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Comparisons of aged samples and modern references provide algorithm for mtDNA analysis in challenging material

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Based on results of over 12 years of research, we performed a comparative analysis of haplotypes from 70 to 80 years old bone samples with modern high quality references. Whole mitochondrial genomes were obtained for the purpose of human identification cases conducted by the Polish Genetic Database of Victims of Totalitarianisms—Pomeranian Medical University Research Centre, using Thermo Fisher Scientific's Precision ID line and Ion GeneStudio S5. Converge 2.2 and IGV 2.12.3 were used for secondary sequence analysis and their parameters were altered to construct a new variant calling algorithm. We have found neither a simple change in thresholds, nor removing contaminant reads significantly decreased the number of discrepancies found between haplotype pairs, and conclude that standard analysis settings can rarely be used for poor quality DNA data. The study confirmed some limitations of the analysis of low-quality samples, and of the familial comparisons themselves. Still, the algorithm we developed helps to decide which calls to accept when dealing with difficult material, reducing manual labour, based on Converge-generated Status and EMPOP state of the variants. Additional step for samples with low region coverage is introduced. This protocol can be used in other areas where DNA quantity and quality are low.

Keywords Whole mitochondrial genome sequencing, Variant calling, Human identification, DNA degradation, Massively parallel sequencing

Mitochondrial DNA (mtDNA) analysis is burdened with numerous interpretational issues. The occurrence of length heteroplasmy (LH), particularly within C- and AC-stretches, presents a significant difficulty, especially when assessing which calls originate from an increased number of identical nucleotides and which are the result of the limitations of a specific sequencing method[1,2]. The interpretation of point heteroplasmy (PH) must be based on a threshold that protects the expert from accepting calls that result from artifacts or contamination while simultaneously allowing for the identification of actual heteroplasmic positions[3]. The sequencing of challenging DNA samples often reveals characteristic patterns of damage that vary depending on the library preparation method used. The damage itself is associated with the deamination of cytosine to uracil in the proximity of ends of DNA fragments. As uracil pairs with adenine during amplification, leading to C◊T and G◊A changes in sequencing data, this process introduces errors in sequencing[4,5]. To mitigate the effects of deamination, strategies such as enzymatic repair or computational filtering can be used, however some commonly-used repair techniques may result in elevated C◊T (5' ends) and G◊A (3' ends) misincorporations[6]. Nuclear mitochondrial DNA segments (NUMTs) can be co-amplified with mitochondrial targets, especially when contigs are generated from sequences resulting from short target amplification, particularly in samples high in nuclear DNA template[3,7,8]. Additionally, sequencing mistakes can be introduced, some of which are platform-dependent[9,10], and degraded DNA matrices can lead to the introduction of biased, incorrect calls due to stochastic effects[11].

These issues can result in non-homogeneous, overlapping reads that are difficult to interpret and can lead to incorrect haplotype calling and haplogroup estimation, thereby hindering the human identification process. While many of these concerns have been addressed by the International Society for Forensic Genetics (ISFG)[12] and the Scientific Working Group on DNA Analysis Methods (SWGDAM)[13], the growing number of mtDNA

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studies based on MPS—including those aimed at whole-mitogenome sequencing—makes understanding the impact of these issues on NGS-based analyses essential. Thus, validation studies are continually being conducted. Unfortunately, even though they usually include tests on artificially degraded DNA, the number of real-case samples tested is often limited[10,14–18]. The fragmentation of DNA in biological material studied in forensic cases renders otherwise highly reliable long-range PCR[19] unsuitable for analyses based on real-life samples. As a result, alternative methods are utilized, including the gold standard Sanger sequencing with short amplicons and NGS using various sequencing platforms[9]. These results help elucidate some differences between haplotypes called by different protocols; once again, they do not provide a complete picture of the haplotype being studied when the protocol is not challenged by DNA degradation.

Phylogenetic analysis-based haplotype assessment plays a critical role in mtDNA studies. Not only forensic guidelines[12,13], but also numerous research papers[3,5,8,10,17,20–24] underline the importance of phylogenetic analysis when assessing the correctness of haplotype calls. The European Mitochondrial DNA Population Database (EMPOP) is an indispensable tool in this context, offering a robust framework for accurate haplotype classification and quality control in mtDNA sequence data. EMPOP's incorporation of phylogenetic alignment and fine-tuned haplogrouping[20,21], ensures its utility across diverse applications, extending beyond forensic genetics to fields such as anthropological research, evolutionary biology, and medical genetics. Its use of quasi-median network analysis filter sharpens error detection capabilities[25], allowing precise resolution of ambiguous variants in mtDNA datasets. Additionally, tools like EMMA 2 leverage Phylotree nomenclature for post hoc deconvolution of mixed mtDNA samples, further broadening EMPOP's applicability[26,27]. The next-generation database search algorithm embedded within EMPOP not only supports forensic casework but also facilitates population-wide mitogenome analyses with enhanced speed and accuracy[28]. As EMPOP utilizes an algorithm that converts mtDNA sequences into alignment-free nucleotide strings (SAM2), facilitating unbiased database searches and phylogenetic alignment, it guarantees that haplotypes are accurately identified within the database, regardless of their initial alignment.

