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Hairy matters: MtDNA quantity and sequence variation along and among human head hairs

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ABSTRACT

Hairs from the same donor have been found to differ in mtDNA sequence within and among themselves and from other tissues, which impacts interpretation of results obtained in a forensic setting. However, little is known on the magnitude of this phenomenon and published data on systematic studies are scarce. We addressed this issue by generating mtDNA control region (CR) profiles of >450 hair fragments from 21 donors by Sanger-type sequencing (STS). To mirror forensic scenarios, we compared hair haplotypes from the same donors to each other, to the corresponding buccal swab reference haplotypes and analyzed several fragments of individual hairs. We also investigated the effects of hair color, donor sex and age, mtDNA haplogroup and chemical treatment on mtDNA quantity, amplification success and variation. We observed a wide range of individual CR sequence variation. The reference haplotype was the only or most common (≥75%) hair haplotype for most donors. However, in two individuals, the reference haplotype was only found in about a third of the investigated hairs, mainly due to differences at highly variable positions. Similarly, most hairs revealed the reference haplotype along their entire length, however, about a fifth of the hairs contained up to 71% of segments with deviant haplotypes, independent of the longitudinal position. Variation affected numerous positions, typically restricted to the individual hair and in most cases heteroplasmic, but also fixed (i.e. homoplasmic) substitutions were observed. While existing forensic mtDNA interpretation guidelines were found still sufficient for all comparisons to reference haplotypes, some comparisons between hairs from the same donor could yield false exclusions when those guidelines are strictly followed. This study pinpoints the special care required when interpreting mtDNA results from hair in forensic casework.

1. Introduction

Human head hairs are common crime scene evidence [1–6]. Morphological comparison may provide investigative leads but is not trivial, especially for small fragments [7–9]. Hairs can also yield nuclear (nuc)DNA profiles when hair roots in anagenic growth phase or adhering tissue are available, but to a much lesser extent from hair shafts [1,7]. NucDNA content in general varies between individuals [10]. Most hairs found at the crime scene were shed, which is why usually only telogenic hairs or hair fragments lacking roots are available that contain only minute or no detectable nucDNA [3,5,11–14]. Still, successful STR typing has been demonstrated also from challenging hair samples and specific DNA screening assays have been developed [10,15], however, resulting STR profiles are often incomplete [16–19]. Here, mitochondrial (mt)DNA presents a valuable genetic marker. Intact mitochondria are commonly observed in hair shafts in small numbers [7,20,21] but disappear later in development [14]. MtDNA hair casework has high success rates in specialized laboratories (*cf.* [22]), where ≥90% full or partial haplotypes were reported [2,3,5,9], even for specimens smaller than 2 mm [23]. Despite degradation [13,24], mtDNA from hairs can successfully be analyzed even in historic cases and "museomics" [9,21,25], and sophisticated strategies have been presented [24,26]. MtDNA base

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changes *post mortem* in hairs have been discussed [27], but hairs are extremely resistant to exogenous DNA or can successfully be decontaminated [28]. In 1988, a single human head hair was proven to contain enough mtDNA to reveal an individual's haplotype [12]. Other early mtDNA investigations determined hair as useful tissue in population studies, genetic screenings and amenable to analytical automation [29–31]. Hair mtDNA typing entered forensic casework in the mid-nineties, being introduced to courts in 1996 in Sweden and the US [3,32–36]. The significance of mtDNA in this regard is not unambiguous identification, but (non-) exclusion for samples to derive from the same individual/maternal lineage.

Earlier studies described mtDNA point heteroplasmy (PHP), the presence of more than one haplotype in a single hair. Initially it was detected at disease related positions [37,38], where selection was discussed for being the driving force. Later PHP was also observed at "neutral" coding region [39] and CR positions [6]. Numerous further investigations found high mtDNA PHP rates in hairs and provided insights into particular aspects, such as familial transmission and site-specific rates, e.g., [4,9,20,40–52]. Several factors were considered to contribute to the regular occurrence and highly variable levels of mtDNA PHP in hairs. Hair follicles develop independently from different sets of stem cells. Multiple cell types, whose proportions can vary, "feed" the growing shaft and give rise to mitochondria. This might cause different ratios of mtDNA populations visible only when PHP is present. Cell contributions during initial growth, when distal hair fragments are produced, are thought to differ from the telo- or catagenic buildup of proximal parts. The extreme bottlenecks along hair shafts might cause variant ratio shifts that are better visible than in other tissues. The high energy demand during proliferation and keratinization, the long growth cycle, apoptosis, environmental effects (possibly greater for distal segments), including exposure to radiation and cosmetics, combined with the lack of a repair mechanism have been linked to a high mtDNA mutation rate in hair [4,7,22,40,41,43,44,53,54].

