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Methodology article

IMPROVED DNA-BASED IDENTIFICATION OF CERVIDAE SPECIES IN FORENSIC INVESTIGATIONS

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The main reasons for wildlife forensic research are animal poaching, illegal trade, and falsified game meat products. Small trace amounts, old and degraded materials present the most common samples in revealing criminal activities in this field. This is the reason why it is crucial to use adequate and reliable methods and samples to identify animal species killed outside the hunting season or species protected by law. In this study, different endpoint PCR and real-time PCR protocols were compared in the identification of three Cervidae species (*Capreolus capreolus, Cervus elaphus, Dama dama*) from old and damaged material found in an enclosed area where the animals were kept. From a total of 129 samples, end point PCR provided results for 119 samples, while real-time PCR was successful in all cases. Also, we created and tested a protocol for simultaneous analyses of different types of samples, which is of great importance as when the amplification is carried out simultaneously it is more cost efficient and speeds up the process.

Keywords: Cervidae species, forensics, molecular analysis, poaching

INTRODUCTION

The main reasons for veterinary forensic investigation regarding wildlife conservation are species identification, identification of geographic origin, individual and familiar identification [1]. When it comes to forensic research, the most common reason in the field of wildlife protection refers to animal poaching, illegal trade, and falsified game meat products. It is considered that wildlife crime and illegal trade of animals and animal products cause annual losses of about US$20 billion [2,3]. The main reason to identify species origin of meat products is to prevent incorrect labeling which

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represents commercial fraud and can cause serious health issues [4]. The other reason is illegal hunting of wild animals during the closed hunting season. Veterinary forensic medicine, which is related to the identification of wild animals, is a multidisciplinary science compared to human medicine and requires a much greater range of different methods and detection capabilities, depending on the nature of the allegations [5]. Forensic tests which refer to wild animals often require analysis of different types of trace material which is important for the control of game populations and protection of wildlife in a particular territory [6]. Commonly used animal biological samples for morphological and DNA analyses are: muscles, hair, skin, feces, urine, horns and internal organs [7]. Materials such as fur, feathers, wool or traces of animals (imprints, saliva, and blood) can also be used as evidence material [8,9].

There are a lot of different methods for species identification, but DNA-based techniques are quick, reliable and most precise [10,11]. End point PCR is a highly reliable method for DNA species identification from different types of material. A specific set of primers designed by Fajardo et al. [12] enables identification of different Cervidae species by amplifying one DNA segment (~712 bp) of the 12S rRNA gene. In the next step, differentiation of red deer, fallow deer and roe deer from other Cervidae has been enabled by developing primers that amplify a shorter region of 170-175 bp [12].

There are different approaches for identification of wildlife species depending on DNA target. Mitochondrial DNA (mtDNA) is often used as a genetic marker over nuclear DNA because mtDNA is easier to extract from highly degraded tissue [2]. DNA analysis is the key method for the elucidation of fraudulent activities related to meat products when physical and chemical features of meat have been changed [14]. These analyses are also of great importance for the disclosure of protected species from the biological trace material and may help in the identification of illegal hunting or other wildlife crimes [6,15]. It is worth to emphasize the importance of wild animals for public health, not only as a valued food source, but also because of their ecological, economic and socio-cultural impact. Hunting, as it is done nowadays, is not sustainable because forest areas become smaller, and the number of animals is also reduced [16]. This leads to extinction of different animal species and loss of biodiversity [17]. Although DNA analyses today are very accurate, molecular forensics of wildlife is still complex because there are no standardized laboratory protocols for species identification [13].

Common problems in DNA forensics are related to the size and age of the trace material, so in this study, samples different in amount and age were used for PCR species identification. End point PCR and real-time PCR were compared in order to elucidate their efficacy for obtaining reliable results from small and degraded trace material. A new PCR protocol for simultaneous analysis of several Cervidae species has been optimized in order to make this method time- and cost-efficient.

METHODS

Animals and samples collection

The samples were collected from three different even-toed ungulates: Roe deer (*Capreolus capreolus*), Red deer (*Cervus elaphus*) and Fallow deer (*Dama dama*). The samples included hair, feces, pelt, spleen, blood and buccal swabs. The material was sampled from the enclosed area of Belgrade and Palic zoos. Hair and feces were taken from the ground. Hair samples were divided into three groups: samples which included one hair, five hairs, and more than ten hairs. The tissue samples (pelt and spleen) were archival. Blood and buccal swabs were taken from zoos' archives (sampled during standard health control procedures). This study included a total of 129 samples: 45 samples of hair (five samples with one, five with five and five with more than 10 hairs, as totals to 15 hair samples from each of three species), nine samples of pelt (three from each species), 30 feces samples (10 from each species), 15 spleen, 15 blood and 15 buccal swab samples (five from each species). The samples were stored at -20°C pending analysis.

