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Forensic Evidence Based on mtDNA from Dog and Wolf Hairs*

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ABSTRACT: In six forensic cases involving murder, bank robbery, theft and poaching, evidence material comprising shed hairs supposedly originating from dogs or wolves was analyzed by mitochondrial (mt) DNA sequencing. A 79 bp segment of the control region was amplified, sequenced, and compared with an established database of the domestic dog and wolf populations. In three murder cases exclusions of all eight suspects could be made. Furthermore, two of the murders could be linked to each other by a rare sequence variant, and the breed of the dog was indicated. In a theft case and a bank robbery a link could be established between the evidence material and the suspects. In a case of suspected wolf poaching, it could be established that the evidential material was of dog rather than wolf origin. We conclude that single hairs from common pets are suitable for DNA analysis and that the described method has proved to be a valuable tool for forensic investigations.

KEYWORDS: forensic science, DNA typing, dog, wolf, *Canis familiaris*, mitochondrial DNA, sequence analysis, hair

Shed human hairs are one of the most commonly secured biological evidence materials at crime scenes. Hairs from dogs and other animals are also frequently found. However, the potential of this source of evidence so far has not been fully exploited. The morphological methods used to date can usually distinguish between species but can never give a positive identification of an individual, and only rarely can be used to exclude an individual or a breed (1). Therefore, the application of DNA analysis to these samples could provide a great improvement to forensic investigations.

However, as different hairs found at the scene of a crime may originate from different sources, they have to be treated separately. As single shed hairs and hair shafts contain only minute amounts (2,3) of undegraded DNA, analysis of nuclear markers is mostly unsuccessful (3,4). Instead, mitochondrial (mt) DNA offers a more suitable target for analysis, as it is present in more than 1000 copies per cell (5), thereby giving a higher probability of finding intact DNA copies in samples that contain small amounts of DNA. In the last few years mtDNA analysis has been used in an expanding range of forensic applications, including analysis of single human hairs, teeth, fragments of bone, and saliva (4,6,7).

In a previous report the sequence variation in the mitochondrial control region of the Swedish domestic dog population was studied in order to estimate the frequencies of the different sequence variants present in the population (8). A 257 bp segment spanning hypervariable region 1 (HV1) was analyzed. Among 102 individuals of 51 different breeds, 19 mitochondrial sequence variants were found. The frequencies of the sequence variants varied from 1% to 21%. Overall, a low degree of correlation between breeds and sequence variants was found. However, one sequence variant was restricted only to a group of Northern Scandinavian Spitz breeds, Norwegian Elkhound, Jämthund and Finnish Spitz. In a comparative study of HV1 in domestic dogs and wolves throughout the world (9), 27 sequence variants were found among wolves and 26 among dogs. Of these sequences, only one was shared between dogs and wolves. It also has been shown that all Swedish wolves carry a single mtDNA variant, which has not been found in the domestic dog population (8,10).

Materials and Methods

All analyses were performed in a laboratory with dedicated areas for the different steps of analysis; multiple negative controls were run in parallel with the samples from the DNA extraction and onwards, and at least two independent PCR amplifications were carried out for each sample.

DNA Extraction

One to two centimeters of the root end of the hairs were washed by putting the hair in a 1.5 mL tube containing distilled water and shaking it. The hair was then transferred to a new tube containing 95% ethanol and finally to a tube containing distilled water. Extractions were performed using two different methods for evidence materials and reference materials, respectively.

The evidence materials were extracted using proteinase K and SDS treatment followed by phenol extraction and Centricon 30 ultracentrifugation according to Higuchi and co-workers (11) with some modifications. Briefly, the hair was placed in a 1.5 mL test tube with screw cap (Saarstedt, Nuernbrecht, Germany) containing 150 μL of hair digestion mixture: 10 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 100 mM NaCl, 39 mM DTT, 2% (w/w) SDS, 500 μg/mL proteinase K and 60 μg/mL carrier tRNA. The mixture was incubated at 56°C for at least 2 h and thereafter vortex mixed 10 s to pulverize the partly digested hair. Additional proteinase K (60 μg) was added and the mixture was incubated at 56°C overnight. After incubation at 95°C for 10 min, vortexing and centrifugation, the supernatant was extracted with 200 μL phenol (phenol : chloroform : isoamylalcohol 25 : 24 : 1, pH 8.0). The aqueous phase was collected and the phenol was back-extracted with 120 μL 1 × TE (10 mM Tris-HCl (pH 7.5) and 1 mM EDTA). The two aqueous phases were pooled and extracted with 250 μL phenol. The aqueous phase was extracted with 250 μL n-butanol

