ACTA ABP BIOCHIMICA POLONICA

Vol. 64, No 4/2017
705–708
https://doi.org/10.18388/abp.2017_2304

Regular paper

Evaluation of the suitability of mitochondrial DNA for species identification of microtraces and forensic traces

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The objective of the study was to demonstrate how mitochondrial DNA (mtDNA) can be used to determine the species origin of animal microtraces. The study included pieces of cat and dog hair without the root, a fragment of cooked chicken bone (0.1g), three goose down samples (0.028 g), a pork swab, a pork scratching (5×5×5 mm), and pork lard (0.22 g). DNA was isolated from all of these samples using the method appropriate for the particular source material. The extracts had DNA concentration exceeding 5.4 ng/µl with $A_{260/280}$ purity range of 1.14–1.88. Next, the samples were subjected to PCR and real-time PCR with species-specific primers and primers complementary to mitochondrial DNA (mtDNA). Control reactions based on the amplification of eukaryotic-specific fragment (18S rRNA) were additionally performed. PCR and real-time PCR products for detection of species-specific mtDNA were obtained for all templates, whereas during the detection of eukaryote DNA no product was obtained for dog and cat hair only. The poor quality of the obtained DNA did not prevent the analysis. The results showed that mitochondrial DNA is suitable for identification of small or highly processed samples, in which genomic DNA often cannot be analyzed.

Key words: biological traces, forensic DNA analysis, species identification of forensic DNA, species identification of biological traces, mtDNA

Received: 17 August, 2017; revised: 17 November, 2017; accepted: 24 November, 2017; available on-line: 13 December, 2017

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Abbreviations: mtDNA, mitochondrial DNA; PCR, polymerase chain reaction; PTC, positive control; NTC, negative controls; NFQ-MGB, nonfluorescent quencher-minor groove binder

INTRODUCTION

Degradation of biological material due to heat treatment, exposure to the weather, or other factors, can cause many problems in species identification. Similar difficulties are often encountered when testing material that is naturally poor in nuclear DNA. In cases like these it seems advisable to analyze mitochondrial DNA (mtDNA). This analysis can be performed in both farmed and wild animals. It provides valuable information about the possibility methods of analyzing raw, processed, natural, or degraded tissues. Hard, soft and liquid samples can be used. Methods that detect animal tissues at 0.1% are now routinely used. However, more detailed determinations can be hindered by insufficient amount of the analyzed template. At the same time it is known that this type of analysis is useful for testing forensic traces (swabs, hair, cooked bones) in the investigations related to poaching, road accidents involving animals, illegal trade of products originating from endangered species or potentially allergenic products (hair, down), or trade of illegal pseudo-medical products (e.g. lard from domestic animals). Therefore, it is appropriate to:

– determine the possible use of mtDNA for species identification of biological microtraces of animal origin.

– designate the method of analysis containing the type of DNA isolation/PCR/qPCR

– test the repeatability of the used method.

MATERIALS AND METHODS

The following criteria were used when choosing the sample types:

1) non-commercial samples, the analysis of which is in high demand;

2) samples with limited amount of nuclear DNA or samples with degraded DNA.

The study used pieces of hair without the root of four cats and dogs, a fragments of three cooked chicken bones from which samples of around 0.1 g were prepared, down from three geese (0.028 g), a pork swab, scratching (5×5×5 mm) and lard (0.22 g) from which two analytical samples were prepared, as well as pork swabs. DNA was extracted from these samples using the methods appropriate for the particular source (Table 2). The obtained DNA was subjected to PCR and Real-Time PCR (DNA from goose down and pork scratching – AX Food) according to the presented methodology (Table 1 – references) using primers flanking the region specific for the analyzed species as well as primers universal for eukaryotes (Table 1). Positive (PTC) and negative controls (NTC) were additionally run for each species. PTC controls were commercial DNA samples of different species with components declared by the manufacturer or samples from proficiency tests. The PCR reaction was performed using HotStarTaq DNA Polymerase (Qiagen), and in real-time PCR Quantum Probe (Syngen) and a NFQ-MGB (nonfluorescent quencher-minor groove binder) probe (ThermoFisher) were used.

The results were analyzed electrophoretically in 3% agarose gel. The length of separated DNA fragments was determined as the absolute number of base pairs (bp), by comparison with a DNA marker with known length of the fragments (25 bp DNA – Promega).

RESULTS AND DISCUSSION

The parameters of obtained DNA isolates (Table 2) showed that the chosen extraction methods were efficient. The kits produced samples with DNA concentration exceeding 5.4 ng/µl in all the templates and with $A_{260/280}$ purity ranging between 1.14 and 1.92.

