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Results of the 2003–2004 GEP-ISFG collaborative study on mitochondrial DNA: Focus on the mtDNA profile of a mixed semen-saliva stain

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Abstract

We report here a review of the seventh mitochondrial DNA (mtDNA) exercise undertaken by the Spanish and Portuguese working group (GEP) of the International Society for Forensic Genetics (ISFG) corresponding to the period 2003–2004. Five reference bloodstains from five donors (M1–M5), a mixed stain of saliva and semen (M6), and a hair sample (M7) were submitted to each participating laboratory for nuclear DNA (nDNA; autosomal STR and Y-STR) and mtDNA analysis. Laboratories were asked to investigate the contributors of samples M6 and M7 among the reference donors (M1–M5). A total of 34 laboratories reported total or partial mtDNA sequence data from both, the reference bloodstains (M1–M5) and the hair sample (M7) concluding a match between mtDNA profiles of M5 and M7. Autosomal STR and Y-STR profiling was the preferred strategy to investigate the contributors of the semen/saliva mixture (M6). Nuclear DNA profiles were consistent with a mixture of saliva from the donor (female) of M4 and semen from donor M5, being the semen (XY) profile the dominant component of the mixture. Strikingly, and in contradiction to the nuclear DNA analysis, mtDNA sequencing results yield a more simple result: only the saliva contribution (M4) was detected, either after preferential lysis or after complete DNA digestion. Some labs provided with several explanations for this finding and carried out additional experiments to explain this apparent contradictory result. The results pointed to the existence of different relative amounts of nuclear and mtDNAs in saliva and semen. We conclude that this circumstance could strongly influence the interpretation of the mtDNA evidence in unbalanced mixtures and in consequence lead to false exclusions. During the GEP-ISFG annual conference a validation study was planned to progress in the interpretation of mtDNA from different mixtures.

Keywords: mtDNA; Standardization; Collaborative exercise; Mixed samples; Control region; SNPs

1. Introduction

The participation in collaborative exercises is a valuable tool to check and compare methodologies, results and statistical analyses among laboratories. At the same time, participation in such exercises is an essential part of control–quality procedures in many laboratories aimed to guarantee the quality of results submitted for a laboratory.

The Spanish and Portuguese working group (GEP) of the International Society for Forensic Genetics (ISFG) comprises forensic genetic laboratories from Spain, Portugal, and several Latin–American countries. With the aim of comparing different laboratory technical strategies and statistical approaches towards a standardization of methodologies and procedures, once a year, the GEP-ISFG organizes a collaborative exercise on DNA profiling [1–6]. During the last 7 years the exercise includes a mtDNA test. The number of participating laboratories in this mtDNA exercise has increased progressively from five in the first 1997–1998 [1] exercise to 34 in the present one.

The 2003–2004 exercise consisted of both a simulated paternity case and a criminal case. In the present edition of the GEP proficiency testing trial, seven samples were submitted to the participating laboratories: five bloodstains from different donors (M1–M5), a mixture sample (M6) and four hair shaft fragments from the same individual (M7). The exercise proposed to investigate the following questions: (a) could the donors of samples M1 and M2 be the children of the donor of M3? (b) could M1 and M2 share the same paternal lineage? (c) could M6 be compatible with a mixture of samples M4 and M5? and finally (d)

could the sample M7 belong to any of the donors of M4 or M5 bloodstains?

We will particularly discuss the mtDNA results obtained with the mixed stain (saliva/semen) that were in apparent contradiction with those obtained with the autosomal STR markers.

2. Materials and methods

Participating laboratories analyzed the samples according with their own lab standard protocols and strategies. By means of a questionnaire, participant laboratories were requested to provide detailed information on the processing of the samples. Table 1 summarizes technical details related to the analysis.

2.1. Samples

The organization and coordination of the GEP-ISFG exercise 2003–2004 was carried out by the Quality Assurance Unit (Instituto Nacional de Toxicología y Ciencias Forenses, Ministerio de Justicia, Madrid, Spain).

Five bloodstains (M1–M5), a body fluid mixture sample (M6) and four hair shaft fragments (M7) were submitted to all participants. Bloodstains were prepared by applying 100 μl of whole blood from healthy donors onto absorbent Whatman paper (Whatman Bioscience) and air-dried before distribution. To prepare the mixed sample M6, 100 μl of saliva were applied onto Whatman paper and, after air-dried, an additional aliquot of 50 μl of a 1:20 dilution of semen was added.