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and transferred to a passivated (the devices were soaked overnight with 1 mg/mL BSA and 100 μg/mL tRNA in 1×TE) Centricon 30 microconcentrator (Amicon Inc., Beverly, MA) containing 1.7 mL 1×TE. The sample was concentrated and rediluted to 2 mL, once with 1×TE and once with H_2O, and finally concentrated to ~35 μL according to the manufacturer's directions.

The reference materials were extracted using a one-step proteinase K treatment as described previously (12). Briefly, the hair was placed in a 1.5 mL tube containing 200 μL of hair digestion buffer: 10 mM Tris-HCl pH (8.5), 0.9% polyoxyethylene 10 lauryl ether, 35 mM DTT and 50 μg/mL proteinase K. The mixture was incubated at 56°C overnight, at 96°C for 10 min, and finally subjected to vortex mixing and centrifugation. The supernatant was used directly in the PCR amplification.

DNA Amplification

Initially, amplification in a nested configuration spanning 348 bp (including primers) was performed (8). However, some of the shed hairs did not yield product and therefore a semi-nested amplification spanning 148 bp was tried with higher success rate. The analyzed region was thus reduced to 79 bp. The outer primer pair (D5: 5′-TGATGGTTTCTCGAGGCATGG-3′, D8: CCCCATGCATATAAGCATGTAC) generated a 148 bp fragment and the inner primers (D7: 5′-ATTAAGCCCTTATTGGACTAAGTG-3′, D8: 5′-CCCCATGCATATAAGCATGTAC-3′) generated a 125 bp fragment. Both inner primers were made in two variants, one biotinylated to enable solid phase sequencing, and the other containing a handle consisting of the M13 −21 sequence (5′-TGTAAAACGACGGCCAGT-3′) to render possible the use of dye-labelled M13 −21 sequencing primers. One primer of each sort was next utilized in pairs to enable sequencing in both directions. To the outer amplification, 5–10 μL of DNA template was added. The PCR mixture consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Tween 20, 2 μg/mL BSA, 0.2 mM of each dNTP, 0.1 μM of each primer, and 2 units of Ampli*Taq* DNA polymerase (Perkin Elmer, Norwalk, CT) in a total volume of 50 μL. To the inner amplification 2 μL of the outer amplification product was added as template. The PCR mixture of the inner amplification was identical to that of the outer amplification but contained no BSA and only 1 unit of Ampli*Taq*. The mixtures were covered by ~50 μL mineral oil and the PCR reactions were performed in a Perkin Elmer 9600 thermocycler. The outer amplification program consisted of a predenaturation step (94°C, 2 min) followed by 25 cycles of denaturation (94°C, 15 s), primer annealing (66°C, 30 s) and extension (72°C, 1 min). The inner amplification program consisted of 25 cycles of denaturation (94°C, 15 s), primer annealing (65°C, 30 s) and extension (72°C, 1 min) followed by a final extension step (72°C, 10 min). Four microliters of the amplification product were analyzed by agarose gel electrophoresis and ethidium bromide staining for the presence of the desired fragment.

Solid-Phase Sequencing and Sequence Analysis

Immobilization of the PCR products onto solid support and sequencing reactions were performed on an ABI Catalyst robotic workstation (Applied Biosystems Inc., Foster City, CA) using dye-primer and T7 DNA polymerase chemistry according to Savolainen et al. (8). The sequence reactions were analyzed on an ABI 373A or 377 DNA Sequencer (Applied Biosystems) according to the manufacturer's directions. The sequences were studied for the presence of polymorphic nucleotide positions using the SeqEd software (Applied Biosystems).

Results

This study describes six cases in which sequence analysis of mtDNA derived from dog hairs were used. A total of 24 hairs were analyzed as evidence material and results were obtained for 14 hairs. Regarding the general level of success it should be noted that the hairs were of very varying quality and age, and that some could not unequivocally be morphologically defined as dog hairs. Furthermore, the methods for extraction and amplification were modified during the study (see Materials and Methods). After the establishment of the final version of the protocol, 13 of 16 analyses have been successful. Of the hairs used as reference material, which were either plucked directly from the dogs or collected from furniture and which were of better and more uniform quality, 23 of 26 were successfully analyzed.

