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Editorial

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

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DNA Commission of the International Society for Forensic Genetics.

1. Introduction

Sequence analysis of human mitochondrial DNA (mtDNA) is being used widely to characterize forensic biological specimens, particularly when there is insufficient nuclear DNA in samples for typing. Hair shafts, bones, teeth and other samples that are severely decomposed may be subjected to mtDNA analysis, e.g. [1–5]. Although many of the quality assurance, quality control and interpretational guidelines used for PCR-based nuclear DNA analyses apply to mtDNA analysis, there are some features of mtDNA that warrant specific consideration: (1) mtDNA is maternally inherited; (2) heteroplasmy; and (3) the greater sensitivity of detection of mtDNA typing. It is imperative that guidelines consider the features of mtDNA and that practices do not exceed the state-of-knowledge on mtDNA. In a effort to refine previously published guidelines [6] and to assist those currently using mtDNA protocols and those considering implementing mtDNA analysis, the DNA Commission of the ISFG met on 16th August 1999 in San Francisco to develop current guidelines. The following are the recommendations by the DNA Commission on the use of mtDNA analysis.

2. Background

Mitochondria are subcellular organelles that contain an extrachromosomal genome

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that is separate and distinct from the nuclear genome. MtDNA is present in much greater copy number per cell compared to nuclear DNA [7]. It has a small (~16.5 kb) genome that is circular, is maternally inherited, and generally does not undergo recombination [8,9], but see [10,11]. A higher success rate is achieved with mtDNA compared with nuclear DNA, from old bones, severely decomposed or charred remains, or single hair shafts.

Excluding mutations, a mtDNA sequence is identical for all maternally linked relatives. The feature of maternal inheritance can be useful to support or refute the identity of putative samples by comparison with reference samples from known maternal relatives. In general, the transmission of a mtDNA type is consistent across many generations. Relationships several generations removed may be evaluated by mtDNA typing, e.g. [12].

The mitochondrial genome has a higher mutation rate than the nuclear genome [13]. Some regions of the mtDNA genome appear to be evolving at 6 to 17 times the rate of single copy nuclear genes [14–17]. The highest degree of variation in the mtDNA among individuals is found within the non-coding control region in hypervariable regions I and II (HVI and HVII, respectively). In fact, for the HVI and HVII regions combined, Caucasian Europeans differ on average at 8 nucleotide positions [18].

In general, there does not appear to be recombination in mtDNA. A mtDNA type is defined as a sequence or haplotype and is treated as a single locus. Since mtDNA is maternally inherited, the mtDNA essentially is monoclonal in an individual. However, it is possible for more than one mtDNA type to be present in an individual; this is called heteroplasmy and it is now thought that all individuals are heteroplasmic at some level. The two types in an individual usually differ at only one base. However, heteroplasmy at two or more sites is expected to occur at lower frequencies. Heteroplasmy may be observed in several ways: (1) individuals may have more than one mtDNA type in a single tissue; (2) individuals may exhibit one mtDNA type in one tissue and a different type in another tissue; and/or (3) individuals may be heteroplasmic in one tissue sample and homoplasmic in another tissue sample. Length heteroplasmy, which typically manifests itself as variation in the number of bases residing within a homopolymeric stretch (i.e., C stretches), is prevalent in the population and much more so than point heteroplasmy.

Although there are several strategies for mtDNA sequencing, inter-laboratory comparative exercises have proven PCR based mtDNA typing by automated sequencing to be a valid, robust and reliable means of forensic identification [19]. A review of mtDNA and its applications to forensic casework can be seen in reference [20].

3. Guidelines

3.1. Quality assurance/quality control

Although there are differences in mtDNA compared with nuclear DNA, human identity testing is fairly straightforward and many practices are similar. The same

recommendations previously proffered by the ISFG [21,22] regarding sample handling and the use of PCR still apply to mtDNA.