Through 10 years of research conducted by the Polish Genetic Database of Victims of Totalitarianisms—Pomeranian Medical University Research Centre (Pol. *Polska Baza Genetyczna Ofiar Totalitaryzmów—Centrum Badań Pomorskiego Uniwersytetu Medycznego w Szczecinie*, PBGOT, see: http://pbgot.pl), the methods used in bringing back the identities to the victims evolved with the changing times and the rise of new technologies. The DNA isolation protocols were optimized to obtain the highest DNA concentration possible[29]. The number of autosomal Short Tandem Repeat (STR) loci included in commercially available kits rose from 15 (AmpFLSTR™ NGM, Thermo Fisher Scientific—TFS, which included also the amelogenin marker) to 21 (GlobalFiler™, TFS, also amplifying the amelogenin marker, one Y-chromosomal insertion/deletion marker, and one Y-chromosomal STR marker), and the number of Y-chromosomal STRs—from 17 (AmpFLSTR™ Yfiler™, TFS) to 27 (Yfiler™ Plus, TFS)[30,31]. We have also implemented Next Generation Sequencing (NGS)/Massively Parallel Sequencing (MPS) analyses into our investigations[32,33]. And finally, the scope of mitochondrial DNA analysis was broadened from hypervariable regions I (HVI) and II (HVII)[34–36] to whole mitogenome sequencing[37]. Each new step enforces the update of the database, based on meticulous method configuration with analysis validation. In the era where WGS becomes more and more widely-used, manual variant analysis becomes one of the biggest issues for laboratories that process as many samples. Despite the introduction of numerous bioinformatics tools to assist in haplotype verification, a secondary analysis that incorporates expert review remains essential for a comprehensive evaluation of MPS-derived mitogenomes in forensic genetics[38]. Having been presented with the unique possibility of looking at haplotypes from over 70-year old samples low in DNA quantity through the modern high-quality samples of maternal relatives, we established an internal semi-automatic analysis algorithm that would help reduce the hands-on time of manual data interpretation. With the approval of the Institute of National Remembrance we are able to share the results of this validation after anonymization of samples.

In this study, we show the development of an algorithm that helps assess the correctness of a call based on available sequencing metrics when using Ion Torrent sequencing Converge software, and EMPOP, even when dealing with haplotypes obtained from highly degraded, low-template material.

Results and discussion

DNA quality and quantity

For DNA samples obtained from bone material originating in 1940s and 1950s (from this point on referred to as "evidence material"), low DNA quantity and the presence of DNA degradation was expected. 24 extracts (53.33%) did not contain the recommended amount of DNA, as per small amplification target parameter (Fig. 1). The quantification results also showed significant DNA degradation (Degradation Index—DI—calculated as the proportion of small to large autosomal target concentration—greater than 10) in 8 samples (17.78%), and slight to moderate degradation (DI between 1 and 10) in remaining 37 (82.22%) (S1).

Discrepancies between evidence and reference material

The results were retrieved from the Converge software as Multi variant Excel files and haplotypes obtained from bone samples were compared with haplotypes from buccal swabs of relatives of the deceased. 85 changes in reference to the revised Cambridge Reference Sequence (rCRS) not observed in the modern reference material were noted in haplotypes obtained from the evidence material (referred to as discrepancies). Those included calls corresponding to point heteroplasmy (36, 42.35%), SNPs (5, 5.58%), and heteroplasmy within C-stretches in HVI (3, 3.53%), HVII (13, 15.29%), and throughout the remaining positions of the mitogenome (28, 32.94%). To investigate if the numerous PH calls were not in fact a result of systemic contamination, the percent of minor allele frequency (%MAF) levels for samples containing apparent PHs were verified; although two samples showed signs of severe degradation, none of the seven samples that included PH discrepancies showed a pattern suggesting an obvious low-level mixture (e.g. %MAF consistently 10% or higher). This high number of discrepancies shows

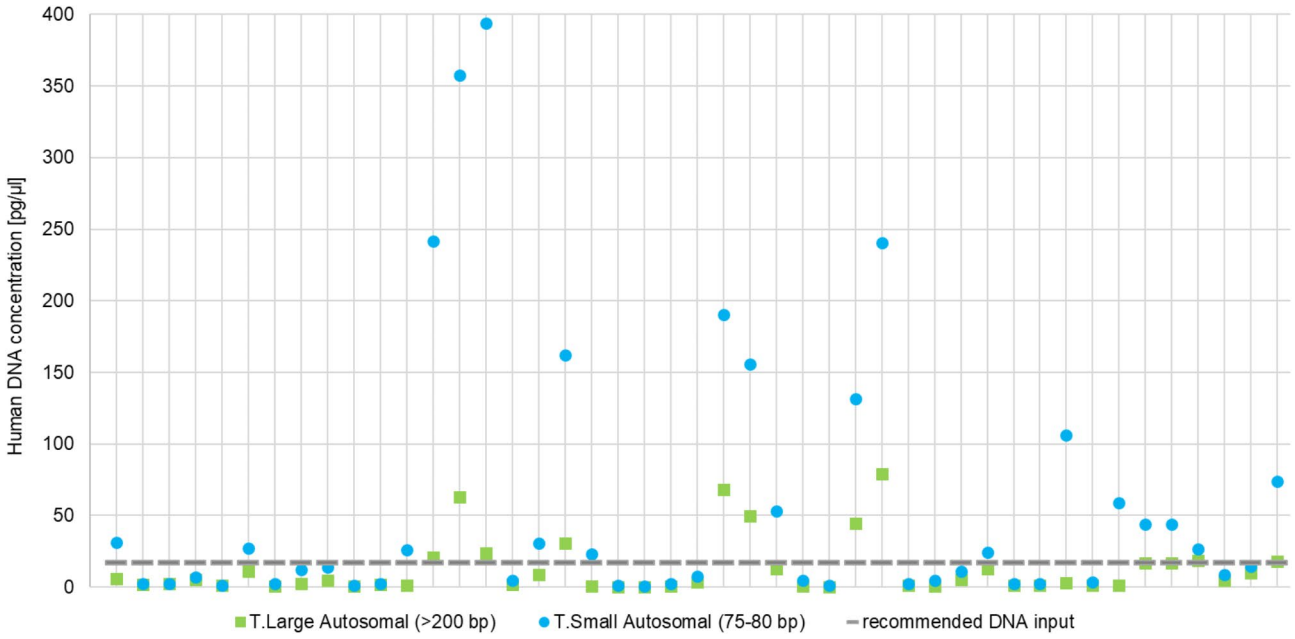

Fig. 1. Human DNA concentration within evidence material extracts [pg/µl].

the amount of labour and the level of expertise of the analyst needed, rather than proves the method itself unfit for the analysis of poor quality samples. Although some of the two-nucleotide mixtures regarded as PHs were situated in the 10 outermost bases at the ends of amplicons, pointing to possible deamination[6], it cannot be said this was true for a considerable portion of those changes (S2). Thus we do not differentiate if those differences occur as results of sequencing artifacts, DNA fragmentation, cytosine deamination, or extremely low DNA input. Regardless of the origin of those changes, they would need to be manually excluded in the process of data analysis and interpretation, also with the use of additional software[8–11,17].

