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MitoMetrics: Incorporation of mtDNA profile discrepancies in likelihood ratio calculations

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ABSTRACT

Interpretation of mitochondrial DNA (mtDNA) evidence in a forensic context faces challenges, particularly when evaluating mtDNA profiles from different tissues. In hair, for example, the segregation of mtDNA shows tight bottlenecks that can result in different mtDNA profiles between and along hair shafts, and between hair and other reference tissues of the same donor. Current forensic interpretation guidelines for mtDNA are based on conventions on the number of discrepant positions observed between the two compared profiles. Most legislations consider two discrepancies between samples as an exclusion, and one discrepancy as an inclusion or as an inconclusive result. More data are needed to understand the variation and occurrence of discrepancies in samples from the same donor, to be able to incorporate this knowledge into a mathematical approach that effectively quantifies the probability of these events. This study reports the first project of the MitoMetrics collaborative initiative. In this study, data were generated from several participating laboratories, and previously published data, along with data generated from casework. mtDNA profiles from blood/buccal reference samples were compared to those from hair shafts from the same individuals. Results report the levels of heteroplasmy detected in the different tissues, the number of differences observed between the tissue comparisons, and the number of discrepant positions observed. We suggest a preliminary model for calculating the evidential value of mtDNA-based evidence using a likelihood ratio approach, that takes into consideration the occurrence of discrepancies between profiles from the same donor. This work represents the first attempt to quantify the probability of finding discrepant events between tissues and to incorporate these when reporting mtDNA in a forensic context.

1. Introduction

Mitochondrial DNA (mtDNA) analysis can be an important element of casework investigations, especially in situations where only small amounts of nuclear DNA (nuDNA) are collected from the crime scene [1]. If the amount of nuDNA is too low for STR typing (e.g. less than 100 pg), it may still be possible to obtain information on mtDNA due to its high number of copies per cell. mtDNA is also present in trace samples

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consisting of keratinized tissues such as hair and nails (commonly found in crime scenes), and in partly degraded tissues like bones [2,3]. The amplification of nuDNA in this type of biological material is often unsuccessful with traditional methods [4].

The analysis of mtDNA only provides information on the maternal lineage (and to some extent, biographic origin); it does not allow identification at the individual level [1]. However, mtDNA can still be very useful when identifying missing persons or in case of mass disasters since it is possible to use maternal relatives, even from several generations apart, as reference profiles to identify the victims [4]. mtDNA can also be used in cold cases since these often contain very old and degraded material that might not produce a reliable DNA STR profile [1].

In 2000, the DNA Commission of the International Society for Forensic Genetics (ISFG) developed guidelines for the interpretation of mtDNA typing results generated by Sanger sequencing [5]; these guidelines were later refined in 2014 [6]. More recently, in 2019 and 2024, the Scientific Working Group on DNA Analysis Methods (SWGDAM) published updated guidelines to include information on Massively Parallel Sequencing (MPS) data [7]. These guidelines base the profile interpretation (and subsequent statistical interpretation) on the number of discrepancies observed between mtDNA profiles (no discrepancy – cannot exclude/inclusion; one discrepancy – inconclusive; two or more discrepancies – exclusion). This rule-based approach does not take phylogenetic information nor (low-level) heteroplasmy into account.

Heteroplasmy is generated during cell division and refers to the presence of different, albeit very similar, mtDNA molecules within a tissue [8]. In different tissues from the same individual, tight bottlenecks in the segregation of mtDNA molecules during development may lead to different levels of heteroplasmy [6,9–11]. Sometimes, the presence of a variant in a tissue differs from the base call observed in other reference tissues of an individual (such as blood and saliva) [2,8,12–19]. This is especially common in hair shafts, where differences between hairs from the same individual, and even along the same hair shaft, have been observed [2,12,20]. The presence of heteroplasmy can have consequences when interpreting mtDNA-based evidence between a trace sample recovered at a crime scene, and a reference sample collected from a person of interest, for example.

Most studies on mtDNA heteroplasmy have focused on the control region or its hypervariable segments. Only a few studies targeting the mtDNA coding region have been performed [3,17,18,20,21], and heteroplasmic levels in the mtDNA coding region in the hair are generally unknown [21].

There is a general need for more data to understand how frequently mtDNA variation occurs within tissues from the same donor. This knowledge can then be incorporated into a statistical model to report mtDNA-based evidence, a model that is more data-driven and more reflective of the biological phenomena.

This broad goal led to the establishment of MitoMetrics, an *adhoc*-forming group of scientists and practitioners working with mtDNA in the field of forensic genetics, aiming to work towards a more regular use of mtDNA in forensic genetic casework. The first project within the MitoMetrics initiative presented here focuses on the detection and interpretation of mtDNA heteroplasmy and the incorporation of mtDNA profile discrepancies in the calculation of the weight of mtDNA evidence.

In this study, samples from hair, blood, and buccal swabs were investigated for mtDNA sequence variation. The distribution and frequency of differences observed between the profiles and the levels of heteroplasmy across tissues from the same individual were investigated. Although lineage-specific differences may exist, the limited sample size of this study precludes reliable conclusions about a potential phylogenetic signature of heteroplasmy. Comparisons that resulted in discrepant profiles were used to develop a method for reporting the weight of mtDNA evidence using a likelihood ratio approach that quantifies the probability of finding discrepant events between tissues from the same donor.

2. Material and methods

2.1. Biological samples and data

2.1.1. Previously generated data

2.1.1.1. Casework data. One participating laboratory contributed data from casework. This constituted 13 cases (comprising two blood, 11 buccal swab and 34 hair samples) where hair samples were retrieved from the scene, and reference samples were collected from the suspect. The hair root was available, and an autosomal STR profile was obtained, which strongly supported that the individual was the same donor of the hair and reference sample. Samples were sequenced for the control region (CR) (16024–576 bps) or parts of it (16024–16569, 16024–16365, 49–576) with the Sanger method.