Cases 1 and 2

A female was found murdered and dog hairs were found on the body. Shortly after, another female was found murdered and several dog hairs were recovered from objects connected with the crime. Because of similar modes of procedure and because dog hairs with similar morphology were found in connection with the bodies, it was hypothesized that the crimes had been performed by the same individual. The mtDNA analysis showed that the evidence material was of sequence variant 8 in both murder cases (Fig. 1). As this is a relatively rare sequence variant, it is probable that the hairs came from the same dog. Reference hairs were collected from dogs associated with seven suspects. The reference hairs were either plucked directly from the dogs or collected from furniture. The mtDNA from the reference materials was of variants 1, 3, 4, 5, 5, 5 and 5, respectively, and it could therefore be concluded that the reference materials did not originate from the same source as the evidence materials. Importantly, the mtDNA variant that was found in the evidence materials has so far been found only in three breeds (Jamthund, Norwegian Elkhound, and Finnish Spitz) belonging to a group of closely related Spitz breeds. Therefore it seems probable that the evidence materials originate from a dog belonging to this group of breeds.

Case 3

A female was reported missing and was believed to have been murdered. The female's body has not been found, but eight hairs believed to belong to the dog of the female's family were found in the car of a suspect. These hairs were collected as evidence material, while hairs plucked from the dog were used as reference material. The mtDNA of the reference material was of variant 5, while the hairs found as evidence were of mtDNA variant 6 and mtDNA variant 3 (Fig. 1). It was therefore concluded that the evidence material originated from at least two individuals and that an exclusion could be established between evidence material 1 and the reference material. Also, evidence material 2 and the reference material had different mtDNA sequence variants and originated most probably from different individuals. However, as the mtDNA variants differed in only one nucleotide position, a categorical exclusion could not be made, because of the possibility of genetic mosaicism (see Discussion).