The final report edited by the coordinators included the compilation of data and the methodological strategies employed by all participants, as well as additional information about the origin of the samples: (a) M1 and M2 bloodstains belonged to two brothers, (b) M3 donor was the biological mother of M1 and M2 donors, (c) M4 and M5 samples were donated by unrelated individuals, a woman and a man, respectively, (d) the mixed stain M6 consisted of a mixture of saliva from the same donor of M4 and semen from the donor of M5 and finally, (e) the hair shafts M7 belonged to the donor of M5.

2.2. DNA extraction and amplification

The amplification and sequencing strategies are summarized in Table 1. To obtain DNA from bloodstains and hair (70 and 64%, respectively) most laboratories used a classical phenol–chloroform procedure followed in some cases (37%) by an additional purification and concentration step using Microcon-100 or Centricon-100 centrifugal filter devices (Millipore, Billerica, MA, USA). DNA extraction using chelating resin such as Chelex-100 (Bio-Rad, CA, USA) was used by 20% of the laboratories. The remaining participants carried out alternative methods or commercial extraction kits such as FTA purification reagent (GIBCO-BRL), QIAmp (Qiagen, Hilden, Germany) or DNA IQ (Promega Corporation, Madison, WI, USA).

Three out of 19 participants that submitted mtDNA data for mixed stain M6 used the preferential lysis procedure [7]. All the participants amplified separately both hypervariable regions I and II (HVRI and HVRII). The primers described previously by Wilson et al. [8] were used by a high number of laboratories (65%), while 20% of participants employed the primers described by Vigilant et al. [9]. AmpliTaq Gold (AB, Applied Biosystems, Foster City, CA, USA) was the most frequently DNA polymerase used in the exercise. In addition, we observed a great variety of PCR protocols. Purification protocols of PCR products previous sequencing reaction involved the use of several commercial devices (see Table 1).

2.3. Sequencing

The majority of the laboratories applied dye terminator cycle sequencing; ~75% of the laboratories used BigDye chemistry (AB) for cycle sequencing, whereas 23% used dichloro-rhodamine terminators (AB). Only one participant (Lab 15) used dye primers. All laboratories were consistent with the use of primers used during HVRI and HVRII amplifications in all the samples. Most of the laboratories (97%) used the automated DNA sequencers of Applied Biosystems (ABI 310, ABI 3100, ABI 377). Electrophoresis was performed using both, slab-gels and capillaries, but the preferred analytical method was the capillary electrophoresis (73%).

3. Results and discussion

GEP-ISFG laboratories reporting partial or complete mtDNA results increased from 18% in 1998 to 37% in 2004 (34 out 93). Table 2 summarizes the level of participation for the different samples included in the exercise, as well as the consensus rates obtained per sample, and Table 3 shows the sequences reported by each laboratory obtained for the different samples. The GEP-ISFG working group considers that a sequence result is consensual when there are at least five participant laboratories involved and at least 70% of them report exactly the same result.

3.1. MtDNA sequence data from bloodstains M1–M5

The results are summarized in Table 3. A high rate of coincidence was observed: 97% in M1–M2–M3, 93% in M4, and 94% in M5. Five laboratories reported some level of heteroplasmy at the polycytosine region of HVRII in samples M1, M2, M3 and M5.

Two types of errors were found:

(a) *Edition mistakes*: lab 1 omitted the common insertion 315.1C and reported base shift in M4 and M5 samples. Until now the design of the collaborative exercise, not