2.1.1.2. Previously published data. Previously published data from Desmyter et al. [2] and van der Gaag et al. [19] were considered. These consisted of data from 21 individuals (21 buccal swab reference samples and 459 hair fragments) analysed for the CR with Sanger sequencing using BigDye Terminator v1.1 chemistry [2] and data from 26 individuals (26 buccal swab reference samples and 462 hair fragments) analysed for the CR using an adjusted mitominis protocol for MPS [22] and sequenced in the MiSeq FGx instrument (Verogen, Inc.) [19]. Please see the original publications for more details on sample processing and sequencing. Ten individuals (ten buccal and 163 hair samples) overlapped in both studies and were therefore sequenced with both Sanger and MPS methods.

2.1.2. Newly generated data

Each participating laboratory collected samples from unrelated individuals. For each individual, one buccal swab, one blood sample on FTA, and a minimum of three hair shafts were collected. Each hair shaft was further divided in 2 cm fragments (Supplementary Figure S1). The root section was excised at 0.5 cm from the proximal hair end and was not included in this study.

DNA extraction and quantification were done by each participating laboratory according to the protocols implemented as part of their own routine or research work. Supplementary Table S1 presents an overview of the techniques utilized in this work. A total of 551 samples comprising 53 blood, 53 buccal swabs and 445 hair fragments were included in the study.

Two participating laboratories contributed with Sanger sequencing data representing ten individuals (ten blood on FTA, ten buccal swabs, and 100 hair fragments). One laboratory reported data for HVI+HVII (16024–340) (five blood samples, five buccal swab samples and 66 hairs fragments). The other laboratory reported CR data for five individuals (five blood samples, five buccal samples, and 34 hairs fragments with HVI+HVII data (34 hair fragments) and HVI or a smaller region (24 hair fragments)). One of the laboratories additionally sequenced the same 76 samples for HVI+HVII using MiSeq Reagents Kits v3 (150-cycle) in a MiSeq FGx™ (Illumina) instrument, with the ForenSeq mtDNA Control Region Kit (five blood, five buccal samples and 66 hair fragments).

Three participating laboratories contributed with MPS data for the whole mtDNA molecule (N = 431 samples in total). Of these, two laboratories used the Precision ID mtDNA whole genome panel (N = 408) (Thermo Fisher Scientific, TFS) and 530 chips (TFS) on the Ion GeneStudio™ S5 instrument (TFS). One laboratory used a long-range PCR approach for mtDNA amplification of blood (N = 4) and buccal samples (N = 4) as reported by Fendt et al. [23], and the strategy reported by Parson et al. [3] for hair fragments (N = 15). Library preparation was done with DNA Prep (M) Tagmentation kit (Illumina), and sequencing was carried out in the iSeq100 instrument (Illumina) using iSeq 100 i1 Reagent v2 (300-cycle) (Supplementary Table S1).

An overview of all data included in the study is provided in

Supplementary Table S2.

2.1.3. Ethical statement

Participants were responsible to comply with ethical regulations according to their local requirements.

Local approvements were provided by the Ethics Committee of the Juntendo University School of Medicine, Tokyo, Japan (approval no. 2019185), the Bioethics Committee of the Nicolaus Copernicus University, Collegium Medicum in Bydgoszcz, Poland (statement no. KB 322/2023), the Research Ethics Committee of the Università Politecnica delle Marche, Ancona, Italy (Prot.n.0212568 of 11/28/2022). Informed consent was obtained from participants.

In Spain, the consent followed the requirements of Law 14/2007, Organic Law 3/2018 as well as the European General Data Protection Regulation (GDPR). The study follows the policies from the Danish National Center for Ethics (https://nationaltcenterforetik.dk/; accessed on 7 November 2025) and the Danish Research Ethics Committees (https://videnskabsetik.dk/; accessed on 7 November 2025) and complies with the rules of the GDPR (Regulation (EU) 2016/679). Explicit ethical review and approval were waived for this study because the study was not health-related research. According to Danish ethical guidelines and legislation, only health-related research projects are subject to mandatory ethical approval by a research ethics committee.

Samples from casework were processed in this study (University of Copenhagen joint registry of research projects processing personal data, 514–1264/25–3000). The processing of pseudonymized newly generated data is registered at the University of Copenhagen joint registry of research projects processing personal data (514–1262/25–3000).

2.2. Detection of heteroplasmy, differences, and discrepancies between hair and reference profiles

Sequence variation was reported relative to the rCRS and according to ISFG recommendations [6].

For Sanger sequencing data, only the presence of heteroplasmy was annotated whenever the secondary peak exceeded a height of 10% of the primary peak. For MPS data, a minimum coverage of 20 reads, and a variant calling threshold of 10% were applied when reporting the variants. Variants with $\geq$ 90% frequency were not reported as heteroplasmy. Additionally, for Precision ID mtDNA whole genome data, a variant strand bias threshold of $\leq$ 0.6 was considered.

Point heteroplasmic (PHP) variation above the analytical threshold was annotated with the respective IUPAC codes. The mtDNA profiles obtained for hair samples were compared to the buccal/blood reference mtDNA profiles of the relevant individual (Supplementary Table S3). Sequence variation between hair and reference samples was considered discrepant (Supplementary Table S3, 'DD') when (i) two non-matching, non-heteroplasmic calls were observed at the same position (e.g., 152C vs T152); (ii) when both profiles displayed heteroplasmy involving different nucleotides (e.g., Y and R); and (iii) when a heteroplasmic call was present in one profile but the constituent nucleotides differed from those of that position in the other profile (e.g., 152C and 152R). Differences in calls involving PHP, e.g., 152Y vs 152C or 152Y vs T152, were noted as differences between the profiles, but not considered discrepant (Supplementary Table S3, 'CD'), in accordance with the ISFG guidelines [6]. Length heteroplasmies in the following regions were not considered for analysis: 301–315, 523–524, 573–576, 16183–16193. The Integrative Genome Viewer (IGV, Broad Institute) was used for manual visualization and inspection of sequence alignments to verify ambiguous variant calls [24].