Alternative variant calling protocols

To identify an approach that minimizes the labour intensity of manual sequence analysis, different analysis settings for the HIDGenotyper plugin were applied. As proposed by Ring and co-authors[7], the "remove contaminant reads" (RCR) option was used. This allows for the software to automatically filter out read blocks that contain many correlated mutations (which might imply degradation) or are suspected to be NUMTs. As in the aforementioned study this step precedes additional bioinformatical read removal and variant filtering methods, the exact efficiency of the RCR was not described there. Our results showed an insignificant reduction in discrepancies using this approach (S3), which may stem from the low-level presence of NUMTs in the sequencing data. In our case, the nuclear DNA was sparse (Fig. 1), and many samples were still diluted. Other alterations included: modified thresholds for PH: 5%—referred to as "T5" and 20%—"T20", and minimum total read coverage per position (50) and minimum variant coverage to call (10), referred to as "50-10", and another setting with 20 and 5 reads, respectively, referred to as "20-5". Only two of those showed a significant level of reduction of the number of discordant changes: "T20" in case of the number of incorrectly called PHs, and "20-5" for apparent SNPs (S3). These results prove that thresholds set based on high-quality DNA sequencing do not translate strictly into the analysis of degraded, sparse DNA, and underline the need to form further instructions for the successful data interpretation from difficult material.

Moreover, almost all of the altered analysis methods lead to the introduction of mutations not existing in reference material and thus none of them was considered as helpful in the process of assessment of correctness of a call from difficult material (Fig. 2). Although the widely-used 20% PH threshold ("T20")[39] did not lead to introduction of incorrect changes, it allowed for reduction of only 18 out of 85 initial discrepancies (21.18%). As the 10% PH threshold seems to be too low for distinguishing actual and apparent PHs when they occur in primer-binding sites[3], "T20" still seems to be a safer approach for semi-automatic mtDNA analysis. Naturally, the elevation of thresholds may cause an oversight of low-level heteroplasmy[7]. However, bearing in mind the biological mechanism behind the mtDNA heteroplasmy itself (namely not only the occurrence of spontaneous mutations, discussed further in a later section, but also the limited number of mitochondria being passed from mother to child[40]), it has to be understood that discrepancies in heteroplasmic positions, and in the levels of those heteroplasmies, are a natural result of this process. The number of additional discrepancies introduced by using the lower thresholds (Fig. 2) showed these method alterations to be highly unreliable. Especially, the extremely sensitive "T5" (5% threshold) protocol used by Kim and co-authors[41] aimed to reduce the risk of erroneous exclusions between mitotypes derived from hair shafts and buccal swabs, resulted in 173 conflicting positions. Those changes may result from DNA damage leading to sequencing errors, and together with the low DNA copy number, cause stochastic effects[42]. Thus, for forensic purposes, even though cases where heteroplasmy levels