Table 1
Amplification and sequencing strategies used by the participants

Lab n°	HVRI/ HVRII primers	Edited	Polymerase	N° cycles blood/hair	Post-PCR purification	Sequencing chemistry	DNA sequencing
1	Other	15983–16410/117–426	Taq polymerase (invitrogen)	31/31	Wizard (Promega)	BigDye term (AB)	ABI 310
2	[8]	16028–16365/73–340	AmpliTaqGold (AB)	32/36	Microspin (Amersham)	Dichlo-rhod (AB)	ABI 3100
3	[9]	16022–16410/56–510	FIREPol (Gentaur)	40/40	Centricon-100 (Millipore)	BigDye term (AB)	ABI 310
4	[9]	16033–16385/30–360	AmpliTaqGold (AB)	30/36	Microcon-100 (Millipore)	Dichlo-rhod (AB)	ABI 310
5	Other	16024–16569/1–580	Taq polymerase (invitrogen)	36/36	Microspin (Amersham)	Dichlo-rhod (AB)	ABI 3100
6	Other	16023–16400/50–410	AmpliTaqGold (AB)	38/38	Microspin (Amersham)	BigDye term (AB)	ABI 310
7	[8]	16024–16325/73–340	AmpliTaqGold (AB)	38/36	Exo-SapIT (USB)	BigDye term (AB)	ABI 310
8	[8]	16024–16365/73–340	AmpliTaqGold (AB)	36/36	Centricon-100 (Millipore)	Dichlo-rhod (AB)	ABI 377
9	[8]	16110–16401	Taq polymerase (invitrogen)	35/35	‘QIAquick (Qiagen)	BigDye term (AB)	ABI 377
10	[8]	16024–16365/73–340	AmpliTaq (AB)	30/36	Microcon-100 (Millipore)	BigDye term (AB)	ABI 310
11	[8]	16024–16310/73–340	AmpliTaqGold (AB)	38/40	QIAquick (Qiagen)	BigDye term (AB)	ABI 3730
12	[8]	16000–16401/58–480	AmpliTaq (AB)	32/32	Centricon-100 (Millipore)	BigDye term (AB)	ABI 310
13	[8]	16024–16365/73–340	AmpliTaqGold (AB)	36/36	Microcon-100 (Millipore)	BigDye term (AB)	ABI 377
14	[8]	15998–16391/49–407	AmpliTaq (AB)	Non-specified	Microcon-100 (Millipore)	BigDye term (AB)	ABI 3100
15	[8]	16024–16365/72–340	Non-specified	35/35	Wizard (Promega)	Dye primers (non-specified)	Visible Gentics
16	[8]	16024–16383/66–319	EcoTaq polymerase (ecogen)	30/30	Microcon-100 (Millipore)	BigDye term (AB)	ABI 3100
17	[8]	16025–16365/73–340	AmpliTaqGold (AB)	30/35	Microcon-100 (Millipore)	Dichlo-rhod (AB)	ABI 310
18	[8]	16024–16365/73–240	AmpliTaqGold (AB)	36/36	Microcon-100 (Millipore)	BigDye term (AB)	ABI 310
19	Other	16070–16400/80–340	Non-specified	39/39	Non-specified	BigDye term (AB)	ABI 310
20	[8]	16024–16365/73–340	Biotools polymerase (biotools)	36/36	UltracleanPCR (MoBio)	BigDye term (AB)	ABI 310
21	[8]	16024–16365/73–340	AmpliTaq (AB)	36/36	Microcon-100 (Millipore)	Dichlo-rhod (AB)	ABI 310
22	[8]	16024–16365/73–340	Biotools polymerase (biotools)	36/36	Bioclean columns (Biotools)	BigDye term (AB)	ABI 310
23	[8]	16033–16391/57–408	AmpliTaqGold (AB)	30/35	QIAquick (Qiagen)	BigDye term (AB)	ABI 310
24	[8]	16024–16365/73–340	AmpliTaqGold (AB)	32/36	Microcon-100 (Millipore)	BigDye term (AB)	ABI 310
25	[8]	16024–16365/72–340	AmpliTaqGold (AB)	36/32	QIAquick (Qiagen)	BigDye term (AB)	ABI 377
26	[8]	15997–16395	Taq polymerase (invitrogen)	Non-specified	Microcon-100 (Millipore)	BigDye term (AB)	ABI 377

Table 1 (*Continued*)

Lab n°	HVRI/ HVRII primers	Edited	Polymerase	N° cycles blood/hair	Post-PCR purification	Sequencing chemistry	DNA sequencing
27	[8]	16075–16362/111–340	Taq polymerase (fermentas)	Non-specified	Exo-SapIT (USB)	BigDye term (AB)	ABI 310
28	[8]	16024–16365/73–340**	AmpliTaqGold (AB)	36/36	Microspin (Amersham)	BigDye term (AB)	ABI 3100
29	[8]	16024–16365/73–340	AmpliTaqGold (AB)	35/35	Microcon-100 (Millipore)	Dichlo-rhod (AB)	ABI 310 y 377
30	Other	16011–16370/55–349	Non-specified	32/32	Wizard (Promega)	BigDye term (AB)	ABI 377
31	[8,9]	16017–16380/49–409	DyNAzyme (finnzymes)	30	QIAquick (Qiagen)	BigDye term (AB)	ABI 310
32	[9]	16023–16400/50–430	Biotools polymerase (Inm Diag)	Non-specified	QIAquick (Qiagen)	BigDye term (AB)	ABI 3100
33	[8]	15978–16419/29–429	AmpliTaq (AB)	Non-specified	Centricon-100 (Millipore)	BigDye term (AB)	ABI 310
34	[8]	16024–16383/73–340	TaqDNA polimerase (Promega)	35/35	Microspin (Amersham)	BigDye term (AB)	ABI 3100

include the submission of raw data therefore is difficult to clarify if the errors happen during edition process or when the laboratories submitting the report (typographical error).