DNA extraction

DNA from the hair and pelt was extracted using the commercial set "KAPA Express Extract Kit" (Kapa Biosystems, USA) according to manufacturer's instructions, with the addition of 20 μl 1M DTT (dithiothreitol). DNA from the fecal samples was extracted using "ZR Fecal DNA MiniPrep" (Zymo Research, California, USA). From the spleen, blood and buccal swabs DNA was extracted using "GeneJET Genomic DNA Purification Kit" (Thermo Fisher Scientific, Waltham, MA). After extraction, DNA concentration of all samples was measured on BioSpec-nano spectrophotometer (Shimadzu, Kyoto, Japan) in order to compare DNA yield of samples.

End point PCR

All PCR amplifications were performed using a Multigene Gradient Thermal Cycler (Labnet International Inc., Edison, NJ, USA). PCR reaction mix was prepared using KAPA Taq Polymerase. DNA amplifications were carried out in a final volume of 25 μl containing: 14.9 μl of nuclease-free water, 2.5 μl of PCR-buffer, 0.5 μl of dNTP, 1.0 μl of each primer, 0.1 μl of Taq-polymerase and 5 μl of DNA template. To detect the specific regions, all samples were subjected to PCR using reverse primer (12SCERV-REV) common to all targeted species and one of three forward species-specific primers (Tab. 1): 12SCE-FW for Red deer, 12SDD-FW for Fallow deer or 12SCC-FW for Roe deer, designed to amplify specific fragment of approximately 170–175 bp in the 12S rRNA gene from each deer species.

The thermal protocol involved 2 min of initial denaturation at 93ºC, followed by 45 cycles of denaturation (93ºC, 30 sec), primer annealing (59 - 65ºC depending on

species-specific primers, 15 sec), extension (72ºC, 45 sec), and a final extension step at 72ºC for 5 min [4]. PCR products were visualized with UV light after staining the 2% agarose gel with Midori Green Direct (Nippon Genetics, Tokyo, Japan). A commercial O'RangeRulerTM 100bp DNA Ladder (Thermo Fisher Scientific, Waltham, MA) was used as size marker. Positive and negative controls were included in each run.

Table 1. DNA sequences of the primers used in this study (Fajardo et al., 2007)

Primers	Sequence (5' to 3')
12SCERV-REV	AAAGCACCGCCAAGTCCTT
12SCE-FW	CAAAAACATATAACGAAAGTAACTTTCCGACC
12SDD	FWTAAACAACGAAGGTAACCTTATCG
12SCC-FW	TGAAAATAGATAACGAAAGTAGCTTTGAACTA

In order to reduce costs and time, a protocol for simultaneous detection of three Cervidae species was tested. Because of different annealing temperatures (Ta) required for each species-specific primer, gradient PCR was employed to test the possibility of simultaneous detection of all species. This protocol provides different Ta in each column of the thermal block which enables to choose proper Ta for each species-specific primer.

Real-time PCR

Real-time quantitative PCR amplification was performed in a 20 µl reaction mixture using the KAPA SYBR® FAST Master Mix (2X) Universal (KAPA Biosystems, USA) according to manufacturer's instructions. The PCR reactions were carried out in a 36-well rotor using Rotor-Gene Q 5plex (Qiagen, Valencia, CA). For quantitative real-time PCR we modified the existing thermal protocol [18] as follows: 95ºC for 3 min followed by 45 cycles of 94 ºC for 20 s, 59 – 65 ºC for 30 s (depending on species) and 72 ºC for 30 s. Fluorescence was measured repeatedly in each cycle during the annealing step. This procedure was followed by a melt-curve dissociation analysis to confirm product size.

No ethical approval was obtained because this study did not include experiments on animals and involved only non-invasive procedures (collecting samples from the ground and usage of archival samples).

RESULTS AND DISCUSSION

Samples which present important evidence material in forensic research are usually degraded, contaminated with co-extracted DNA and contain different PCR inhibitors

which can cause a large number of problems during molecular analysis [19]. Although molecular genetic studies have shown that it is possible to extract DNA from old and small samples [20] it still remains a challenge to work with this kind of materials.