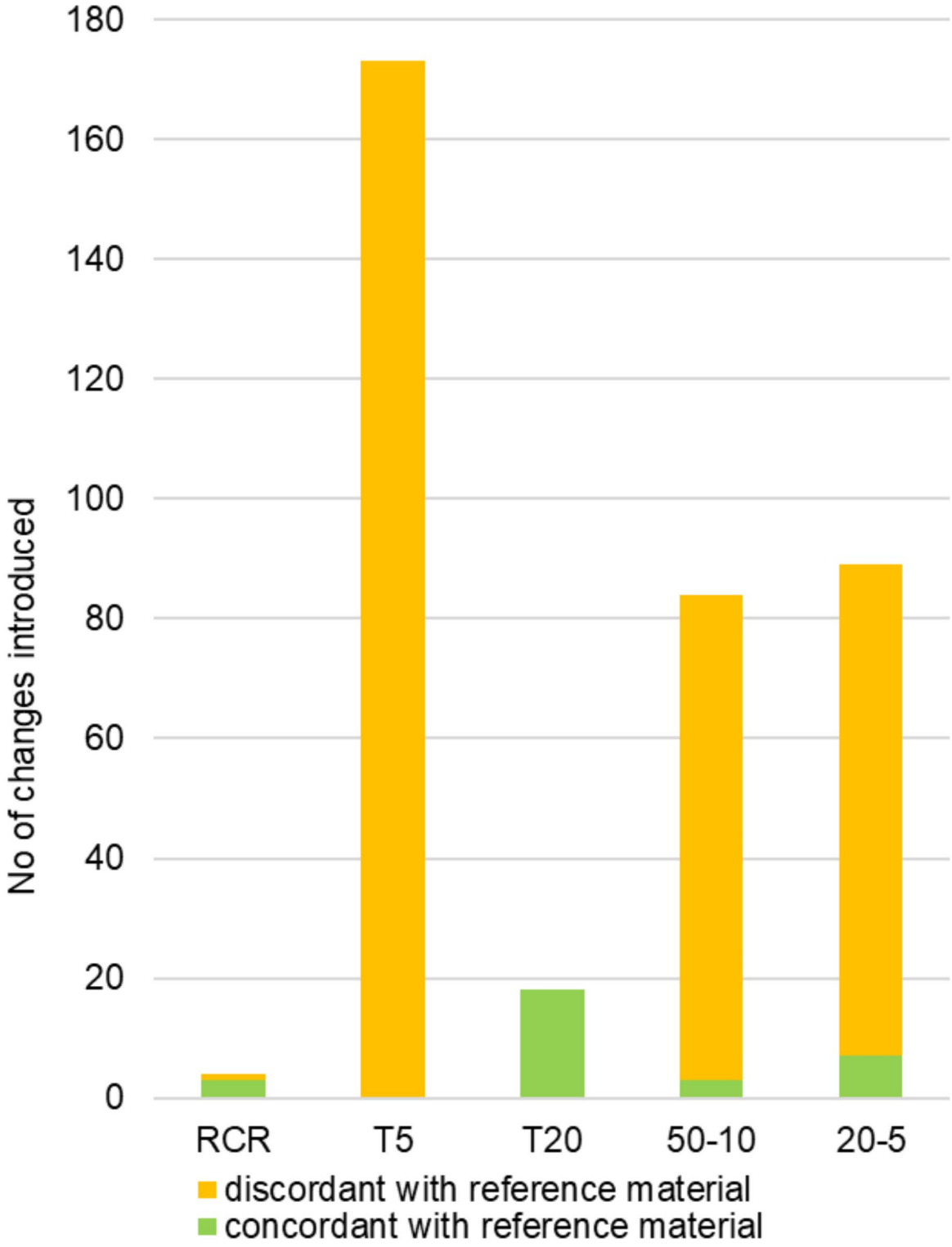

Fig. 2. Concordance of mutations introduced by altered analysis parameters with haplotypes called from reference material.

have ben shown to be of importance are known[5,43], we advise to prioritize the establishment of a haplotype that encompasses confidently identified mutations rather than pursuing a more intricate haplotype that incorporates low-frequency heteroplasmic variants. Still, in some cases of clinical research, minor heteroplasmy variants may be of importance. A study by Rathbun et al.[5] describes how the analysis of the transition: transversion (TS: TV) ratio of SNP positions in a sample haplotype may help predict damage when assessing heteroplasmy in MPS

data. Still, to ensure accurate heteroplasmy analysis, replicate data analysis and phylogenetic assessment should be implemented.

Even though the exclusion of the NUMTs in highly degraded material, potentially low in nuclear DNA copy, may not be an issue for many samples, there is no quantity threshold for their presence, and so the possibility of their existence should still be considered. While it is true that some NUMTs are universal through the population[44], many are extremely rare, with an estimated de novo creation rate at every 10^4 births[45]. Strobl, Cihlar and co-authors[3] proposed an algorithm to establish if an apparent PH is actually a NUMT based on variant strand bias, the number of SNPs in phase, homology to a nuclear chromosome, the mutation being on a list of known NUMTs, and consistency of the SNP with haplogrouping results of the mitotype.

Discrepancies disregarded for algorithm construction

In 15 haplotypes obtained from evidence samples and 7 from reference material, LH and PH calls within different positions in the range 8247 and 8256 were indicated by the Converge software. As none of these changes (8247.1G, 8254del, 8256del) were ever noted in EMPOP database v4/R13 (as of 29.10.2024), the origin of those changes was investigated. This region is covered by two amplicons: 8197–8365 (Pool 1) and 8081–8254 (Pool 2). Indeed, the alignment of those sequences to the location of primer-binding sites in Integrative Genomics Viewer (IGV) 2.11.9 showed that primer RV_79 attaches in the nearest proximity to those changes (S4), suggesting a possible alignment issue. Although some authors mention a misalignment risk in mitochondrial DNA analysis in clinical studies[46,47], this is usually associated with state-of-the-art algorithms. Another possible explanation includes sequencing artifacts, as proposed by Strobl and co-authors[24]. These positions were disregarded in further analysis. Also calls concerning the presence of length heteroplasmy within C-stretches in HVI (16182–16193) and HVII (303–310) were disregarded, as discrepancies within these ranges are frequently observed across different tissues[41] and between family members[48,49]. Therefore, their impact on haplotype calling for the identification process is limited[13]. The DNA Commission of the International Society for Forensic Genetics also advises to ignore the polymorphisms in the C-stretches in database searches[12].

Algorithm development

The Converge software describes variant calls with several parameters, including universally used metrics as variant strand bias, frequency or coverage, while also showing the call's Status and EMPOP state. To evaluate which of those metrics could be helpful for call assessment, the correlation between call concordance within the evidence material-reference material pairs (from this point on called simply "concordance") and sample quality, quantity, library quantity, and sequencing metrics was studied and is shown in Table 1.

The concordance of a call was found to be very strongly correlated with the variant strand bias (Table 1). Despite this very strong correlation, no threshold concerning this metrics allowed for the assessment of a concordance of a call (S5). Although this stands in opposition to the results obtained by Ring and co-authors[7], and partially with Cihlar and co-authors[3], it can be explained by the level of degradation of the material and overall low DNA input, which lead to the occurrence of stochastic effects and thus occurrence of strand bias regardless of a correctness of a call. Thus, in potentially degraded DNA samples, we do not advise to use a fixed variant strand bias setting.

The call's consistency strongly correlated with its Status and EMPOP state. While the EMPOP state is established based on the presence of the variant in EmPOP database[28] (unknown—not present in the database—a private mutation, NUMT or degradation; unexpected—present, but not in the haplogroup assigned to the sample; unchecked—fits into the haplogroup, but has not been manually verified; confirmed—fits into haplogroup and has been manually verified), the Converge's Status of variant is calculated based on more factors. Those include: variant strand bias, coverage, frequency, contamination, homopolymer length, the variant being on the list of known NUMTs or at typical sequencing artefact positions and its aforementioned EMPOP state. Based on this formula, the call is assigned a value in the range of 0.0 to 1.0, and then described as unlikely (0.16–0.31), unclear (0.32–0.48), possible (0.49–0.65), likely (0.66–0.81) or confirmed (0.82-1.0). Additionally, individual variance reports include calls described as false (0.0-0.15), the one status that cannot be manually changed by the analyst, and for this reason these calls were disregarded from any analysis. All calls assigned "likely" and "confirmed" Status, as well as "unchecked" and "confirmed" EMPOP states were concordant with calls from reference material, as well as almost all (86.14%) of calls with an "unexpected" EMPOP state. Close to none "unclear" (5.88%), "unlikely" (4.76%) Status and "unknown" EMPOP states (13.64%) variants were concordant with reference material calls for these positions (Fig. 3).