2.3. Statistical analyses and modelling

All mtDNA profiles were originally reported as variants relative to the rCRS. To avoid reference bias, the profiles were converted back to nucleotide sequences. The following regions were considered: HVI+HVII (16024–340), CR (16024–576) and whole mtDNA (1–16569). Statistical analyses were conducted in R version 4.5.1 [25].

The minor allele frequency (MAF) of PHPs observed in the different sample types (blood, buccal, and hair) were compared using a Kruskal–Wallis rank-sum test due to non-normal distribution and an unequal number of data points per sample type. Paired *t*-tests were performed to assess whether the number of PHP events differed significantly between the reference (blood and buccal) and hair samples. To account for the multiple hairs and hair fragments analysed per individual, a mean PHP value was calculated per individual and used in the test. This approach ensured a direct and paired comparison between the tissues, while accounting for the non-normal distribution of the PHP event counts. Furthermore, to investigate the relationship between the number of PHP events and the fragment location along the hair shaft, a categorical Linear Model (LM) was applied to the dataset. The categorical LM was selected to test whether the PHP event counts significantly differed across hair segments, without assuming any specific trend of PHP occurrence along the hair shaft. A chi-square test based on the number of differences observed between buccal and hair profiles was used to assess if casework, previously published, and newly generated data (for HVI+HVII and CR) could be combined.

Hair and reference mtDNA profiles from the same donor were compared. The number of differences and discrepancies observed between the profiles were annotated, and the expected frequencies of these events under the Poisson distribution were estimated.

3. Results

3.1. Heteroplasmy observed in blood, buccal, and hair samples

Point heteroplasmy was reported at a MAF of 10% for MPS data, regardless of the sequencing method used for analysis. Supplementary Table S4 presents a more detailed overview of PHPs identified and their MAF. No statistically significant difference was observed in MAF across tissues (Kruskal-Wallis test: $\chi^2(2) = 1.47$, $p = 0.48$).

The frequency of the observed number of PHP events detected in reference (blood and buccal swabs) and hair samples is presented in Fig. 1 and Supplementary Figure S2. Data were separated into four categories: i) whole mtDNA genome data sequenced with MPS, ii) CR data sequenced with MPS, iii) HVI+HVII region sequenced with MPS and iv) HVI+HVII region sequenced with Sanger sequencing.

For the whole mtDNA genome data sequenced with MPS (Fig. 1), blood and buccal swab samples showed similar results, with ~70% of the samples containing no PHP events and presenting a similar number of average PHP events of 0.279 (standard deviation (SD) 0.549) and 0.326 (SD: 0.566), respectively. In hair, roughly 50% of the samples presented at least one PHP. The average number of PHPs observed in hair samples was 0.693 (SD: 0.875). There was a statistically significant difference in the number of PHP observed in hair samples compared to reference samples (paired *t*-test, 95% confidence intervals (CI) of 0.235 to 0.655 for comparisons between hair and blood samples and 95% CI of 0.219 to 0.579 between hair and buccal swab samples). One hair fragment presented five heteroplasmies. Further inspection on IGV could not detect the presence of contamination, nuclear mitochondrial sequences (NUMTs), or sequencing artefacts. The observation of more than three PHPs in a hair sample is very rare but has been reported by others [15,26,27]. Observations show fewer PHP events detected in the current study compared to previous studies. Kim et al. [18] reported that, on average, 60% of blood and buccal samples had no PHP, while 50% of hair samples had at least one PHP, in a dataset of 20 blood, 20 buccal and 40 hair shaft samples. In a study for the CR, van der Gaag et al. [19] found an average of 1.8 PHP events in the buccal sample in a total of 26 buccal and 475 hair samples investigated. The difference in the number of detected PHP events is most probably due to the lower detection thresholds used in these studies: Kim et al. applied a MAF of 5% with a minimum read count of 100, while van der Gaag et al. used a MAF of 3%

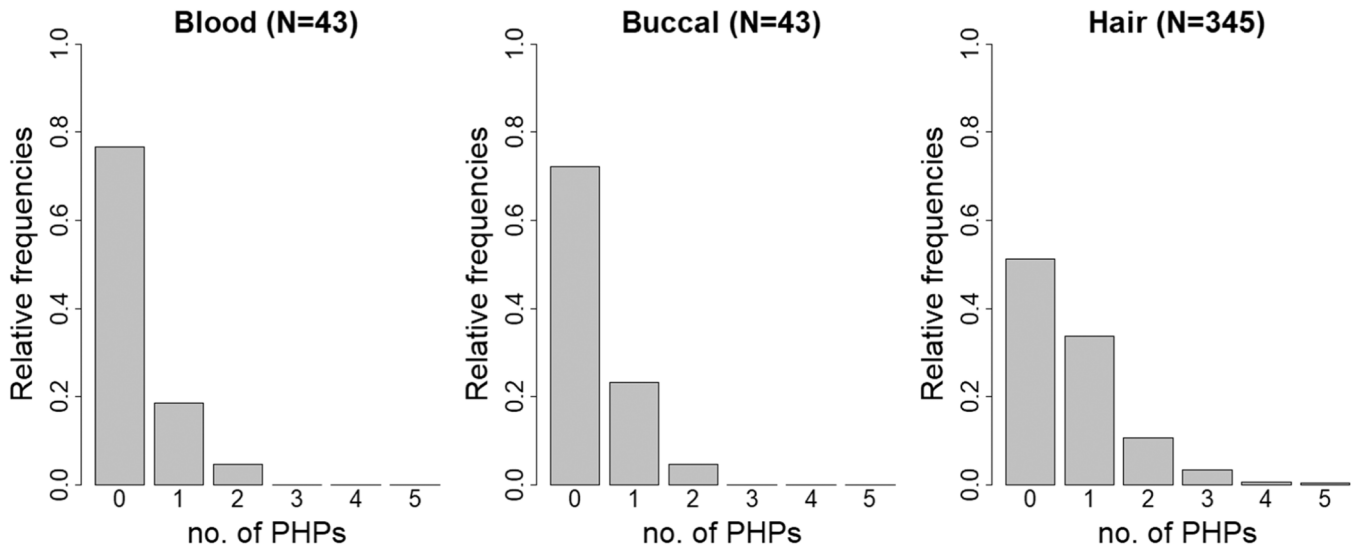

Fig. 1. Relative frequencies of the number of observed point heteroplasmies (PHPs) per sample in each tissue type (blood, buccal swab or hair) considering the newly generated data for the whole mtDNA genome, analysed with Massively Parallel Sequencing.

with a minimum read count of 30 reads, and only focused on the CR.