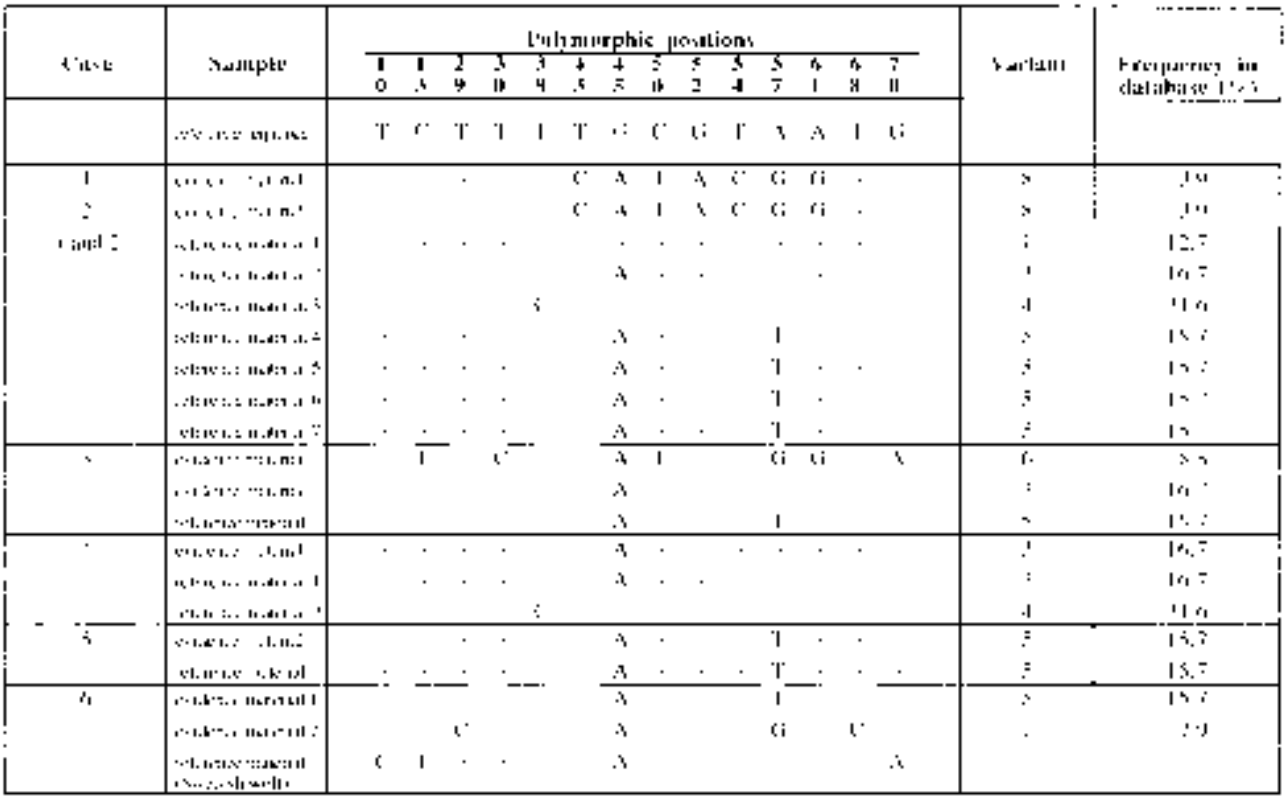

FIG. 1—*Summary of results. Source of DNA, mtDNA sequence, and observed frequencies of mtDNA variants (8) for the analyzed materials. The sequences are compared with a reference sequence (sequence variant 1) according to Savolainen et al. (8), and only differences from this sequence are indicated. The numbering of the sequences starts at the 3′-end of primer D8.*

Case 4

A medieval Bible was stolen from a public exhibition but later retrieved. Two hairs believed to derive from dog were found in the cover of the Bible. Reference material was obtained from dogs in two apartments where suspects had resided. mtDNA was isolated from one of the hairs (evidence material) found in the Bible and was shown to be of variant 3 (Figs. 1 and 2). The mtDNA from reference material 1 was also of sequence variant 3 while that from reference material 2 was of sequence variant 4. The analysis was therefore exclusive for reference material 2, whereas it was inclusive for reference material 1 in that it could not be excluded that reference material 1 could originate from the same individual as the evidence material.

Case 5

A number of bank robberies were carried out by a group of four men. In connection with one of the crimes a car was stolen from a family who owned a dog. At the arrest, dog hairs were found on the clothes of one of the suspects. To investigate whether the hairs could originate from the dog of the family, an mtDNA analysis was performed. The mtDNA of the evidence material was of sequence variant 5 (Fig. 1). Reference hairs were plucked from the dog and were also shown to contain mtDNA of variant 5. The investigation was therefore inclusive in that it could not exclude that the hairs could have originated from the same individual.

Case 6

A case of suspected poaching of protected wolves was investigated. Two samples of hair were collected from items belonging to the suspects to determine whether the hairs derived from a wolf. Previous studies have shown that all Swedish wolves have a single mtDNA sequence variant which has not been found among domestic dogs (8,10). Furthermore, the immigration of foreign wolves is very infrequent (two reported cases in 20 years). The mtDNA from evidence material 1 was of sequence variant 5, and that from evidence material 2 was of variant 7, both common sequences in the domestic dog population (Fig. 1). Therefore, it was concluded that the evidence material most probably originated from domestic dogs.

Discussion

DNA analysis of human cellular material is now routinely used in forensic investigations for discrimination between individuals. However, although DNA analysis of animal tissue is extensively used to determine species identity (13,14), discrimination of individuals has been performed to date mainly by morphological methods despite that DNA analysis has a considerable potential of information in these cases. In this study we show a range of important information derived from sequence analysis of mtDNA obtained from dog hairs. The analysis was used to investigate four types of crime: murder, robbery, theft, and poaching. Furthermore, five kinds of inferences could be drawn from the obtained genetic information: individuals were included or excluded as potential sources of hairs, a possible link was shown between two crimes, the breed of a dog was indicated by its sequence variant, and hairs were shown not to originate from a wolf but from dogs.

