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Subtyping mtDNA haplogroup H by SNaPshot minisequencing and its application in forensic individual identification

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Abstract Sequence variation of the hypervariable segments (HVS) I/II of mitochondrial DNA (mtDNA) and the haplogroup affiliation were determined in a sample of 271 Italian subjects. This analysis showed that 42% of the individuals could be ascribed to H, the most frequent haplogroup in European Caucasian populations. This fraction was then screened for specific single nucleotide polymorphisms located in the coding region to identify H subclades H1–H15. We set up two multiplex polymerase chain reactions and specific SNaPshot assays to investigate the frequency distribution of these subgroups in our population sample and to examine their usefulness in discriminating among commonly shared HVS I/II sequences. This allowed the assignment of a large portion of the mtDNAs (~70%) to specific subhaplogroups, with H1 and H5 being the most represented. About two-thirds of the individuals sharing common HVS I/II sequences were subdivided and ascribed to specific H subhaplogroups with a significant reduction of the frequencies of the most common mtDNA haplotypes. Haplogroup H subtyping could thus be extremely useful in forensic identification when many samples have to be analysed and compared, avoiding excessive time-consuming and labor-intensive sequencing analysis.

Keywords MtDNA · SNPs · Haplogroups · Primer extension methods · HVS

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Introduction

Mitochondrial DNA (mtDNA) has been widely analysed in human evolution/population genetic studies [1–3] and in molecular medicine to correlate pathological mutations to a variety of human diseases [4].

In the forensic field, mtDNA typing is particularly important in human identification where the amount of highly degraded genomic DNA recovered from skeletal remains and hair shafts is extremely reduced or totally absent. The presence of multiple copies (several hundreds or thousands) of mtDNA in individual cells can represent the last chance to get a result where typing of conventional short tandem repeats has failed or given unreliable results. However, the non-mendelian pattern of inheritance (maternal transmission), the absence of recombination and the high mutation rate are limiting factors in the forensic application of mtDNA typing, as genetic analysis can only identify maternally related individuals sharing a common sequence.

The analysis of the genetic variation in the hypervariable segments (HVS) I/II of the control region is the most common approach to mtDNA characterisation in the forensic field. Many individuals show rare mtDNA haplotypes but

the most common HVS I/II sequence is shared by about 7% of the Caucasian population [5].

It could be useful to characterise other mtDNA polymorphisms, such as single nucleotide polymorphisms (SNPs) found in the coding region to increase the discriminating power of the common sequences for forensic purposes. These binary markers define haplogroups that often seem to have a distinctive geographical distribution, probably because of past human migrations [6]. Recent publications [7–9] investigated the distribution of the major European Caucasian haplogroups in different population samples by multiplex SNaPshot minisequencing assays or sequencing the entire mtDNA genome. The results are in agreement with previous population genetic studies [6, 10] showing that haplogroup H is the most common haplogroup in all European populations (40–50%) with a wide geographic distribution. Whilst at low resolution haplogroup H analysis is barely informative, subclades of haplogroup H seem to have more restricted geographic distributions.

Specific mutations identifying many H subhaplogroups have been characterised [8, 10, 11], but the most detailed molecular dissection of this haplogroup was performed by Achilli et al. [12] with the definition of 15 subclades (H1–H15). When the frequency distributions for only half of the H subclades (H1–H7) were compared in Europe, a different geographic frequency pattern was seen [11] with individual branches relatively frequent in some populations and rare in others.

Since no frequency distributions of subclades H8–H15 have been described in European population samples and only a few Italians were characterised for subhaplogroups other than H1 and H3, we report here the distribution pattern at the most in-depth level on haplogroup H samples. This pattern was identified by restriction fragment length polymorphisms (RFLP) analysis (−7025*Alu*I) in the course of a progressing Collaborative Exercise of the Italian working group (GeFI) of the International Society for Forensic Genetics (ISFG) on mtDNA. Two specific SNaPshot assays have been set up to detect H1–H7 and H8–H15 subhaplogroup diagnostic mutations. The forensic usefulness of this approach has been evaluated for the ability to discriminate individuals sharing a common mtDNA haplotype (HVS I/II).

Materials and methods

DNA samples and extraction

DNA samples from 271 unrelated northern-central Italian individuals were analysed. The geographic origin and the number of samples for each participating group was: Turin 50; Pavia 49; Modena 49; Florence 49 and Ancona 74.

