Polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) as a molecular discrimination tool for raw and heat-treated game and domestic animal meats

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Summary
Evaluation of two polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) methods for the identification of different kinds of meat samples was performed. Both PCR methods amplified a region of the gene encoding for the subunit 12S of mitochondrial ribosomal RNA. The first method amplified an approx. 714 bp long DNA fragment. The second PCR-RFLP method was newly developed and it was able to amplify a smaller DNA fragment (around 269 bp). The PCR products of both methods were digested with two different restrictases (*Ase*I and *Fat*I). The two methods allowed a clear discrimination of all 87 meat samples of different animal origin: beef (*Bos taurus*), goat (*Capra hircus*), sheep (*Ovis aries*), pork (*Sus scrofa domestica or Sus scrofa fera*), red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*) and fallow deer (*Dama dama*). The newly developed PCR assay permitted the identification of heat-treated meat samples.

Keywords
species identification; PCR-RFLP; 12S rRNA; meat

Identification of the species origin of meat is a non-detachable part of food regulatory control. The game meat products are often an object for deceitful labelling, because of different prices between game meats and meats from domestic animals. Meat from game animals is considered a delicacy which has a special flavour, lower fat and cholesterol contents, and it a higher level of polyunsaturated fatty acids. All the above mentioned features cause a high demand for game meat and also a higher price compared to the other kinds of meat [1]. Improper labelling might be a serious problem from several aspects. Apart from economic profit, incorrectly labelled food product may bring about health risks for individuals who may have specific food allergies, or problems to individuals with religious dietary restrictions.

Several analytical methods have been developed for meat species identification, which are mainly based on the analysis of proteins by electrophoretic [2–4], chromatographic [5] or immunochemical assays [6].

Because accurate and precise methods are required to establish fair labelling, more commonly methods based on DNA analysis are used. In recent years, many works concurred that polymerase chain reaction (PCR) is a promising and useful method for this kind of screening. The PCR approaches were mainly targeted to amplify the regions of cytochrome b gene [7–10] or 12S rRNA gene [11, 12]. Different approaches are used for species-specific identification. For example, one of the possible strategies is to identify the various types of meats in one sample by multiplex PCR employing a universal forward primer for all kinds of meats and a series of species-specific reverse primers. Therefore, the recognition of the different kinds of meat present in the sample is based on the different length of the amplicons [10, 11, 13].

Polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) is another PCR-based method, which combines amplification of a conserved DNA region with digestion of the PCR products by one or more restric-

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Tab. 1. PCR primers used.

Primer sequence	Reference
12S-FW; 5´ – GGTAAATCTCGTGCCAGCCA – 3´	[14]
12S-REV; 5´ – TCCAGTATGCTTACCTTGTTACGAC – 3´	[14]
12SF2; 5´ – CTTGCTAATACAGTCTATATA – 3´	This work
12SF3; 5´ – ACGTTAGGTCAAGGTGTAACC – 3´	This work

tion endonucleases. The result is that each meat species displays its own typical restriction profile [9, 14]. This method is also suitable to discriminate closely related species, for instance *Capreolus capreolus*, *Cervus elaphus* and *Dama dama*.

As considerably unfavourable are taken species-specific PCR methods oriented to each species separately [8]. In this case, a great number of PCR assays is necessary to determine the origin of meat. Methods of this kind are useful for the verification of results obtained by alternative assays or to distinguish closely related species [15].

The aim of our work was the evaluation of two different PCR-RFLP approaches at identification in a simple and rapid way the origin of different meat samples at a species level.

MATERIAL AND METHODS

Meat samples preparation and DNA extraction

The meat samples (beef, goat, sheep, pork, red deer, roe deer and fallow deer) were mainly obtained from various butchers' shops in Bratislava, Slovakia. In addition, some goat and sheep meat samples from breeding, and roe, red and fallow deer meat samples from hunters.