(b) *Nomenclature deficiencies*: there are a number of laboratories that do not use the nomenclature recommended by the ISFG mtDNA guidelines [10,11] to describe point heteroplasmy and length variants (see lab 28 in Table 3). In addition, 45% of laboratories reported the double HVRII C-insertion in the homopolymeric tract simply as ''309.2C'' instead of ''309.1C, 309.2C''.

3.2. mtDNA sequence data from a saliva/semen mixture (M6)

In comparison with the number of participating laboratories for the M1–M5 samples, the analysis of the mixture M6 (saliva from M4 donor/semen from M5 donor) was lower (19 labs). This low participation could be due, in part, to the fact that many laboratories do not consider mtDNA as an appropriate marker for DNA mixtures. In fact, in routine forensic casework mtDNA typing analysis is not frequently employed for DNA typing from body fluid stains. Moreover, many laboratories only participate in the paternity exercise and, normally, just analyse blood samples.

Table 2
Number of participants in the 2003–2004 GEP-ISFG exercise

	No. of participant labs	Results	
		Consensus	Non-consensus
M1	30	29 (97%)	1 (3%)
M2	30	29 (97%)	1 (3%)
M3	30	29 (97%)	1 (3%)
M4	30	28 (93%)	2 (7%)
M5	31	29 (94%)	2 (6%)
M6	19	13 (68%)	6 (32%)
M7	28	23 (82%)	5 (18%)

All laboratories carried out autosomal STRs typing in this sample. Some of them extracted the DNA by complete digestion and others by preferential lysis. In both cases, the consensus result was an unequal DNA mixture (3:1) compatible with the M4 and the M5 genetic profiles (data not shown), but the male component was by far the predominant one. This is a logical finding since there are more cells in semen than in saliva fluid. The laboratories performing Y-STR analyses only obtained a single haplotype which matched those obtained from M5.

Therefore, the expected consensus mtDNA sequence of the mixed stain M6, should have been 16,266Y 263G 309.1C 315.1C including length heteroplasmy in HVRII (tracts 303–309). Nevertheless, mtDNA sequencing analysis of the saliva/semen mixture M6 produced an unexpected consensus result (13/19 laboratories ~70%) because only the saliva profile (M4) was detected, either after preferential lysis or complete DNA digestion. Six (30%) laboratories reported the expected mixed mtDNA sequence result. Analyzing the participant's data related to extraction, amplification and sequencing strategies, no methodological differences were observed between these two groups. Therefore, the absence of additional information such as detailed data of extraction procedure, microscopic analysis (if performed) or electropherograms turns difficult to infer the cause why several laboratories detected both components of the mixture. In relation to this question, at the annual GEP-ISFG 2004 meeting the members expressed the utility and the need

Table 3
Results reported by the 34 participating laboratories

	M1	M2	M3	M4	M5	M7
CS	16298C 195C 263G 309.1C 309.2C 315.1C	16298C 195C 263G 309.1C 309.2C 315.1C	16298C 195C 263G 309.1C 309.2C 315.1C	263G 315.1C	16266T 263G 309.1C 315.1C	16266T 263G 309.1C 315.1C
Lab						
1	–	–	–	262G 309.1C	16265T 262G 309C 309.1T 309.2C	16265T 262G 309C 309.1T 309.2C
2	CS	CS	CS	CS	16266T 263G 309.1C 309.2C 315.1C	16266T 263G 309.1C 315.1C
3	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	CS	CS	263G 315.1C
4	CS + 309.3C	CS + 309.3C	CS + 309.3C	CS	16266T 263G 309.1C 309.2C 315.1C	16266T 263G 309.1C 309.2C 315.1C
5	CS	CS	CS	CS	CS	CS
6	CS	CS	CS	CS	CS	CS
7	16298C 195C 263G 309.2C 315.1C	16298 C 195C 263G 309.2C 315.1C	16298 C 195C 263G 309.2C 315.1C	CS	CS	CS
8	CS	CS	CS	CS	CS	CS
9	–	–	–	CS[a,b]	CRS[a,c]	CS[a,c]
10	CS	CS	CS	CS	CS	CS
11	CS	CS	CS	263G 309.1C 315.1C	CS	CS
12	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	CS	CS	CS
13	CS	CS	CS	CS	CS	–
14	CS	CS	CS	CS	CS	CS
15	16298C[a]	CS	CS	–	–	–
16	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	CS	CS	263G 315.1C
17	CS	CS	CS	CS	CS	CS
18	CS	CS	CS	CS	CS	CS
19	CS	CS	CS	CS	CS	–
20	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	CS	CS	CS
21	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	CS	CS	CS
22	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	CS	CS	–
23	CS	CS	CS	CS	CS	CS
24	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	CS	CS	CS
25	CS	CS	CS	CS	CS	CS
26	16298Ca	16298Ca	16298Ca	–	16266T[a]	16266T[a]
27	–	–	–	CS	CS	16266T[a]