DNA was recovered from fresh blood or buccal swabs using different extraction methods: phenol-chloroform, Chelex 100 purification [13] and QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

mtDNA amplification and sequencing

Polymerase Chain Reaction (PCR) amplification was carried out for 30 cycles by adding 5 ng DNA in a 20 μl final volume with 1 U AmpliTaq Gold (AB, Applied Biosystems, Foster City, CA) and 0.25 μM of the following primers: HVS/I: L15997 and H16401; HVS/II: L29 and H408. PCR reactions were purified using QIAquick PCR Purification Kit (Qiagen) and the amplified products were sequenced with the BigDyes Terminator Cycle Sequencing Ready Reaction kit (AB). Unincorporated dye terminators were removed using Performa gel filtration cartridges (Edge BioSystems, Gaithersburg, MD). Sequences were separated by capillary electrophoresis on ABI PRISM 310 (AB) automatic sequencers.

RFLP analysis

The DNA samples were screened by RFLP analysis and assigned to one of the major European Caucasian haplogroups, using a phylogenetic approach. PCR amplification conditions and primer sequences are according to Torroni et al. [14]. RFLP typing was carried out in two laboratories (Pavia and Ancona) during the collaborative exercise on mtDNA.

PCR-multiplex amplification

Subtyping of haplogroup H samples was performed by setting up two multiplex PCR reactions. The first one (multiplex A) included the primers described by Quintans et al. [8] to amplify PCR fragments containing H1–H7 polymorphisms. An improved multiplex A PCR was set up, adding the primer sequences for haplogroup H amplification according to Quintans et al. [8]. The second one (multiplex B), defining subclades H8–H15 [12] and a new polymorphic site for H5, was set up in our lab designing primers with Primers 3 software, and checking them with the program Autodimer (http://www.cstl.nist.gov/biotech/strbase/AutoDimerHomepage/AutoDimerProgramHomepage.htm). SNPs at np 13020 and 13101, very close in the mtDNA sequence, were co-amplified in the same amplicon.

Multiplex B produced 8 amplicons with lengths from 113 to 193 bp, a molecular weight range useful for the characterisation of degraded forensic samples.

The PCR multiplex reaction was carried out in a total volume of 10 μl using 5 μl of Multiplex PCR Master Mix (Qiagen), 1 ng of DNA template and 1 μl of 10X premixed primers. Primer sequences and concentrations in the reaction mix are shown in Table 1.

Multiplex PCR thermal cycling conditions were: 95°C pre-incubation step for 15 min, then 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 90 s and extension at 72°C for 60 s, with a 30 min final extension at 60°C. The size of the PCR products was checked on 8% polyacrylamide gels by silver staining.

Table 1 Primer sequences and concentrations for multiplex PCR amplification of SNPs from H8 to H15 (multiplex B)

SNPs	Amplicon size (bp)	PCR primer sequences (5 '-3')	FC (μM)
456F	125	CACCAGCCTAACCAGATTTCA	0.10
456R		CGGGGGTTGTATTGATGAGA	0.10
3936F[a]	158	TAGCAGAGACCAACCGAACC	0.20
3936R[a]		GAAGATTGTAGTGGTGAGGGTGT	0.20
4745F[a]	118	CCGGACAATGAACCATAACC	0.10
4745R[a]		TGGGTAACCTCTGGGACTCA	0.10
6253F	113	TTTCCCCGCATAAACAACAT	0.15
6253R		GGAGGGTAGACTGTTCAACCTG	0.15
8448F	170	ATGGCCCACCATAATTACCC	0.15
8448R		GCAATGAATGAAGCGAACAG	0.15
11377F	193	CTTCCCCTACTCATCGCACT	0.10
11377R		CGACATGGGCTTTAGGGAGT	0.10
13020–13101F[b]	182	AAACGCTAATCCAAGCCTCA	0.10
13020–13101R[b]		GGTGGAAGCGGATGAGTAAG	0.10
14470F	154	CCCCATGCCTCAGGATAC	0.10
14470R		TGATTGTTAGCGGTGTGGTC	0.20

FC Final concentration of the primers in the reaction mix
[a]The primers we used to amplify the SNPs at np 3936 and 4745 are the same selected by Quintans et al. [8] to amplify H4 and H2, respectively
[b]Two SNPs co-amplified in the same amplicon

SNaPshot minisequencing reaction

After PCR amplification, 1 μl ExoSAP-IT (USB, Cleveland, OH) was added to 2.5 μl of the PCR products, in order to remove primers and unincorporated deoxynucleotides, was incubated for 15 min at 37°C followed by 15 min at 80°C for enzyme inactivation.