Based on this observation, an algorithm for the assessment of a possible call concordance was created. While initial, separate algorithms for Status and EMPOP states had both a concordance rate of 99.81% (S6), the algorithm based on combined states showed a 100% concordance between variants called from evidence material in relation to reference material (Fig. 4).

The high effectiveness of the use of the EMPOP state of the variant and the combined variant Status is consistent with other studies, including the aforementioned paper by Cihlar and co-authors[3]. The EMPOP itself is a free tool widely recognized in the field. Beyond serving as a database search platform, EMPOP offers advanced capabilities for analysing observed haplotype variants in the context of their phylogenetic compatibility. It identifies the most likely haplogroup for a sample and evaluates the likelihood of individual mutations (or rather, the numerical value for the possibility of their presence in the haplotype—*cost*—based on the mutation type), including those absent not only in the entire database but also in the reference and universal PhyloTree framework, which spans multiple disciplines. This robust functionality underscores EMPOP's pivotal role in ensuring accuracy and reliability in mitochondrial DNA studies in every area that utilizes this uniquely inherited and exceptionally degradation-resistant marker. Thus, in line with other forensic genetics studies[3,5,8,10,17,20–24], we propose using the widely-recognized, free tool, for assessing the correctness of calls in cases with low quality

Parameter	*R* Spearmann's
Percent of genome covered	0.155
Median coverage	0.147
Total number of reads	0.069
Number of reads used	0.088
Number of confirmed	0.085
Number of unconfirmed	− 0.213
Number of NUMTS	− 0.008
Var freq	0.371
Major freq	0.363
Read coverage+	0.107
Read coverage−	0.113
Allele coverage	0.288
Allele coverage+	0.276
Allele coverage−	0.286
Status	**0.505**
Frequency	0.371
Var strand bias	**− 0.707**
Read strand bias	− 0.110
EMPOP	**0.567**
Score	0.459
Library quantity	0.096
T.IPC	− 0.001
T.large autosomal	− 0.049
T.small autosomal	− 0.007
Degradation index	0.057
Mean quantity	− 0.011

Table 1. Correlation between various sample and sequencing metrics for calls from the evidence material and the concordance of variants called with the reference material. Strong and very strong correlation values are bolded.

and or quantity DNA template material. Analogically, phylogeny analysis has shown to be helpful in finding contamination within the apparent mitotype[50]. The phylogenetic approach is implemented also in mtDNA mixture deconvulsion tools like MixtureAceMT™ that assigns likelihoods for each read to belong to a particular haplogroup from the Phylotree database[51], and MMDIT, which leverages a graph algorithm and haplotype estimation approach, utilizing a population reference database to support mixture deconvolution[52].

Apart from discrepancies resulting from changes in reference to the rCRS present in evidence material, which were absent in reference material, 15 variants were observed in the reference material and not in the evidence material. Within those were four LH calls, six PH calls and five SNPs. All of the LH and PH calls were assigned "unclear" Status and "unexpected" or "unknown" by EMPOP states and thus were disregarded. From the group of five samples for which SNPs were observed in the reference material and not in the evidence material, four shared extremely low coverage in the discordant regions (S7, samples 1, 2, 4, 5), thus the sequence was manually inspected in IGV 2.11.9. In three of those samples (2, 4, 5), the mutation observed in the reference material was clearly visible in the evidence material. For these samples using the 20 – 5 variant reporting protocol allowed to uncover the variants, while, after disregarding PH and LH changes resulting from that adjustment, not contributing to additional discrepancies. Hence, we propose to use the 20 – 5 protocol for samples with low region coverage (Fig. 5).

Additional analysis

Sample pairs 1 and 3 were investigated further. For evidence sample 1 the discordant SNP was assigned "confirmed" Status and an "unexpected" EMPOP state, with the region coverage median 33 (region covered by one amplicon only), while for evidence sample 3 the categories assigned were "possible" and "unexpected", respectively, with the region covered by two amplicons of median coverage 2588 and 3437. Both sample pair 1 and 3 consisted of first-degree relatives (sisters).

Sequences of the 16,126–16,304 region of evidence sample 1 differed between the two technologies used (S7), however, all sequences showed a considerable amount of apparent heteroplasmy (Sanger: 16126T/C-15%, 16271T/C-13%, 16294 C/T-19%, 16296 C/T-19%, 16304T/C-16%; MPS: 16126 C/T-19.3%, 16271 C, 16294 C/T-27.3%, 16296T/C-27.3%, 16304 C/T-27.3%). These differences seem to be the result of an extremely low DNA quantity within the sample (0.0007 ng/ul—T. large, 0.002 ng/ul—T. small), leading to the introduction of stochastic effects, or DNA deamination, as all of those changes consisted of T/C mixtures[11]. The low coverage of the reads for this sample further supports this reasoning. Moreover, when analysing heteroplasmic sites through

