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The role of mitochondrial DNA heteroplasmy in the interpretation of forensic analyses

O papel da heteroplasmia do DNA mitocondrial na interpretação de análises forenses

El papel de la heteroplasmia del ADN mitocondrial en la interpretación de análisis forenses

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Abstract:
Introduction: Mitochondrial DNA (mtDNA) is highly relevant to forensic genetics, especially in human identification analyses involving degraded biological samples or samples with a low amount of genetic material. However, the occurrence of heteroplasmy, characterized by the presence of different mitochondrial DNA variants within the same individual, may pose a challenge to the interpretation of forensic results. Objective: To analyze the influence of mitochondrial DNA heteroplasmy on the reliability of human identification in forensic analyses. Methodology: This is an integrative literature review, conducted through searches in the PubMed, Virtual Health Library (BVS), SciELO and PubMed Central (PMC) databases, using the descriptors “Mitochondrial DNA”, “Heteroplasmy”, “Forensic Genetics”, “Human Identification” and “Forensic Science”, combined with Boolean operators. Scientific articles published between 2021 and 2026 were considered, and ten studies were selected after applying the inclusion and exclusion criteria and analyzing the full texts. Results: The studies showed that heteroplasmy may vary between tissues and individuals and is influenced by biological and methodological factors. Differences in variant detection capacity were also identified according to the sequencing techniques employed, highlighting the importance of appropriate technical and interpretative criteria. Conclusion: Heteroplasmy must be considered when interpreting mitochondrial profiles, since its inadequate assessment may lead to inconsistencies in genetic comparisons and misinterpretations. Thus, understanding this phenomenon contributes to improving forensic genetic analyses and to greater reliability in human identification processes.

Keywords: Mitochondrial DNA. Heteroplasmy. Forensic genetics. Human identification. Genetic sequencing.

Resumo:
Introdução: O DNA mitocondrial (mtDNA) apresenta grande relevância para a genética forense, especialmente em análises de identificação humana envolvendo amostras biológicas degradadas ou com baixa quantidade de material genético. Entretanto, a ocorrência de heteroplasmia, caracterizada pela presença de diferentes variantes de DNA mitocondrial em um mesmo indivíduo, pode representar um desafio para a interpretação dos resultados periciais. Objetivo: Analisar a influência da heteroplasmia do DNA mitocondrial na confiabilidade da identificação humana em análises forenses. Metodologia: Trata-se de uma revisão integrativa da literatura, realizada por meio de buscas nas bases de dados PubMed, Biblioteca Virtual em Saúde (BVS), SciELO e PubMed Central (PMC), utilizando os descritores “Mitochondrial DNA”, “Heteroplasmy”, “Forensic Genetics”, “Human Identification” e “Forensic Science”, combinados por meio de operadores booleanos. Foram considerados artigos científicos publicados entre 2021 e 2026, sendo selecionados dez estudos após a aplicação dos critérios de inclusão e exclusão e a análise dos textos completos. Resultados: Os estudos demonstraram que

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Ano VII, v.1 2026 | submissão: 08/11/2026 | aceito: 15/11/2026 | publicação: 20/11/2026

a heteroplasmia pode apresentar variações entre tecidos e indivíduos, além de sofrer influência de fatores biológicos e metodológicos. Também foram identificadas diferenças na capacidade de detecção de variantes conforme as técnicas de sequenciamento empregadas, evidenciando a importância de critérios técnicos e interpretativos adequados. Conclusão: A heteroplasmia deve ser considerada na interpretação de perfis mitocondriais, uma vez que sua avaliação inadequada pode levar a inconsistências nas comparações genéticas e a interpretações equivocadas. Dessa forma, a compreensão desse fenômeno contribui para o aprimoramento das análises de genética forense e para maior segurança nos processos de identificação humana.

Palavras-chave: DNA mitocondrial. Heteroplasmia. Genética forense. Identificação humana. Sequenciamento genético.

