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Regular paper

The use of cytochrome *b* and ryanodine polymorphism to identify DNA of animal and human origin

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The aim of this study was to determine a match between DNA recovered from evidence material, such as knocked down red deer, and from comparative material in form of two brown traces on the bonnet of a car driven by a person suspected of knocking down the animal. The spots coming from the car provided no DNA profile, which questioned that they originated from a red deer and ruled out performance of a comparative DNA analysis. For this reason, the material obtained from the blood smear was analyzed for species identification. The method applied can discriminate between cattle, red deer and roe deer based on restriction analysis (Tsp509I) of PCR product (195 bp), obtained by amplifying a fragment of the cytochrome *b* coding gene. Because the obtained restriction profile confirmed the match with red deer DNA for one trace, and in the second case ruled out that the biological traces originated from the species mentioned above, the PCR products were subjected to sequencing. In both cases, 195 bp PCR products that were 98% homologous with red deer DNA sequence-NC_007704.2-trace1 and with the gene coding for the human ryanodine receptor-NC_008799.2-trace2. The quantity and quality of DNA obtained from the traces collected from the car bonnet did not allow confirmation of the involvement of a specific animal in the event, but the applied method made it possible to determine the species from which the obtained traces originated. Furthermore, the applied method, which was used earlier to determine cervine DNA, was successfully used to detect human DNA.

Key words: individual identification, STR, species identification, real-time PCR, sequencing

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Abbreviations: PCR, polymerase chain reaction; mtDNA, mitochondrial DNA; RFLP, restriction fragments length polymorphism; STR, short tandem repeats

INTRODUCTION

The study presented here dealt with individual and species identification of biological traces in the form of brown spots, which were the subject of examination initiated by law enforcement authorities. Analysis of mitochondrial DNA (mtDNA) and DNA profiling at microsatellite loci are routinely used in criminological studies of species identification (Galimberti *et al*., 2013; D'Amato *et al*., 2013) and individual identification. Traces in the form of blood spots are the most common biological traces found at the scene of an incident. mtDNA analysis can be performed using highly degraded traces (Jaiprakash, 2016). This analysis is very helpful and most often used when analyzing material whichis deficient in nuclear DNA due to its nature. This analysis uses various fragments of the mitochondrial genome as necessary. The cytochrome *b* coding gene is most often used to identify conservative fragments of several species (Galimberti *et al*., 2013; D'Amato *et al*., 2013). The nucleotide sequence of this gene shows high homology in the group of mammals, and at the same time it is species specific. These properties enable the conservative fragment to be amplified using one pair of primers, and later to distinguish them by using sequencing or restriction enzymes.

Microsatellite sequences (short tandem repeats, STR) have found wide application for individual identification; when automatically analyzed in DNA sequencers, they are currently the most effective and fastest methods for individual identification of both, farmed and wild animals (Socratous *et al*., 2009; Radko *et al*., 2014; Szabolcsi *et al*., 2014).

The study presented here dealt with individual and species identification of biological traces in the form of brown spots, which were the subject of judicial examination to determine whether the evidence material taken from the knocked down red deer matches the traces secured on the car of a person suspected of participating in a vehicle crash.

MATERIALS AND METHODS

The study material consisted of muscle tissue collected from a knocked down red deer (*Cervus elaphus*), as well as comparative traces in the form of brown spots collected from a vehicle driven by a person suspected of knocking down the animal. DNA from the tissue and from the spots was isolated using the Sherlock AX kit (A&A Biotechnology) according to the manufacturer's protocol. The concentration and quality of the obtained DNA were determined using a Nanodrop (NanoDrop 2000, Thermo Scientific, USA). All of the analyses were performed under sterile conditions (laminar flow cabinet, disposable gloves, disposable laboratory equipment, solution for DNA decontamination of laboratory surfaces).

Individual identification. The individual identification tests were conducted with 12 STR markers: BM1818, OarAE129, OarFCB5, OarFCB304, RM188, RT1, RT13, T26, T156, T193, T501, TGLA53. Multiplex PCR was carried out in a 12µl reaction mixture using Master Mix reagents (Qiagen) and primer sequences labeled with 6-Fam, VIC, NED and PET fluorescent dyes. The primer sequences were synthesized by *BIONOVO*.

The obtained PCR products were electrophoresed in a 3130xl sequencer on a 7% denaturing polyacrylamide gel (POP-7) in the presence of a GeneScan 500-LIZ length standard. The results of electrophoretic separation were analyzed by GeneMapper® Software 4.0.

Individual identification. The analysis of STR markers determined the complete DNA profile at 12 microsatellite loci (Table 2) for the evidence material. However, no DNA profile was obtained for the comparative material.

