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Mitochondrial DNA error prophylaxis: assessing the causes of errors in the GEP'02–03 proficiency testing trial

Antonio Salas[a,*], Lourdes Prieto[b], Marta Montesino[b], Cristina Albarrán[c], Eduardo Arroyo[d], Miguel R. Paredes-Herrera[e], Ana Ma Di Lonardo[f], Christian Doutremepuich[g], Isabel Fernández-Fernández[h], Alberto González de la Vega[i], Cíntia Alves[j], Carlos M. López[k], Manolo López-Soto[l], José A. Lorente[m], Antònia Picornell[n], Rosa M. Espinheira[o], Alexis Hernández[p], Ana Ma Palacio[q], Marta Espinoza[r], Juan J. Yunis[s], Anna Pérez-Lezaun[t], José J. Pestano[u], Juan Carlos Carril[v], Daniel Corach[w], Ma Conceiçao Vide[x], V. Álvarez-Iglesias[a], M.F. Pinheiro[y], Martín R. Whittle[z], Antonio Brehm[A], Josefina Gómez[B]

[a]*Unidad de Genética, Facultad de Medicina de la Universidad de Santiago de Compostela, Instituto de Medicina Legal, A Coruña, Galicia-Spain*
[b]*Comisaría General de Policía Científica, Sección de Biología-ADN, Madrid, Spain*
[c]*Departamento de Madrid, Sección de Biología, Instituto de Toxicología, Madrid, Spain*
[d]*Toxicología y Legislación Sanitaria, Facultad de Medicina, Universidad Complutense, Madrid, Spain*
[e]*Departamento de Barcelona, Sección de Biología, Instituto Nacional de Toxicología, Barcelona, Spain*
[f]*Banco Nacional de Datos Genéticos, Hospital Dr. C.G. Durand, CF Buenos Aires, Argentina*
[g]*Laboratoire D'He'matologie, Bordeaux, France*
[h]*DataGene, Sondika, Bizkaia, Spain*
[i]*ADF TecnoGen, SL, Madrid, Spain*
[j]*Instituto de Patología e Inmunología, Molecular de la Universidad de Porto, Porto, Portugal*
[k]*Jefatura de Investigación y Criminalística, Dirección General de la Guardia Civil, Laboratorio de ADN, Madrid, Spain*
[l]*Sección de Biología, Departamento de Sevilla, Instituto de Toxicología, Sevilla, Spain*
[m]*Departamento de Medicina Legal, Laboratorio de Identificación Genética, Universidad de Granada, Granada, Spain*
[n]*Laboratori de Genètica, Institut Universitari d'Investigacions en Ciències de la Salut i Departament de Biologia, Universitat de les Illes Balears, Spain*
[o]*Serviço de Genética e Biologia Forense, Delegação de Lisboa do Instituto, Nacional de Medicina Legal, Lisboa, Portugal*
[p]*Departamento de Canarias, Instituto Nacional de Toxicología y Ciencias Forenses, La Cuesta, Santa Cruz de Tenerife, Spain*
[q]*Centro de Análisis Genéticos C.A.G.T., Zaragoza, Spain*
[r]*Departamento de Ciencias Forenses, Unidad de Genética Forense, Poder Judicial, Costa Rica, Spain*
[s]*Servicio Médico Yunis Turbay y Cía, Santafé de Bogota, DC, Colombia, Spain*
[t]*Unitat de Biología Evolutiva, Universidad Pompeu Fabra, Barcelona, Spain*
[u]*Servicio de Genética Forense, Universidad de Las Palmas de Gran Canaria, Las Palmas, Spain*
[v]*Departamento de Genética Forense, GENÓMICA, S.A.U., Madrid, Spain*
[w]*Servicio de Huellas Digitales Genéticas, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina*
[x]*Servicio de Biología Forense, Instituto de Medicina Legal, Coimbra, Portugal*
[y]*Instituto de Medicine Legal, Servicio de Biología Forense, Oporto, Spain*
[z]*Genomic Engenharia Molecular LTD A, Sao Paulo, Brazil*

[*] Corresponding author. Tel.: +34-981-582-327; fax: +34-981-580-336.
E-mail address: apimlase@usc.es (A. Salas).