Resumen:
Introducción: El ADN mitocondrial (mtDNA) es de gran relevancia para la genética forense, especialmente en análisis de identificación humana que involucran muestras biológicas degradadas o con escasa cantidad de material genético. Sin embargo, la heteroplasmia, caracterizada por la presencia de diferentes variantes de ADN mitocondrial en un mismo individuo, puede dificultar la interpretación de los resultados periciales. Objetivo: Analizar la influencia de la heteroplasmia del ADN mitocondrial en la fiabilidad de la identificación humana en análisis forenses. Metodología: Se realizó una revisión integradora de la literatura mediante búsquedas en PubMed, Biblioteca Virtual en Salud (BVS), SciELO y PubMed Central (PMC), utilizando los descriptores “Mitochondrial DNA”, “Heteroplasmy”, “Forensic Genetics”, “Human Identification” y “Forensic Science”, combinados mediante operadores booleanos. Se consideraron artículos científicos publicados entre 2021 y 2026 y se seleccionaron diez estudios tras aplicar los criterios de inclusión y exclusión y analizar los textos completos. Resultados: Los estudios demostraron que la heteroplasmia puede variar entre tejidos e individuos y verse influida por factores biológicos y metodológicos. También se identificaron diferencias en la capacidad de detección de variantes según las técnicas de secuenciación empleadas, evidenciando la importancia de criterios técnicos e interpretativos adecuados. Conclusión: La heteroplasmia debe considerarse al interpretar perfiles mitocondriales, ya que su evaluación inadecuada puede generar inconsistencias en las comparaciones genéticas e interpretaciones erróneas. Por tanto, comprender este fenómeno contribuye a mejorar los análisis de genética forense y a aumentar la seguridad de los procesos de identificación humana.

Palabras clave: ADN mitocondrial. Heteroplasmia. Genética forense. Identificación humana. Secuenciación genética.

1. Introduction

Forensic genetics is a fundamental field of forensic science, applying knowledge of molecular biology and genetics to criminal investigation and human identification. DNA analysis has enabled significant advances in forensic examination, allowing the genetic identification of individuals from biological traces such as blood, saliva and tissues with a high degree of precision. In this context, genetic material has become an important tool for solving

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Ano VII, v.1 2026 | submissão: 08/11/2026 | aceito: 15/11/2026 | publicação: 20/11/2026

crimes and identifying victims, particularly with the development of new molecular analysis techniques (CAVALCANTI et al., 2024).

The development of genetic analysis techniques over recent decades has significantly expanded the possibilities for human identification in forensic science. Traditionally, nuclear DNA is widely used in forensic analyses because of its high discriminatory power and its ability to distinguish genetic profiles with considerable precision. However, obtaining complete profiles may be difficult when samples are degraded, old or contain little biological material, compromising the reliability of the analyses. In such cases, mitochondrial DNA provides an important alternative: its high copy number per cell increases the likelihood of recovering genetic material and supports human identification under adverse conditions (TANG et al., 2021; GUPTA et al., 2023).

Mitochondrial DNA is a circular, double-stranded molecule located in mitochondria and has distinctive characteristics that contribute to its application in forensic analyses. These include its predominantly maternal inheritance, high copy number per cell and widespread tissue distribution, allowing the analysis of biological material in which nuclear DNA may be absent or significantly degraded. These characteristics favor its use in samples such as bones, teeth and rootless hairs, making mtDNA a relevant tool for human identification in different forensic contexts (FERREIRA; RODRIGUEZ, 2024; CAVALCANTI et al., 2024).

Despite the advantages of mitochondrial DNA, its analysis has particular features that may affect the interpretation of results, notably heteroplasmy. This phenomenon consists of the simultaneous presence of two or more mitochondrial DNA variants within one individual, whether between different cells or tissues or within a single cell. The proportions of these variants may differ between biological tissues, making mitochondrial profile interpretation more complex. In forensic analyses, this variability may hinder comparisons between samples from the same individual and influence the reliability of the results obtained (SLAPNIK et al., 2024; BURY et al., 2024; MCELHOE et al., 2022).

In this context, understanding mitochondrial DNA heteroplasmy and its effects on genetic profile interpretation is relevant to forensic genetics, particularly in analyses intended for human identification. Accordingly, this study aimed to analyze the influence of mitochondrial DNA heteroplasmy on the reliability of human identification in forensic genetic analyses.