When considering the CR (Supplementary Figure S2) the average number of PHP events decreased to 0.140 (SD: 0.351) for blood samples, 0.186 (SD: 0.394) for buccal samples and 0.246 (SD: 0.506) for hair samples, resulting in non-significant differences between the references and hair samples (paired *t*-test 95% CI's of −0.101 to 0.254 and −0.050 to 0.203 for comparisons between hair and blood and between hair and buccal swab samples, respectively). When focusing on the HVI+HVII region only (Supplementary Figure S2), the average number of PHP events decreased even further to 0.125 (SD: 0.334) for blood samples, 0.167 (SD: 0.377) for buccal samples and 0.229 (SD: 0.485) for hair samples, but comparison of the occurrence of PHP in reference and hair samples remained non-significant (paired *t*-test 95% CI of −0.079 to 0.238 and −0.034 to 0.194 for comparisons between hair and blood and between hair and buccal swab samples, respectively). For the HVI+HVII region sequenced with the Sanger method (Supplementary Figure S2), fewer PHP events were observed than in the MPS data. The profiles generated with Sanger sequencing data presented non-significant differences between the tissues (paired *t*-test 95% CI of −0.096 to 0.224 for the comparison of hair and blood samples and 95% CI of −0.232 to 0.160 for the comparison of hair and buccal swab samples) and an average number of PHP events of 0.100 (SD: 0.316) for blood samples, 0.200 (SD: 0.421) for buccal samples and 0.170 (SD: 0.403) for hair samples. For the subset of samples that were sequenced with both Sanger and MPS methodologies (HVI+HVII region), the sequencing data presented similar results, with the same PHP positions being reported in both methodologies.

Considering the region where the PHP events occurred, in hair samples, more PHPs were found in the coding region (64%), while in blood and buccal samples, PHP events were more evenly distributed between both regions (50%/50% for blood and 43%/57% for buccal - coding/control). The trends described were observed in the MPS datasets for which the whole mtDNA molecule was analysed, regardless of the methodology used to generate the mtDNA profiles. In total, 285 PHP positions were observed in the dataset. Supplementary Table S5 presents a list of PHP positions reported per tissue observed in more than one individual. No recurring PHP positions were observed in the blood sample dataset. For the buccal samples, PHP position 16093Y was observed twice in the dataset. This position has previously been reported as a known hotspot for PHP [15]. In hair samples, ten recurring PHP positions were observed. Position 16093Y was the most frequently observed in hair samples, with 34 observations in six individuals. This position has also been previously reported as a hotspot for PHP in hair samples [2]. Eight of the 11 recurring PHP positions were located in the mtDNA CR. As reported by Just et al., 2015 [28], the PHP events observed in the CR seem to be associated with specific hotspots, whereas in the coding region, the PHP events are more randomly distributed [28]. The mutation rate in the coding region is lower than in the CR, as the DNA in the coding region is almost completely genic [29,30]. In the coding region, heteroplasmy is more frequently observed in intergenic areas, since mutations affecting functional genes may lead to disease or even be lethal to the donor [31]. In this dataset, three PHP positions located in the coding region (2706R, 3243R and 9565Y) were observed in more than one individual; all positions were present in hair samples only.

3.1.1. *Heteroplasmy positions observed along the hair*

As described in the methods section, the root ends of the hair shafts were excised, and the remaining hair was cut into 2 cm fragments. Selected fragments along the hair shaft were sequenced to understand if the heteroplasmy detected varied with the distance to the root, and if the same PHP positions were observed in hair fragments from the same individual. When generating the data, it was observed that for some of the fragments further distant from the root (fragment ten and up, equalling a hair shaft longer than 20 cm), the quality of the mtDNA profiles decreased, and partial profiles were reported due to failed amplification of some of the amplicons. One possible explanation is the low quality and quantity of mtDNA present in these fragments. A quantification of the number of mtDNA copies was not available for most samples. For a subset of samples for which mtDNA copy number was available, it was indeed possible to observe a decrease in the number of mtDNA copies with an increase in the distance to the root (data not shown). A similar observation was previously reported, stating the decrease in mtDNA in hair fragments that are more distal from the root [2,20,32–34].

As described before, 445 2 cm hair fragments were analysed in this study (345 with MPS, 34 with Sanger sequencing and 66 with both MPS and Sanger sequencing), representing overall hair lengths up to 40 cm (hair fragment 20). The distribution of PHPs observed per hair fragment and the number of fragments analysed for the whole mtDNA genome with MPS, considering the distance to the root, are presented in Fig. 2. For the MPS dataset, sequence data up to fragment 20 were available, whereas for the Sanger dataset, the maximum distal fragment available for analysis was fragment 9. Taking into account the number of hair