Prior to the extraction of DNA, the samples were washed with ethanol and deionized water. This treatment permitted the elimination of potential surface contaminants. DNA from meat samples was extracted by chaotropic solid-phase extraction using Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), which employed proprietary silica columns. The protocol for animal DNA extraction was used. Small meat pieces (from 25 mg to 30 mg) were mixed with 180 μl of lysis buffer ATL and 20 μl proteinase K, vortexed, and incubated at 56 °C until the tissue was completely lysed (3 h proved sufficient). After the incubation, 4 ml of RNase A (100 mg·ml^{-1}) were added to the mixture, mixed by vortexing, and incubated for 2 min at room temperature. Consequently, 200 μl of buffer AL included in the kit and 200 μl of ethanol (96–100%) were added to the sample, and mixed thoroughly by vortexing. The new mixture was pipetted into the DNeasy Mini Spin Column and the DNA washing procedure recommended by the manufacturer was performed. DNA was eluted with two consequent elution steps with 50 μl of AE buffer included in the kit.

The meat samples were also heat-treated; each kind of meat was cut to small pieces with a size of approximately 1.5 × 1.5 cm and weight from 3 g to 7 g, and then they were boiled for 10 min, 30 min and 60 min. After boiling treatment, DNA was extracted using the same method as described above.

PCR-RFLP

Two different PCR detection methods were employed and both were based on the amplification of a DNA fragment of the mitochondrial gene encoding for the ribosomal subunit 12S rRNA.

The first method was that proposed by Fajardo et al. [14] using primers 12S-FW and 12S-REV. The PCR mixture contained 15 pmol of each primer (Tab. 1), 200 μmol·l^{-1} of each dNTP, 1× PCR buffer, 2 mmol·l^{-1} of Mg^{2+}, 1.25 U Taq DNA polymerase (HotStarTaq, Qiagen) and 3 μl of the template DNA in the total reaction volume of 30 μl. The PCR programme included a hot start step at 95 °C for 15 min, 40 cycles comprising denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s and polymerization at 72 °C for 45 s, and a final extension at 72 °C for 8 min. Then, 10 μl of PCR product were digested by three restriction enzymes *Ase*I, *Fat*I and *Mse*I (Fermentas, St. Leon-Rot, Germany), in a total reaction volume of 20 μl. The fragments were separated by electrophoresis using 3% agarose gel in TBE buffer (containing 0.089 mol·l^{-1} Tris, 0.089 mol·l^{-1} borate and 0.002 mol·l^{-1} EDTA).

For the second PCR-RFLP detection method, two new forward primers (12SF2 and 12SF3) were designed. The new forward primers were combined with the reverse primer 12S-REV. The combination of the primers 12SF2 / 12S-REV amplified a fragment of 348-350 bp, while the combination 12SF3 / 12S-REV gave a PCR product of 267–269 bp.

The PCR assay with primers 12SF2/12S-REV was not able to amplify all the meat samples, therefore a suitable PCR identification method was developed using the pair of primers 12SF3/12S-REV. PCR was performed in a total volume of 30 μl containing 15 pmol of each primer (12SF3/12S-REV), 200 μmol·l^{-1} of each dNTP, 1× PCR buffer, 3 mmol·l^{-1} of Mg^{2+}, 1.25 U Taq DNA polymerase (HotStarTaq) and 3 μl of the template DNA solution. The PCR programme consisted of initial denaturation at 95 °C for 15 min, 40 cycles with a denaturation at 94 °C for 45 s, annealing at 53 °C for 45 s and polymerization at 72 °C for 45 s, and a final polymerization at 72 °C for 8 min. Ten μl of the PCR product were digested with the restriction endonucleases *Ase*I, *Fat*I and *Mse*I (Fermentas) for 5 h in a total reaction volume of 20 μl. The DNA fragments were separated by electrophoresis in a 12% polyacrylamide gel in TBE buffer and visualized under UV light after ethidium bromide staining.