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Ano VII, v.1 2026 | submissão: 08/11/2026 | aceito: 15/11/2026 | publicação: 20/11/2026

2. Materials and methods

This study consisted of an integrative literature review. The literature search was conducted on November 1, 2026, in PubMed, the Virtual Health Library (BVS), SciELO and PubMed Central (PMC), without language restrictions. Scientific articles published between 2021 and 2026 were considered, using the descriptors “Mitochondrial DNA”, “Heteroplasmy”, “Forensic Genetics”, “Human Identification” and “Forensic Science”, identified in the DeCS and MeSH vocabularies and combined using the Boolean operators “AND”, “OR” and “NOT”.

The inclusion criteria selected complete scientific articles available in full that addressed the application of mitochondrial DNA and heteroplasmy in forensic genetics and human identification. Original studies, literature reviews and systematic reviews covering mitochondrial variability, sequencing techniques and forensic genetic interpretation were included.

Duplicate articles, brief abstracts, incomplete studies, dissertations, theses, letters to the editor, publications outside the established period and studies unrelated to the topic were excluded. Studies on mitochondrial diseases without a connection to forensic genetics and animal studies were also excluded.

After applying the selection criteria, the articles underwent exploratory and analytical reading and were organized and critically analyzed to identify evidence on the influence of mitochondrial DNA heteroplasmy on the reliability of human identification in forensic analyses.

3. Results and discussion

3.1 Results

The structured search conducted in PubMed, the Virtual Health Library (BVS), SciELO and PubMed Central (PMC) identified 599 scientific records. After duplicate removal and the application of inclusion and exclusion criteria through title, abstract and full-text assessment, 10 scientific articles were selected to form the analytical corpus of the integrative review, as shown in Figure 1.

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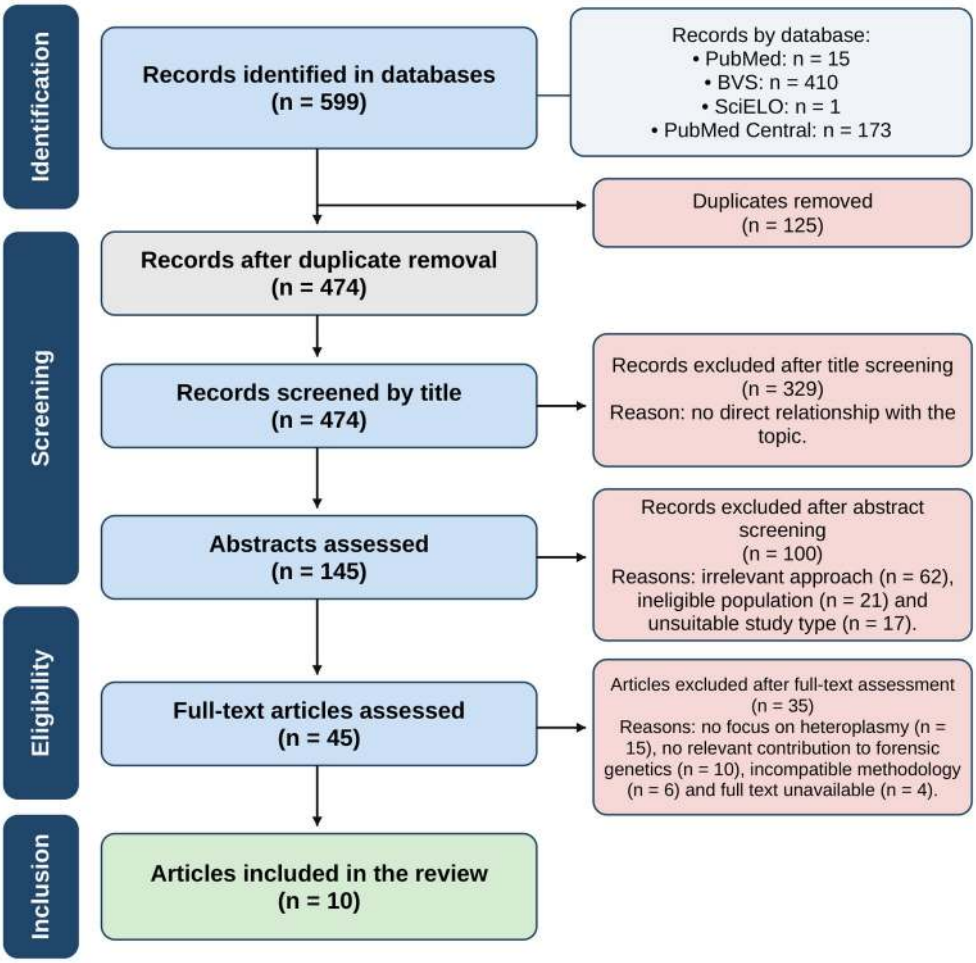
Ano VII, v.1 2026 | submissão: 08/11/2026 | aceito: 15/11/2026 | publicação: 20/11/2026

