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# Results of the 1999–2000 collaborative exercise and proficiency testing program on mitochondrial DNA of the GEP-ISFG: an inter-laboratory study of the observed variability in the heteroplasmy level of hair from the same donor

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**Abstract**

The Spanish and Portuguese working group (GEP) of international society for forensic genetics (ISFG) 1999–2000 collaborative exercise on mitochondrial DNA (mtDNA) included the analysis of four bloodstain samples and one hair shaft sample by 19 participating laboratories from Spain, Portugal and several Latin-American countries. A wide range of sequence

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results at position 16,093 of the HV1 (from T or C homoplasmy to different levels of heteroplasmy) were submitted by the different participating laboratories from the hair shaft sample during the first phase of this exercise. During the discussion of these results in the Annual GEP-ISFG 2000 Conference a second phase of this exercise was established with two main objectives: (i) to evaluate the incidence of the HV1 sequence heteroplasmy detected in Phase I across different sample types from the same donor including blood, saliva, and hair shafts, (ii) to perform a technical review of the electropherograms to evaluate the relative levels of heteroplasmies obtained by the different laboratories and also to examine the source of possible errors detected in Phase I. Anonymous review of the raw sequence data permitted the detection of three transcription errors and three errors due to methodological problems. Highly variable levels of heteroplasmy were found in the hair shaft and more stability in blood and saliva. Three laboratories found variable levels of heteroplasmy at position 16,093 across adjacent fragments from the same hair shaft. Two laboratories also described more than one heteroplasmic position from a single hair. The relevance of these findings for the interpretation of mtDNA data in the forensic context is also discussed. 

*Keywords:* Mitochondrial DNA (mtDNA) polymorphism; Standardization; Collaborative exercise; Forensic science heteroplasmy; Hair shafts

## 1. Introduction

Since 1997, the Spanish and Portuguese working group (GEP) of the international society for forensic genetics (ISFG) has been organising collaborative exercises on mitochondrial DNA (mtDNA) profiling with the main aim of making progress on standardization, technical approaches, and the interpretation of evidence. This is therefore the third consecutive year that the GEP-ISFG has included the mtDNA test in its annual proficiency-testing program. Since 1997, the number of laboratories participating in the mtDNA exercise has increased progressively: five in GEP 1997–1998, 16 in GEP 1998–1999 and 19 in GEP 1999–2000 [1,2].

The present work is a review of the fourth mtDNA exercise undertaken by the GEP-ISFG corresponding to the period 1999–2000. This exercise has shown important progress in standardization and reliability in the mtDNA test, as well as an increasing interest from the GEP-ISFG in giving answers to some important questions related to mtDNA biology and affecting the forensic tasks; for instance, heteroplasmy, intra-individual variability in hair samples, and its consequences for the interpretation of evidence.

A total of 19 laboratories belonging to the GEP-ISFG submitted results for the mtDNA test of the 1999–2000 collaborative exercise and proficiency testing program on DNA typing. The present exercise was developed in two main phases. Phase I: four blood stains belonging to four individuals and hair shafts (two fragments of ca. 4 cm long) of one individual, were submitted to each laboratory in order to perform the mtDNA test. Phase II: it was organised as a consequence of some discrepancies in sequence results at position 16,093 of the HV1 (from T or C homoplasmy to different levels of heteroplasmy) submitted by the different participating laboratories from a hair shaft sample during Phase I. It incorporated anonymous reviewing of the results (including visual inspection of the electropherograms by a scientific committee of the GEP-ISFG group) obtained by the different laboratories, and the remittance of new biological samples (blood, saliva and hair shaft) of the hair donor in Phase I to the different participating laboratories. This latter phase had two main aims: to examine the source of possible errors of Phase I, and to analyze in depth the individual (donor of hair in Phase I) whose mtDNA results had revealed the existence of high levels of heteroplasmy.

## 2. Material and methods

### 2.1. Samples

A total of four bloodstains belonging to four healthy individuals and two hair shafts (two fragments of ca. 4 cm long) of one individual, were submitted to each laboratory in order to perform the mtDNA test in Phase I of the exercise. Each bloodstain was prepared by applying 200 μl of whole blood onto absorbent paper (Fitzco Inc.) and was air-dried before distribution. The exercise simulated both, a paternity case and a criminal case including bloodstain samples from the mother, the child and two putative fathers (samples M1, M2, M3, and M4, respectively) and two hair shafts fragments (sample M5, only for mtDNA analysis). The proposed questions were: (1) who is the father?; (2) investigate if samples M2, M3 and M4 could share the same Y-haplotype by Y-STRs analysis and (3) investigate who can be the donor of the hair shaft by mtDNA analysis.

