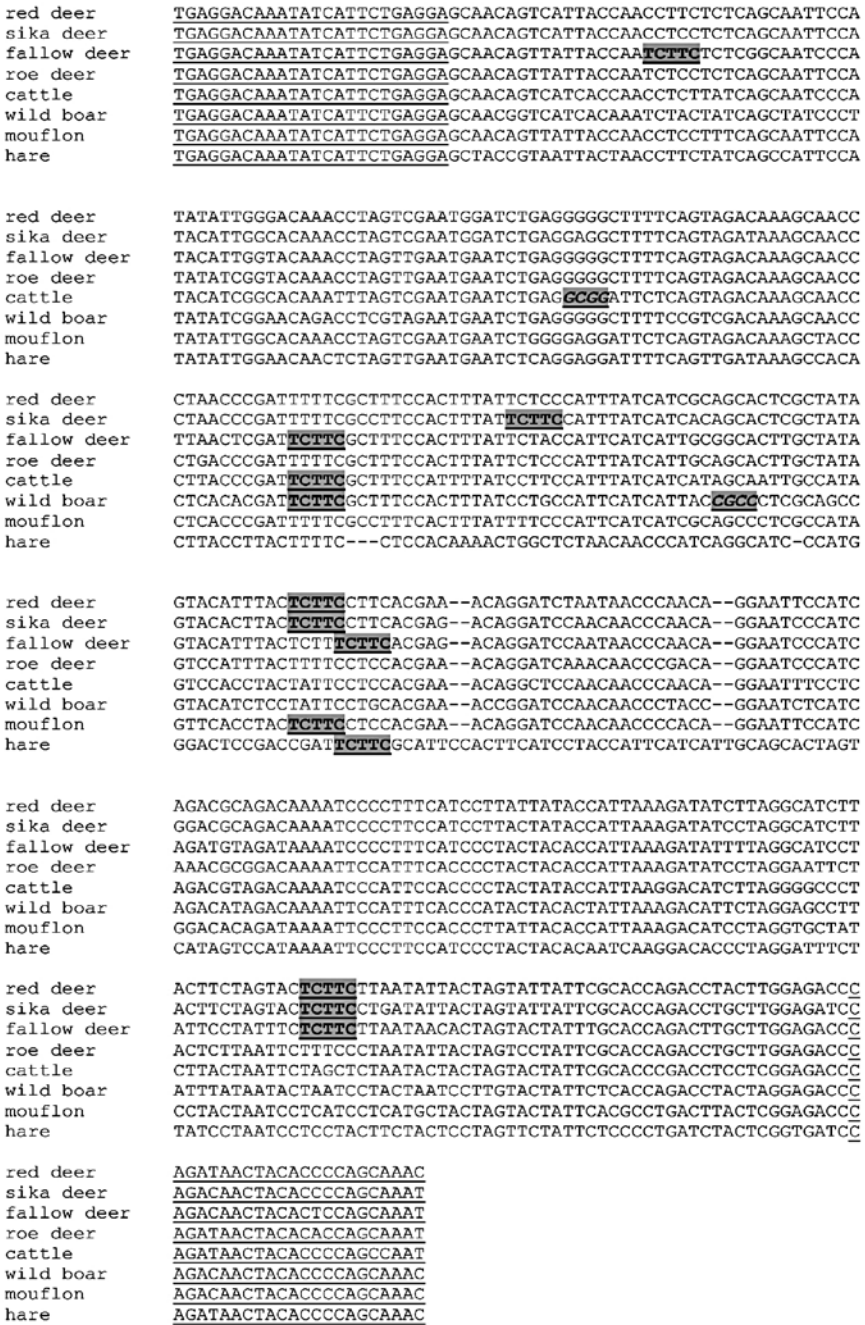

Fig. 1. Multiple alignments for *CYTB* gene sequences in the animals tested in this study. Underlined sequences indicate the primer-binding sites and bold underlined sequences indicate the the recognition sites against MboII (bold) and AciI (bold italic) restriction enzymes.

deer, roe deer, fallow deer, mouflon, wild boar and hare) and beef meat was successfully achieved in all the samples using RFLP analysis with the two restriction enzymes MboII and AciI.