Figure 1. Article selection flowchart, 2026.



The ten articles selected for the integrative review included studies investigating the occurrence and variability of mitochondrial DNA heteroplasmy, its distribution across human tissues and the technical factors affecting its identification by sequencing methods. Research on mitochondrial genetic profile interpretation in forensic contexts was also assessed, particularly regarding the reliability of genetic matches, possible discrepancies between samples and the application of statistical methods to evaluate results. The main aspects investigated included point and length heteroplasmy, the frequency of heteroplasmic variants, laboratory detection limits and their implications for human identification. Information on the titles, authors, publication years, objectives and principal findings of the studies is presented in Table 1.

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Ano VII, v.1 2026 | submissão: 08/11/2026 | aceito: 15/11/2026 | publicação: 20/11/2026

Table 1. Characteristics of the selected scientific articles by title, authors, publication year and findings, 2026.

Study title	Authors	Year	Main findings
Forensic evaluation of mitochondrial DNA heteroplasmy in Gujarat population, India	Alqaisi et al.	2022	In 104 individuals, point heteroplasmy was identified in 7.69% and length heteroplasmy in 46.15%; the study also evaluated differences between blood and buccal mucosa.
Evaluating the suitability of current mitochondrial DNA interpretation guidelines for multigenerational whole mitochondrial genome comparisons	Connell et al.	2022	Analysis of 2,339 pairs of individuals from maternal lineages identified a risk of false exclusions when interpretation guidelines were applied inappropriately; eight false exclusions (0.34%) were reported.
Exploring statistical weight estimates for mitochondrial DNA matches involving heteroplasmy	McElhoe et al.	2022	In 1,301 samples, point heteroplasmy was identified in 37.7% using a minimum minor allele frequency of 2%; the authors proposed a statistical framework for evaluating matches.
Nuclear genetic control of mtDNA copy number and heteroplasmy in humans	Gupta et al.	2023	Data from 274,832 individuals identified genetic factors associated with mtDNA copy number and heteroplasmy, including somatic variation and the influence of aging.
The effect of library preparation protocol on the efficiency of heteroplasmy detection in mitochondrial DNA using two Massively parallel sequencing Illumina systems	Daca-Roszak et al.	2024	Comparison of library preparation protocols demonstrated differences in coverage uniformity and the efficiency of heteroplasmic variant detection.
Mitochondrial point heteroplasmy: insights from deep- sequencing of human replicate samples	Korolija et al.	2024	Deep sequencing of replicate samples identified low-frequency variants and highlighted the importance of technical and biological replicates in validating the findings.
The quality and detection limits of mitochondrial heteroplasmy by long read nanopore sequencing	Slapnik et al.	2024	Under the conditions evaluated, Nanopore sequencing had a detection limit close to 12%, demonstrating the influence of technology and analytical parameters.
MitoMetrics: incorporation of mtDNA profile discrepancies in likelihood ratio calculations	Claessens et al.	2026	The authors presented an approach for incorporating mitochondrial profile discrepancies into likelihood ratio calculations, accounting for possible differences between samples.
Probability of mitochondrial DNA heteroplasmy in different tissues from European populations	Cuesta-Aguirre et al.	2026	Analysis of blood samples and data from other tissues identified differences in heteroplasmy frequency according to the biological tissue analyzed.
The EDNAP collaborative study on mtDNA heteroplasmy detection using Sanger and Massively Parallel Sequencing technologies	Ewers et al.	2026*	The collaborative study observed general agreement in point heteroplasmy identification, but differences in low-frequency and length variant detection between methods and laboratories.