Species identification. PCR-RFLP. Primers flanking a fragment of the cytochrome *b* encoding gene were used for analysis. PCR was performed using universal primers for cattle, goat, pig, sheep, red deer and roe deer DNA (Pfeiffer *et al*., 2004), as well as HotStarTaq DNA Polymerase (Qiagen) at annealing temperature of 54°C for 32 cycles, using standard amounts of reagents recommended by Qiagen. The PCR product was fragmented with Tsp509I restriction enzyme detecting the AATT sequence, which was aimed to distinguish between DNA fragments from red deer, sheep, cattle, roe deer, and goat. Restriction patterns for different species are as follows: red deer – 20/54/121, sheep – 13/77/105, cattle – 13/68/114, roe deer and goat – 182 bp) (Pfeiffer *et al*., 2004).

The obtained results were analyzed by electrophoresis in a 3% agarose gel. The lengths of separated DNA fragments were determined as absolute base pair (bp) numbers, by comparing them with a DNA marker with known fragment lengths (25 bp DNA).

Sequencing. Both strands of PCR product were sequenced by ABI Prism BigDye Terminator v1.1 Cycle Sequencing Kit in the presence of 5 ng PCR. The sequencing products were isolated and subjected to capillary electrophoresis on an ABI 3130×1 sequencer (Applied Biosystems) using POP7 polymer (Applied Biosystems), the rapid sequencing module and a set of filters E. The separation was conducted for 30 min at 50°C and at 15 kV. The results were analyzed by Finch TV software (Geospiza, Inc).

Identification of PCR product. The sequence determined from the analyzed DNA was searched among sequences deposited in GenBank using the BLAST algorithm (Altschul *et al*., 1997). The product obtained was subjected to in-silico restriction analysis using the NEBcutter software (http://tools.neb.com/NEBcutter2/index.php).

Verification of human DNA identification method. To validate identification of human DNA with the primers used in the present study, PCR was performed using the same thermal profile and reagents kit with DNA isolated from human blood. The PCR product was subjected to sequencing.

RESULTS

DNA isolation from the evidence material (knocked down red deer) and the comparative material (biological traces secured from the vehicle of the person suspected of the incident) resulted in DNA with parameters presented in Table 1.

Individual identification

Analysis of STR markers enabled complete DNA profiles to be determined in 12 microsatellite loci (Table 2) for the evidence material. No DNA profile was obtained for the comparative material. Because of the doubts that the material obtained from the traces really comes from the red deer, it was decided to analyze it in direction of the species identification.

Table 1. DNA isolation parameters

DNA sample	c [ng/µl]	A260/280
Evidence material (knocked down red deer)	33.9	1.73
Comparative material (trace 1)	18.1	1.53
Comparative material (trace 2)	19.3	1.48

Species identification

Primers reported by Pfeifer amplified a 195 bp fragment for DNA isolated from brown spots. Restriction analysis with Tsp509I enzyme for the first trace (Fig. 1, RFLP sample) indicated a pattern that did not match any of the identifiable species (red deer, sheep, cattle, roe deer and goat) (Pfeiffer *et al*., 2004) and, for the second trace, a pattern matching red deer DNA (Fig. 1, RFLP 1 sample). To eliminate the problem, and further determin the origin of traces, the obtained PCR products were subjected to sequencing reactions and gave products 195 bp in size with the following sequence:

>trace 1

CGTACGCAACCTTACGATCAATCCCTAACAAATT-
AGGAGGCGTATTAGAACTCATATCTTCCTTTCT-
CATTCTAGCAGTTATTCCCATACTTCACA-
CATCTAAACAACAAAGGGTAATATTCCAGC-
CATTCAGTCACTGCCTATTCTGAATCCTAGTGGCT-
GACTTATTCACACTCACATGAATTGGAGGCCAGA

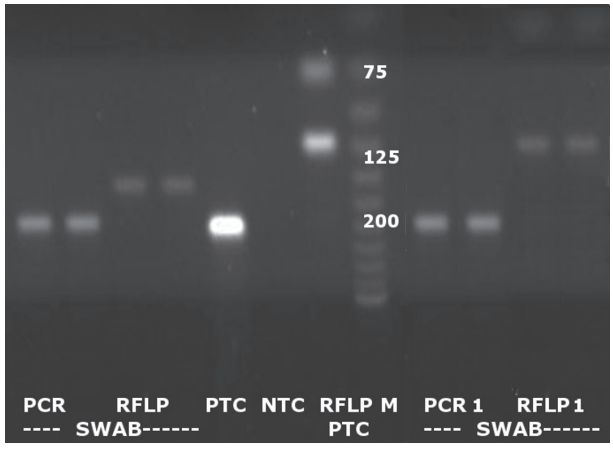

Figure 1. Results of PCR reaction and its restriction analysis. PCR-products of PCR reaction of trace 1, RFLP – its restriction analysis with Tsp509I; PTC – positive control for PCR for bovine DNA; NTC – negative control for PCR; RFLP PTC – RFLP for PTC; PCR1 – products of PCR reaction of trace 2; RFLP1 – its restriction analysis with Tsp509I. M – size marker 25 bp (Promega)

Table 2. DNA genotype obtained for the evidence material

loci	BM1818	OarAE129	OarFCB5	OarFCB304	RM188	RT1
evidence material	239/245	154/	85/101	139/143	127/	265/271
loci	T26	T156	T193	T501	RT13	TGLA53
evidence material	352/360	163/169	194/	248/260	296/	157/163