Multiplex primer extension reactions were carried out in a total volume of 7 μl, containing 2 μl of SNaPshot Ready Reaction mix, 1 μl of purified PCR product and 0.7 μl of primers extension mix 10X. Primer sequences and final concentrations for H1–H7 are exactly the same as described by Quintans et al. [8], whilst those for H8–H15 and the new polymorphic site H5 are shown in Table 2. The extension primer to probe haplogroup H polymorphic site is the same as designed by Quintans et al. [8]. Thermal cycling conditions for single base extension reactions were performed according to the SNaPshot multiplex kit user's manual (AB): 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s and extension at 60°C for 30 s.

The extension primers were designed using Primer3 software and HPLC purified to remove incomplete synthesis products (Invitrogen, Carlsbad, CA). Resulting minisequencing product sizes ranged from 23 to 56 bp.

After the SNaPshot reaction, the excess fluorescence-labelled dideoxynucleotides (ddNTPs) were inactivated by adding 1 U of shrimp alkaline phosphatase (SAP) (USB) to 3 μl of the minisequencing reaction. Incubation was performed at 37°C for 60 min followed by 15 min at 80°C for enzyme inactivation.

The minisequencing SAP treated products (1 μl) were mixed with 13 μl of Hi-Di formamide and 0.5 μl of LIZ-120 internal size standard (AB). The samples were separated by capillary electrophoresis on an ABI PRISM 310 Genetic Analyser (AB) after denaturation. Data were analysed using the GeneScan ver. 3.1 software (AB).

Results and discussion

Sequence variation of the mtDNA hypervariable regions HVS I/II was investigated in 271 unrelated individuals from north-central Italy, in the course of a collaborative project on mtDNA. The HVS I/II sequences went through a

Table 2 Sequences of the extension primers used to target haplogroups H8–H15. The nonspecific primer tail of poly(GACT) is underlined

SNP	Sequence variation	Tail and extension primer sequence	Size (bp)	Chain	Added ddNTPs	FC (μM)
456[a]	C-T	ATTAGTAGTATGGGAGTGGGAGGG	24	H	G-A	0.08
3936	C-T	$\underline{(GACT)_2}$AGGGGAGTCCGAACTAGTCTCAGG	32	L	C-T	0.30
4745	A-G	CAATGAACCATAACCAATACTACCAATCA	29	L	A-G	0.08
6253	T-C	$\underline{ACT(GACT)_3}$TCCTACTCCTGCTCGCATCTGCTA	39	L	T-C	0.25
8448	T-C	$\underline{CT(GACT)_3}$AGGTAGGTGGTAGTTTGTGTTTAAT	39	H	A-G	0.08
11377	G-A	$(GACT)_6$TCATAAGTGGAGTCCGTAAAGAGGTAT	51	H	C-T	0.25
13020	T-C	GCAGGCAAATCAGCCCAATTAGG	23	L	T-C	0.08
13101	A-C	$\underline{ACT(GACT)_3}$CCTACTCCACTCAAGCACTATAGTTGTAGC	45	L	A-C	0.08
14470	T-A	$\underline{T(GACT)_6}$GATACTCCTCAATAGCCATCGCTGTAGTATA	56	L	T-A	0.25

FC Final concentration in the minisequencing reaction
[a]Primer targeting the new H5 SNP

multi-step review process to avoid interpretation errors and/or artificial recombination artefacts of the kind described by Parson et al. [15] and Bandelt et al. [16]. A phylogenetic analysis according to Bandelt et al. [17] was performed on the Italian samples, by RFLP typing of specific mutations identifying the major European Caucasian mtDNA haplogroups.

The results showed that about half of the samples (42%) was assigned to haplogroup H by the absence of the *Alu*I restriction site at position 7025 (−7025*Alu*I), in agreement with the distribution found in other European population samples [6, 10, 18]. Out of 115 haplogroup H samples, 25 shared the same HVS I/II sequence (CRS, 263G, 315.1C, not considering the length variation at position 309), which is the most frequent sequence even in the European populations.

We analysed a number of SNPs located in the mtDNA coding region represented in Fig. 1, defining the subclades H1–H15 at the deepest known level, in order to investigate the frequency distribution of the H subhaplogroups in our Italian population sample and to discriminate between shared mitotypes. The methodological approach we used to detect mtDNA mutations was a primer extension assay with fluorescence labelled ddNTPs followed by capillary electrophoresis of the extended products. This minisequencing reaction (SNaPshot) is widely used in the forensic practise to type SNPs selected for individual identification [19] as it shows high accuracy and robustness for degraded casework samples.