The main concern for mtDNA typing is to control and monitor contamination within the laboratory. The practices include use of dedicated lab areas, lab coats, disposable gear (such as gloves, caps or sleeves) and positive displacement or aerosol resistant pipette tips. Only one case and only one item from that case should be investigated in a laboratory room at a time, items of evidence (when possible) should be extracted and amplified prior to known reference samples or in a physically separate room in the laboratory. Pre and post-amplification areas should be physically separated, work surface areas should be thoroughly cleaned before and after use, and workspaces under dedicated hoods should be employed when possible. In addition, exposing to UV light all appropriate materials and reagents should be carried out.

Because of the sensitivity of detection of mtDNA analysis, low levels of exogenous DNA contamination can be observed; yet low levels of contamination can be tolerated because reliable results can be obtained in the presence of contamination. Contamination must be monitored. Reagent blanks and negative controls (samples containing all reagents, except template DNA) are one way in which contamination levels are monitored. An extraction reagent blank should monitor contamination from the extraction through the analysis. A PCR negative control should monitor the presence of exogenous DNA from amplification through analysis. Both of these controls should be processed in a similar manner to that of an evidence sample. In order to trace potential sources of contamination, all laboratory personnel involved in the mtDNA analysis should be typed, and those mtDNA types should be maintained on file.

Although single analyses can produce reliable results, it is desirable to carry out analysis twice on separate occasions to better interpret the effects of contamination. The choice for single (in lieu of double analysis) should be based in part on contamination observed in the case, consumption of evidence, number of PCRs carried out and controls run.

If either the extraction reagent blank or the PCR negative control yields a sequence that is the same as that of the evidence sample, the results from the evidence sample must be rejected and the analysis repeated. If either control yields a sequence that is different from that of the evidence sample, the results should be interpreted in the light of all of the results in the case, including positive and other negative controls, results from duplicate analyses, and relative strength of the negative result versus the evidence sample. A positive control (known DNA sample) must yield the appropriate result.

When appropriate, a sample from an apparently unstained portion of the substrate, or an unstained similar nature sample, should be analyzed.

3.2. Nomenclature

All nomenclature recommendations are intended to be compatible with IUPAC (International Union of Pure and Applied Chemistry) codes. The first entire human mtDNA sequence (i.e., the light strand sequence) was described by Anderson et al. [23] and this sequence is used as a reference standard to facilitate nomenclature of mtDNA types. When a difference between an individual's sequence and that of the Anderson et

al. sequence is observed, only the site (which is a designated number) and the nucleotide differing from the reference standard are recorded. For example, at site 73 (in HVII), the Anderson sequence has an A; however, a large portion of the population carries a G at site 73. Such an individual's mtDNA sequence is described as 73G. If no other bases (or sites) are noted, then it is understood that the particular mtDNA sequence is identical to the Anderson sequence, except as noted at site 73. If an unresolved ambiguity is observed at any site, the base number for the site is listed followed by an 'N' (e.g., 16125N). Insertions are described by first noting the site immediately 5′ to the insertion followed by a decimal point and a '1' (for the first insertion), a '2' (if there is a second insertion), and so on, and then by the nucleotide that is inserted. In the case of homopolymeric tracts, where the exact position at which the insertion has occurred is unknown, the assumption is always made that the insertion has occurred at the highest numbered end of the homopolymeric region. For example, a homopolymeric region, at which insertions are common, occurs between nucleotide positions 311 and 315 (inclusive). The polymorphism, a C insertion, is assumed to occur after site 315, so the nomenclature used is 315.1C. Deletions are recorded by listing the missing site followed by a 'd' (i.e., 220d). At confirmed heteroplasmic sites, IUPAC codes for base calling can be applied. If heteroplasmy is suspected but not confirmed, an N can be used. For example, an A/G heteroplasmy can either be designated as N or as R.

3.3. Heteroplasmy

The occurrence of heteroplasmy does not invalidate the use of mtDNA for forensic analysis. In fact, heteroplasmy is often observed during hair analyses. The existence of heteroplasmy is considered to be confirmed if two bases clearly above the background level of the sequence can be observed in sequence from both strands of the DNA. The background level is likely to be different for sequences in each case. Heteroplasmy may be observed in both known and questioned samples, observed in known but not in questioned sample (or vice-versa), or may not be detected in either sample, yet be responsible for an apparent difference between the samples. When heteroplasmy is observed in the questioned sample but not in the known sample or vice-versa, or in cases where questioned and known samples differ by a single nucleotide, it may be useful to obtain and process additional known samples; during such additional analysis, heteroplasmy may be revealed.