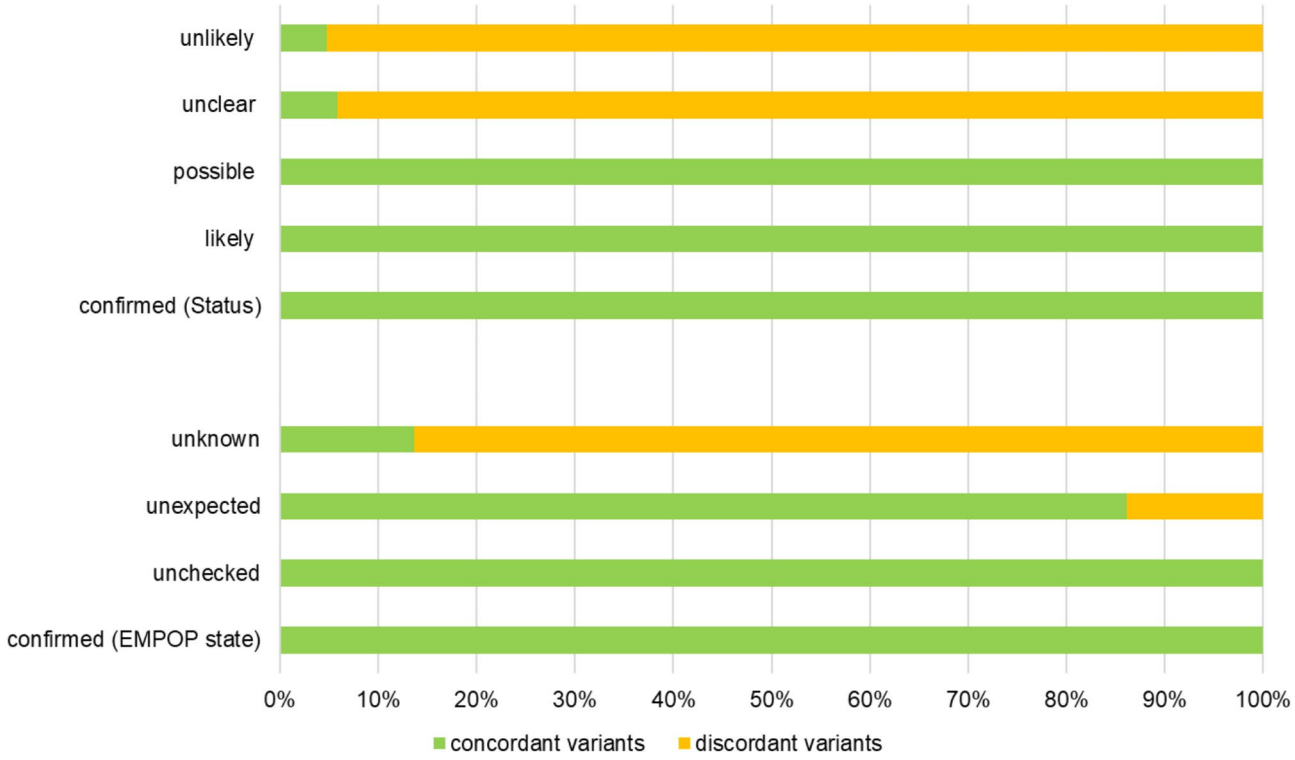

Fig. 3. Concordance of variants with different variant states assessed by Converge's Status (top) and EMPOP states (bottom) from evidence material with variants from reference material.

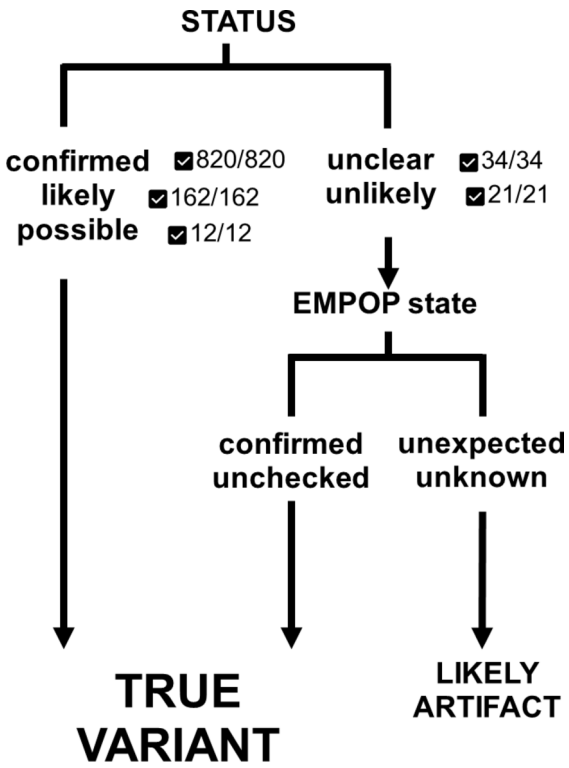

Fig. 4. Concordance of changes called with the algorithm for variant calling from evidence material with the modern reference, based on Converge's Status and EMPOP state.

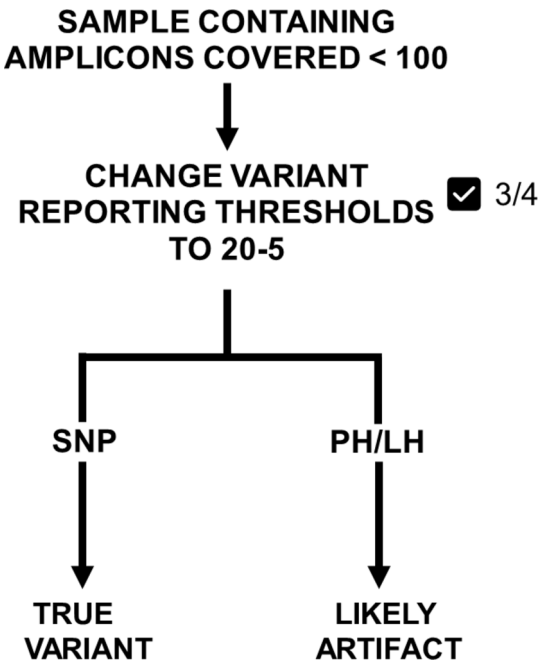

Fig. 5. Additional steps for samples with low coverage (< 100 reads).

Sanger sequencing, different peak height ratios are observed when different primers are used, and so the ratios do not necessarily reflect the proportional presence of the contributing sequence variants[12]. Sanger sequencing of the reference sample 1 confirmed the MPS-derived mitotype, while none of the sample-derived sequences were fully concordant with it.