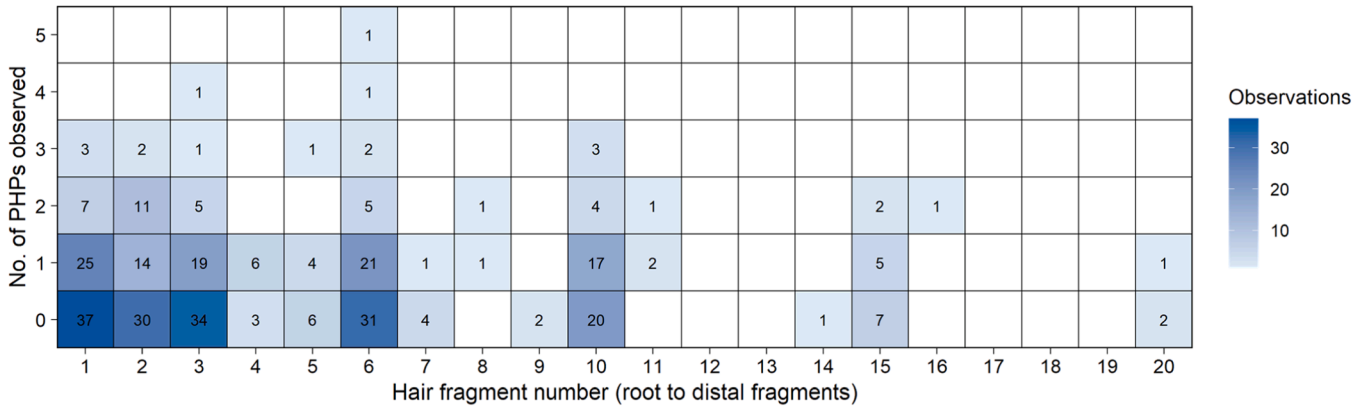

Fig. 2. Number of observations of point heteroplasmy (PHP) per hair fragment, considering newly generated data for the whole mtDNA genome analysed with Massively Parallel Sequencing.

fragments analysed, the average PHP occurrence was similar along the hair shaft ~ 0.6 for whole mtDNA data and ~ 0.2 in the CR and HVI+HVII region (data not shown).

A categorical LM was considered to investigate the relationship between the number of PHP observed and the fragment location along the hair shaft, based on the average number of PHP events observed in each fragment. For all datasets, the categorical LM reported non-significant p-values (Fig. 3). While the average number of PHPs appears slightly higher in more distal fragments, this pattern is not statistically supported, indicating that there was no statistically significant correlation between the average number of PHPs across the fragments along the hair.

3.2. Comparison of mtDNA profiles from different tissues of the same donor

3.2.1. Newly generated data

The evaluation of mtDNA-based forensic evidence is done by comparing the profiles of trace samples collected at a scene (here represented by hair samples) and the reference samples retrieved from the person of interest (blood and/or buccal swabs in this study).

Variant positions in the mtDNA profiles were reported relative to the rCRS. The 627 profiles newly generated in the scope of this study (corresponding to 58 blood, 58 buccal and 511 hair fragments) were compared for each individual. As previously stated, all positions that were different between the two profiles were annotated. Forty-five individuals presented no differences when their blood and buccal swab mtDNA profiles were compared. These included eight individuals who presented PHPs, where the positions were observed in blood and in buccal swab samples (Supplementary Table S6). Eight individuals presented differences between blood and buccal mtDNA profiles due to PHPs being present in one tissue and absent in the other (Supplementary Table S6).

For each individual, the mtDNA profiles obtained from each hair fragment were then compared to the mtDNA profiles from blood and buccal reference samples. As reported by Gaag et al. [19], some PHPs observed in blood and/or buccal swab samples were present in one or more hairs of the same donor. In our dataset, of the 13 PHPs positions observed in blood, nine were are also present in at least one hair fragment from the same donor, while for the 16 PHPs reported for buccal samples, 14 were also present in hair fragments from the same donor. These observations are in agreement with what has been previously reported by Gallimore et al. [17] where buccal samples were found to be better proxies than blood samples to estimate the PHPs expected in hair samples.

While most PHPs detected in reference tissues were also observed in hair, the reverse was not necessarily observed, with some of the PHPs reported in hair samples not being present in the reference samples. mtDNA profiles also varied between hairs from the same donor, and even amongst fragments along the same hair shaft.

3.2.2. Combined dataset of published, casework, and newly generated data

No statistically significant differences were detected when considering the number of differences (defined in Supplementary Table S3 as CD and DD) observed between buccal and hair profiles in the casework, previously published, and newly generated data for the HVI+HVII and

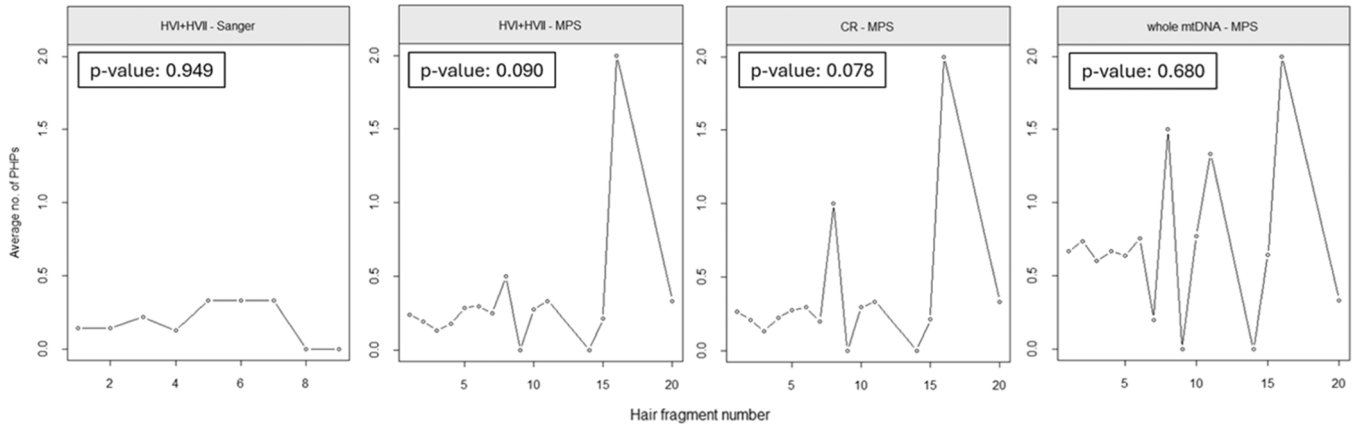

Fig. 3. Categorical linear model fitted for the average number of point heteroplasmies (PHPs) along the hair shaft for newly generated data considering HVI+HVII (Sanger and MPS data), CR, and the whole mtDNA molecule (MPS data only).