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[A]Centre of Biological and Geological Sciences, University of Madeira, Campus of Penteada, 9000 Funchal, Portugal
[B]Instituto de Toxicología, Unidad de Garantía de Calidad, Madrid, Spain

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Abstract

We report the results of the Spanish and Portuguese working group (GEP) of the International Society for Forensic Genetics (ISFG) Collaborative Exercise 2002–2003 on mitochondrial DNA (mtDNA) analysis. Six different samples were submitted to the participating laboratories: four blood stains (M1–M2–M3–M4), one mixture blood sample (M5), and two hair shaft fragments (M6). Most of the labs reported consensus results for the blood stains, slightly improving the results of previous collaborative exercises. Although hair shaft analysis is still carried out by a small number of laboratories, this analysis yielded a high rate of success. On the contrary, the analysis of the mixture blood stain (M5) yielded a lower rate of success; in spite of this, the whole results on M5 typing demonstrated the suitability of mtDNA analysis in mixture samples. We have found that edition errors are among the most common mistakes reported by the different labs. In addition, we have detected contamination events as well as other minor problems, i.e. lack of standarization in nomenclature for punctual and length heteroplasmies, and indels. In the present edition of the GEP-ISFG exercise we have paid special attention to the visual phylogenetic inspection for detecting common sequencing errors.

Keywords: mtDNA; Standardization; Collaborative exercise; Heteroplasmy; Hair shafts; Sequence errors; Phylogenetic

1. Introduction

Since 1997, the Spanish and Portuguese Working Group (GEP) of the International Society for Forensic Genetics (ISFG) included mtDNA profiling as part of the GEP proficiency testing program [1–5]. Along the years, the number of participating labs has progressively increased.

Here we review the results of the sixth mtDNA trial carried out by the GEP-ISFG group corresponding to the period 2002–2003. This exercise shows an important progress in standardization and reliability on the mtDNA analysis, as well as an increasing interest for the GEP-ISFG group to answer important questions concerning mtDNA forensic caseworks. Particularly, in the present exercise, we point out the convenience of using visual phylogenetic inspection of the final sequence report as a prophylactic tool to detect sequencing errors.

Two different cases were included in the GEP proficiency testing trial: a forensic and a paternity case. It is important to note that not all the labs in the group are interested in criminalistic casework, and this is the main reason why mtDNA is generally performed by a small number of labs (27/85). As part of the present exercise, five blood stains (M1, M2, M3, M4, and M5), and two hair shaft fragments (M6) were distributed to all participants. M1 and M2 stains were taken from a father and a mother, respectively. M5 consisted of a blood stain made of a mixture 2:1 from a woman maternally unrelated with the rest of the donors and blood from the M4 donor. Information on the characteristics of M3, M4, M5 and M6, was not provided to the participants till the completion of the exercise. The posed questions for the whole exercise were: to examine the possibility of relationship of M3 and M4 donors with the mother (M1) and the father (M2), and determine if M1, M2, M3, and M4 donors could have been the biological source of M5 and the two hair shafts fragments (M6) as well. Therefore, M1 to M4 were submitted for the paternity exercise, whilst M5 and M6 were part of the forensic simulated case; M6 was submitted exclusively for mtDNA typing.

2. Materials and methods

2.1. Samples

The Quality Control Office (Unidad de Garantía de Calidad, Instituto Nacional de Toxicología, Ministerio de Justicia, Madrid, Spain) provided all the participants with a total of five bloodstains as well as two hair shafts from the same individual (two fragments of approximately 3 cm long). M1, M2, M3, and M4 were prepared by applying 100 μl of whole blood onto Whatman surface (Whatman Bioscience) and were air-dried before distribution. The exercise simulated a traffic accident with several victims. The case consisted of identifying the son of a mother and father (the donors of M1 and M2, respectively) among the donors of M3 and M4. The two hair shafts fragments were submitted in order to evaluate their

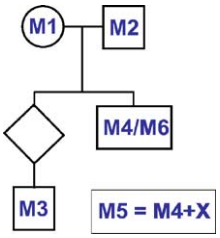

Fig. 1. Diagram showing the genetic relationships between the samples analyzed in 2002–2003 GEP-ISFG Collaborative Exercise. An X indicates a donor not maternally related to M1, M2, M3, M4/M6.

potential biological sources as coming from M1, M2, M3, and M4 donors.