The products of the digestion are shown in Figs 2–4, demonstrating that the generated bands correspond to the predicted sizes shown in Table 1, and are characteristic for all eight species. According to expectation, the restriction enzyme MboII generated fragments of 195 bp, 117 bp and 66 bp in red deer; 154 bp, 117 bp, 66 bp and 41 bp in sika deer; no fragments in roe deer; 113 bp, 89 bp, 66 bp, 64 bp and 46 bp in fallow deer; 195 bp and 183 bp in mouflon, and 243 bp and 135 bp in both wild boar and European hare. The wild boar and European hare had the same RFLP patterns using MboII as cattle whose meat may be mistaken for game meat. However, these species can be separated from each other based on the RFLPs produced by the AciI enzyme. AciI generated fragments of 208 bp and 170 bp in wild boar, a fragment of 378 bp in European hare, and 280 bp and 98 bp in cattle.

The PCR-RFLP studies were conducted on meat and meat juice processed at 120 °C, 100 °C, 70 °C and 40 °C for 10 min. The cooking process did not affect the RFLP pattern and the results were similar in meat samples processed and cooked at various temperatures (Figs 3–4).

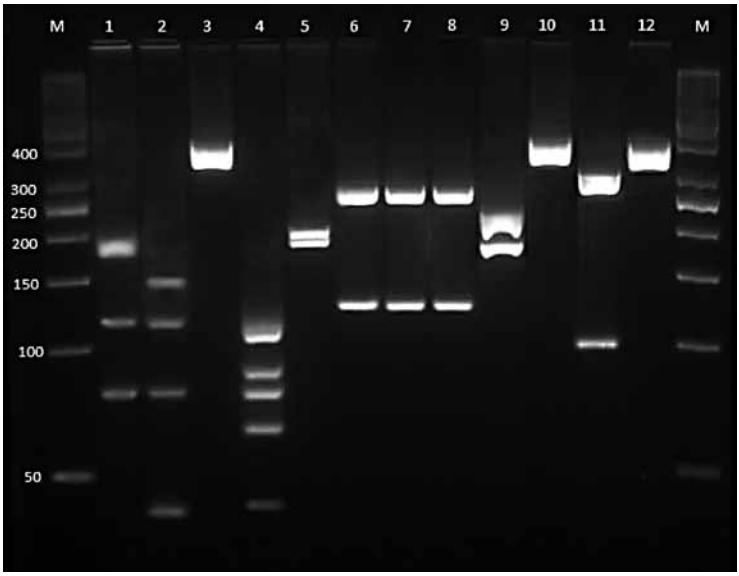

Fig. 2. Agarose gel electrophoresis of PCR-RFLP products of DNA extracted from raw meat. Polymerase chain reaction amplicons were subjected to restriction analysis with MboII in red deer (lane 1), sika dear (lane 2), roe deer (lane 3), fallow deer (lane 4), mouflon (lane 5), wild boar (lane 6), hare (lane 7), cattle (lane 8) and restriction analysis with AciI in wild boar (lane 9), hare (lane 10) and cattle (lane 11). The PCR product is shown in lane 12 and 50 bp DNA ladder in lane M.

Discussion

Authentication methods based on molecular biology provide much higher accuracy, sensitivity and reliability than the morphological or spectrometric techniques. Markers based on DNA analysis are the same for all the types of tissue and they are stable and not affected by age or physiological conditions (Lo and Shaw 2018). Mitochondrial DNA sequences are commonly used as genetic markers for species identification,