CR (non-significant chi-square test $p > 0.05$, data not shown). Therefore, all the information from these datasets was combined for the HVI+HVII (1437 comparisons) and CR (1291 comparisons). Results for the whole mtDNA comprised only MPS data, corresponding to 345 comparisons.

Supplementary Figure S3 represents the frequency distribution of the number of differences observed between buccal and hair fragment profiles. Most comparisons in the HVI+HVII and CR had no differences between the two profiles (77% and 76%, respectively). When the whole mtDNA molecule was considered, the number of comparisons with one difference was almost as frequent as situations where no differences between the profiles were observed (35.9% vs. 44.0%, respectively). One comparison presented four differences and another five differences, representing a combined frequency of 0.6%.

Results considering comparisons between blood and hair samples, are presented in Supplementary Figure S4. Here, only casework and newly generated data were considered, as published data did not report mtDNA profiles for blood samples. While the overall tendencies were similar to those observed for buccal vs hair comparisons, the number of comparisons presenting one difference between blood and hair profiles was slightly higher (23.5% for HVI+HVII, 26.7% for CR, and 36.8% for the whole mtDNA molecule).

3.3. *Incorporation of mtDNA profile discrepancies in the statistical evaluation of mtDNA forensic evidence*

Since the interpretation of mtDNA forensic evidence only takes into account the number of observed discrepancies between the profiles, the

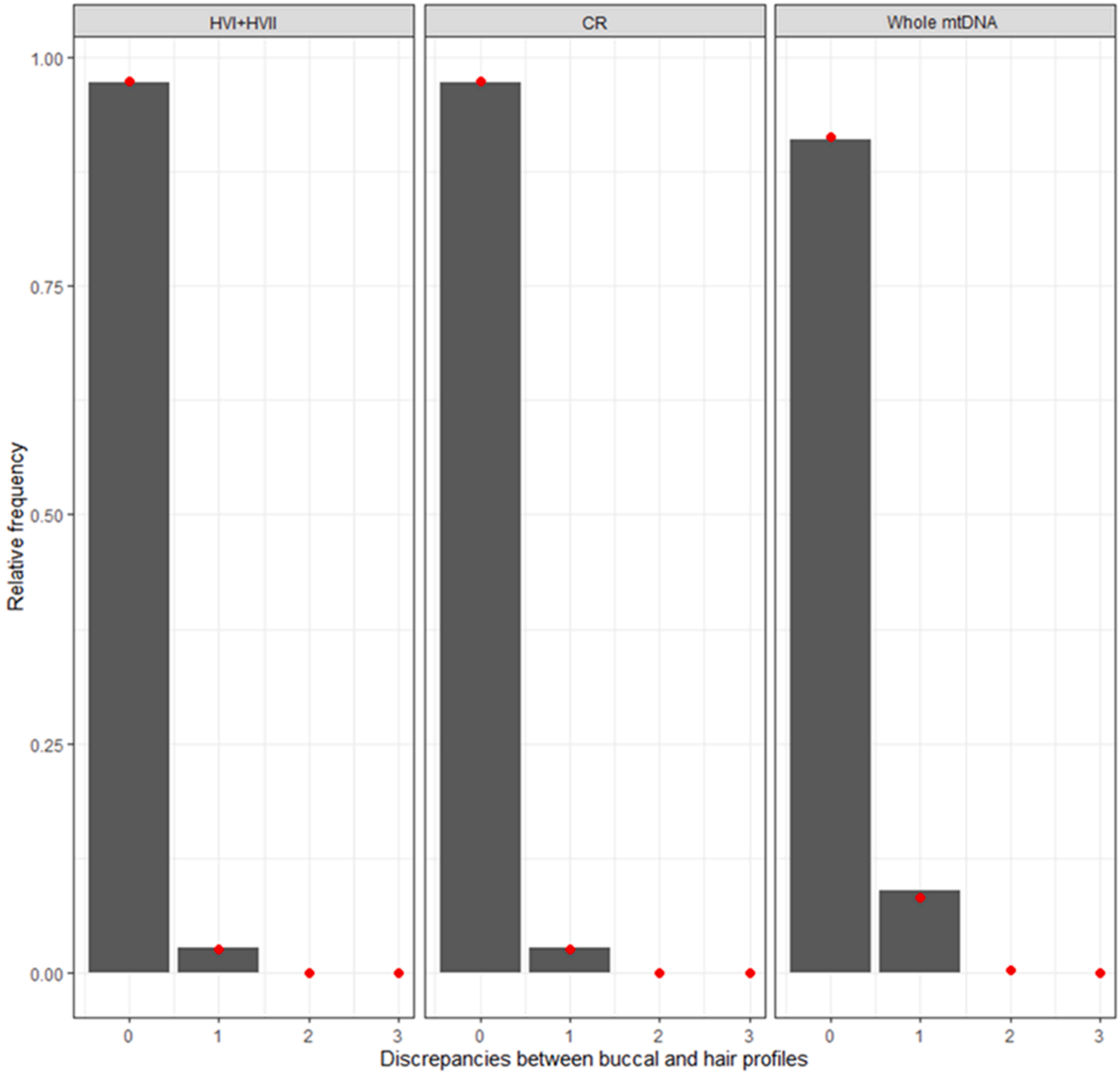

Fig. 4. Histogram of the relative frequency distribution of the number of discrepancies observed whenever buccal and hair shaft mtDNA profiles were compared, considering HVI+HVI (996 comparisons), CR (958 comparisons) (Sanger and MPS data) and the whole mtDNA molecule (122 comparisons) (MPS data only). The red dots represent the values of the Poisson distribution.

dataset was investigated to understand how many of the differences observed were indeed discrepancies between the profiles (marked in Supplementary Table S3 as DD) that could potentially lead to inconclusive results (or even exclusions) in a forensic setting.