Posterior to the completion of the analysis and submission of the results to the Quality Control Office, labs were informed about the origin of M3, M4, M5 and M6. M4 donor was the son of M1 and M2. M3 donor was a grandson of the donors of M1 and M2 (and maternal nephew of the M4 donor). Blood stain M5 was prepared as a mixture of two blood samples at proportion 2:1, taken from a woman unrelated to M1, M2, M3, M4, and M6, and from the same donor of M4. The mtDNA profile of this unrelated individual was also determined independently of the rest of the exercise in order to evaluate the a posteriori admixture-profile of sample M5 in the whole quality control. Hair shaft fragments were taken from the donor of M4. Fig. 1 displays a diagram showing the familial relationships among samples.

Following the routine established in previous trials, all labs were given an anonymous number and were requested to fill in a questionnaire with all the technical details related with the analysis.

2.2. DNA extraction and amplification

For the bloodstains samples and hair shafts, phenol–chloroform was used by the majority of laboratories (66 and 79%, respectively) usually followed by microcon or centricon-100 purification, and 26 and 16% (respectively) used a Chelex-100 extraction. Most of the labs used the primers described by Wilson et al. [6] and Vigilant et al. [7] for the amplification of the mtDNA HVS-I and HVS-II segments.

2.3. Sequencing

All the labs used automated sequencers (mainly ABI systems of Applied Biosystems: ABI377, ABI3 10, and ABI 3100). Most of the participants used the same primers as those used for the amplification of HVS-I and HVS-II. Rhodamine or BigDye terminators, and Thermosequenase were the most common chemistries used for sequencing.

Table 1
Participation of laboratories in the mtDNA exercise

Samples	Number of labs that analyzed mtDNA	Number of labs that reported the consensus result (%)
M1, M2, M3, M4	21[a]	18 (∼86)
M1, M2,M3, M4, M5	18	8 (∼44)
M1, M2, M3, M4, M6	18	13 (∼72)
M1–M6	13	6 (∼46)

[a] Although 27 labs partially analyzed these samples, only 21 of them reported HVS-I and HVS-II.

3. Results and discussion

3.1. MtDNA sequence results

A total of 27 labs out of 85 (∼32%) reported partial or complete mtDNA results for the samples submitted. This level of participation was slightly higher than in the previous edition of the quality control (30%).

HVS-I and HVS-II sequence results of the blood samples M1, M2, M3, and M4, were submitted by 21 out of 27 labs (∼77%; 89% submitted HVS-I or HVS-II). However, the number of labs reporting results for both hypervariable regions decreased significantly when considering M5 and M6 samples: only 18 labs reported results for M1, M2, M3, M4, and M6, 18 labs reported results for M1–M5, whereas only 13 reported results for the whole set of samples (M1–M6) (see Table 1). A few labs did not consider mtDNA the most appropriate marker for DNA profiling of mixture samples (in fact, this M5 mixture was perfectly resolved by most participating labs by using STRs markers); this explains in part the low participation of labs in mtDNA typing of sample M5.

The inspection of Table 1 shows that 18 out of 21 labs (∼86%) reported the consensus HVS-I/II result for M1–M2–M3 and M4, while only 6 out of 13 (∼46%) reported consensus result for the six samples submitted. M5 sample was responsible for this low rate of success: only ∼44% (8/13) were consensus results. Analysis of the hair shafts (M6) was significantly more successful: 13 out of 19 labs (∼68%; when contrasting this frequency with the ones from Table 1, note that (Table 2), there is one lab that did not analyze M2, but shows results for M6). This clearly contrasts the low rate of success yielded by the previous edition of the GEP mtDNA exercise due to the low quantity and quality of DNA present in those hair samples (see [5]).