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Ano VII, v.1 2026 | submissão: 08/11/2026 | aceito: 15/11/2026 | publicação: 20/11/2026

* Published online in 2026; included in volume 86, dated 2027.

3.2 Discussion

The studies analyzed show that mitochondrial DNA heteroplasmy is relatively common in the human population and that its identification depends on biological and methodological factors. Investigating 1,301 individuals, McElhoe et al. (2022) identified point heteroplasmy in 37.7% of samples using a minor allele frequency threshold of 2%. This percentage represents the minimum criterion used to classify a variant as heteroplasmic: the minor variant had to account for at least 2% of the sequences identified at that mitochondrial DNA position. Thus, this value refers to the detection threshold adopted in the analysis, rather than the percentage of individuals with heteroplasmy. Deep sequencing techniques, which perform multiple reads of the same genetic region, increase the ability to identify variants present at low proportions and contribute to more sensitive heteroplasmy assessment (MCELHOE et al., 2022; KOROLIJA et al., 2024).

Consistently, Gupta et al. (2023), analyzing genetic data from 274,832 individuals, identified genetic factors associated with variation in mitochondrial DNA copy number and heteroplasmy. The authors observed that point heteroplasmic variants may arise in somatic tissues and accumulate over a person's lifetime, while the distribution of certain variants may also be influenced by hereditary factors. These findings show that heteroplasmy is dynamic and may vary with age, genetic composition and the tissue analyzed.

The differences between the findings of McElhoe et al. (2022) and Alqaisi et al. (2022) also demonstrate the influence of the criteria used to identify and report heteroplasmic variants. Whereas McElhoe et al. (2022) considered variants with a minimum frequency of 2%, Alqaisi et al. (2022) identified point heteroplasmy in 7.69% of the individuals analyzed using a different methodological approach. Differences in the observed frequencies should therefore not be interpreted exclusively as population variation: detection limits, sequencing depth and classification criteria also affect the results. Heteroplasmy should thus be understood as a dynamic characteristic of mitochondrial DNA, influenced by biological mechanisms and technical analytical conditions.

Another relevant aspect identified in the studies was the heterogeneous distribution of heteroplasmy across body tissues. Comparing blood and buccal mucosa samples from 104 unrelated individuals, Alqaisi et al. (2022) identified point heteroplasmy in 7.69% of the

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Ano VII, v.1 2026 | submissão: 08/11/2026 | aceito: 15/11/2026 | publicação: 20/11/2026

population and length heteroplasmy in 46.15%. Point heteroplasmy consists of different nitrogenous bases at the same mitochondrial DNA position, whereas length heteroplasmy involves the coexistence of mitochondrial DNA molecules with different numbers of nucleotides in particular regions, especially repetitive sequences. These two forms of heteroplasmy exhibit distinct variations and may introduce different levels of complexity into genetic analysis.

Although Alqaisi et al. (2022) observed a higher frequency of point heteroplasmy in blood, no statistically significant difference between the two tissues was found for this type of variant. In a broader investigation, Cuesta-Aguirre et al. (2026) analyzed more than 400 blood samples from Spanish individuals and integrated sequencing data from ten other human tissues. The results demonstrated differences in heteroplasmic variant distribution, with higher frequencies in skeletal muscle, kidney and liver and lower frequencies in intestine, skin and cerebellum. This distribution shows that mitochondrial genetic composition may vary by tissue and is not necessarily uniform throughout the body.