Table 1. Methods applied and the fragment size of amplified nucleic acids

Species	Primers/Probes 5′	Amplicon size [bp]	Amplified fragment	Method	References
cat	F: atctcagccttagcaggagtacac R: tggatcggagaattgcgtatgcga	286	Cyt B	PCR	Natonek-Wiśniewska, 2009
dog	F: 5′-cgtcgtgcattaatggtttg R: 5′-gtttctcgaggcatggtgat	163	d-loop	PCR	Natonek-Wiśniewska, Krzyścin 2012
chicken	F:aacctcctccagcggataataat R: tttgttggtggctgcttgaa	66	16SrRNA	PCR	Natonek-Wiśniewska *et al*., 2013
goose	F: tcaaggtatagcctatggagtcga R:ctaaatccgccttccagaaatg	98	12SrRNA	Real-time PCR	Pegels *et al*., 2012
	P: atagggcacacggaaa				author's own data
	F: acaggacataccctaaca R:gtccaggcttagattgtg	387	d-loop	PCR	Hou *et al*., 2015
pig	F: gacatcggcaccctgtacct R: gctcaaggcagtgcccacta P: ctatttggtgcctgagcag	53	COX1	Real-time PCR	Natonek-Wiśniewska, Krzyścin, 2015
	F: ggagcagtgttcgccattat R: tttttgctcatgcttggttg	85	COX1	PCR	Natonek-Wiśniewska *et al*., 2013
eukaryotes	F:agcctgcggcttaatttgac R:caactaagaacggccatgca	120	18SrRNA	PCR	López-Andreo *et al*., 2005

The proteinase K method, which was used to isolate hair DNA, is one of the most common methods employed for this type of templates (Iniesta *et al*., 2013; Muñoz-Madrid *et al*., 2013; Pfeiffer *et al*., 2004). The obtained DNA amount ranging from 108 to 144 ng (in 20 µl of extract) was objectively small, but much higher than reported in the literature; by way of comparison, the literature on the subject mentions that 0.4 ng was extracted from an elephant's hair (Heywood *et al*., 2003).

Likewise, obtaining satisfactory DNA concentrations from a scratching and lard was not difficult, and the amount of DNA obtained with the Wizard kit was almost twice as high as for AX Food. However, comparison of purity spoke in favor of the latter kit. It should be stated that the obtained results confirmed the suitability of both kits for isolation from animal fats, which is consistent with the findings of other workers (Di Pinto *et al*., 2007), even though they used greater amounts of the source material for the isolation. The kits used in the present study allowed obtaining more extract compared to the other known kits (Aida *et al*., 2005; Mafra *et al*., 2008; Vietina *et al*., 2013).

For both templates under discussion, regardless of the isolation method, the obtained DNA extracts were contaminated with polysaccharides ($A_{260/230}$<1.4) and RNA ($A_{260/280}$<1.4).

Unlike the hair, DNA obtained from down was not only in sufficient quantity, but also of good quality. DNA was obtained from very small amounts of the source material that have never been reported in the literature (Bello *et al*., 2001; Yannic *et al*., 2011), which suggests that the kit used for DNA isolation is extremely efficient.

The next templates were a cooked chicken bone and a pork swab. In both cases, unlike hair and fat, DNA of very good quality was obtained. The amount of analyzed bone tissue was indicative of its high efficiency in DNA isolation, with as little as 10 mg of the tissue allowing effective identification.

Although the DNA isolates differed in quality, these extracts were further successfully analyzed with PCR (Fig. 1). As a result, species-specific products were obtained for the dog (1–4), chicken (5–7), goose (8–10), pig (11–14) and cat (15–18) and their size was typical

Table 2. Amplified templates, extraction methods, and parameters of the obtained DNA

Species	Template	Method	DNA		
			c [ng/µl]	$A_{260/280}$	$A_{260/230}$
cat	hair	Sherlock AX*	5.7–7.2	1.46–1.52	1.34–1.55
dog	hair	Sherlock AX*	5.4–6.3	1.60–1.68	1.36–1.48
chicken	cooked bone	Sherlock AX*	63.8–66.0	1.84–1.92	1.8–1.92
goose	down	Sherlock AX*	55.2–65.3	1.88–1.92	1.80–1.86
pig	swab	Sherlock AX*	15.7	1.49	1.40
	scratching	AX Food**	64.7	1.18	0.86
	scratching	Wizard***	80	1.16	0.50
	lard	AX Food**	44.0	1.29	0.7
	lard	Wizard***	75.5	1.14	0.29

*Sherlock AX (A&A Biotechnology) **AX Food (A&A Biotechnology) ***Wizard Genomic DNA Purification Kit (Promega)