Following the routine established in previous collaborative exercises, all laboratories were given an anonymous number and they were requested to fill in a questionnaire with all the technical details related to the analysis (extraction and quantitation methods, PCR-amplification details, detection systems, ladders, population databases employed, . . .). The whole process was previously discussed and agreed to by the GEP-ISFG assembly and organization, and the proficiency testing validation was carried out by the Quality Assurance Unit (National Institute of Toxicology, Ministry of Justice, Madrid, Spain).

Table 1
Different methods for DNA extraction, amplification and sequencing used by the participating laboratories[a]

| Number of laboratories | | | | |
|---|---|---|---|---|
| DNA extraction | P:C | P:C & centricon-100 | Chelex | Other |
| | 5 | 6 | 5 | 3 |
| Amplification (primers and conditions) | Wilson et al. [3] | Vigilant et al. [4] | Holland et al. [23] | Other |
| | 11 | 5 | 1 | 2 |
| Sequencing | | | | |
| Dye terminator | DR | BD | | Other |
| | 6 (ABI377) 4 (ABI310) | 4 (ABI377) 1 (ABI310) | | 1 |
| Dye primer | BD | | | Other |
| | 1 (ABI310) | | | 1 (visible genetics) 1 (ALFexpress) |

[a] P:C, phenol-chlorophorm digestion; DR, rhodamine terminators; BD bigdye terminators.

### 2.2. DNA extraction and amplification

For the bloodstain samples and hair shafts, phenol-chloroform was used by a majority of laboratories (58%) followed in some cases (32%) by centricon-100 purification, and 26% used a Chelex-100 extraction. Most of the laboratories used the primers described by Wilson et al. [3] and by Vigilant et al. [4] to separately amplify the HVI and HVII region of the mtDNA control region (Table 1).

### 2.3. Sequencing

All the laboratories used automated sequencers (mainly Applied Biosystems ABI377 and ABI310 systems, one laboratory used the ALFexpress of amersham pharmacia biotech (APB) and one laboratory the MicroGene Blaster from VisibleGenetics. Most of the laboratories used the same primers as were used for the amplification. HVI and HVII were sequenced using mainly Rhodamine or BigDye terminator cycle sequencing (Table 1). All the laboratories carried out sequencing in both directions.

The nomenclature for mtDNA variability used followed the recommendations of the DNA Commission of the ISFG [5].

## 3. Results and discussion

### 3.1. mtDNA sequence results

All 19 laboratories submitted results for the four bloodstains (HVI and HVII regions), and most of them (15 laboratories, 79%) for the hair shaft fragments. Consensus results for all the samples are shown in Table 2.

With respect to the bloodstains, 13 out of 19 laboratories had participated in previous mtDNA exercises of the GEP-ISFG. From these, there was a total consensus in the results obtained for the HVI region, whilst for HVII, only minor

Table 2
The consensus mtDNA sequence results obtained from bloodstain samples (M1–M4) and the variable mtDNA sequence results obtained from the hair sample (M5)[a,b]

| Sample | HVI region | | | | HVII region | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 16093 | 16224 | 16298 | 16311 | 72 | 73 | 152 | 195 | 228 | 263 | 309.1 | 315.1 |
| CRS | T | T | T | T | T | A | T | T | G | A | – | – |
| M1 | C | C | – | C | – | G | C | – | – | G | – | C |
| M2 | C | C | – | C | – | G | C | – | – | G | – | C |
| M3 | – | – | C | – | C | – | – | C | A | G | C | C |
| M4 | – | – | C | – | C | – | – | C | A | G | C | C |
| M5-1 | C | – | – | – | – | – | Y | – | – | G | – | C |
| M5-2 | – | – | – | – | – | – | – | – | – | G | – | C |
| M5-3 | Y | – | – | – | – | – | – | – | – | G | – | C |
| M5-4 | Y | – | – | – | – | – | – | – | – | G | – | C |

[a] The mtDNA segments analysed by almost all the laboratories were from position 16,024 to 16,365 (HVI) and from 73 to 340 (HVII). Some laboratories sequenced a wider fragment of mtDNA and could detect additional variability (position 72 at the HVII region). A dash in the table indicates the presence of the same nucleotide as in the CRS [22]. Numbers on top indicate CRS's numeration.