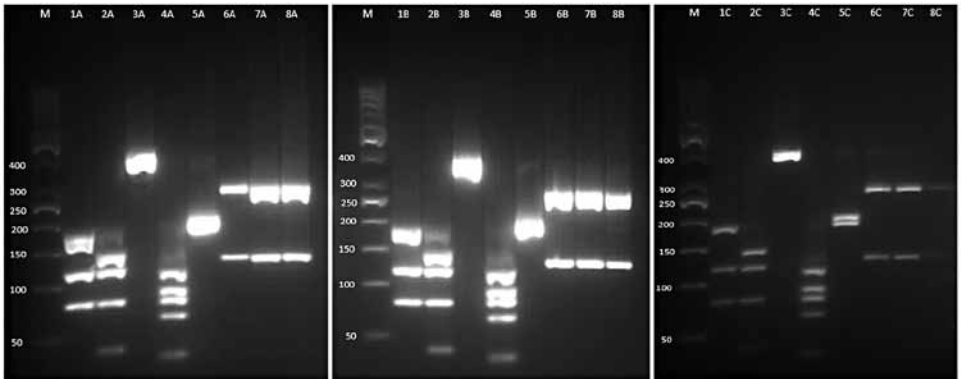

Fig. 3. Agarose gel electrophoresis of PCR-RFLP products of DNA extracted from meat cooked at 70 °C/10 min (lanes A), 100 °C/10 min (lanes B) and 120 °C/10 min (lanes C). Polymerase chain reaction amplicons were subjected to restriction analysis with MboII in red deer (lane 1), sika dear (lane 2), roe deer (lane 3), fallow deer (lane 4), mouflon (lane 5), wild boar (lane 6), cattle (lane 7) and hare (lane 8); 50 bp DNA ladder is shown in lane M.

Fig. 4. Agarose gel electrophoresis of PCR-RFLP products of DNA extracted from raw meat juice (lane 1–5), cooked meat juice at 70 °C/10 min (lane 6–10), cooked meat juice at 100 °C/10 min (lane 11–15). Polymerase chain reaction amplicons were subjected to restriction analysis with MboII in red deer (lane 1, 6, 11), sika dear (lane 2, 7, 12), roe deer (lane 3, 8, 13), fallow deer (lane 4, 9, 14), and wild boar (lane 5, 10, 15); 50 bp DNA ladder is shown in lane M.

because mtDNA is more conserved than nDNA and because it accumulates mutations at a × 10 higher rate. Thanks to the point mutations in mtDNA, even closely related species can be accurately discriminated after amplification of a section of mtDNA by an appropriate set of primers. Specific mtDNA sequences fitting for species identification have been published for many species (Kumar et al. 2014). The main advantage of mtDNA is its abundance in the samples – unlike nDNA, mtDNA is present in multiple copies in each cell and can be

isolated from samples with very low quantity and/or quality of DNA due to its degradation. Moreover, interpretation of the results is very simple thanks to the maternal inheritance of mtDNA. When sperm mtDNA enters an egg, it gets diluted or degraded, and only maternal mtDNA stays in the fertilized egg in most species (Yang et al. 2014). The common targets in mtDNA for species identification are represented by the D-loop region and genes like *CYTB* and *mt rRNA* (5 s, 12 s, 16 s, 18 s etc). The *CYTB* gene is a preferred target not only for taxonomic and phylogenetic studies, but also for meat speciation (Kumar et al. 2014).

Various molecular methods of meat authentication have been developed, all using the DNA polymorphism for distinguishing between species and allowing for sensitive, accurate and reliable results from analysis of even a trace amount of DNA. Foodstuffs usually undergo various processing steps during production, which may lead to fragmentation of DNA or even its removal (Lo and Shaw 2018). During the heat treatment of meat, high temperatures and prolonged treatment lead to a gradual DNA degradation into smaller fragments. The heat treatment duration and temperature were shown to strongly correlate with the DNA quantity and the length of amplicon. Amplification of shorter fragments should be preferred, as the amplicon size shows a strong correlation with PCR success (Şakalar et al. 2012; Lo and Shaw 2018).