Table 3 (*Continued*)

	M1	M2	M3	M4	M5	M7
28	16298C 195C 263G 309CC -CCCC 315C	16298C 195C 263G 309CC-CCCC 315C	16298C 195C 263G 309CC-CCCC 315C	263G 315C ins	16366T 263G 309C-CC 315C	16189T + C 16223C + T 16266C + T 16278C + T 16294C + T 16309A + G 146T + C 152T + C 19 T + C 263A + G 309C–CC 315 C
29	CS	CS	CS	CS	CS	CS
30	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	CS	CS	CS
31	CS	CS	CS	–	–	–
32	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	CS	CS	263G 309.1C 315.1C
33	–	–	–	CS	CS	CS
34	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	16298C 195C 263G 309.2C 315.1C	–	–	–
n	29/30	29/30	29/30	28/30	29/31	23/28

CS: consensus sequence for the reading frames 16024–16365 in HVRI and 73–340 for HVRII; (–) result not reported.

[a] Only HVRI was analyzed and/or reported.

[b] (16149–16394) edition.

[c] (16149–16402) edition.

to attach electropherograms for confirmation of the results and evaluation, in such cases where discrepancies were observed.

There was only a single difference (at site 16,266) between M4 and M5 samples. With the aim of finding additional polymorphisms that might make it possible to discriminate the M4 and M5 samples, laboratories 5 and 25 analyzed other D-Loop segments outside HVRI and HVRII, namely nucleotide positions 16,159 to 17 and 340 to 576. However, these samples showed the same sequence profile at this fragment (16,519C). Therefore, this information was not useful to distinguish between the two samples. In order to clarify this supposed contradictory result between mitochondrial and nuclear DNA several laboratories proposed alternative hypothesis and carried out additional experiments.

3.2.1. Hypothesis 1: primer binding site mutation in the mtDNA from the semen

Laboratories 5 and 25 performed HVRI amplifications using different primer sets (L15997/H16395; L16159/H16395; L16159/H00017) in order to know whether a mutation in the primer binding sites in the sperm DNA could explain the paradox. Nevertheless, all analysis yielded the same result in M6.

3.2.2. Hypothesis 2: mutation in 16,266 nucleotide position in the mtDNA from semen

Lab 25 reproduced the same scenario of the M6 stain using other saliva and semen donors different from the ones used in the collaborative exercise (100 μL of saliva from donor A plus 50 μL of semen diluted 1:20 from donor B). In agreement with the result obtained with M6 sample, only the saliva haplotype was detected, thus indicating that the problem was not caused by the semen of M5 donor.

3.2.3. Hypothesis 3: lower mtDNA copy number in semen than in saliva

(i) Test 1: PCRs from serial dilutions of semen and saliva DNAs. Lab 25 analyzed semen and saliva fluids separately. DNA was extracted from 10 μl of semen from a donor different from the one in the exercise and from 10 μl of saliva from another donor. The amount of nuclear DNA was quantified by slot blot hybridization (Quantiblot, AB) and the results showed that the nuclear DNA concentration from saliva was lower than the DNA concentration from semen. This could explain the results obtained in M6 by autosomal STR analyses, where the male component was the predominant one.

In order to estimate the amount of mitochondrial DNA in each fluid this laboratory carried out a semi-quantitative PCR consisting of HVRI amplifications using decreasing serial DNA dilutions from the semen and saliva fluids. The results were contrary to those obtained with nuclear DNA. As indicated in Fig. 1, HVRI amplicons were detected up to 0.2 pg/μL nuclear DNA from saliva, while no PCR product was detected in the ten times more concentrated extract from semen (2 pg/μL). This finding clearly suggested that the amount of mitochondrial DNA in saliva was higher than in semen.

(ii) *Test 2*: analysis of mtDNA coding SNPs by SNaPshot technology, a more sensitive method than sequencing.