[b] CRS (Cambridge reference sequence) [22]; Y (C/T).

Table 3
The mtDNA sequence results reported by the different laboratories at position 16,093 as well as other heteroplasmic positions reported by the two laboratories[a]

| Number of laboratories | Sequence results reported at position 16,093 |
|---|---|
| 8 | 16,093T |
| 1 | 16,093C |
| 3 | 16,093Y |
| 1 | 16,093T and 16,093C (two independent extracts) |
| 1 | 16,093Y and 16,093T (two independent extracts) |
| 1 | 16,093C (in one extract) and 16,093Y (in other three extracts) |
| 1 | 16,162R[b] |
| 1 | 16,092Y[b] (one extract) and 152Y (one extract) |

[a] Y (C/T); R (A/G).
[b] These are the other heteroplasmies found in hair shafts.

errors (probably due to miswriting; see Section 3.2) were found in two laboratories.

Six laboratories participated for the first time in this exercise. From these, only one error was detected in one laboratory (transcription error), and another laboratory emitted a result out of consensus, probably due to a methodological problem (sequence artifacts).

The consensus results (which means the result reported by the majority of laboratories) for these samples are shown in Table 2. Only three laboratories reported a length heteroplasmy in the homopolymeric tract of the HVII region for samples M3 and M4.

Fifteen laboratories sent results for the two hair shafts (belonging to the same donor) submitted by the organization (sample M5). Twelve of them (80%) had participated in previous exercises, and two of these emitted two apparent miswriting errors for the HVI, while three laboratories emitted results out of consensus for the HVII region. Only three out of the six new participants in the mtDNA proficiency test GEP 1999–2000 submitted results for the hair shaft, but only one error was reported for the HVII region. The results for the hair shafts are shown in Table 3.

Different laboratories have submitted results for either only one of the two hair shafts, or the two hair shafts, or even, results for different extracts from each hair shaft.

The different participating laboratories submitted a wide range of sequence results at position 16,093 of the HVI from the hair shaft sample (Table 2: M5-1, M5-2, M5-3 and M5-4), some laboratories which performed different extractions from the same hair fragment have found sequence differences among different extracts from the same hair fragment (Fig. 1). These findings are discussed in Section 3.2.

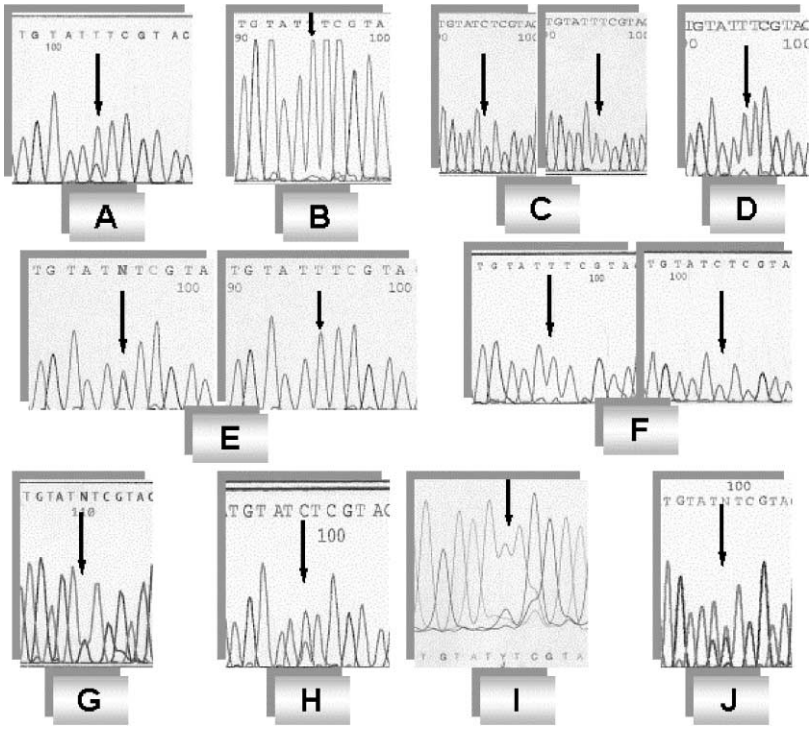


Fig. 1. Sequence data around position 16,093 (indicated by an arrow) obtained from the hair shaft sample and submitted by ten different laboratories (A–J). A wide range of sequence results was observed from T or C homoplasmy to different levels of heteroplasmy. Laboratories C, E and F submitted different mtDNA sequence results at position 16,093 from independent DNA extracts obtained from adjacent portions of the same hair shaft.