Interestingly, we have detected that a high number of labs reported HVS-I/II consensus results for those blood samples belonging to the same lineages, that is, 20/27 M1, 21/27 M3, and 21/27 M4. However, a minor number of them (18 out of 27) reported consensus results concerning the only sample (M2) that belonged to a different mtDNA lineage than the one shared by M1, M3, and M4. Note that the information related to the degree of familial relationship could be

Table 2
Non-consensus results for the five blood samples (M1, M2, M3, M4, M5) and the two hair shafts fragments (M6) analyzed in the 2002–2003 edition of the GEP-ISFG quality control

	M1–M3–M4	M2	M5	M6
Consensus	HVS-I: 16189C 16256T 16270T 16362C HVS-II: 73G 185A 204C 263G 309.1C 315.1C	HVS-I: 16189C 16265C HVS-II: 152C 263G 309.1C 315.1C	HVS-I: 16051G/A 16189C 16256C/T 16270T 16362T/C HVS-II: 73G 146C/T 150T/C 185G/A 204T/C 263G 309.1C 315.1C	HVS-I: 16189C 16256T 16270T 16362C HVS-II: 73G 185A 204C 263G 309.1C 315.1C
Lab 1	HVS-I: 16189C 16256T 16270T HVS-II: =	HVS-I: – HVS-II: –	HVS-I: – HVS-II: –	HVS-I: 16189C HVS-II: 309.1C 310C/T 315.1C
Lab 2	HVS-I: = HVS-II: 73G 185A 204C 263G 309.1C	HVS-I: = HVS-II: 152C 263G 309.1C	HVS-I: – HVS-II: 73G 146C + T 150C + T 185A + G 204C + T 263G 309insC	HVS-I: – HVS-II: –
Lab 3	HVS-I: 16189C HVS-II: 185A 204C 263G 309.1C 315.1C	HVS-I: 16189C HVS-II: 152C 309.1C	HVS-I: 16189C HVS-II: 146C/T 150T/C 185A/G 204C/T 263G 309.1C 315.1C	HVS-I: 16189C HVS-II: 185A 204C 263G 309.1C 315.1C
Lab 4	HVS1: – HVS-II: 185A 204C 263G 309.1C 315.1C	HVS-I: – HVS-II: 152C 303 + 1 309 + 1	HVS-I: – HVS-II: 146C 185A 204C 263G 303 + 1C 311 + 1C	HVS-I: – HVS-II: –
Lab 5	HVS-I: 16189C 16256A 16270A 16362G HVS-II: =	HVS-I: 16189C 16265G HVS-II: 152C 263G	HVS-I: 16051A/G 16189C 16256A/G 16270A 16362A/G HVS-II: =	HVS-I: 16126C 16186T 16189C 16294T HVS-II: 73G 263G 309insC 315insC
Lab 6	HVS-I: = HVS-II: =	HVS-I: = HVS-II: 152 263 309.1C 315.1C	HVS-I: – HVS-II: –	HVS-I: – HVS-II: –
Lab 7	HVS-I: = HVS-II: =	HVS-I: = HVS-II: 152C 263G 309.1C 310C/T 315.1C	HVS-I: – HVS-II: –	HVS-I: – HVS-II: –
Lab 8	HVS-I: = HVS-II: =	HVS-I: = HVS-II: =	HVS-I: = HVS-II: 73G 146C/T 150T/C 185C/T 204T/C 263G 309.1C 315.1C	HVS-I: 16189C 16193.1C 16216G 16223T HVS-II: –
Lab 9	HVS-I: = HVS-II: =	HVS-I: = HVS-II: =	HVS-I: 16051G 16189C 16256T 16270T 16362C HVS-II: 73G 185A 204C 263G 309.1C 315.1C	HVS-I: 16189C 16193.1C 16256T 16270T 16362C HVS-II: =
Lab 10	HVS-I: = HVS-II: =	HVS-I: = HVS-II: =	HVS-I: 16048R 16189C 16256Y 16270T 16362Y HVS-II: =	HVS-I: – HVS-II: –

Table 2 (*Continued*)