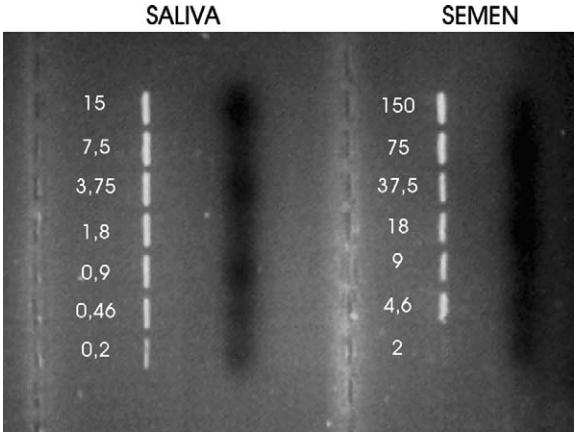

Fig. 1. HVRI amplifications from serial dilutions of saliva and semen DNAs. Numbers indicate the nuclear DNA concentrations in pg/μl.

Lab 5 investigated 15 nucleotide positions in the mitochondrial coding region (3010, 3915, 3992, 4216, 4336, 4529, 4580, 4769, 4793, 6776, 7028, 10,398, 10,400, 10,873 and 14,766) by two PCR multiplexes and SNaPshot methodology in samples M4, M5 and M6 following the protocol of Quintáns et al. (2004). The electropherograms showed a difference at position 3010 between the M4 and M5 samples corresponding to different haplogroup affiliations of samples M4 (haplogroup H1) and M5 (haplogroup H*) (see Fig. 2).

The analysis of M6 showed the expected results, namely, a G/A nucleotide mixture at site 3010, being the adenine the predominant variant (which corresponds with to the saliva component). The same lab also performed the analysis of the 3010 position in a singleplex PCR reaction in order to confirm this result. Therefore, this methodology proved to be very useful because it is highly sensitive in comparison with sequencing. This test allowed confirming the predominant mtDNA component of the saliva in the mixture sample.

3.3. Hair (M7)

Twenty-eight laboratories reported complete or partial HVRI/II mtDNA results for the hair shaft sample. Twenty-four out of 28 laboratories submitted results for both hypervariable regions. The analysis of hair shaft yielded a high rate of success since the majority of them (23 out of 28 laboratories) reported the consensus sequence 16266T 263G 309.1C 315.1C (see Table 3). Lab 1 reported the same erroneous base assignment (due to a base shift) as for the bloodstains (see above), while three laboratories (3, 16 and 32) reported a sequence lacking the 16,266 polymorphism (note that a mutation reversion at the site [e.g. heteroplasmic in the donor sample M7] is unlikely since this site is not considered to be a mutational hotspot [12]); inspection of the original sequence electropherograms from the corresponding laboratories will help to corroborate this potential error. Finally, lab 28 reported a sequence that was clearly contaminated with a sample belonging to haplogroup L2a1 [13,14].

The majority of the participating laboratories agreed that the donor of the sample M5 could not be excluded as the contributor of the hair (M7), rejecting M4 as a potential contributor. In agreement with their artifactual results, two laboratories (3 and 16), concluded that M4 was the donor of the sample M7.

Strikingly, only four laboratories considered the possibility of a mutational event in the hair sample at position 16,266. Several studies [4,15,16] have demonstrated that sequence heteroplasmy is a phenomenon commonly observed in hair shafts. Nevertheless, when reporting their conclusions, only four laboratories mentioned the issue. Following the recommendations of the ISFG DNA Commission, a single difference between an unknown sample and a reference sample cannot exclude a common maternal origin. Although 16,266 is not considered a mutation hotspot (see above; [12]), M4 could not be excluded as the source of M7. Besides, the recommendation of analyzing several fragments of a single hair has been also considered by the DNA Commission in order to evaluate possible heteroplasmies. In the present exercise only two laboratories reported to have analyzed more than one fragment independently. Lab 5 rejected the hypothesis of common maternal origin on the basis of an additional nucleotide difference at position 3010 between samples M4 and M5–M7 (see Table 4).