Despite its relatively low exclusion capacity, the method has proved useful in excluding a large number of suspects from the

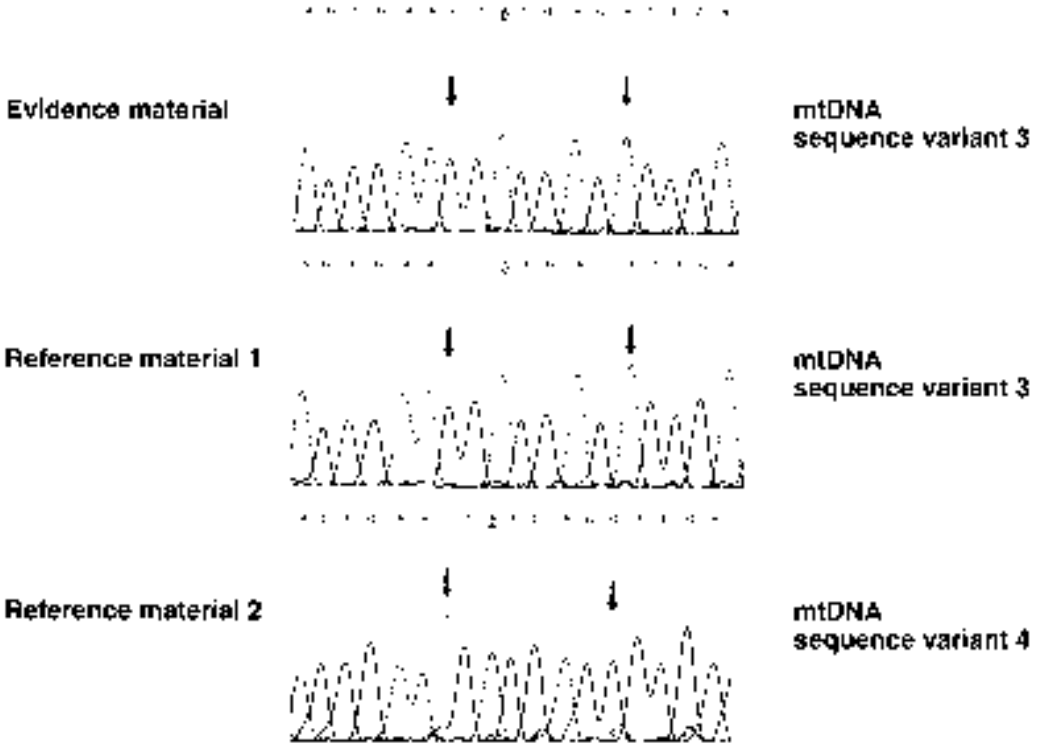

FIG. 2—*Example of mtDNA sequencing results corresponding to the evidence and reference materials from case 4. The distinguishing positions are indicated by arrows.*

investigations. In cases one and two, all seven suspects could be excluded. Also in case three the evidence and reference materials most probably originate from different individuals. However, the interpretation of the results is complicated by the fact that the mtDNA variants obtained from the reference material and one of the evidence materials differ in a single nucleotide position. Recent data have demonstrated heteroplasmy and mosaicism in human mtDNA, caused by point mutations (15,16). As a consequence, although this is a rare phenomenon, categorical exclusions of individuals based on single nucleotide differences are not possible. Inclusions of individuals were obtained in two cases: 4 and 5. However, the frequencies of the sequence variants found in these cases are high (Fig. 1), and the results can only be used as weak circumstantial evidence.

As dogs usually shed more hairs than humans, the probability of finding hairs originating from a dog (directly or indirectly, e.g., from the owner's clothes), which could be used to link different crimes, is often higher than finding biological material from the owner. This is exemplified by cases one and two, which could be linked by the presence of dog hairs containing sequence variant 8 in both cases.

Generally, only a limited correlation between breed and mtDNA sequence variant is found among dogs (8), but in the case of variant 8 it has been found so far only in a group of Scandinavian Spitz breeds. The hairs found in cases one and two therefore probably derive from a dog belonging to this group of breeds. It is possible that studies of other geographically isolated breeds may show other distinctive sequence variants.

Poaching of protected animals such as some species of carnivores and birds of prey is a major problem in many countries, and the use of DNA markers to indicate the species of animal remains would be valuable in many cases. The fact that Swedish wolf and domestic dog mtDNA sequences are different (8,10) implies that it is possible to distinguish between these species in the case of suspected poaching. In case six it could be shown that the hairs obtained did not originate from a wolf but from domestic dogs.