	M1–M3–M4	M2	M5	M6
Lab 11	HVS-I: = HVS-II: =	HVS-I: = HVS-II: =	HVS-I: = HVS-II: 73G 146T/C 150C/T 185A 204C 263G 309.1C 315.1C	HVS-I: = HVS-II: =
Lab 12	HVS-I: = HVS-II: =	HVS-I: = HVS-II: =	HVS-I: 16051G > A 16189C 16235A ∼ C 16256T > C 16270T 16362T > C HVS-II: 73G 146C > T 150T > C 185G > A 204T ∼ C 263G 309.1C 315.1C	HVS-I: = HVS-II: =
Lab 13	HVS-I: – HVS-II: 185A 204C 263G 309.1C 315.1C	HVS-I: = HVS-II: 152C 263G 309.1C 315.1C	HVS-I: – HVS-II: HVS-II: 146C 185A 204C 263G 303 + 1C 311 + 1C	HVS-I: – HVS-II: –
Lab 14	HVS-I: = HVS-II: =	HVS-I: = HVS-II: =	HVS-I: 16051G 16189C 16256C/T 16270T 16273A/G16362C/T HVS-II: =	HVS-I: = HVS-II: 73G 185A/G 204C 263G 309.1C 315.1C

The results are shown as reported to the Unidad de Garantía de Calidad. This allows to illustrate some problems concerning to, i.e. difference in nomenclature, especially at heteroplasmies and insertions at homopolimeric stretches. Note that labs reported exactly the same sequence errors for samples M1, M2, and M4 (the three donors belonged to the same matrilineage; see text): (=) indicates ''in agreement with the consensus sequence''; (–) indicates ''not reported''.

indirectly obtained by all the labs (as part of the same quality control exercise) through the analysis of autosomal markers. In conclusion, it seems that the labs are more confident in their mtDNA report when it is supported on the basis of consensus results obtained through the analysis of different samples belonging to the same mtDNA lineage. In support of this argument is the fact that some labs repeated exactly the same sequence mistakes (some of them concerning the omission of polymorphisms positioned at the sequences ends) in the three samples belonging to the same lineages (Table 2; see errors in samples M1, M3, and M4, reported by labs number 1, 2, 3, 4, 5 and 13). This approach is not adequate in the forensic genetic practice since each electropherogram must be independently evaluated. Therefore, this fact was informed and discussed during the GEP-ISFG meeting in order to aware the participants about avoiding this type of errors in future trials.

A lower number of labs submitted complete HVS-I/II results for samples M5 (18/27) and M6 (19/27), and the success rate was significantly worse in the case of sample M5: only 8 out of 18 participants (∼44%) reported the consensus sequence for HVS-I/II segments. This is a poor result, especially if we compare it with the success reached in the analysis of the rest of samples for HVS-I/II: 75–77% for M1, M2, M3, and M4, and 72% for M6.

In the present edition of the control, we have also discussed the advantages of analyzing the complete control region in the forensic casework and in future quality controls, since many polymorphisms of forensic interest are positioned out of the HVS-I and HVS-II segments commonly analyzed. This obviously would lead to increase the discrimination power of the mtDNA test. Nevertheless, we also considered that this issue should be discussed in terms of updating guidelines for mtDNA testing, perhaps in the context of the ISFG.

3.2. Assessing the causes of error

Visual inspection of the whole mtDNA GEP-ISFG report has allowed us to assess the cause of most of the non-consensus results (Table 2). Most of the labs performed both hypervariable regions (HVS-I and HVS-II) and it was not detected a significant correlation between the hypervariable region studied and the number or type of errors.

3.2.1. Edition mistakes

The most common mistakes are due to errors during edition process. Table 2 testifies for several examples: (a) lab 2 omitted the common insertion 315.1C in HVS-II in all the samples; (b) in samples M1, M3 and M4, lab 5 edits the H chain variants instead of the L chain ones in HVS-I segments after position 16189, that is, 16256A 16270A 16362G variants instead of 16256T 16270T 16362C. This is probably due to the fact that this lab performed two separated

amplifications of HVS-I segment (a practical choice when length heteroplasmy occurs at the homopolymeric track around 16189) and just H reading of the 3′ HVS-I segment was done; in M2, error at position 16265C in lab 5 probably responds to the same explanation; (c) in M5, lab 8 edits 185C/T instead of 185G/A; (d) lab 10 edits an erroneous three base pair shift in HVS-I of M5 sample: position 16048R instead of 16051G/A, etc.

3.2.2. Electrophoregrams quality at the 3′ and 5′ ends of the sequences

Also striking is the fact that some labs do not report certain positions, although using primers that would permit to do so. This is probably due to the fact that these labs have some problems in reading and interpreting the 5′ extremes of their sequences (where the quality of the electrophoregram, close to the sequence primer, is usually poorer). This deficiency can be properly corrected by modifying some critical step of the sequence protocol (i.e. purification of the PCR fragments) and performing forward and reverse sequence of the same amplicons.