Table 4
Results obtained by lab 5 in mtDNA coding region SNPs

Sample	SNP profile
M1	7028T 14766C 4529A 4580G 10400C 4216T 10873T 3010G 3915G 3992C 4336T 4769G 4793A 6776T
M2	7028T 14766C 4529A 4580G 10400C 4216T 10873T 3010G 3915G 3992C 4336T 4769G 4793A 6776T
M3	7028T 14766C 4529A 4580G 10400C 4216T 10873T 3010G 3915G 3992C 4336T 4769G 4793A 6776T
M4	7028C 14766C 4529A 4580G 10400C 4216T 10873T **3010A** 3915G 3992C 4336T 4769G 4793A 6776T
M5	7028C 14766C 4529A 4580G 10400C 4216T 10873T **3010G** 3915G 3992C 4336T 4769G 4793A 6776T
M6	7028C 14766C 4529A 4580G 10400C 4216T 10873T **3010A** > **G** 3915G 3992C 4336T 4769G 4793A 6776T
M7	7028C 14766C 4529A 4580G 10400C 4216T 10873T **3010G** 3915G 3992C 4336T 4769G 4793A 6776T

Note that position 3010 is the only difference between M4 and M5.

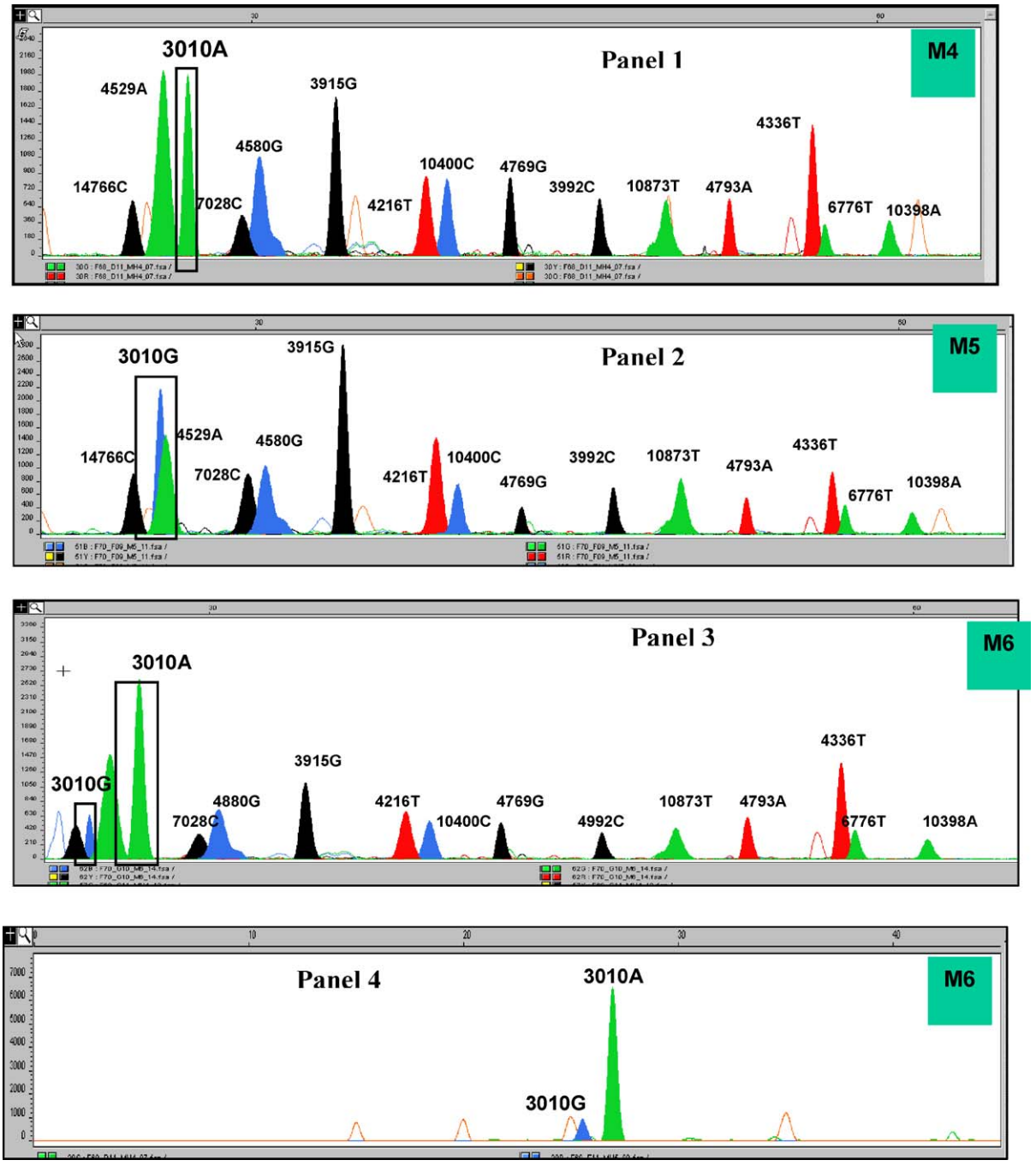

Fig. 2. Coding mtSNPs analysis of samples M4–M6 samples by SNaPshot minisequencing reaction. Panels 1–3: multiplex analysis of M4–M6 samples, respectively. Panel 4: single-plex analysis of M6 sample.