Methods of molecular biology based on DNA analysis offer a rapid and sensitive testing that overcomes a number of the shortcomings of microscopic and ELISA examination. Methods such as PCR and its modifications (PCR-RFLP or real-time PCR) or sequencing are now used successfully for the identification of animal species. The PCR method and its modifications can be used to identify animal species in single-constituent and multi-constituent foodstuffs, in raw foodstuffs and in products that have been subjected to technological processing (the effect of mechanical processing, temperature or pressure in meat products and industrially produced feed that has been heat-processed to a temperature higher than 133 °C) in spite of the fact that the DNA they contain may be highly fragmented. The most reliable method of obtaining information from PCR products and for animal species identification is DNA sequencing. This method is, however, more expensive and time-consuming. In many cases, animal species can be accurately identified using the digestion of DNA fragments with particular restriction enzymes. PCR-RFLP is the most widely used method for identification of meat and meat products from mammals, poultry or fish. After traditional PCR the amplified products are digested via restriction enzymes which can be visualized by agarose or polyacrylamide gel electrophoresis. For each species there is a unique number of products of digestion of a specific length. The banding pattern is dependent on the restriction enzymes used for digestion. The method is very fast, simple, inexpensive and suitable for both raw and cooked animal tissues, albeit only pure and not mixed with other species (Farag et al. 2015; Alikord et al. 2018).

In the present study, we employed the PCR-RFLP assay for identification of meat from red deer (*Cervus elaphus*), sika deer (*Cervus nippon*), roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*), mouflon (*Ovis musimon*), wild boar (*Sus scrofa*), hare (*Lepus europaeus*) and cattle (*Bos taurus*) by amplifying a 378 bp region of the *CYTB* gene and subsequent digestion with MboII and AciI restriction enzymes. In the past, few investigators have used a similar approach to identifying game species. However, those studies used comparatively more restriction enzymes and focused only on a limited number of game species. The technique described in this study can be valuable for food fraud detection, as it provides sufficient discrimination to identify meat of the most common game species marketed in Central Europe and their possible substitution by one another or beef. Moreover, the method requires only basic laboratory equipment for molecular biology, which makes it more affordable and accessible compared to more advanced techniques and instruments. The improvement of this procedure lies in the use of only two restriction enzymes to differentiate eight game species in a two-step process of PCR amplification

followed by RFLP analysis. In a similar report, Fajardo et al. (2006) amplified a 712-bp fragment of *12S rRNA* gene followed by restriction digestion with four enzymes (MseI, MboII, BsIl, and ApoI) to identify meat from red deer, fallow deer, roe deer, cattle, sheep and goat. Pfeiffer et al. (2004) reported a method for differentiation of cattle, sheep, goat, roe deer and red deer meat by amplifying a 195 bp region of the mitochondrial *CYTB* gene using the Tsp509I enzyme. Also Gupta et al. (2008) developed a PCR-RFLP based method for identification of chital or spotted deer, hog deer, barking deer, sika deer, musk deer and sambar. They amplified a fragment of *12S rRNA* gene (440 bp) and subsequently PCR amplicon of these deer species were subjected to restriction digestion with RsaI, DdeI, BsrI and BstSFI endonucleases. Jiang et al. (2018) amplified a 263 bp length fragment from the *CYTB* gene followed by digestion with DdeI restriction endonuclease to differentiate the origin in antler velvet products of red deer and sika deer from other nine cervidae species.

In conclusion, molecular genetic methods based on DNA analysis offer a rapid and sensitive testing overcoming a number of the shortcomings of other methods used (e.g. microscopic examination and ELISA analysis). The PCR method and its various modifications and sequencing have been increasingly used over the last ten years for detection of the muscle tissue of animal species. The PCR method can be used successfully to identify and demonstrate the raw muscle tissue of selected animal species and to demonstrate the presence of food constituents that have been subjected to technological processing (mechanical processing, processing involving temperature or pressure, processing with the addition of further constituents, etc.) in which the DNA contained may be highly fragmented. The falsification or substitution of individual types of meat in meat products with a declared proportion of species muscle tissue in foodstuffs represents a multifactorial problem with implications for public health, religious, lifestyle and dietary customs, and the social and economic interests of the consumers. All this is also accompanied by an enormous impact in the media.

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