The haplogroup H samples were then analysed by setting up two multiplex SNaPshot assays. The first one was the minisequencing reaction defined by Quintans et al. [8], allowing the detection of subhaplogroups H1–H7 whilst the second multiplex, optimised and validated in our lab, identified diagnostic mutations defining subhaplogroups H8–H15. According to the hierarchical principle described by Richards et al. [20], we decided to add to the second multiplex a new set of primers defining the presence of the *456T* site, a specific more ancestral variant identifying subhaplogroup H5, assigning the samples previously characterised by the *4336C* mutation to the subtype H5a (see Fig. 1). Following the same hierarchical approach, the *3915A* nucleotide site identifies the sub-branch H6a whilst haplogroup H6 is now defined by the presence of the mutations *239C* and *16362C*, in the hypervariable regions [10].

This analysis provided an in-depth definition of haplogroup H subclades distribution in our Italian population sample as shown in Table 3, where mutations diagnostic for each H subclade are indicated. A high portion of the samples (~70%) could be assigned to a specific subhaplogroup, with the second multiplex showing a lower discriminating power than the first (H8–H15 samples: 12 out of 82, but with six samples now assigned to H5). Several additional minor subbranches have been defined by Loogvali et al. [10] and Achilli et al. [11]. Some of them can be easily identified by peculiar HVS I/II motifs (H1a: *16162G, 73G*; H1b: *16189C, 16356C*; H2a1: *16354T*; H2c: *16274A*; H3a: *16239G*; H6b: *16300G*). However, even if some of the Italian mtDNA samples could be clearly assigned to one of these subbranches (see Fig. 1), we decided not to proceed with this further subdivision because of the low frequencies of these samples. We also decided to refer all the samples to the major H subclades previously described. All the samples not belonging to one of the H subhaplogroups were placed in the paraphyletic group H*.

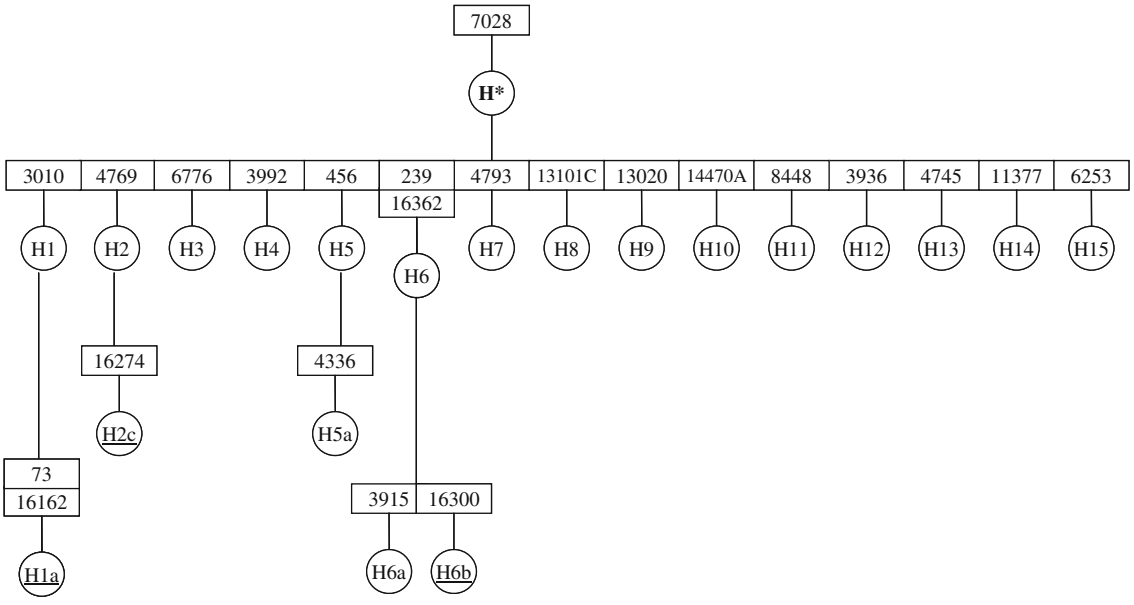

Fig. 1 Phylogenetic tree showing H mtDNA subhaplogroups. Each branch is identified by diagnostic mutation/s (*in box*). Minor subclades [10, 12] found in our population sample (defined by variations in HVS I/II) are *underlined*. All the mutations are transitions unless a base change indicates a transversion