A discordant call between evidence sample 3 and reference sample 3 was confirmed by results of Sanger sequencing (S8). This known phenomenon is the reason for the SWGDAM interpretation guidelines to state that each laboratory must establish the number of differences that makes an exclusion[13]—*de novo* mutations can be found even between monozygotic twins[46]. A recent study estimates mutations in the control region of mitochondrial DNA to occur at a rate of 2.38×10^{-5} mutations per base pair per generation[40]. As mentioned before, even samples collected from the same person but different tissue types can give discordant calls, especially when full mitogenomes are considered[41]. Although comparing mtDNA haplotypes from decades ago with contemporary material allows for observing the effects of true degradation on the haplotype, it is not without limitations.

The context of forensic genetics research

mtDNA analysis is an indispensable tool in the genetic examination of degraded skeletal remains, particularly when nuclear DNA is too fragmented or insufficient for reliable analysis. Leveraging mtDNA control region sequencing and complete mitogenome reconstruction, studies have successfully identified individuals from various historical and forensic contexts, including conflict victims and ancient populations (e.g.[37,43,53–60]). Advanced NGS methodologies, as primer capture enrichment or mitotiling, have enabled the recovery of high-quality data from even severely compromised samples[53,61]. These techniques have been pivotal in identifying remains from events such as the Korean War[59], the Viking Age mass graves[54], and World War II[56].

The study conducted by Ta et al.[53], which analyzed remains buried for 50 years in Southeast Vietnam, demonstrates substantial alignment with our research. The bone samples exhibited a pronounced degree of degradation, and authors employed analytical thresholds comparable to those utilized in our study (minimum coverage of 20× and thresholds for deletions, insertions, and PH calls set at 30%, 20%, and 10%, respectively). Similarly, this resulted in a high number of PH calls attributed to DNA damage or sequencing artifacts, and so to mitigate false positives, the PH threshold was adjusted to 20%, which allowed for over 25-fold decrease in PH calls (94% of initial PH calls were C/T mixtures). However, in our analysis, all observed variations within the hotspot regions 303–315 and 16,183–16,193 are classified as LH, which in our view more adequately represents the phylogenetic characteristics of these variations, whereas Ta et al. described some of them as PHs, which makes the comparison between our datasets less straightforward. Notably, Ta et al. lacked access to reference data to validate their findings, which compelled them to raise the PH threshold to 20%. In contrast, our real-case-based results demonstrate that the 10% threshold can still be applied even when the DNA matrix is degraded. This is achieved through the application of our newly developed algorithm, which prioritizes phylogenetic assessment. By addressing this limitation in the present study, we successfully retained the 10% PH threshold, without the need for manual verification of each variant call.

In addition to individual identifications[43,55], optimized mtDNA analysis protocols, as demonstrated in the phylogeographic identification of Jewish Holocaust victims[37], or the analysis of ancestry of victims found in

a Slovenian mass grave[56] offer significant insights into biogeographical ancestry, population history, and anthropological contexts. Such protocols align closely with the goals of this study, which integrates mtDNA sequencing and bioinformatics tools to analyse degraded skeletal samples, shedding light on individual identities and broader population structures from past conflicts.

Broader implications for other research areas

After internal validation, we believe our findings have potential use not only in forensic sciences (including mass disaster victim identification, and trace evidence analysis [42]), but also in other fields where DNA quality and quantity are usually low. Recent studies have shown that the persistence of mtDNA in challenging conditions, such as in formalin-fixed paraffin-embedded tissues, or cell-free DNA, can yield valuable genetic insights even decades post-preservation[62–65]. In this context, our work resonates with a broad spectrum of clinical research, including those addressing mitochondrial diseases (MD), which are the most common group of inherited metabolic disorders with a prevalence of at least 1 in 5000 live births[66], with ongoing studies investigating the possibility of mitochondrial-based mechanism of growing number of diseases, as Parkinson's[67] or myotonic dystrophy type 2[68], to name a few. Understanding heteroplasmy and mtDNA variant distributions is crucial not only for diagnosis but also for therapeutic interventions. Exploring biogeographic ancestry within anthropological studies[54,58], further underscore the importance of robust mtDNA variant-calling methods that can handle degraded or low-template material. Our findings align also with broader anthropological investigations, such as tracing human migratory patterns or unraveling haplotype diversity in extended populations[69]. These studies highlight the pivotal role of mtDNA in reconstructing settlement histories and identifying evolutionary links[70], which extend well beyond the forensic realms of naturally degraded samples. Our algorithm provides a methodological foundation applicable across these diverse fields, ensuring more reliable genetic conclusions under suboptimal conditions.

The algorithm we developed minimizes the hands-on time of manual sample analysis, and helps to eliminate artifacts prior to more detailed analysis, if needed. It bypasses fixed threshold settings to the extent possible, adjusting itself to the analysed sample's parameters.

Conclusions

Whole mitochondrial genome analysis from material low in DNA copy still proves to be a challenge. Our study shows that those samples frequently do not contain the recommended amount of DNA, while additionally being burdened with degradation, both of which may impact the haplotyping process. We have found 85 changes in the evidence material not present in modern reference samples; these included SNPs, PHs and LHs. None of the initially applied analysis protocol modifications (RCR, T5, T20, 50 – 10, 20 – 5) lead to a significant rise of the concordance of haplotypes between sample types.

We were able to develop an algorithm that would help assess the correctness of a SNP or PH call based on metrics available in the Converge-generated reports: namely the Status and EMPOP state of a variant called. With limited exceptions, the algorithm allowed for obtaining haplotypes from historical samples concordant with modern reference samples. Those exceptions were: a case of extreme degradation leading to an apparent variety of heteroplasmy levels shown both in MPS-generated data and Sanger sequencing electropherograms, and a spontaneous mutation between relatives.