In the present control, this mainly affects variants at sites 16362 (i.e. lab 1 in M1, M3, and M4 samples) and 73 (i.e. lab 3, 4 and 13 in M1, M3, and M4 samples).

3.2.3. Contamination

We have detected several instances of contamination. Lab 3 reported the same HVS-I sequence in all the samples (16189C), but different (although with errors) HVS-II profiles. The most plausible explanation for this result is contamination of HVS-I amplification primers. All the results reported by this lab contained some additional mistake; therefore, in the GEP-ISFG meeting we stressed the need for an urgent solution to these critical blunders. Lab 5 provides a clear instance of contamination of the hair shaft sample (M6): a genuine U5 profile of M6 sample (‘‘16189C 16256T 16270T 16362C 73G 185A 204C 263G 309.1C 315.1C’’) has been accidentally contaminated with a biological source harbouring the following profile ‘‘16126C 16186T 16189C 16294T 73G 263G 309insC 315insC (Table 2)’’, which clearly identifies a sequence belonging to haplogroup T. In addition, labs 1 and 8 show two additional likely instances of contamination of sample M6.

3.2.4. Nomenclature deficiencies

Table 2 reveals a common problem concerning mtDNA sequence nomenclature. There are a number of labs that do not use the consensus nomenclature recommended by the ISFG mtDNA guidelines [8] to describe punctual heteroplasmies and length variants.

We have detected a common mistake in the report of HVS-II homopolymeric variation. Position 310 in HVS-II is phylogenetically stable and should not mutate frequently. However, when length heteroplasmy affects the homopolymeric C track 303–309, the electropherogram displays a point heteroplasmy-like pattern at this position 310. Thus, a 310C/T pattern (i.e. lab 1) is really a length heteroplasmy provoked by the combination of molecules of different homopolymeric lengths.

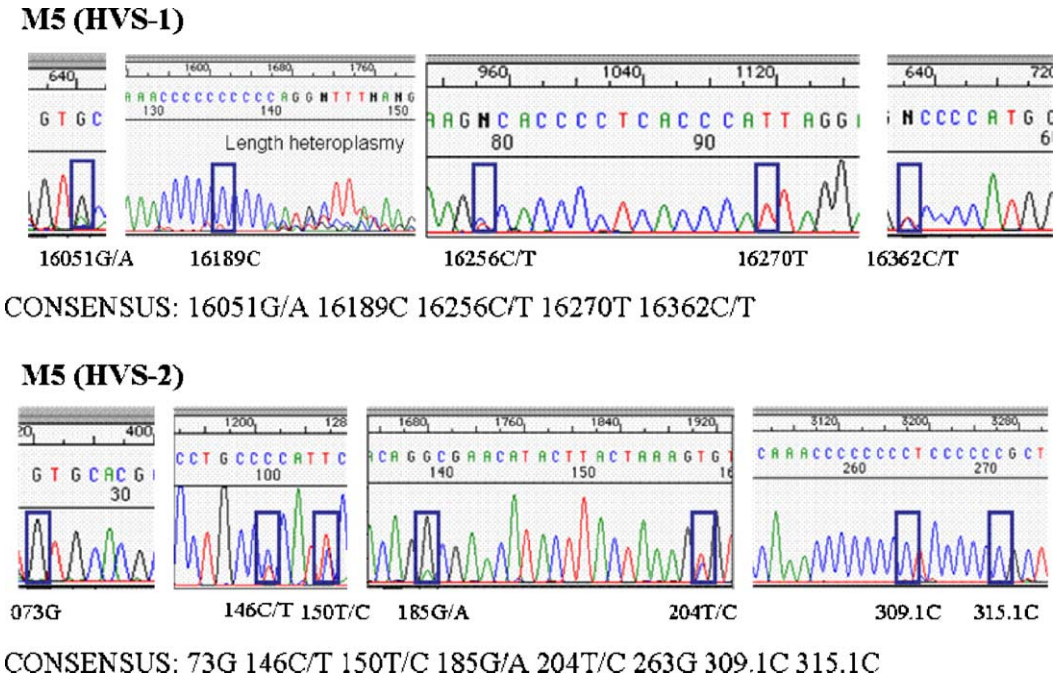

Fig. 2. Different segments of the electrophoregrams corresponding to the mixture sample M5.