Table 3 SNPs variation and frequencies of the H subhaplogroups in the Italian population sample. The SNPs analysed in multiplex B are in boldface. Nucleotide differences from the Cambridge Reference Sequence (rCRS) are in boxes (transitions) or underlined (transversions)

rCRS	H*	H1	H2	H3	H4	H5[b]	H5a	H6a[c]	H7	H8	H9	H10	H11	H12	H13	H14	H15
456C	C	C	C	C	C	T	C	C	C	C	C	C	C	C	C	C	C
3010G	G	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
3915G	G	G	G	G	G	G	G	A	G	G	G	G	G	G	G	G	G
3936C	C	C	C	C	C	C	C	C	C	C	C	C	C	T	C	C	C
3992C	C	C	C	C	T	C	C	C	C	C	C	C	C	C	C	C	C
4336T	T	T	T	T	T	T	C	T	T	T	T	T	T	T	T	T	T
4745A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	G	A	A
4769A	G	G	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G
4793A	A	A	A	A	A	A	A	A	G	A	A	A	A	A	A	A	A
6253T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	C
6776T	T	T	T	C	T	T	T	T	T	T	T	T	T	T	T	T	T
8448T	T	T	T	T	T	T	T	T	T	T	T	T	C	T	T	T	T
11377G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	A	G
13020T	T	T	T	T	T	T	T	T	T	T	C	T	T	T	T	T	T
13101A	A	A	A	A	A	A	A	A	A	C	A	A	A	A	A	A	A
14470T	T	T	T	T	T	T	T	T	T	T	T	A	T	T	T	T	T
N individuals	35	35	4	5	3	16	10	3	2	2	2	2	/	/	4	/	2
frequency[a]	0.129	0.129	0.015	0.018	0.011	0.059	0.037	0.011	0.007	0.007	0.007	0.007	/	/	0.015	/	0.007

[a]Frequencies referred to 271 samples
[b]Subclade H5 now includes subclade H5a (see text)
[c]Subclade H6a is now included in the more ancestral subclade H6 (n=5 individuals) defined by HVS I/II sequence variations (see text and Fig. 1)

Contingency table analysis indicates a substantial geographical uniformity in the frequency distribution of the H subclades among the five northern-central Italian sub-populations analysed (χ^2, p value=0.655). A significant statistical difference (χ^2, p value<0.001) was found in the distributions by comparing our H1–H7 frequency data to those from population samples from Iberia [8, 11, 21]. When the contribution of the different haplogroups to the χ^2 was examined, it could be appreciated that H*, H1 and H3 mtDNA samples were mainly responsible. This confirms that H1 and H3 are well represented in Iberia but show lower frequencies in other European populations [11, 12].

The power of discrimination of these SNP panels was then evaluated to test the ability of the assay to distinguish among individuals sharing common mtDNA HVS I/II sequences.

Two-thirds of the individuals sharing common HVS I/II sequences were subdivided and assigned to specific H subhaplogroups, with about half of these samples belonging to H1, the most frequent subclade in the European populations. The SNaPshot analysis of the 12 individuals sharing the most common haplotype in our database (CRS, 263G, 309.1C, 315.1C) resulted in the assignment of six samples to subhaplogroup H1. Whilst two individuals, subtyped H3 and H15 respectively, are now unique in our database.

We observed that all but one of our Italian samples characterised by the most common HVS I/II haplotype (CRS, 263G, 315.1C, not considering the length variation at position 309) belonged to haplogroup H. This striking correlation could be extremely useful in terms of a strategic approach to the following H subtyping, when such a poorly informative sequence is obtained from the analysis of a forensic casework sample. In such a case, the next approach could be the subtyping of haplogroup H, as it is very likely that the mtDNA belongs to that clade. In order to be sure about the haplogroup assignment, we modified the first most informative SNaPshot assay (H1–H7) with the addition of the primer targeting the 7028C site, diagnostic of haplogroup H, already utilised by Quintans et al. [8]. We suggest that this "improved" SNaPshot assay should be used when the sequencing analysis of a forensic sample shows the aforementioned common haplotype.

Subtyping haplogroup H can contribute to understanding the history of modern human populations and much other relevant information may come from further molecular dissection of the undetermined H subtype (H*), a rather abundant fraction in our samples (~30%). In addition, this analysis could be extremely useful in forensic identification casework, for example when a large number of suspects have to be compared to an evidence profile, as described by Pfeiffer et al. [22]. This approach allows rapid screening when many samples (for example hair) have to be compared to a single individual, identifying only the matching ones and avoiding time-consuming sequencing analysis of all of them. Moreover, this analysis could be helpful in discriminating among relatives of missing persons included in a specific database, when unidentified human skeletal remains are recovered from single or mass graves [23, 24].

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