The analyses were conducted considering the aggregated information from the hair shafts analysed for each individual, to avoid an overrepresentation of these events in the cases where the individuals' hair shafts had been split into multiple fragments. This represented 115 buccal swab and 996 hair profiles that were compared for HVI+HVII region, 102 buccal swabs and 958 hairs for the CR, and 43 buccal and 122 hair profiles for the whole mtDNA molecule. Fig. 4 presents the relative frequency of discrepancies in such comparisons.

Most of the comparisons did not present discrepancies, leading to 'inclusion/cannot exclude' scenarios. However, between 3% (HVI+HVII and CR) and 9% (whole mtDNA) of the profile comparisons presented discrepancies involving non-matching, non-heteroplasmic positions that could potentially be interpreted as inconclusive results. In all the profile comparisons, not more than one discrepancy was observed. A list of the discrepancies observed is presented in Supplementary Table S7.

Once the information about the underlying distribution of these events was described, the observed data were compared to a model based on the Poisson distribution to assess its fit (Fig. 4). The Poisson model provided a reliable representation of the data. As per the ISFG guidelines, a comparison with two or more discrepancies would lead to an exclusion scenario in the forensic setting; using the Poisson model here described, it is possible to quantify the very low probabilities of these events. In this study, the Poisson λ were 0.0271 (HVI+HVII), 0.0271 (CR), and 0.0902 (whole mtDNA). Using these parameters, the probabilities of observing two or more discrepancies were 0.00060 (HVI+HVII and CR) and 0.00360 (whole mtDNA). With the proposed model, the probability of discrepancies can then be incorporated in the evaluation of the weight of mtDNA-based evidence, using a likelihood ratio (LR) approach. The standard calculation for the evidential weight by LR is given by [35]:

$$LR = \frac{P(E|H_1)}{P(E|H_2)}$$

In the context of mtDNA data, *E* would represent the evidence - in this case, the observed mtDNA profile of the trace sample, *H1* would represent the hypothesis that the person of interest is the donor of the mtDNA profile in the trace sample, and *H2* would represent the hypothesis that a random individual is the donor of the mtDNA profile obtained from the trace sample.

If the reference sample retrieved from a person of interest, and a trace sample collected from the crime scene originate from different biological material from the same individual (buccal reference and trace sample from hair, e.g.) then the following expression can be considered:

$$LR = \frac{P(D)}{P(X_Q)}$$

where $P(D)$ represents the probability of discrepancies observed between buccal swabs and hair samples from the same donor in a population, and $P(X_Q)$ is the frequency of the mtDNA profile of the trace in a population. Several methods have been described to estimate $P(X_Q)$; the DNA commission of the ISFG has published a comparison between these methods [6], reviewing both their pros and cons. For illustrative purposes and simplicity, in this study, an example was considered where $P(X_Q)$ is the frequency of a profile observed once in the Westeurasian metapopulation from the EMPOP v4/R14 database profiles containing the CR (database size of 20,162 individuals, last assessed 27 November 2025).

Assuming that $P(D)$ follows the Poisson distribution presented in Fig. 4 for the CR of mtDNA, and that $P(X_Q)$ is equal to 4.96×10^{-5} (1/20,162), if no discrepancies were observed in a comparison between two mtDNA profiles from a reference buccal swab sample and a hair trace (i.e., D = 0) then:

$$LR = \frac{P(D=0)}{P(X_Q)} = \frac{0.973}{4.96\times 10^{-5}} = 19,617.63$$

Similarly, when a comparison between two mtDNA profiles presents one discrepancy (i.e., D = 1):

$$LR = \frac{P(D=1)}{P(X_Q)} = \frac{0.0264}{4.96\times 10^{-5}} = 532.28$$

Once comparisons involving two or three discrepancies between the profiles were considered, the LR values decrease to 1.82×10^{-8} and 6.09×10^{-9}, respectively. With the current ISFG guidelines [6] these values would have been zero, excluding the person of interest as a potential donor of the trace.

Besides the number of discrepancies, the LR will also be highly dependent on the frequency of the observed haplotype: if a haplotype is observed more times in the database instead of the single observation in the presented example, the LR would decrease.

4. Discussion

Hair samples represent one of the most common traces in crime scenes. To date, it is still difficult to obtain viable autosomal STR profiles from hair samples without the root, and in this context, the analysis of mtDNA can provide further information about a suspect. Nevertheless, even when a person of interest is identified, biological reference material is collected, and a mtDNA profile is retrieved, the presence of mtDNA heteroplasmy or a single discrepancy between samples can complicate the interpretation of results. As seen in this and other previous studies, heteroplasmy is a common phenomenon in mtDNA and this is particularly so in hair samples, where the bottlenecks during cell division are more pronounced, and the consequences of the mutated positions along the mtDNA molecule might not be as severe as in more functional tissues such as blood, for example.

Current guidelines for forensic interpretation of mtDNA evidence rely on the number of discrepancies observed between the reference and trace profiles to assess whether the comparison should be considered an inclusion, an exclusion, or inconclusive. These rules, although practical, do not necessarily reflect the biology of the different tissues. This work investigated heteroplasmy and the number of differences observed when comparing mtDNA data from blood, buccal swabs and hair fragments from the same donor. The aim was to assess how often differences occurred between these tissues, and how many of these differences were discrepancies that could impact the forensic interpretation of the evidence, to expand on the knowledge for the interpretation of forensic evidence based in ground data.

The work confirmed that while mtDNA profiles from blood and buccal swabs from the same individual tend to not present differences, the same was not observed when comparing these references to hair samples. Hair samples frequently presented more PHPs compared to the reference profiles from the same individual. Similar trends have been described by others [2,19].