3.3. Evaluating mtDNA as a marker to detect sample mixtures

Sample M5 consisted of a mixture of two different blood samples at 2:1 proportions.

- M4 donor provided with the minor component of the mixture: "16189C 16256T 16270T 16362C 073G 185A 204C 263G 309.1C 315.1C".
- A maternally unrelated donor to M1, M2, M3, and M4 represented the major component of the mixture and carries the following profile "16051G 16189C 16270T 073G 146C 150T 263G 309.1C 315.1C".

Then, the expected profile of such mixture is 16051G > A 16189C 16256C > T 16270T 16362T > C for HVS-1 and 73G 146C > T 150T > C 185G > A 204T > C 263G 309.1C 315.1C for HVS-II. Due to the complexity of the electropherogram, the following HVS-II profile "16051G/A 16189C 16256C/T 16270T 16362T/C and 73G 146C/T 150T/C 185G/A 204T/C 263G 309.1C 315.1C" was accepted as M5 consensus (Fig. 2).

Many labs did not detect the admixture in M5, and many other reported several edition mistakes (Table 2). Only one lab reported the existence of the expected profile as indicated above; that is, indicating the correct proportions of both the major and the minor component of M5. This lab used a phylogenetic approach to interpret the electrophoretic pattern of M5: the admixture profile could be explained as a product of an admixture of two mtDNA haplotypes, both belonging to a typical western European haplogroup, namely U5. However, it is important to keep in mind that the power of the mtDNA genome for the determination of mixed forensic samples depends on the phylogenetic nature of the participating profiles in the mixture. In addition, the typing of additional suspected diagnostic positions (coding or non-coding polymorphisms) can help to further define the participating lineages.

Moreover, two other labs reported the consensus result for both HVS-I and HVS-II (as indicated above), this time without providing information on the proportion of the different components (that is, no information on the intervening haplotypes, but a report which consists of a list of the polymorphisms detected in the electrophoregrams). Some other labs identify an admixture but did not report the consensus result.

In conclusion, since three labs reported the consensus result (and taking into account that this fact is unlikely by chance), we interpret that mtDNA is an appropriate marker for detecting DNA admixtures. However we recognize that mtDNA is not the best marker for this purpose, but still can be useful in those contexts where autosomal or Y chromosome STRs marker cannot be typed (degraded samples or low copy number samples). We find additional support to the suitability of mtDNA for mixture DNA typing in Szibor et al. [9], although they used restriction enzyme analysis instead of sequencing typing.

Results on M5 sample have also shown that there were different ways of reporting heteroplasmies (or heteroplasmy-like variants). We encourage the labs to use the recommended nomenclature as proposed by the DNA Commission of the International Society for Forensic Genetics [8].

4. Final remarks

Most of the errors detected in the GEP 2002–2003 mtDNA exercise could be assigned to the following categories: (a) deficient electrophoregrams; (b) edition errors; (c) contamination; (d) deficiencies in terms of detection of heteroplasmies; and (e) other minor problems, i.e. nomenclature.

As for other markers, it is interesting to highlight the fact that the worst and the most evident mistakes were concentrated in a few labs.

Apart from these problems, in general we noted that the technical state of the mtDNA analysis has reached a high level of reliability. Although the present exercise did not yield significant progress in terms of number of errors, we have noticed that most of the labs have gained experience. We have also stressed the fact that an important percentage of mistakes could have been easily corrected, such as edition errors, improving significantly the whole results of the GEP exercise. In the present edition we have made an important effort in order to show the usefulness of the phylogenetic approach for mtDNA prophylaxis: the cause of all the mistakes reported in Table 2 could be detected by direct visual—in cases phylogenetic—inspection of the mtDNA profiles. More details concerning phylogenetic approaches for forensic profilaxis can be seen in [10–12; among others]. In general, the Quality Control Program has again proved to be extremely valuable to the GEP-ISFG group in order to address important questions concerning mtDNA test.

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