These findings have important implications for forensic genetics, especially when a questioned sample is compared with a reference sample from another biological tissue. For example, differences between mitochondrial profiles obtained from blood, buccal mucosa and hair may reflect an uneven distribution of heteroplasmic variants and do not necessarily mean that the samples belong to different individuals. Claessens et al. (2026) also addressed this issue, investigating discrepancies between mitochondrial profiles from reference samples, such as blood and buccal mucosa, and hair samples from the same individuals. The authors proposed a statistical approach to account for such differences when evaluating the weight of genetic evidence. Taken together, the studies demonstrate that tissue origin is a relevant factor in interpreting results and establishing the reliability of human identification.

Advances in sequencing technologies have increased the ability to identify heteroplasmic variants, especially those present at low frequencies. Among the methodologies assessed in the selected studies, massively parallel sequencing (MPS), also known as next-generation sequencing (NGS), permits simultaneous analysis of multiple DNA regions and generates large numbers of reads, favoring the identification of minor variants. However, its sensitivity depends on factors such as sequencing depth, sample quality, library preparation and the analytical criteria employed; no single platform can therefore be considered superior under all experimental conditions.

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Ano VII, v.1 2026 | submissão: 08/11/2026 | aceito: 15/11/2026 | publicação: 20/11/2026

Daca-Roszak et al. (2024) compared different library preparation protocols used in mitochondrial DNA sequencing. The results showed that one protocol provided more uniform coverage across the genome and favored heteroplasmic variant identification, whereas the other produced a more uneven read distribution. This difference matters because regions with lower coverage may hinder variant detection or change the estimated frequency, demonstrating that sample preparation also influences result quality.

Complementing these findings, Korolija et al. (2024) used deep sequencing to investigate point heteroplasmy in replicate blood and buccal mucosa samples. In this analysis, each DNA region is sequenced repeatedly; coverage of 7,700-fold therefore means that each analyzed position was read approximately 7,700 times on average. This strategy increases the ability to identify variants present at low proportions by allowing more detailed observation of sequence differences. The study also demonstrated the importance of analyzing technical and biological replicates to distinguish true variants from potential sequencing noise.

Technical limitations were also demonstrated by Slapnik et al. (2024), who assessed heteroplasmy detection through long-read sequencing using Nanopore technology. Under the experimental conditions evaluated, the authors identified a detection limit close to 12%, demonstrating that variant detection capacity can vary significantly according to the technology and parameters employed. This finding reinforces the need to assess analytical sensitivity under the specific conditions of each methodology; considering the sequencing platform alone is insufficient.

Furthermore, the collaborative study conducted by Ewers et al. (2026) compared heteroplasmy identification and interpretation across forensic laboratories using Sanger, Ion Torrent and Illumina sequencing. The results showed general agreement in point heteroplasmy identification, but also showed that low-frequency variants may go unreported when they fall below laboratory thresholds. Together, these findings demonstrate that more sensitive technologies do not eliminate the challenges of interpreting heteroplasmy. Rather, they demand more rigorous method validation, detection threshold establishment and laboratory procedure standardization.

The main implication of heteroplasmy for forensic genetics is the possibility of differences between mitochondrial profiles of individuals sharing a maternal lineage, or even between tissues from the same individual. In this context, inappropriate variant interpretation may lead to erroneous conclusions, particularly when heteroplasmic differences are treated as genetic incompatibilities without assessing their nature, frequency and possible biological

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Ano VII, v.1 2026 | submissão: 08/11/2026 | aceito: 15/11/2026 | publicação: 20/11/2026

origin. A discrepancy between two samples therefore does not necessarily indicate that they belong to different individuals: whether the observed difference can be explained by heteroplasmy must be investigated.

Assessing comparisons of complete mitochondrial genomes between individuals from different generations of the same maternal lineage, Connell et al. (2022) demonstrated that inappropriate application of interpretation guidelines may lead to incorrect exclusions. Among the 2,339 pairs analyzed, eight false exclusions were observed, corresponding to 0.34%. This finding shows that heteroplasmy-related differences must be assessed in light of mitochondrial DNA-specific guidelines, preventing biologically possible variations from being automatically interpreted as evidence against a maternal relationship.