A major advantage of DNA analysis over morphological comparison of hairs is the possibility of a database query to yield an estimate of the probability of a match of the questioned hair haplotype with that of a randomly selected person [8]. Large and reliable databases are a prerequisite for forensic queries [55]. The limits of microscopic hair examination have been outlined in [56], where 9 of 80 associations were excluded by mtDNA. In 2009, a US National Research Council report claimed imprecision of microscopic hair analyses and reporting when considering statistics about the distribution of particular characteristics in the population and standards on the number of features "matching" hairs must agree on [57]. After reviewing ~500 of its microscopic hair comparison cases prior to 2000, the FBI concluded in 2015 that ≥90% of trial transcripts contained statements that led to exaggeration of data significance [58]. However, this does not flaw the results of hair (mt)DNA analyses, but instead pinpoints the importance of investigations as the current one: with this study, we provide the first systematic and comprehensive insight into mtDNA variation among and along single human head hair shafts applicable to the forensic practitioner. We performed *(i)* a "latitudinal" hair study by comparing 25–50 hairs *per capita* from eleven individuals to gain better insight into intra-individual hair mtDNA variation, and *(ii)* a "longitudinal" hair study on 20 hair shafts from ten individuals to investigate mtDNA quantity and sequence variation along single hairs. We address shortcomings of previous investigations in mimicking forensic casework by including a reasonable number of individuals and hairs, reporting entire CR sequences from single hair shaft fragments generated by STS, the current gold standard in forensic DNA analyses, and comparing them to buccal swab reference haplotypes from the same donors. Our aims were to investigate the degree of mtDNA variation and the existence of mutational patterns in human head hairs and to assess the validity of current forensic mtDNA interpretation guidelines that derive mainly from blood/buccal swab data.

2. Materials and methods

This two-center study combines data from the National Institute of Criminalistics and Criminology, Brussels, Belgium (NICC) and the Institute of Legal Medicine, Medical University of Innsbruck, Austria (GMI).

2.1. Sample collection

Plucked or shed head hairs and reference buccal swabs were collected from 21 individuals of European origin after informed consent. Donor age, sex, natural hair color and current cosmetic treatments were recorded at sampling (Table S1). The proximal centimeter of each hair including the root and possible adhering cells was removed. At NICC, 25–50 hairs *per capita* were collected from eight female and three male donors (23–65 years old, named P1-P11) resulting in a total of 348 hairs for testing. The first proximal 2 cm fragment of each of the 348 hairs was used for the investigation of mtDNA variation across hairs (latitudinal study). At GMI, two hairs were collected from ten women (24–36 years old, named A-J) and cut into 2 cm fragments numbered in consecutive order. This resulted in seven to 20 fragments per hair (244 in total) for a comparison of mtDNA sequence and mtDNA quantity along hairs (longitudinal study). Two cm was also the average fragment length available in 691 casework hairs [9] and is recommended in [33].

2.2. Decontamination and DNA extraction

For decontamination, the hair fragments were incubated at (*a*) 56 °C for 1 h in 1,250 μl preferential lysis buffer A (10 mM Tris-HCl at pH 8.0, 100 mM NaCl, 10 mM EDTA, 0.5% SDS, 400 μg/ml proteinase K) and rinsed in MilliQ water at NICC, or (*b*) at 56 °C for 2 h in 800 μl and washed thrice in 500 μl of preferential lysis buffer B (10 mM Tris-HCl at pH 8.0, 100 mM NaCl, 1 mM $CaCl_2$, 2% SDS, 800 μg/ml proteinase K) at GMI, respectively. Total DNA was extracted (*a*) using the Tissue and Hair Extraction kit (for use with DNA IQ) and the DNA IQ casework sample kit on a Maxwell 16 instrument (all: Promega, Madison, WI, USA) at NICC, or (*b*) on a BioRobot M48 workstation (Qiagen, Hilden, Germany) after digestion in 320 μl lysis buffer B containing 20 μl 1 M DTT for 2 h or until they were completely dissolved at GMI (extraction volume: 50 μl). DNA from control buccal swabs was extracted using (*a*) the NucleoMag kit (Macherey-Nagel, Düren, Germany) on a KingFisher96 purification platform (Thermo, Breda, The Netherlands) at NICC, or (*b*) Chelex-100 (Bio-Rad, Hercules, CA, USA) [59] at GMI.

2.3. MtDNA quantitation

A 143 bp-mtDNA fragment was assessed in all longitudinal hair extracts in a modular real-time quantitative PCR assay [60] on an AB7500 Fast Real-Time PCR Instrument (AB, Oyster Point, CA, USA) at GMI to compare mtDNA content along hairs and to evaluate amenability to amplification.

2.4. MtDNA amplification

Complete CRs (nps 16024-576) were amplified as single fragments from the buccal swab extracts, while the amplicon