In this study, most differences observed were caused by the presence of heteroplasmy in the mtDNA profiles, but one cannot discard that other events, such as sequencing errors, artefacts of the methodology, and degradation due to the condition of the biological material can occur in a forensic context. Differences in the number of observed PHPs should also be considered in light of the genomic regions analysed. The whole mitochondrial genome is substantially larger than the CR, thereby increasing the likelihood of detecting heteroplasmic positions. In addition, the coding region is under a stronger functional constraint than the CR, which may affect the expected level of true biological variation. The observed differences were identified based on the interpretation thresholds defined in this study (minimum variant frequency of 10%). However, it is important to acknowledge that the number of differences

reported, and consequently - the number of discrepancies that could lead to inconclusive or exclusion scenarios - will inherently be dependent on these thresholds. Sensitivity levels for reporting mtDNA heteroplasmy are currently changing with the emergence of MPS technologies [28, 36–38] and as such, it is expected that the frequency and the distribution of differences (and discrepancies) will also change, highlighting the need for careful consideration of the thresholds to use.

Previous work by McElhoe et al. [27] has focused on the evidential weight of mtDNA, proposing a statistical framework to incorporate the frequency of PHP events into LR calculations, as a way to strengthen the evidential value of matches. Their work provided a better understanding of PHP rates across populations and main haplogroups. The current study considers the probability of observing discrepancies between different tissues from the same donor, addressing a different but equally important aspect for calculating the value of mtDNA-based evidence.

As demonstrated, the strength of the mtDNA evidence is highly dependent on the number of discrepancies between profiles. Compared to the traditional way of calculating LR considering inclusion vs. exclusion, in this model, when no discrepancies are observed between E and X_Q (i.e., inclusion), the LR values will be lower (the numerator will present values lower than 1, instead of 1 using the traditional LR model described in the current ISFG guidelines [6]). A single discrepancy (D=1) reduced the LR to approximately 37-fold, reflecting a marked decrease in the evidential weight, and providing lower support for identity. Nevertheless, the described model adjusts the LR so that it better reflects the uncertainty attributed to minor, biologically plausible differences. With the proposed model, the LR values will always be higher in situations where discrepancies have been observed compared to the traditional approach to calculate LR [35] (i.e., the LR will have values higher than 0 in the numerator, instead of 0 in the traditional model).

The presence of two or more discrepancies resulted in dramatic reductions in the LR effectively lowering the probability of a common origin of the mtDNA profiles. These findings underscore the critical importance of accounting for discrepancies in mtDNA profile interpretation and provide a quantitative framework for assessing the strength of forensic evidence in cases of partial matches. While in general alignment with the current ISFG guidelines that would also consider an exclusion for profile comparisons with more than two discrepancies, the results from the proposed model would be particularly helpful in situations where LRs from different evidence types would be combined by providing low values, but above 0.

5. Limitations of the study

The study contributed to adding knowledge and data necessary to enhance statistical calculations based on mtDNA comparisons. However, some limitations should be acknowledged: i) although many participants have contributed data to this initiative, the sample sizes and populations represented are still limited, especially if phylogenies are to be accounted for, highlighting the need for larger and more representative datasets; ii) donor-specific variation regarding hair maintenance habits (e.g. the use of hair dyes or styling products) was not systematically documented; iii) variation in chemistries across the laboratorial workflows (from extraction to sequencing methods) may have influence on the detection of PHP; iv) variation in the analytical pipelines could also contribute to the variability detected. Future studies incorporating standardised methodology on different population groups will be necessary to extend these findings.

6. Conclusion

Although still in its infancy, this approach aims to move the interpretation of mtDNA forensic evidence from a rule-based approach to one more grounded on biological phenomena.

Future work (and future statistical models) should aim to assess the impact of common vs. rare discrepant positions and the frequency of the haplotype in the LR calculations, as the dataset in the current study was quite limited to fully encompass the complexity of these events.

Along the same line, it would be interesting to explore whether some phylogenetic lineages/haplogroups are more prone to present discrepancies when two mtDNA profiles are compared. Finally, it should be investigated if mtDNA reference databases that contain data generated from different biological tissues can be used to estimate the weight of the evidence when data is generated in other tissues, or if, on the other hand, correction factors are needed to accommodate differences observed between the tissues. With the data produced in this work, it was observed that maybe the use of such databases can be applied whenever buccal and blood samples are compared, since most often, both tissues presented similar profiles.

As mentioned, to address these questions, it is necessary to have a great amount of data generated for many individuals, from different populations, representing different phylogenetic lineages, to obtain more reliable estimates. Such efforts are only possible to achieve through scientific collaborations and by leveraging technical infrastructure and databasing to host this type of information.

CRediT authorship contribution statement

Floor Claessens: Writing – original draft, Writing – review & editing, Data curation, Formal analysis. **Mikkel Meyer Andersen:** Writing – review & editing, Conceptualization, Methodology, Formal analysis. **Christina Amory:** Writing – review & editing, Data curation. **Cristina Arévalo:** Writing – review & editing, Data curation. **Stijn Desmyter:** Writing – review & editing, Data curation. **Sophie Dognaux:** Writing – review & editing, Data curation. **Kristiaan J van der Gaag:** Writing – review & editing, Data curation. **Tomasz Grzybowski:** Writing – review & editing. **Gabriela Huber:** Writing – review & editing, Data curation. **Filomena Melchionda:** Writing – review & editing, Data curation. **Hiroaki Nakanishi:** Writing – review & editing, Data curation. **Tóra Olsen:** Writing – review & editing. **Cristina Pie:** Writing – review & editing, Data curation. **Lourdes Prieto:** Writing – review & editing, Data curation. **Katarzyna Skonieczna:** Writing – review & editing, Data curation. **Chiara Turchi:** Writing – review & editing, Data curation. **Walther Parson:** Writing – review & editing, Supervision, Validation, Methodology, Conceptualization. **Vania Pereira:** Writing – original draft, Writing – review & editing, Supervision, Conceptualization, Project administration, Funding acquisition, Data curation, Formal analysis, Methodology.

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Given his role as Editor-in-Chief, Walther Parson had no involvement in the peer review of this article and had no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to another journal editor.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsigen.2026.103477.

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