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Use of Cytochrome b Polymorphism for Species Identification of Biological Material Derived from Cattle, Sheep, Goats, Roe Deer and Red Deer

Małgorzata NATONEK-WIŚNIEWSKA, Ewa SŁOTA, and Beata KALISZ

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The objective of this study was species identification of the following biological trace material: skin, blood stains, meat samples and jawbone with a tooth, which were the subject of expert opinion ordered by a court. The expert appraisement was conducted by an analysis of a cytochrome b fragment. The choice of mtDNA fragment for analysis was based on its conservation in mammals which enabled several farm and wild species to be identified with one pair of primers. The PCR product was differentiated by *Tsp509I* and *AluI* enzymes. Due to problems with amplification of roe deer DNA, primers specific to this species only, flanking a cytochrome b fragment (Y14951.1), were designed. On the basis of this analysis, it was concluded that the skin sample was derived from a goat, dried blood from a roe deer, the jawbone from cattle, and two meat samples from a roe deer and red deer. This method allowed rapid and efficient identification of several species of mammals using diverse biological material.

Key words: Cytochrome b, PCR-RFLP, species identification, expert opinion.

Małgorzata NATONEK-WIŚNIEWSKA, Ewa SŁOTA, Department of Immuno- and Cytogenetics of Animals, National Research Institute of Animal Production, Krakowska 1, 30-083 Balice, Poland.
E-mail: mnatonek@izoo.krakow.pl
Beata KALISZ, Department of Biotechnology, University of Agriculture, Mickiewicza 21, 31-120 Kraków, Poland.
E-mail: kaliszbeata@poczta.fm

Analysis of mitochondrial DNA (mtDNA) is a routine method used in forensic research for species identification. This method is useful in the case of poaching, road accidents involving animals, illegal trade in products derived from endangered species, and illegal food products. Tests can be made using trace material damaged by heat or environmental conditions, or those that naturally contain little genomic DNA. One of the mtDNA fragments used for this type of analysis is the gene encoding cytochrome b (BRANICKI *et al*. 2003; PARSON *et al*. 2000). The nucleotide sequence of this gene is highly conserved among mammalian species while being simultaneously species specific. These properties make it possible to amplify a conserved fragment using one pair of primers and then to distinguish among them using sequencing or restriction enzymes. This study presents the application of a cattle, sheep, goat, red deer and roe deer identification method for identifying biological trace material that were the subject of expert opinion ordered by a court.

Material and Methods

The following material was analyzed: dried blood spots on a leaf, a sample of skin with hair, two samples of meat, a jawbone with teeth (the subject of expert opinion ordered by a court) and a sample of ovine and roe deer blood (PCR/RFLP control). DNA was isolated from dried blood using the Sherlock AX kit (A&A Biotechnology), from samples of meat, blood and skin using the Wizard kit (Promega), and from teeth using NaOH/Tris-HCl (ZIMOWSKI *et al*. 2004).

For this analysis we used primers (pair A) flanking a fragment of the gene encoding cytochrome B (annealing temperature 54°C) and *Tsp 509I* restriction enzyme (PFEIFFER *et al*. 2004). The *AciI* enzyme (http://tools.neb.com/NEBcutter2/index.php)

was used for roe deer and goat. Moreover, primers for roe deer (pair B), limiting a 490-628 (139 bp) region of cytochrome b (Y14951.1): F:5'- ATCTGAGGGGGCTTTTCAGT-3 and R: 5'- CTGTCGGGTTGTTTGATCCT-3, were designed using *Primer3* software (ROZEN & SKALETSKY 1998). Prior to use, species specificity of the primers was analysed using FastPCR software (KALENDAR 2009), and PCR products were tested *in silico* with BLAST (ALTSCHUL *et al.* 1997) to detect possible conservation with other species. The primers were also checked experimentally for all analysed species and were shown to be specific for roe deer.

The results were analysed electrophoretically in a 2.5% agarose gel. The length of the separated DNA fragments was determined as the absolute number of base pairs (bp), by comparing with a DNA marker (Invitrogen) of known fragment lengths (25 bp DNA).

Results

The primers reported by Pfeiffer were used to amplify a fragment of 195 bp for DNA isolated from skin, blood stain, two meat slices, a jawbone with a tooth, and a control sample of ovine and roe deer blood (Fig. 1). The products obtained were treated with the *Tsp509I* enzyme. Restriction analysis (http://tools.neb.com/NEBcutter2/index.php) showed 20/54/121, 13/77/105 and 13/68/114 patterns characteristic for red deer, sheep and cattle, respectively. Both roe deer and goat PCR products had restriction sites at 182 bp (13/182 pattern). To distinguish between these two species, restrictase *AciI* was used. This enzyme cut the goat product into 72 and 123 bp fragments and did not cut the roe deer product. Comparison of the test results with *in silico* analysis demonstrated that the skin sample originated from a goat (Fig. 2/1; Fig. 3/2), dried blood from a roe deer (Fig. 2/5; Fig. 3/1), meat sample from a red deer (Fig. 2/4), and jawbone from cattle (Fig. 2/2). For our primers, specific for a roe deer, a product of 139 bp was obtained for dried blood, meat and control sample of roe deer blood (Fig. 4).

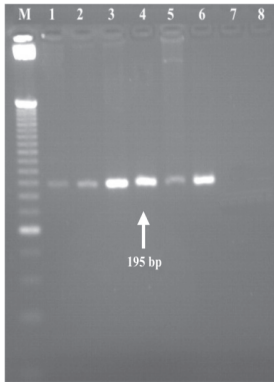

Fig. 1. Electrophoresis of PCR products (primers pair A). PCR products amplified from DNA isolated from dried blood (1), meat (2, 5), tooth (3), skin (4), ovine blood (6), and roe deer blood (7). Negative control (8). M – size marker.