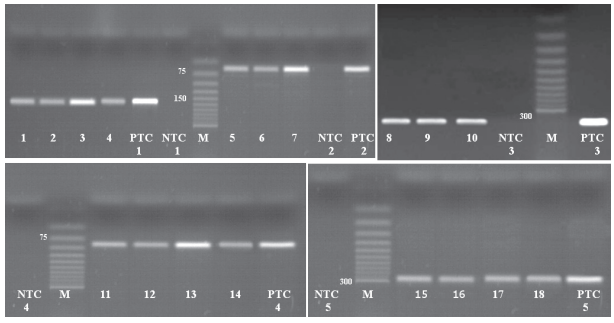

Figure 1. Result of species identification.
PCR product specific for the hair of dog (1–4), cooked bone of chicken (5–7), down of goose (8-10), swab of pig (11), lard – Ax Food (12), lard – Wizard (13), scratching – Ax Food (14), scratching – Wizard (14), hair of cat (15–18). PCR controls – positive for particular species (PTC1–PTC5). negative (NTC1–NTC5). M – 25 bp marker (Promega)

for the particular species (Table 2). For all the templates, band intensities were sufficient to interpret the results.

In addition, all the DNA isolates were examined to detect the DNA of eukaryotes. As a result, PCR products were obtained for all tissues (2–8) except hair (cat and dog, 1 and 9, respectively) (Fig. 2). The lack of PCR product in this cases was due to the fact that mtDNA predominates in the hair cores, while the 18S subunit belongs to the cytoplasmic rRNA.

The real-time PCR reaction resulted in a species-specific product amplification for both goose down (Fig. 3A) and pork swap and scratching (Fig. 3B). The ct from analysis of down for different animals differed very little – up to two cycles. For pig traces, the results were also reproducible, the amplification time for all tested samples was similar. The difference for individual DNA isolation from scratching was 1.5 cycles, and about 5 cycles for the DNA of the swab.

The mtDNA of the examined templates, regardless of quality and quantity, allowed obtaining a PCR product. Both long products (dog, cat, goose – PCR) and short products (pig, chicken, goose – real-time PCR) were obtained. Good amplification was due to the presence of mtDNA in many million copies per cell as well as its high resistance to unfavorable external conditions. Meanwhile, no PCR product could be obtained by amplification of the fragment of a gene encoding ribosomal RNA from tissues with limited amounts of DNA.

Conclusively, the present results confirmed the suitability of mitochondrial DNA for analysis of low DNA content samples due to their small size or highly processing, in which genomic DNA often cannot be analyzed. An analytical pathway including isolation with AX Food, Scherlock AX (A & A Biotechnology), Wizard Genomic DNA Purification Kit (Promega), and primers and probes was effective for sensitive and specific species identification. All presented methods were reproducible.

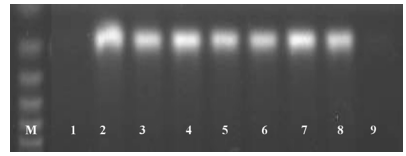

Figure 2. Result of eukaryote DNA presence.
PCR product for DNA from cat hair (1), goose down (2), pig blood smear (3), lard – Ax Food (4), lard – Wizard (5), scratching – Ax Food (6), scratching – Wizard (7), cooked chicken bone (8), dog hair (9). M – 25 bp marker (Promega)

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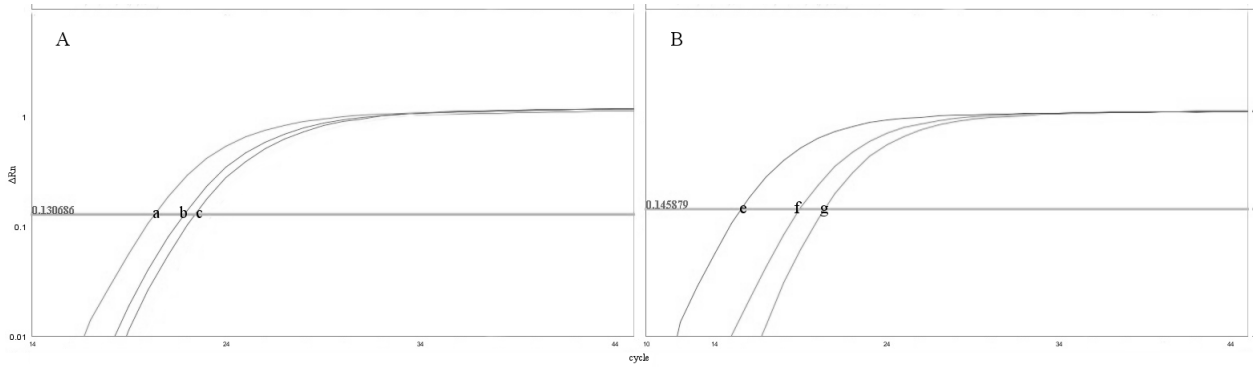

Figure 3. Amplification graph for three goose down (Fig. 3A) samples from different individuals (a–c) and pork (Fig. 3B) swab (e), scratching – AxFood (f), scratching – Wizard Food (g) ΔR_n – fluorescence delta. cycle – reaction cycle.

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