4. Final remarks

The results of the GEP 2003–2004 mtDNA exercise showed again an improvement in quality with respect to previous editions. The number of participating laboratories has slightly increased with respect to the last exercise [6], but this circumstance has not involved a decrease in the quality of the results. The main causes of error were documentation mistakes and nomenclature deficiencies. Contamination or sample mix-ups were also the cause of a few errors. Parti-

cularly important were the good results obtained for the hair shafts (M7) since most of the laboratories (only one exception) reported the consensus results. It is worth mentioning that a scrutiny of the results using a phylogenetic approach [6,12,16–19] could easily recognize most of the causes of errors.

A remarkable aspect constitutes the fact that a significant proportion of laboratories do not follow the international nomenclature recommended to describe the presence of insertions in the homopolymeric track of HVRII. This mistake was already acknowledged during the previous edition of the exercise and once again we emphasize the need of using a common nomenclature and encourage the laboratories to report the data following the recommended nomenclature of the ISFG.

Once again, the quality exercise here reported indicates that additional efforts are needed to improve mtDNA evidence interpretation. For instance, only few laboratories did take into account the fact that there was a single difference between sample M4 and M5–M7 sequences (16,266 nucleotide site). The mtDNA evidence interpretation is not always easy and depends on several factors such as tissue type (e.g. blood–blood or blood–hair comparisons), number of specimens involved (e.g. single hair–several hair analysis), the sequences themselves (e.g. full match, a single or few differences in hotspot positions, a single difference in a mutational stable position, sequence or length heteroplasmy, etc.) and the biological relationship between the samples under analysis (relatives or two samples from a single individual). Although the nucleotide site 16,266 is not a recognized hotspot, it would be desirable to observe additional nucleotide differences in order to safely conclude that samples M4 and M7 come from a different biological source. Here, the useful role of coding region variants seems to be of primordial importance.

The result obtained with the mixture M6 sample is also noteworthy. Although the expected result was a mixture sequences belonging to M4 and M5, the consensus result yield exclusively the M4 profile for both HVRI and HVRII. The mtDNA from sperm was not detected. Different experiments carried out by several laboratories contributed to partially solve the puzzle: the existence of different relative amounts of nuclear and mtDNAs in saliva and semen. Depending on tissue type the number of mtDNA molecules in somatic cells is variable, ranging from about 250 to about 1700 mtDNA copies [20,21]. On the other hand, the number of mtDNA copies in each spermatozoon is in the range of 50–100 [22,23]. The exclusive use of mtDNA for mixture samples analysis could in this case lead to a false exclusion.

The results obtained when analyzing the female and male fractions of the differential extractions are also of interest. Theoretically, when a preferential lysis is performed, the mitochondria located in the mid-pieces of spermatozoon and those situated in the female epithelial cells remain in the first (or female) fraction, whereas the heads of the sperm (without mitochondria) are carried to the second fraction.

Therefore, in this particular case, after preferential lysis, the expected result should be a mixture of M4 and M5 profiles in the first fraction and the lack of any mitochondrial amplicon in the second fraction. Many laboratories detected the mitochondrial female component in the male fraction, which would indicate that the lysis was not carried out correctly. As previously described [24], three possible scenarios could be expected in the second fraction: no mtDNA sequence detected, the presence of (only) the female profile, or the possible amplification of an insert of mtDNA present in the nuclear genome.

The analysis of the results obtained for the M6 sample turned out to be extremely interesting. A GEP-ISFG collaborative study was planned and is now in progress aimed to the complex interpretation of mtDNA when analyzing different body fluid mixtures.

Finally and concerning to mixture interpretation, it seems evident that the inferior participation in the analysis of the M6 sample shows the majority idea that mtDNA in not the marker of choice in the resolution of profiles in mixed samples (saliva:semen). STRs are most clearly the method of choice. Nevertheless, in a number of cases, it could be interesting to know the mitochondrial DNA (mtDNA) haplotypes that contributed to the mixture (e.g. degraded reference samples, exclusion of a maternal relationship between the victim and suspect in rape cases...) and in this type of cases we consider that the interpretation should be making with extreme care.

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