### 3.2. Assessing the possible causes of error

Anonymous examination of the electropherograms submitted by all participants has allowed identifying the origin of almost all the above mentioned errors which resulted mainly due to transcription mistakes and occasionally to methodological questions or to the low quality of the sequences obtained (one laboratory).

On the other hand, comparison of the electropherograms allowed us to evaluate correctly the relative levels of heteroplasmies obtained by different laboratories. This is of great importance to rule out the impact of subjectivity in the personal evaluation of heteroplasmy.

The reported results have shown that the technical state of mtDNA analysis has reached a high level of reliability and the importance for the laboratories to focusing their attention on the reading of the electropherograms, and on the transcription of results to the final report.

### 3.3. Different levels of heteroplasmy detected in the hair shaft and more stability in blood and saliva

Heteroplasmy is the coexistence of two or more populations of mtDNA molecules in a single mitochondrion, cell or individual. This biological state is expected to be more widespread than has been reported in initial investigations ([6–9]). In adittion, mtDNA analysis is increasingly being used in forensic casework, and in many cases, many biological samples, especially hair, are found at the scene of a crime. Hair being a common evidential finding in criminal cases, the knowledge of the mtDNA variation pattern is of special interest from a forensic-genetic perspective. The present work tries to contribute to the knowledge on heteroplasmy and how it operates in the hair.

As we showed earlier, Phase I of the GEP-ISFG exercise allowed the identification of different degrees of heteroplasmy in the hair shaft samples distributed to all laboratories and derived from the same individual (Table 3; Fig. 1).

Phase II also allowed evaluation of the incidence of the HVI sequence heteroplasmy detected in Phase I across different sample types from the same donor including blood, saliva and hair shafts. After examining position 16,093 of the HV1 region, hair shaft samples exhibited a wide range of sequence results, from T or C homoplasmy to different levels of heteroplasmy (Table 4). By contrast, blood and saliva samples always exhibited a Cytosine at position 16,093 except for a heteroplasmic result obtained from saliva that contained predominantly Cytosine:Tymine (80:20) (Table 4). Therefore, the levels of heteroplasmy observed in single hair shafts differed substantially from the levels detected in blood and buccal cells. These results are in agreement with the findings reported by Bendall et al. [10] Sullivan et al. [11], Wilson et al. [12], Hühne et al. [13] and Calloway et al. [14]. A development bottleneck in the generation of individual hair was proposed to account for the high variable levels of heteroplasmy found in individual hair samples [4,10]. On the contrary, the multiple cellular origins of blood and buccal epithelial cells could explain the more homogeneous mtDNA sequence results obtained from these samples [10].

Table 4
Results reported by the six participants in phase II for the biological samples of the donor of hair at the 16,093-nucleotide position

| Type of sample | Polymorphism detected at position 16,093 (number of laboratories) |
|---|---|
| Blood | 16,093C (all the laboratories) |
| Saliva | 16,093C (five laboratories)<br>16,093C/T, 80:20 (one laboratory) |
| Hair shafts | 16,093C (one laboratory)<br>16,093C/T, 80:20 (three laboratories)<br>16,093T/C, 80:20 (three laboratories)<br>16,093T (one laboratory) |

### 3.4. Variable levels of heteroplasmy across different fragments from the same hair shaft

During Phase I we have also identified an additional level of heterogeneity in the mtDNA sequences generated from hair samples. Three participant laboratories reported different mtDNA sequence results at position 16,093 from independent DNA extracts obtained from adjacent portions of the same hair shaft. As can be seen in Fig. 1, the variability of the sequence results at position 16,093 obtained from different portions of the same hair shaft is similar to that obtained from different hair shaft samples. These results confirm the possibility of finding different proportions of heteroplasmy in a single hair at a single nucleotide position. As suggested by others [9,10], the most likely explanation for this result could be the existence of narrow bottlenecks in mitochondrial transmission due to the stochastic segregation of mtDNA types during hair growth.