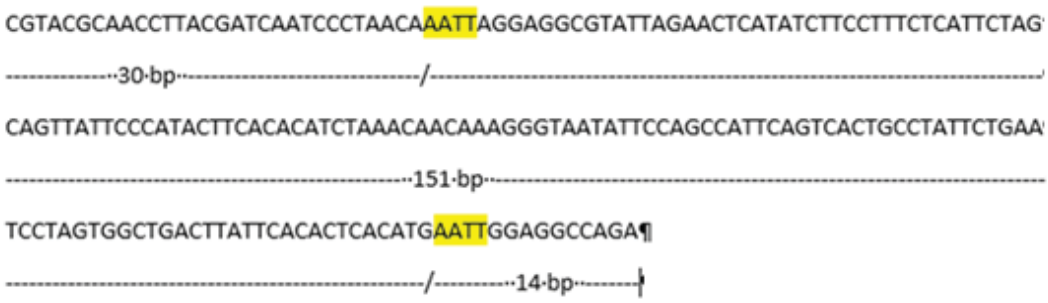

Figure 2. Result of sequencing of PCR product for trace 1

>trace 2

TGCGTACGCAATCTTACGATCAATTCCTAACAAAC-
TAGGAGGAGTACTAGCCCTAATCTCATCAATC-
CTAATCTTGATCCTTATACCCCTCCTCCACA-
CATCTAAACAACGCAGTATAATGTTCCGGCCATT-
TAGTCAATGCTTATTCTGAATCCTAGTAGCTGACCT-
ATTAACACTCACATGAATTGGAGGCCAG

Comparison with the sequences deposited in GenBank revealed 98% homology between the analyzed sequence (trace 1) and the human ryanodine receptor gene (NC_008799.2), and the same homology with a red deer mtDNA fragment – NC_007704.2 (trace 2). The sequencing result for trace 1 subjected to in-silico RFLP analysis was found for TSP509I, where the restriction pattern 30/151/14 is consistent with that in Fig. 1 (Fig. 1, RFLP sample) and Fig. 2. The analytical cycle is shown in Fig. 3.

Verification of human DNA identification method

The method applied resulted in a 195 bp amplification product obtained for human DNA. The sequencing reaction confirmed 100% consistency with the sequence that had been obtained from the analyzed trace 1.

DISCUSSION

The use of mtDNA in criminological analyses has been practiced since the 1990s (Davis, 1998). The current study successfully identified species affiliation of biological traces in the form of brown spots. The issues presented in this study are highly important because blood spots or gastric content are the most frequent evidence or comparative material in addition to feathers, hairs, bones, and stomach contents (Parson *et al.*, 2000; Zehner *et al.*, 1998).

Methods that use restriction enzymes are convenient because they allow testing for several species potentially present in the analyzed sample. What is more, compared to multiplex reactions, the use of restriction enzymes reduces the costs of analysis. On the other hand, Sanger sequencing can determine samples where other methods fail. This procedure determines DNA sequence present in the analyzed sample. The primers used can identify not only bovine, caprine, roe deer and red deer DNA, as shown by Pfeiffer, but also human DNA. This is a novel observation for the primers used, that is they allow the extension of the identification method also to human DNA. In animals, they anneal to mtDNA fragment in the cytochrome *b* section, and for human DNA

Figure 3. The analytical cycle used in this study.

this fragment is compatible with the DNA segment of chromosome 1. The available literature confirms suitability of cytochrome *b* to discriminate between cattle, sheep, goats, and buffaloes (Lanzilao *et al.*, 2005). In addition, it is often typed in barcoding to select sequences for species differentiation (Jaiprakash *et al.*, 2016). Typing such a fragment by sequencing and aligning the obtained sequence to that in GenBank database allows for a rapid, reliable, easy to automate and profitable identification of species. It should be highlighted that the method in which DNA is amplified with universal primers, and then individual species are distinguished by sequencing, is often applied in species identification tests. This type of tests is conducted not only in mammals, but also in fish (Sultana *et al.*, 2018). The application of the method described in this publication was the optimal choice because it allowed to identify the DNA of species that were of interest in the study of the described microtraces. Its advantage was in 100% selection of the marked species for the problem – we were interested in cervids and their analysis was enabled by the proposed method. The extension of the panel of identified species to human DNA is very useful in the analysis of biological traces because human microtraces very often accompany animal traces resulting from road accidents.

Forensic traces are often largely degraded, as a result of which their DNA is strongly fragmented, making amplification difficult. In such cases, amplification of short fragments is the only alternative. Universal primers should be used which minimize PCR deviations caused by variable mismatching of the matrix to different species, so as to ensure that all of the components are detected.

In the described case, the obtained DNA was of inadequate quality to determine the DNA profile, but it proved suitable for sequencing, which allowed us to confirm that one of the spots found on the bonnet comes from red deer. The procedure in respect of the second, human trace, was left for the Court do decide.

Declaration of interest statement

The authors declares that they have no relevant interest(s) to disclose.

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