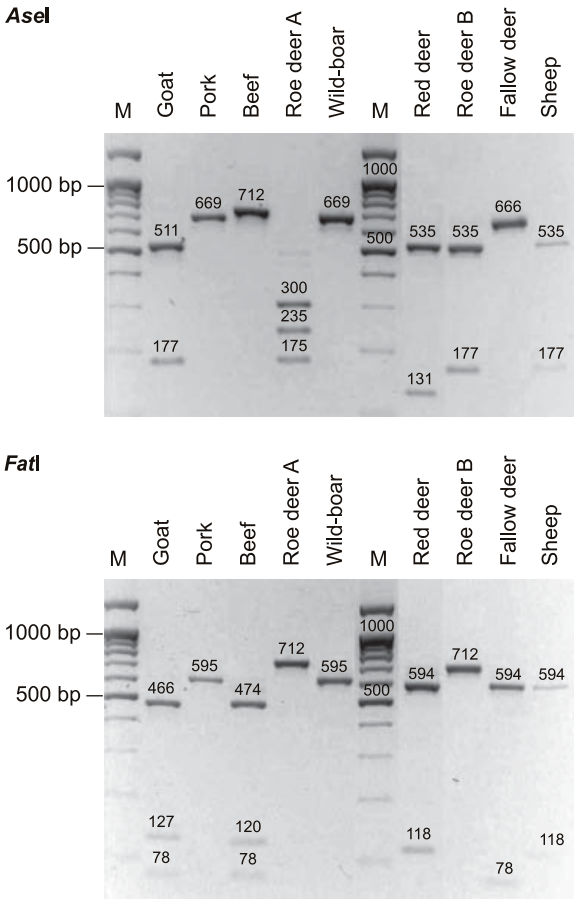

Fig. 1. Agarose gel electrophoresis of PCR products (712 bp) amplified with primers 12S-FW/12S-REV, digested with *Ase*I and *Fat*I.
M – molecular weight marker 100 bp (Promega, Madison, Wisconsin. USA). Numbers at fragments indicate estimated molecular weight.

RESULTS AND DISCUSSION

A new PCR-RFLP assay was developed in order to have a robust and rapid method for the identification of meat samples. The assay couples the amplification of a DNA fragment encoding the subunit 12S of mitochondrial ribosomal RNA to the consequent digestion by two different restriction endonucleases.

Our work was inspired by the study of Fajardo et al. [14], where the authors amplified a DNA fragment of 712–714 bp, and the consequent enzyme restriction permitted the recognition of different kinds of meat: beef, goat, sheep, pork, red deer, roe deer and fallow deer. We analysed the 714 bp DNA portion by Vector NTI 9 software (Invitrogen, Camarillo, California, USA) in order to find restriction enzymes suitable for a more accurate differentiation of the meat samples. This was achieved using the enzymes *Ase*I and *Fat*I.

In order to improve the method, two new forward primers (12SF2 and 12SF3; Tab. 1.) were designed. The new forward primers were combined with the reverse primer 12S-REV. The combination 12SF2/12S-REV amplified a fragment of 348–350 bp, but was not able to detect DNA from pork. The other combination 12SF3/12S-REV successfully amplified all the meat samples and produced a DNA fragment of 267–269 bp. The amplification of such a short fragment allowed also detection of DNA from heat-treated meat samples.

The two PCR-RFLP methods, according to Fajardo et al. [14] with primers 12S-FW/12S-REV and the new one using primers 12SF3/12S-REV, were compared. A total of 87 meat samples (20 of roe deer, 41 of red deer, 4 of fallow deer, 9 of pork, 4 of goat, 4 of sheep, 5 of beef) were tested by both methods. The PCR products of both methods were digested by the enzymes *Ase*I, *Fat*I and *Mse*I.

The restriction fragment profiles of the longer PCR product (712–714 bp) are shown in Fig. 1 (digestion with the enzymes *Ase*I and *Fat*I) and Fig. 2 (DNA restriction analysis with the enzyme *Mse*I). From these results is evident that, when the long fragment was amplified, it was better to use the *Mse*I restrictase or the enzyme combination *Ase*I / *Fat*I.