### 3.5. More than one sequence heteroplasmy in single hair

As it has been already discussed, the results clearly had shown the existence of sequence differences among different hair shafts and among adjacent portions of the same hair shaft at a single position. Moreover, two participants performed a more exhaustive analysis of these samples studying intra-hair sequence differences. In this way, different extracts of the same hair shaft resulted in punctual or heteroplasmic differences. Both laboratories reported different levels of homoplasmy/heteroplasmy for position 16,093; but also heteroplasmies for other variable positions. One of them, reported an additional heteroplasmy at position 16,162 (16, 162R) from two different extracts of one hair (Phase II). The other laboratory reported another

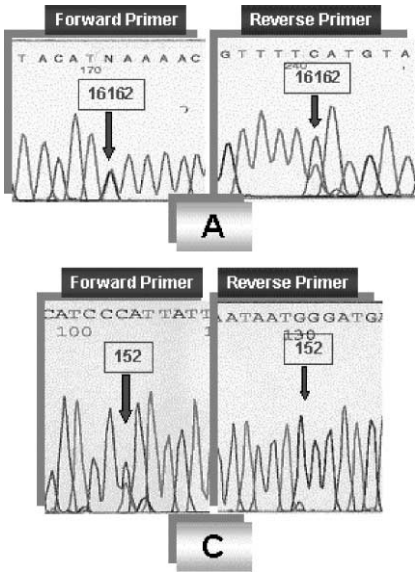


Fig. 2. Sequence results obtained from the hair shaft sample submitted by laboratories A and C showing additional heteroplasmies at position 16,162 (Laboratory A) and at position 152 (Laboratory C).

heteroplasmic position for the HVII region (152Y), from three different extracts obtained from the same hair shaft (Phase I); in addition, one of these extracts showed heteroplasmy at position 16,092Y (95:5) (Fig. 2).

Although these results are in complete agreement with those found by other authors [6,9,11], we couldn't rule out the possibility of contamination as a possible cause of these results. This possibility never should be forgotten in practical mtDNA analysis, especially when small hair fragments (with a small quantity of mtDNA molecules) are being analysed. However, due to the fact that the two laboratories have a wide experience in the analysis of minimum and degraded forensic samples, and in mtDNA testing, contamination is less likely to have been the cause of the present results. Moreover, in both laboratories, the reliability of the results is supported by the use of extreme precaution controls for extraction and amplification (with the use of negative and positive samples during all the analysis) procedures. However, negative controls are only able to detect biased contamination throughout a batch of samples and they can give only limited information at all on punctual contamination. On the other hand, the inter-lab reproducibility is a scientific requirement to exclude the possibility of contamination that was not obtained in this case.

In addition, it is important to note that one extra heteroplasmy found in the present study at position 152 corresponds to a fast-mutating position [15–17], while positions 16,162 and 16,093 have been considered to have theoretical mutation rates of 1.32 and 3.01, respectively higher than the control region average [16,18].

### *3.6. Some comments for the Interpretation of mtDNA data in the forensic context*

On the basis of the present results and those obtained by other authors regarding intra-individual mtDNA variability, instability seems to be especially important in hair probably with important differences among individuals. This fact is of great importance from an interpretative point of view, since minor sequence differences (including not only the situation of one single base difference but also the possibility of two base or heteroplasmic differences as has been showed in this collaborative study) between samples do not rule out the possibility of the same biological or matrilineage origin. Another question that should be taken into account for mtDNA profile interpretation is the mutation or heteroplasmy rate at specific nucleotide positions in the mtDNA genome [16–18]. Since it has been demonstrated that there is a great heterogeneity in mutation rates along the mtDNA control region, this should be taken into account in the evaluation of the evidence. In a situation where mtDNA profiles from evidence and reference samples show minimum differences from each other (i.e. one nucleotide position and/or heteroplasmies), it would be desirable to balance the weight of the evidence depending on the nature of such differences. For instance, a single base difference in a stable position (those which define a mtDNA haplogroup; [17–20]; among others) should be evaluated in a different way to an unstable one. Then, ignoring the existence of heterogeneous mutation rates may yield biased estimates for the inclusion of an evidence sample as potentially originating from a specific biological source. However, until further data are available a conservative criterion has been proposed for the interpretation of heteroplasmy in forensic casework [5]. Therefore, limitations in the knowledge regarding mtDNA genetics and technology must be considered when an interpretation is made (inclusion, exclusion, or inconclusive), or the evaluation of the weight of the evidence must be assessed [5].

### *3.7. General conclusions*

The experience of the GEP exercises have shown that in spite of the increasing number of participants, the quality of the results has been increasing with time. According to the results obtained by previous quality controls [21], the present work demonstrates the ability to obtain consistent results between laboratories despite a diversity of methodologies applied. The quality control program developed by the GEP-ISFG standardization group has proved to be extremely valuable and clearly improves the quality of mtDNA testing, not only from a technical point of view, but also for the evaluation of those aspects affecting the interpretation of evidence

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