In the analysis of materials containing low amounts of partly degraded DNA, the number of copies of the target molecule and the extent of fragmentation of the DNA are critical aspects. Although forensic analysis of cat hairs using micro-satellite analysis has been demonstrated (17), analyses of shed human hairs mostly fail for nuclear markers in cases where analysis of mtDNA routinely yields results (4). Furthermore, when DNA amplification is started from a few copies of nuclear DNA, there is a risk of false assessment of homozygosity to heterozygotes due to allelic dropout (3,4). We therefore believe that analysis of mtDNA offers a more robust system with a higher incidence of useful results in the case of analyzing shed hairs and hair shafts. In this study, DNA amplification was initially performed on a segment spanning 348 bp (including primers) (8). Since some of the evidence materials failed to yield amplification products, a 148 bp segment was tried instead, which resulted in a higher success rate. This is in accordance with observations that mtDNA is fragmented to a small average size under degrading conditions (18). The analyzed region thus was reduced to only 79 bp, but as this segment contains most of the highly variable sites of HV1, the exclusion capacity was reduced only from 0.88 to 0.86.

This study describes a protocol for the extraction and sequence analysis of mtDNA from shed dog and wolf hairs, and its application in six forensic cases. The potential of a hitherto unexploited source of information, mtDNA from animal hairs, is thereby exemplified. Further mtDNA mapping of dogs and other common pets will widen the interest of applying mtDNA analysis of animal hairs in forensic investigations.

Acknowledgments

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ERRATA/CORRECTIONS

We have identified a number of instances in which the authors of work published in the Journal of Forensic Sciences have miscited papers originally published in the Journal of the Forensic Science Society as having been published in the Journal of Forensic Sciences.

The known instances of this error for volume 44 of the Journal of Forensic Sciences are detailed/corrected below. We have not checked other volumes for similar errors. The Journal of Forensic Sciences regrets these errors.

Since 1995 (Volume 35), the Journal of the Forensic Science Society has been published under the title "Science and Justice."

The editors of both journals take this opportunity to remind authors of the necessity for ensuring the accuracy of the references they cite in manuscripts submitted for publication. The Instructions for Authors of both journals make it clear that accuracy of reference citation is the responsibility of authors, and good scholarship demands attention to this matter.

A. R. W. Forrest	R. E. Gaensslen
Editor, Science and Justice	Editor, Journal of Forensic Sciences

The journal citation in reference 7 in Foreman LA, Smith AFM, Evett IW. Bayesian validation of a quadriplex STR profiling system for identification purposes. should read: J Forensic Sci Soc 1992;32:5–14.

The journal citation in reference 5 in Bourel B, Hedouin V, Martin-Bouyer L, Becart A, Tournel G, Deveaux M, Gosset D. Effects of morphine in decomposing bodies on the development of Lucila sericata (Diptera: Calliphoridae). should read: J Forensic Sci Soc 1991;31:469–72.

The journal citation in reference 8 in Hedouin V, Bourel B, Martin-Bouyer L, Becart A, Tournel G, Deveaux M, Gosset D. Determination of drug levels in larvae of Lucila sericata (Diptera: Calliphoridae) reared on rabbit carcasses containing morphine. should read: J Forensic Sci Soc 1994;34:95–7.

The journal citation in reference 15 in Hedouin V, Bourel B, Martin-Bouyer L, Becart A, Tournel G, Deveaux M, Gosset D. Morphine perfused rabbits: A tool for experiments in forensic entomotoxicology. should read: J Forensic Sci Soc 1991;31: 469–72.

The journal citation in reference 10 in McDermott SD, Willis SM, McCullough JP. The evidential value of paint. Part II. A Bayesian approach. should read: J Forensic Sci Soc 1992;32: 333–48.

The journal citations in references 4 and 5 in Infante F, Dominguez E, Trujillo D, Luna A. Metal contamination in illicit samples of heroin. should read for 4: J Forensic Sci Soc 1979;19:203–9. and for 5: J Forensic Sci Soc 1980;20:177–81. [in reference 5 only the volume number is miscited]. And in both references, the lead author's name is "Joyce JR."

The journal citation in reference 1 in Savolainen P, Lundeberg J. Forensic evidence based on mtDNA from dog and wolf hairs. should read: J Forensic Sci Soc 1988;28:335–9.

The journal citation in reference 1 in Kupfer DM, Chaturvedi AK, Canfield DV, Roe BA. PCR-based identification of postmortem microbial contaminants—A preliminary study. should read: J Forensic Sci Soc 1968;8:73–6.

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