McElhoe et al. (2022) also emphasized the importance of statistically assessing mitochondrial matches involving heteroplasmy. The authors proposed a statistical framework to estimate the frequency of profiles exhibiting heteroplasmic variants, taking population information and minor allele frequency into account. This approach demonstrates that heteroplasmy should be treated both as a potential source of discrepancies between samples and as a characteristic that can contribute to evaluating the weight of genetic evidence.

More recently, Claessens et al. (2026) presented MitoMetrics, a proposed approach for incorporating mitochondrial profile discrepancies into likelihood ratio calculations. The proposal accounts for possible differences between tissues from the same individual, contributing to a more appropriate statistical assessment of evidence. Nevertheless, further research and validation are still needed for its application across different forensic contexts.

The findings of Ewers et al. (2026) reinforce this discussion by showing that heteroplasmy interpretation may vary with the technology used and the criteria established by each laboratory. Although point heteroplasmy identification showed general agreement among participants, length heteroplasmy exhibited greater interpretative variability. This situation highlights the need for standardized procedures and trained professionals capable of critically evaluating the results obtained.

Therefore, heteroplasmy alone should not be considered grounds for excluding identity. Its interpretation must consider the variant type, minor allele frequency, tissue analyzed, methodology employed and applicable scientific guidelines. Integrating these factors is essential to prevent false exclusions and increase the reliability of conclusions in forensic investigations.

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Ano VII, v.1 2026 | submissão: 08/11/2026 | aceito: 15/11/2026 | publicação: 20/11/2026

Final considerations

This integrative review analyzed the influence of mitochondrial DNA heteroplasmy on the reliability of human identification in forensic analyses. The ten selected articles showed that heteroplasmy is a relevant biological phenomenon, characterized by the coexistence of different mitochondrial DNA variants within one individual, whose frequency and distribution may vary according to genetic factors, age, biological tissue and the methodology employed.

The studies demonstrated that heteroplasmy may directly affect mitochondrial profile comparisons, particularly when analyzing samples from different tissues or identifying low-frequency variants through highly sensitive sequencing techniques. Heteroplasmic differences should not be automatically interpreted as genetic incompatibilities, since doing so may lead to erroneous conclusions and false exclusions.

Advances in sequencing technologies were also found to have expanded mitochondrial variant detection while introducing new challenges regarding method standardization, detection limits and data interpretation. Analytical reliability therefore depends on both the technological capabilities of the equipment and the validation of laboratory procedures and application of appropriate scientific criteria.

The work of appropriately qualified biomedical scientists is relevant to forensic genetics, particularly in biological sample processing and mitochondrial DNA extraction, quantification and sequencing. Studies by Daca-Roszak et al. (2024), Korolija et al. (2024) and Slapnik et al. (2024) highlight the importance of understanding sequencing methodologies, detection limits and possible interfering factors, reinforcing the need for quality control and careful evaluation of laboratory results.

In addition to technical work, qualified biomedical scientists may contribute to heteroplasmic variant interpretation and the preparation of technical documents, while respecting the limits of their professional competence and the regulations of the Federal Council of Biomedicine, particularly Resolutions No. 78/2002 and No. 391/2025. Knowledge of heteroplasmy thus helps identify possible mitochondrial profile differences arising from biological characteristics or methodological limitations, contributing to safer, more reliable human identification analyses.

The limitations of this review include the small number of selected articles and heterogeneity among the studies in populations analyzed, tissue types, technologies employed and criteria for identifying heteroplasmic variants. Furthermore, some research focuses

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Ano VII, v.1 2026 | submissão: 08/11/2026 | aceito: 15/11/2026 | publicação: 20/11/2026

predominantly on biological or methodological issues rather than directly addressing real forensic cases. These characteristics limit direct comparison between findings and call for caution when generalizing the results.

Finally, further research involving different human tissues, populations and sequencing platforms is recommended, together with the development of standardized criteria for identifying, classifying and interpreting heteroplasmy in forensic analyses. Improved statistical approaches and the integration of laboratory, genetic and forensic knowledge may increase the safety and reliability of mitochondrial DNA analyses, strengthening their application in human identification.

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Ano VII, v.1 2026 | submissão: 08/11/2026 | aceito: 15/11/2026 | publicação: 20/11/2026

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