IDENTIFICATION OF AN ANIMAL CONTRIBUTOR WITHIN MIXTURE TRACES BY INTERPRETATION OF THE MITOCHONDRIAL DNA SEQUENCE

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Introduction

Species identification of non-human traces is well established in the forensic community and supplements the commonly used human specific DNA analysis. The routinely used Sanger sequencing analysis of the cytochrome b (cytb) gene proved to be one of the most suitable methods to infer the species of an unknown sample over the last years as it is robust and in a lot of cases successful due to the high copy number of mitochondrial (mt) DNA per cell.

However, as a consequence of potentially high human background in forensic evidentiary samples the analysis of the cytochrome b gene may occasionally result in a DNA mixture. So far, mtDNA mixtures were either interpreted as inconclusive or the mixture components were separated using laboratory technologies such as cloning that are time consuming and cost-intensive.

We here present two case work reports where we were able to resolve mtDNA components in forensic mixture samples by phylogenetic interpretation of the components. The haplotypes of a minor and a major contributor were assessed based on differences in allele peak height at several mixed positions within the cytb gene. Species identification was then performed using a BLAST (Basic Local Alignment Search Tool) search and the results were confirmed by cloning and sequencing of the control regions (CR) using species specific primers.

Case History 1

In this case a male family member was suspected of sexual abuse of a nine year old girl but the suspect denied to have ever been in the girl´s bed. The trace evidence examiner collected numerous hairs from the linen of the girl´s bed and two of them were suspected to be pubic hairs from the suspect. Mitochondrial DNA typing of should clarify, whether the mtDNA haplotype actually matched the suspect or the victim.

Results

Both hairs had telogen roots and the morphological examination showed that one hair was of non-human origin. The root of this hair was extracted, amplified and sequenced in the mt cytb region according to [1] and gave successful results displaying full double-strand sequence coverage. Sequencing results revealed an mtDNA profile of mixed origin. The facts of the case suggested that this mixture could comprise a human and a non-human component, as the girl´s cat was often sleeping in the bed. As both components were clearly distinguishable both hypothetical mtDNA sequences could be resolved by phylogenetic interpretation (**Fig 1**).The components were interpreted 1) based on the differences in allele peak heights at several mixed positions and 2) based on comparison of the mixture sequence to the Revised Cambridge Reference Sequence (rCRS – human) and the reference sequence of *Felis catus* (Accession number U20753.1). In the following, both hypothetical sequences were searched in internet databases using BLAST. The minor component gave a 98% match with *Felis catus* and the major component gave a 100% match with the human cytb region sequence. It seemed reasonable enough, that it had been cell particles of the girl´s skin that caused the contamination sequence signal. To confirm this assumption the cytb region as well as the CR using feline species specific primers were analysed using a portion of the hair shaft. The surface of this hair shaft was cleaned before DNA extraction and so a non-mixed sequence for the cytb region as well as for the feline mtDNA CR could now be obtained. An internet database search using BLAST revealed a 100% match with the animal species *Felis catus*.

The second hair was of human origin and was investigated in the human mtDNA control region. It could be excluded to origin from the suspect and could be assigned to the girl´s mtDNA haplotype.

Materials and Methods

All samples were extracted using the BioRobot M48 workstation (Qiagen, Hilden, Germany) according to the manufacturer's protocol and the Qiasoft M Operating System software (ver.2.0E001). Hair shafts were cleaned performing a preferential extraction step before lysis. The hair shafts were incubated using Proteinase K at 56 ºC for at least two hours, heated to 95 ºC for three minutes and washed three times using extraction buffer. Amplification and sequencing processes were performed according to [1]. The primer locations flanking the corresponding cytb region are listed in **Table 1**.

Amplification products were subjected to capillary electrophoresis on an ABI Prism 3100 Genetic Analyzer using POP 6, 36 cm capillary arrays and default instrument settings (all Applied Biosystems). The data were analyzed using Sequencing Analysis Version 3.7 (AB) and Sequencher Version 4.10.1 (GeneCodes, Ann Arbor, MI, USA).

PCR products were cloned using the Zero Blunt TOPO PCR cloning kit for sequencing (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Plasmid minipreps were performed with the montáge plasmid miniprep kit (Millipore, Billerica, MA, USA) following the included instructions.