The new PCR assay, producing a shorter fragment of 267–269 bp, was convenient to couple

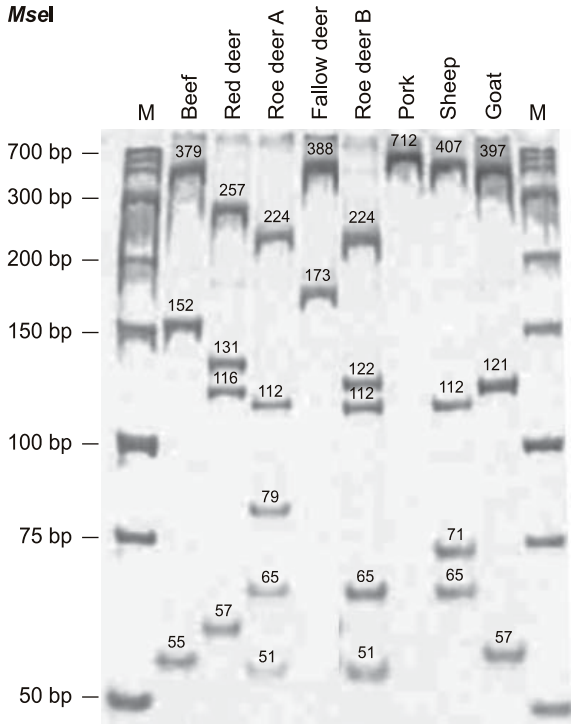

Fig. 2. Electrophoretic analysis (12% PAGE gel) of *Mse*I restriction fragments obtained from PCR products from 12S r RNA gene amplified with primers 12S-FW and 12S-REV (712 bp).
M – molecular weight marker O´GeneRuler 100–700 bp (Fermentas). Numbers at fragments indicate estimated molecular weight.

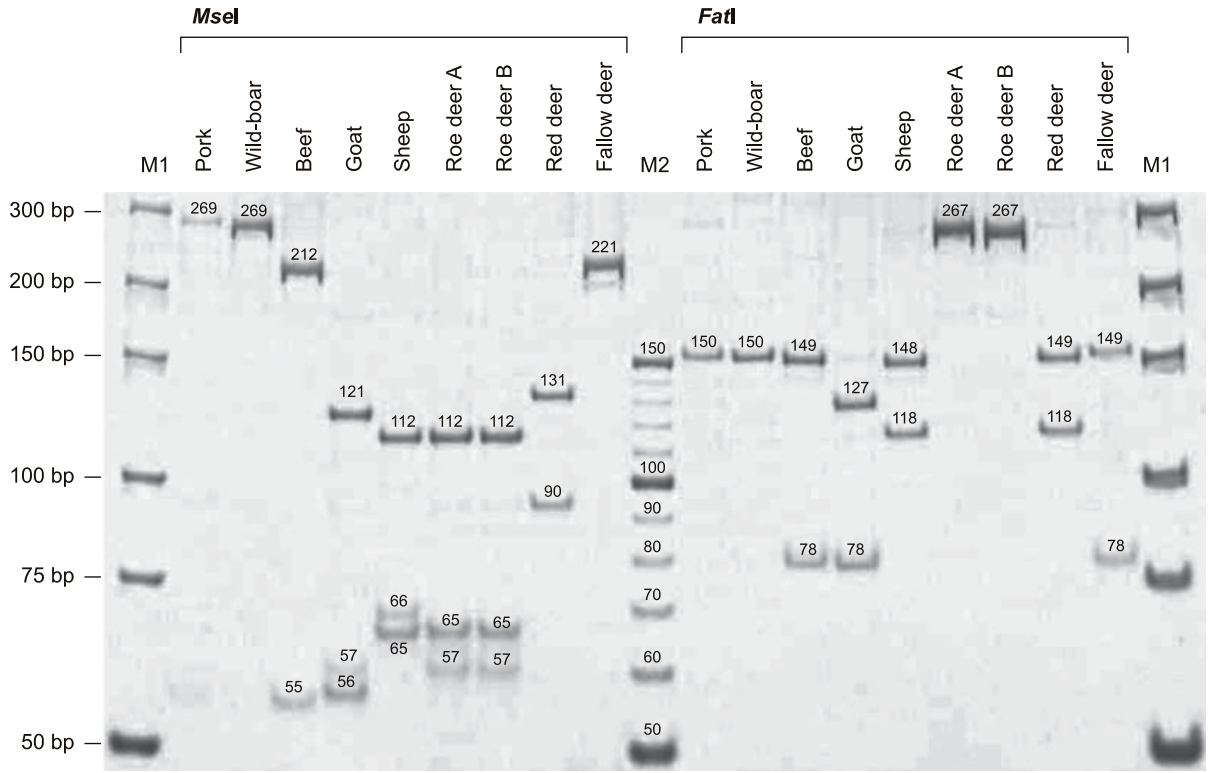

Fig. 3. Electrophoretic analysis (12% PAGE gel) of restriction fragments obtained from PCR products from 12S r RNA gene amplified with primers 12SF3 and 12S-REV.
M1 – molecular weight marker O´GeneRuler 100–300 bp (Fermentas), M2 – molecular weight marker O´RangeRuler 10 bp (Fermentas). Numbers at fragments indicate estimated molecular weight.

