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DNA Commission of the International Society for Forensic Genetics: Revised and extended guidelines for mitochondrial DNA typing

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ABSTRACT

The DNA Commission of the International Society of Forensic Genetics (ISFG) regularly publishes guidelines and recommendations concerning the application of DNA polymorphisms to the question of human identification. Previous recommendations published in 2000 addressed the analysis and interpretation of mitochondrial DNA (mtDNA) in forensic casework. While the foundations set forth in the earlier recommendations still apply, new approaches to the quality control, alignment and nomenclature of mitochondrial sequences, as well as the establishment of mtDNA reference population databases, have been developed. Here, we describe these developments and discuss their application to both mtDNA casework and mtDNA reference population databasing applications. While the generation of mtDNA for forensic casework has always been guided by specific standards, it is now well-established that data of the same quality are required for the mtDNA reference population data used to assess the statistical weight of the evidence. As a result, we introduce guidelines regarding sequence generation, as well as quality control measures based on the known worldwide mtDNA phylogeny, that can be applied to ensure the highest quality population data possible. For both casework and reference population databasing applications, the alignment and nomenclature of haplotypes is revised here and the phylogenetic alignment proffered as acceptable standard. In addition, the interpretation of heteroplasmy in the forensic context is updated, and the utility of alignment-free database searches for unbiased probability estimates is highlighted. Finally, we discuss statistical issues and define minimal standards for mtDNA database searches.

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1. Introduction

Mitochondrial DNA (mtDNA) is present in much higher copy number in the human cell than nuclear DNA. For this reason, mtDNA analysis can provide useful results in (forensic) samples that fail to yield successful nuclear DNA profiles. MtDNA haplotypes are uniparentally inherited and therefore reflect information from a non-recombining maternal lineage that may be shared by numerous people. As a result, mtDNA data cannot be applied to the identification of individuals in the same way that analyses from recombining nuclear DNA markers can. The mtDNA control region (CR), the largest non-coding portion of the molecule, nevertheless represents one of the most discriminatory single genetic markers known to forensics. Previous recommendations on the use of mtDNA sequence data in forensics addressed the need

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for appropriate laboratory practice, the use of negative and positive controls, basic nomenclature guidelines for sequence differences and heteroplasmy, as well as guidance on interpretation, reporting and statistics [1–3]. In this article, we take a closer look at the challenges surrounding mtDNA casework and population databasing that have arisen since the establishment of these original guidelines, and we update the guidelines where appropriate.

Many of the challenges were encountered during the establishment of the EDNAP Mitochondrial DNA Population Database (EMPOP; www.empop.org; [4]) over the past 15 years. In its early stages, EMPOP was envisioned and designed to serve as a reference population database for use in the evaluation of mtDNA evidence worldwide, with its primary goal of providing the highest quality mtDNA data. The architecture of the EMPOP search engine and the various analysis tools provided *via* the website have evolved over the years. However, the emphasis of the EMPOP database continues to be on the importance of mtDNA data quality. As a result, EMPOP not only serves as a reference population database, but also as quality-control tool for scientists in forensic genetics and other disciplines. Though a number of high-quality reference population databases exist for forensic comparisons, EMPOP is the most comprehensive resource from the standpoint of populations represented. We therefore recommend EMPOP for its data quality control tools and its resource of global population data.

While the topics addressed in the following sections are generally applicable to both population genetic studies and forensic evidentiary sample handling, particular matters and recommendations are more relevant to one application than the other. In these cases, we treat forensic casework and population databasing separately, and describe the differences.

2. Generation of mtDNA data, good laboratory practice

2.1. General recommendations

MtDNA testing is extremely sensitive and thus contamination is of greater concern than it is with other forensic PCR-based methods. Pre-amplification measures that should be used to mitigate contamination were put forth in earlier guidelines [2,3,5] and still hold true. The following measures apply to both casework and databasing scenarios unless otherwise noted:

- Appropriate laboratory conditions that include dedicated spaces, instruments, chemistry and lab wear should be established for mtDNA testing.
- The use of controls (negative amplification, reagent blank and positive controls) should be carried all through the laboratory process.
- Low levels of contamination may be tolerated, since reliable results can be obtained in the presence of contamination.
- Redundant sequence information should be obtained for each reported mtDNA position to include both forward and reverse sequencing primers when possible.
- Sample consensus sequences should be determined using dedicated software for raw data alignment to the revised Cambridge Reference Sequence (rCRS; [6]).
- Consensus sequences should be confirmed by a second independent analysis of the raw data.
- Regular participation in proficiency testing programs for forensic mtDNA casework laboratories, such as GEDNAP [7], GHEP-ISFG [8–11], or the U.S. Quality Assurance Standards [12] is highly recommended.

In those cases for which forward and reverse coverage are not possible, replicate coverage from the same strand, preferably obtained from different primers, is acceptable. In the end, the quality of the raw electropherograms should dictate the forward and reverse coverage requirements. EMPOP data review of population datasets has shown that redundant coverage is sometimes missing or has only been performed in a subset of samples [13]. Lack of redundancy can lead to so-called phantom mutations (sequencing artifacts reported as actual mutations; [14]) that compromise data quality in forensic database searches. This has been shown in a systematic analysis of numerous mtDNA research studies [15], which demonstrated that erroneous calls tend to occur at particular positions. In addition, errors were shown to be more frequently observed downstream of homopolymeric sequence regions, such as the C-tracts in the hypervariable segments I and II (HVS-I/II; [13]). In both cases, these errors tend to reflect sequencing artifacts in a single strand of data that generally could have been resolved with data from either the complementary strand or a different primer for the same strand.

Recommendation #1
Good laboratory practice and specific protocols for work with mtDNA must be followed in accordance with previous guidelines.

Recommendation #2
Negative and positive controls as well as extraction reagent blanks must be carried through the entire laboratory process.

Recommendation #3
Reported consensus sequences must be based on redundant sequence information, using forward and reverse sequencing reactions whenever practical.

Recommendation #4
Manual transcription of data should be avoided and independent confirmation of consensus haplotypes by two scientists must be performed.

Recommendation #5
Laboratories using mtDNA typing in forensic casework shall participate regularly in suitable proficiency testing programs.

2.2. Targeted region, amplification and sequencing ranges

The CR harbors the vast majority of quickly evolving sites in the mitochondrial genome (mtGenome) that are relevant for the discrimination of haplotypes in the forensic context. Traditional protocols have targeted the hypervariable segments of the CR (usually delimited as follows: HVS-I, 16024–16365; HVS-II, 73–340 and HVS-III, 340–576) using independent amplification and different combinations of primers. This restricted approach has a number of implications. In population studies, the independent amplification of the hypervariable regions, combined with the manual processing of multiple samples at one time, has been shown to lead to an increased risk of chimeric haplotypes or so-called "artificial recombinants" that are caused by the inadvertent mix-up of mtDNA segments from different individuals [16,17]. Laboratory protocols have been established and improved over the past years that support the amplification of the entire CR in a single amplicon (16024-576, [17–20]) for high-quality samples routinely used for databasing purposes. This strategy eliminates the risk of chimeric sequences in databasing applications, and given the ease with which entire CR data can be developed from high-quality samples, the generation of only HVS-I/II data is no longer accepted for reference population databasing purposes.

In forensic casework, artificial recombination is mitigated by strict sample handling measures that include many or all of the

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