Using BioSpec-nano spectrophotometer we determined the presence of double-stranded DNA in all samples. From a single hair between 57-102 ng/μL of DNA was extracted, from five hairs 52-90 ng/μL and from more than ten hairs 52-105 ng/μL of DNA was extracted. The number of hair can cause the difference between DNA concentrations, and even different hairs from the same human head may contain a different amount of DNA [21]. Although studies have shown that fresh samples have higher quantities of DNA, it is possible to extract DNA from old and small hair samples [20] which are of great importance in forensic analysis as only poor material is usually available and crucial for the case. This is why it is important to successfully extract DNA from damaged or archives samples [22].

In this study, the classical (end point) PCR method provided results from a total of 119 (92 %) samples, while ten samples were negative (4 feces samples, 3 hair samples, 2 swab samples and 1 pelt sample). All blood and spleen samples were positive (Fig. 1). All samples that failed to be amplified by end point PCR were positive using real-time PCR (100%). Unsuccessful reactions of 4 feces samples (13.33% of all analyzed feces samples) are in correlation with the study of Huber et al. (2002) where 15% of feces samples failed. The two main issues with feces samples are low DNA yield and inhibition of polymerase [23], and the presence of a complex of polysaccharides which are PCR inhibitors in the feces [24]. In hair samples, there are also different kinds of inhibitors such as melanin and eumelanin [25,26]. Low DNA yield in hair samples is probably due to the very small quantities of DNA and additionally, even if sufficient quantities of DNA are extracted, the presence of inhibitors can cause negative PCR results [27].

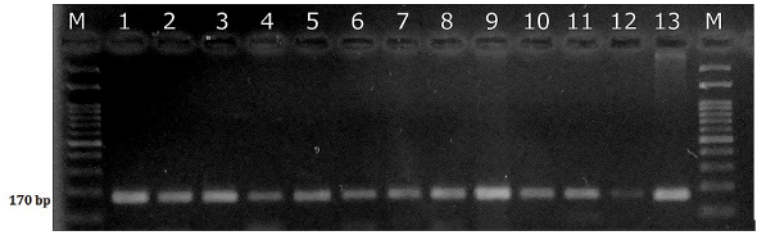

Figure 1. M – ladder; 1 – positive control for Red deer; 2 – hair (Red deer); 3 – positive control for Fallow deer; 4 – hair (Fallow deer); 5 – positive control for Roe deer; 6 – hair (Roe deer); 7 - blood (Red deer); 8 – blood (Fallow deer); 9 – blood (Roe deer); 10 – pelt (Red deer); 11 – feces (Roe deer); 12 – feces (Fallow deer); 13 – spleen (Red deer)

Real-time PCR is a highly sensitive method which allows testing of extremely small samples. It does not require post-PCR manipulation, so the probability of contamination is reduced. The success of real-time PCR in this study corresponds with literature data [18,28]. The role of end point PCR is also of great importance for

forensic purposes, as it appeared as suitable as real-time PCR in analyses such as those where body fluids from forensic stains are examined [29].

In this study, the protocol for simultaneous analysis of samples at different annealing temperatures was made using Multigene Gradient Thermal Cycler. Positive results for Roe deer and Fallow deer were obtained on the same annealing temperatures, which gives a huge contribution in molecular analysis (Tab.2). This protocol can save time and costs because it offers the possibility to differentiate more than one species at the same temperature and at the same time.

Table 2. Same annealing temperatures for Roe deer and Fallow deer provide the possibility to identify this species simultaneously using a Multigene Gradient Thermal Cycler

T (°C)	59	59.3	59.6	60.4	61.3	62.2	62.6	63.3	64.0	64.6	64.8	65
Roe deer	+	+	+	+	+	-	-	-	-	-	-	-
Fallow deer	+	+	+	+	+	-	-	-	-	-	-	-
Red deer	-	-	-	-	-	-	-	-	+	+	+	+

The results obtained by real-time PCR suggest that the number of hairs in the sample is irrelevant for DNA analysis, as similar results were obtained from one, five and more than ten hairs (Fig. 2). Previous studies [30] evidenced 10-50 dog hairs as sufficient for obtaining PCR results in 91% of cases, but less than ten hairs had been a big issue in forensic research. However, as already mentioned, a lot of PCR inhibitors are present in the hair, so more hairs contain a larger amount of inhibitors. For that reason, the effectiveness of DNA amplification is not directly proportional to the number of hairs in a sample. This result is of great importance because it proves that just one hair is enough for resolving the case.