Species identification was then performed using a BLAST (Basic Local Alignment Search Tool).

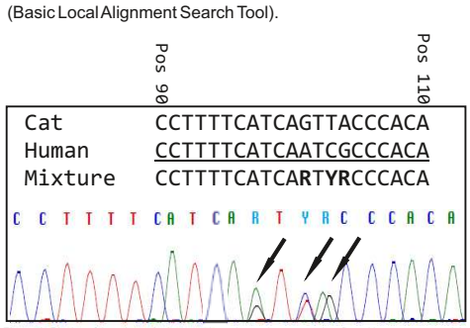

Figure 1 Display of the mixture in the sequence electropherograms (confirmed by reverse strands). The interpretation of the mixture was based on the differences in allele peak heights at several mixed positions and on comparison of these positions to the reference sequences of the species in question. The mixed positions were designated with an International Union of Biochemistry code. In this figure the position 90 corresponds to position 15,222 within the cat reference sequence and to position 14,931 in the human rCRS. The sequencing assay brought a clear dominant variant for human species molecule.

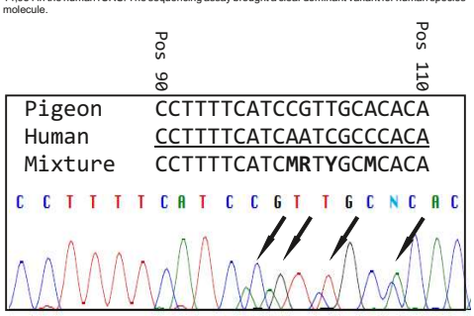

Figure 3 Display of the mixture in the sequence electropherograms (confirmed by reverse strands). The interpretation of the mixture was based on the differences in allele peak heights at several mixed positions and on comparison of these positions to the reference sequences of the species in question. The mixed positions were designated with an International Union of Biochemistry code. In this figure the position 90 corresponds to position 15,496 within the pigeon reference sequence and to position 14,931 in the human rCRS. The sequencing assay brought a clear dominant variant for the species *Columba livia* .

References

[1] Parson et al. *et al.* Int.J Legal Med 114 (2000): 23-28.

Case History 2

In this second case multiple pigeons were decapitated with a knife. The police secured the jacket of a suspect who denied to ever have been in contact with the pigeons of his neighbour. Small quantities of blood were identified on the right sleeve of the suspect´s jacket (**Fig 2**) and were recovered for comparisons with tissues of the dead pigeons using cytb analysis.

	Cat (U20753.1)	Human (rCRS)	Pigeon (GQ240309.1)
primer-F	15107-15132	14816-14841	15381-15406
cytb region	15133-15423	14842-15148	15407-15713
primer-R	15424-15448	15149-15173	15714-15738

Table 1 Primer range of the cytb primer [see ref 1] and sequencing range (in bp) with respect to the reference sequences of the animal species investigated. Within the brackets the accession numbers of the reference sequences used are indicated.

Results

Two blood stains gave successful results for the cytb region displaying full double-strand sequence coverage. The results showed identical mixture sequences for both blood samples composed of a major and a minor component clearly distinguishable by the peak height ratios at mixed positions (**Fig 3**). The circumstances of the case made plausible that these mixtures could include the suspect as human contributor in addition to the non-human component. Therefore we resolved the mtDNA components by phylogenetic interpretation of the mixture as described in case 1. Again, both hypothetical sequences were searched in BLAST. The major component gave a 100% match with the animal species *Columba livia* and the minor component gave a 99% match with the human cytb region sequence. The results were confirmed by cloning the amplification product of one blood sample. The reference tissue samples of two dead pigeons gave successful results for the cytb region. They differed at position 15590 (C/T). The pigeon-specific cytb component of both mixtures gave 15590 C which is why one of the pigeons could not be excluded as donor of the blood samples. CR analysis of the pigeon-specific contribution was not necessary in this case.

Figure 2 Blood stain on the right sleeve of the suspect´s jacket. The DNA of this blood stain was later cloned to evaluate and confirm the sequencing mixture results that could be assigend to a human and a pigeon mitochondrial sequence performing a data base search (BLAST).