Tab. 2. Lengths of DNA fragments generated by digestion of PCR products from 12S r RNA gene with the indicated restriction enzymes.

	Pork	Beef	Goat	Sheep	Roe deer		Red deeer	Fallow deer
					A	B		
	n = 9	*n* = 5	*n* = 4	*n* = 4	*n*' = 18	*n*' = 2	*n* = 41	*n* = 4
PCR product 712–714 bp, primers 12S-FW and 12S-REV								
*Ase*I	669	712	511 177	535 177	300 235 175	535 177	535 131	666
*Fat*I	595	474 120 78	466 127 78	594 118	712	712	594 118	594 78
*Mse*I	712	379 152 55	397 121 57	407 112 71 65	224 112 79 65 51	224 122 112 65 51	257 131 116 57	388 173
PCR product 267–269 bp, primers 12SF3 and 12S-REV								
*Ase*I	269	267	177 66	177 89	177 90		131 90	221
*Fat*I	150	149 78	127 78	148 118	267		149 118	149 78
*Mse*I	269	212 55	121 57 56	112 66 65	112 65 57		131 90	221

When the roe deer samples were analysed by the PCR-RFLP proposed by FAJARDO et al. [14], two different restriction profiles (A and B) were displayed. By the newly PCR-RFLP method, only one characteristic restriction profile was obtained for all roe deer samples.
n – number of samples analysed for each species, *n'* – number of samples showing profile A or B.

to the restriction enzyme combination either *Ase*I / *Fat*I or *Mse*I / *Fat*I (Fig. 3). This new PCR-RFLP method also allowed the analysis of heat-treated meats. Meat samples belonging to each species were tested after different intervals of boiling (10 min, 30 min and 60 min). With these samples, PCR normally amplified and the restriction analysis gave the fragment profile typical for each kind of meat. Unfortunately, by this method it was not possible to distinguish between domestic pig and wild boar. For this purpose, it is necessary to use the method developed by KIJAS et al. [16], which is oriented to the gene encoding for the melanocortin receptor 1.

All the restriction profiles, obtained by the two PCR-RFLP methods, are summarized in Tab. 2.

FAJARDO et al. [14] warned in their work that it is not possible to use the restrictase *Mse*I because it finds too many restriction sites, which leads to profiles with a high number of fragments, and such kind of profiles is difficult to be well visualized on a gel. Therefore, they advised to use the combination of three restriction endonucleases (*Mbo*I, *Bsl*I and *Apo*I). This relevant aspect led us to find new restrictases able to reduce the number of fragments in the restriction profiles and, at the same time, to allow the rapid identification of meat samples. We propose the use of only two restriction enzymes: *Ase*I and *Fat*I.

Another disadvantage of the method proposed by FAJARDO et al. [14] was the length of the PCR product, which did not permit the amplification of heat-treated meat samples. It was shown by other works that, for food baked up to 220 °C, DNA fragments of up to 1 100 bp were amplified [17] and also fragmentation of DNA did not affect amplification of fragments of up to 565 bp, the amplification rates being roughly similar to those of raw samples, regardless of the boiling or baking conditions [18]. We overcame this negative feature by the development of a new PCR assay capable to amplify a small fragment and therefore able to detect the DNA from heat-treated meat samples.

CONCLUSION

Identification of meat samples at the species level was obtained by the application of two PCR-RFLP approaches. The method of FAJARDO

et al. [14] was improved through the use of only two restriction enzymes (*Ase*I and *Fat*I) instead of three. In addition, a new PCR-RFLP method was developed, which by the amplification of a small DNA fragment facilitates also identification of heat-treated meat samples.

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