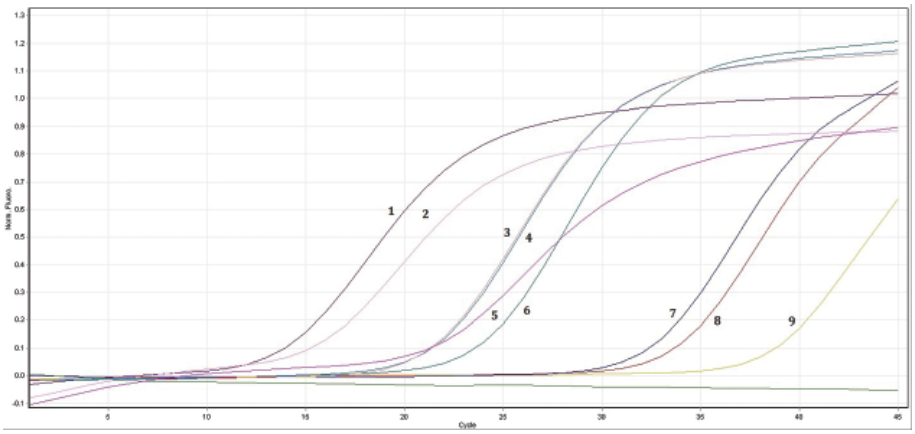

Figure 2. Amplification plots of samples tested on real-time PCR (1, 5, 7 – samples with one hair; 4, 6, 9 – samples with five hairs; 2, 3, 8 – samples with ten hairs)

CONCLUSIONS

Molecular methods enable species identification of animals important for wildlife protection. Adequate protocols for DNA extraction can provide results from different types of samples (hair, pelt, feces, buccal swabs, blood, and spleen) which is of great importance in forensic investigation, due to the fact that evidential material is often very limited and there is no choice of matrices. The here described procedure for DNA-based analysis has shown that the number of hairs is not of great importance for providing satisfactory results. The main contribution of this study is the development of a method for simultaneous detection of different species at the same time. Beside time saving, such methodology enables reducing the analysis costs. Finally, this study confirms that real-time PCR is the most promising technique because of its precision and speed of obtaining results from small and demanding samples, especially when end point PCR is unsuccessful.

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Authors' contributions

DD and GU conceived the study, designed and participated in manuscript writing. NK participated in manuscript writing and its critical revising. DD and VM carried out the experiment and participated in manuscript writing. NV coordinated experiment performance and made substantial contributions to interpretation of data. SZ made substantial contribution to acquisition, analysis and interpretation of data and coordinated experiment performance. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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DNK IDENTIFIKACIJA VRSTA IZ FAMILIJE *CERVIDAE* U FORENZIČKIM ISPITIVANJIMA KORIŠĆENJEM UNAPREĐENOG PCR PROTOKOLA

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Najvažniji razlozi za forenzička ispitivanja u oblasti divljih životinja jesu krivolov, ilegalna trgovina i falsifikovanje proizvoda poreklom od divljih životinja. Tragovi koji se koriste za otkrivanje ovih kriminalnih radnji najčešće su stari, dostupni u maloj količini i degradirani. Ovo je razlog zašto je ključno koristiti adekvatne i pouzdane metode i uzorke za identifikaciju životinjskih vrsta koje su ubijene van sezone lova ili vrsta koje su tokom cele godine zaštićene od lova. U ovom istraživanju vršeno je

poređenje protokola za klasični PCR i real-time PCR u identifikaciji tri vrste iz familije *Cervidae* (*Capreolus capreolus*, *Cervus elaphus*, *Dama dama*) iz starih i oštećenih materijala koji su pronađeni u zatvorenim prostorima u kojim su životinje živele. Od ukupno 129 uzoraka, klasičan PCR je bio uspešan u dobijanju rezultata iz 119 uzoraka, dok je real-time PCR bio uspešan u svim slučajevima. Takođe, kreiran je i testiran novi protokol za simultanu analizu različitih uzoraka, koji je od velikog značaja, jer se amplifikacija odvija u isto vreme, što u velikoj meri štedi vreme i novac.