While the method was not without its limitations, based on our results we advise establishing a haplotype that includes confidently identified mutations when dealing with degraded material, especially for database searches in broad identification studies, rather than attempting to create a more complex haplotype with low-frequency heteroplasmic variants. Using the algorithm developed within this study, it is possible to obtain a haplotype that would be fit for database searches and identification purposes, even when dealing with material low in DNA quantity and quality. The results may be extrapolated to other areas that struggle with suboptimal standard of genetic material.

Materials and methods

Additional sequence analysis was conducted based on results obtained by the Department of Forensic Genetics (now the Department of Genomics and Forensic Genetics) at Pomeranian Medical University in Szczecin, which is part of the Polish Genetic Database of Victims of Totalitarianisms—Research Centre. These results were gathered during historical human identification cases commissioned by regional branches of the Institute of National Remembrance. Victims and their maternal relatives were assigned numbers instead of names, with only their degree of kinship noted. In each case, only the haplotype of the victim's closest maternal relative was selected for the study. These included first-degree relatives (sisters, brothers), second-degree relatives (nephews, nieces), and third-degree relatives (grand-nieces, grand-nephews).

All methods were carried out in accordance with relevant guidelines and regulations. All protocols were approved by the Chairperson of the Bioethics Committee of the Pomeranian Medical University in Szczecin (KB.006.178.2024). Informed consent was obtained from all subjects and/or the legal guardian(s) of the data.

The steps preceding haplotype calling were as follows: material preparation, DNA extraction and quantification methods for bone sample analysis, as well as Sanger sequencing performed for two cases with discrepancies concerning two pairs of relatives were described in[34] (primer pairs for the sites specifically investigated were published by[71]: 15971–16410 for Sample 1, covering HVI, including nucleotide position 16271, and L15—R429 for Sample 3, HVII, position 64); for the high-quality reference material steps leading to DNA extraction are described in[72]. The protocol for completing mitogenome analysis was not yet published: DNA extracts were diluted to 16.7 pg genomic DNA/µl (the recommended target DNA input for the amplification reaction is 100 pg in 6 µl, which corresponds to ca. 3000 mitocopies) based on the mean quantity parameter of the Quantifiler

TRIO, if necessary. Whole mitogenomes were amplified using the 2-in-1 method, indexed, and purified using the Precision ID mtDNA Whole Genome Panel, Precision ID Library Kit, Ion Xpress™ Barcode Adapters, and AMPure™ XP Reagent, per manufacturer instructions. If the total DNA input was lower than optimal, the number of amplification cycles within the target amplification reaction was adjusted accordingly (< 100 pg—22 cycles, < 50 pg—23 cycles, < 25 pg—24 cycles, < 12.5 pg—25 cycles, < 6.75 pg—26 cycles). Library quantities were assessed using the Ion Library TaqMan™ Quantitation Kit. Template preparation and sequencing were carried out on the Ion Chef™ Instrument using the Ion S5™ Precision ID Chef & Sequencing Kit, Ion 520™ Chip Kit, and 25 µl of pooled libraries normalized to 30 pM each, per manufacturer instructions. For the reference material extracts, all the library preparation and sequencing steps were analogical, however the conservative method was used instead of the 2-in-1 method for the target amplification. Primary sequence analysis and reference alignment were performed by Torrent Suite™ Software v.5.10, while secondary analysis was conducted using the HID Genotyper v.2.2 plug-in with Converge Software v.2.2. Converge is a forensic data management software by Thermo Fisher Scientific, designed to analyse a wide range of genetic markers, including STRs, SNPs (Single Nucleotide Polymorphisms), and mtDNA. It integrates tools for forensic data interpretation, such as kinship analysis, biogeographical ancestry estimation, and mtDNA haplogroup assessment. The mtDNA analysis module features visual sequence inspection similar to that of Integrative Genomics Viewer (IGV), and allows for manual verification of variant calls. Apart from the AMPure™ XP Reagent (Beckman Coulter™), all the aforementioned kits and software are produced by Thermo Fisher Scientific.

The initial analysis included a comparison of haplotypes obtained using the default Genotyper mtDNA coverage and threshold parameters (minimum total read coverage per position, minimum variant coverage to call, and coverage threshold to mark region—20; threshold for confirmed call—96; threshold for PH—10; insertion—20; deletion calls—30) with haplotypes obtained from modern reference samples, noting discrepancies within the victim-maternal relative pairs. Next, the Genotyper analysis was repeated using altered genotype calling parameters, which included applying the "remove contaminant reads" protocol (RCR) and using modified thresholds: threshold for PH—5% (from this point on called "T5") and 20% ("T20"); minimum total read coverage per position and minimum variant coverage to call set at 50 and 10, respectively ("50 – 10"), and 20 and 5, respectively ("20 – 5"). Once again, the obtained haplotypes were compared to their respective references, and discrepancies were noted.

For further analysis, calls concerning the presence of length heteroplasmy within C-stretches and the 8247–8256 region were disregarded, as they were not important for the purpose of this work and thus distorted the general data view; concordant and discordant calls were grouped, and the correlation between call concordance and sample quality, quantity, library quantity, and sequencing metrics was studied with Spearmann's correlation test. For these calculations, a concordant call was assigned a value of 1, while an incorrect one was annotated with 0. Converge variant states were assigned values of 2, 4, 6, 8, and 10 for unlikely, unclear, likely, possible, and confirmed, respectively, and EMPOP variant states were assigned 2.5, 5.0, 7.5, and 10 for unknown, unexpected, unchecked, and confirmed, respectively. Based on the results of correlation testing, followed by manual verification, an algorithm was created to predict the concordance of a call from degraded, low-template material with a high-quality modern sequence, based on available parameters.

Data availability

The datasets generated during and/or analysed during the current study are not publicly available. Some additional data may be available from the corresponding author on reasonable request.

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Author contributions

M.S., S.C., J.D., G.Z. and M.D. carried out the experiment. M.S. wrote the manuscript with input from all authors. A.O. supervised the project.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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