Both HV I and HV II of the human mtDNA control region contain segments of sequence which are homopolymeric (i.e., C stretches). Length heteroplasmy is often observed in these regions. In HV I, a homopolymeric region begins at nucleotide number 16184. When a transition at position 16189 (T-C) is observed, length heteroplasmy often occurs. A similar homopolymeric region resides in HVII, at positions 303–315. In order to assess the presence of length heteroplasmy, careful consideration should be given to positions 309 and 310 in both light and heavy strand sequences. In general, length heteroplasmy is commonly observed, as 'out-of-phase sequence carryover' downstream of this homopolymeric region. In such cases in either region, it can sometimes be difficult to unambiguously determine the exact number of cytosine residues present. If this is the case, no attempt should be made to count the number of residues (for

interpretation purposes), and all comparisons will treat this region as having the same number of cytosines.

3.4. Interpretation

Interpretation guidelines to evaluate sequencing results from evidence and reference samples are necessary. These recommendations take into account the limitations of the current state-of-the-art and knowledge regarding mtDNA genetics and technology. Assumptions and limitations should be clearly stated. When rendering an interpretation (e.g. inclusion, exclusion, or inconclusive) and for assessing weight of the evidence in any forensic comparison, one should be careful not to exceed current limitations in knowledge, mtDNA or otherwise.

Sequencing of a mtDNA region should be performed twice. Sequencing of both strands of a mtDNA region is preferable in order to reduce ambiguities in sequence determination. The sequences from a reference and evidence sample are compared. If the sequences are unequivocally different, then the samples can be excluded as originating from the same source. If the sequences are the same, then the reference sample and evidence cannot be excluded as potentially being from the same source. In cases where the same heteroplasmy is observed in both the questioned sample and the known sample, its presence may increase the strength of the evidence. If heteroplasmy is observed in the questioned sample but not the known sample or vice versa, a common maternal lineage cannot be excluded. If the questioned and known samples differ by a single nucleotide, and no evidence of heteroplasmy is present, the interpretation may be that the results are inconclusive. However, one nucleotide difference between two samples may, on occasions, provide evidence against the samples originating from the same source or maternal lineage; in particular, where both the questioned sample and known sample are a tissue such as blood, a single nucleotide difference points towards exclusion of a common maternal origin. In cases where more than a single nucleotide difference exists between the evidence and known sequences, careful analysis of the sequences, which differ, will determine the proper interpretation of the comparison. The sources of the tissues investigated should be taken into consideration, because differences in mtDNA sequences due to mutations seems to be more likely between e.g. hair and blood than between two blood samples taken from the same individual. Also, the knowledge of mutation rates is, presently, limited and may not allow precise estimates but should be taken into consideration. In general, however, the larger the number of differences between samples, the smaller the chance of them being from the same source or having the same maternal origin.

When a mtDNA profile from an evidence sample and one from a known reference sample cannot be excluded as originating from the same source, it is desirable to convey some information about the weight of the evidence. Presently, the practice is to count the number of times a particular sequence (or haplotype) is observed in (a) database(s) and applying a correction for sampling errors (such as a confidence interval, bootstrap value or Balding and Nichols [24] correction). The estimated mtDNA haplotype frequencies should be interpreted in the light of the data available concerning the distribution of the mtDNA haplotypes and the possible subpopulation structures within in the relevant

population(s). As for other DNA evidence it is desirable to communicate the value of the mtDNA evidence using likelihood ratios.

4. Conclusions

In conclusion, mtDNA sequencing provides another useful tool for characterizing biological evidence. The recommendations described here should be useful for those implementing mtDNA sequencing. With high quality practice and careful consideration when evaluating typing data, confidence in the results and interpretational conclusions can be attained. As more genetic information is generated and improvements in technology occur, it is the intention of the DNA Commission of the ISFG to update the guidelines to reflect such changes.

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