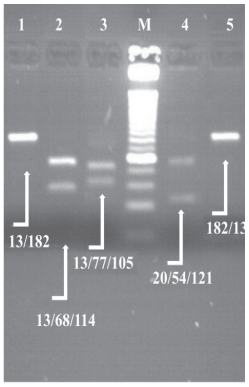

Fig. 2. *Tsp509I* restriction profile of PCR product from different tissues. 1- product 13/182 bp (skin); 2- product 13/68/114 bp (tooth); 3- product 13/77/105 bp (ovine blood); 4- product 20/54/121 bp (meat); 5- product 13/182 bp (dried blood). M – size marker.

Discussion

MtDNA has been used for forensic research since the 1990s (DAVIS 1998). The expert analysis

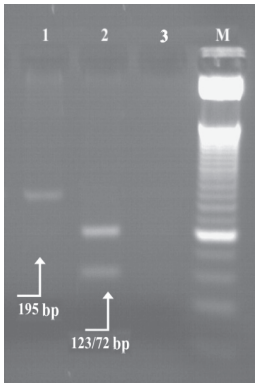

Fig. 3. *AciI* restriction profile of PCR products from dried blood (1)- product 195 bp and skin (2)- products 72/123 bp. Negative control (3). M – size marker.

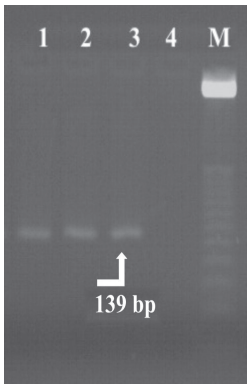

Fig. 4. Electrophoresis of PCR products (primer pair B) from meat (1), dried blood(2) and blood (3). Negative control of PCR reaction (4). M – size marker.

presented above successfully identified the species affiliation of biological trace material such as meat, fur, teeth and blood stains. Together with feathers, hair, bones and gastric content (PARSON *et al*. 2000; ZEHNER *et al*. 1998) these materials are most often used as reference material or evidence in court. Predicting the species from which DNA is present in trace material is difficult. In consideration of this, methods which identify several farm and wild species at the same time are very popular. Most often mtDNA fragments of 12SrRNA or cytochrome b are analysed. Because these two fragments show considerable homology among mammals, one pair of primers that flanks conservative fragments in several species can be designed. For example, a method for identification of the conservative region of 12SrRNA has been known for three years. It identifies cattle, donkey, goat and red deer in a multiplex reaction using a common forward primer complementary to the sequences of all studied species and different reverse primers to obtain a PCR product of different size for every analysed species (HA *et al*. 2006). This method circumvents the need for RFLP. However, because in this case the amount of forward primer may be difficult to assess, the use of common primers for all analysed species seems more beneficial than the multiplex reaction. FAJARDO *et al.* (2006) suggest identifying a 12SrRNA fragment of about 712 bp for red deer, fallow deer, roe deer, cattle, sheep and goats (FAJARDO *et al*. 2006). The species are then identified by cutting the PCR product with three restriction enzymes.

The presented expert report used a fragment of the gene encoding the mitochondrial cytochrome b. The available literature confirms that it is useful for distinguishing cattle, sheep, goats and buffalo (LANZILAO *et al*. 2005) or pigs, cattle, sheep, goats, horses and several species of wild animals (MEYER *et al*. 1995). Unfortunately, in Meyer's method different restriction enzymes are needed for almost every species, which is interesting from a cognitive point of view but can be a nuisance in routine research. Unlike the earlier analysis, our method enabled the identification of five species (cattle, sheep, goat, roe deer, red deer) using just two enzymes. Although the 13 bp band of PCR/RFLP products specific for both sheep and cattle was not visible after agarose electrophoresis, the two other remaining fragments (77 bp, 105 bp and 68 bp, 114 bp) for sheep and cattle, respectively, successfully distinguished both species. However two other problems appeared during the analysis. The first was the identical restriction pattern for the goat and roe deer product, which is why the *AciI* enzyme was used to distinguish between the two. The second problem was a very weak PCR product from DNA isolated from the second sample of meat. The amount of the product obtained was too small to enable restriction analysis. Also the amplification of the control sample of roe deer blood did not succeed. Based on these observations, it was conjectured that this meat may also originate from a roe deer and additional primers specific only to roe deer material were designed. As expected, a product of 139 bp was obtained for all roe deer material (blood stains, meat tissue, blood). The designed primers are specific to roe deer DNA sequence, therefore it is easier to amplify the desired fragment using these primers than those used for several species.

ZEHNER *et al*. (1998) emphasize that there is a risk of contamination samples with human DNA during expert analysis. There is also a possibility that samples can contain material from several different species which can be the result of e.g. the method of collecting material. The analysis of primers used for species identification with FastPCR software enables checking their specificity *in silico*. On the basis on this analysis, it was possible to predict that only mtDNA of roe deer would be amplified.

The application of the presented method allowed rapid and efficient identification of cattle, sheep, red deer, roe deer and goat using diverse biological materials. The smallest fragments of the PCR/RFLP products aren't visible in the gel. Their visualization would be possible in polyacrylamide gel. However, the analysis in agarose gel is quicker, cheaper and also well legible and therefore it is routinely used in our laboratory. In our laboratory we use this type of analysis for species identification of about 30 samples each year.

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