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Federica Alessandrini,[1] B.Sc.; Chiara Turchi,[1] B.Sc.; Valerio Onofri,[1] B.Sc.; Loredana Buscemi,[1] M.D.; Mauro Pesaresi,[1] M.D.; and Adriano Tagliabracci,[1] M.D.

Multiplex PCR Development of Y-chromosomal Biallelic Polymorphisms for Forensic Application*

ABSTRACT: Single-nucleotide polymorphisms of Y chromosome (Y-SNPs) are a class of markers of interest in forensic investigations, because many of them show regional specificity, providing useful information about the geographic origin of a subject or evidence under investigation. A first multiplex with 7 SNPs (M35, M89, M9, M170, M172, M45, M173), which occur in the basal branches of the phylogenetic tree and are able to assign a subject to known most frequent European haplogroups, was designed. SNP genotyping was accomplished by hot-start PCR with primers amplifying fragments between 96 and 136 nucleotides, minisequencing, and capillary electrophoresis of extension products. Ninety seven subjects of known geographic provenance were studied, of which 68 from Europe. Of these, 57 had mutations found more frequently in European haplogroups and 11 more frequent in Asian populations. Subjects from non-European countries were also examined and had haplogroups common in their regions of provenance. Experiments with low molecular weight DNA gave positive amplification from 1 ng of DNA for all seven SNPs.

KEYWORDS: forensic science, Y single nucleotide polymorphism, multiplex-polymerase chain reaction, minisequencing, geographical affiliation

Y-binary polymorphisms, also known as unique-event polymorphisms (UEPs) and single nucleotide polymorphisms (SNPs) are a series of biallelic polymorphisms occurring on the non-recombining region of the Y chromosome (NRY). The absence of recombination, uniparental transmission and low mutation rate make Y-SNPs particularly suitable for the identification of stable paternal lineages and the reconstruction of an ancestral state from which to explore the evolution of humans (1).

Using denaturing high performance liquid chromatography (DHPLC), Underhill et al. (2) recently detected more than 200 biallelic variations screening a sample of 21 populations, many of the markers showing regional specificity (3). The combination of these binary polymorphisms yielded a phylogenetic tree based on the principle of maximum parsimony, defining 10 haplogroups, called Groups I–X. The novel nomenclature proposed by the Y Chromosome Consortium (YCC) to unify the several unrelated and nonsystematic prior nomenclatures, now classifies these major clades of the tree into 18 haplogroups, indicated by capital letters from A to R (4). As well as their use in studies of regional population substructure and gene flow, they may have forensic applications, since the presence of mutations provides information about an individual's origin, addressing identification problems (5). In casework, where decomposed bodies or human remains, or victims of mass disasters, including aircraft or boat accidents involving people from various geographical areas, are analyzed, prediction of population origin may be a powerful aid in forensic investigations. Lastly, the use of short amplicons spanning the single-base mutation potentially may allow positive amplification, even with highly degraded DNA, where microsatellites fail.

[1] Istituto di Medicina Legale, Università Politecnica delle Marche, I-60020 Ancona, Italy.

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Due to the hierarchical structure of Y-SNPs, the only suitable approach for identification means that several loci must be examined in two or more steps. In the first step, we need to explore the markers of the basal branches of the phylogenetic tree, in order to assign evidence to a major clade. In the second step, we need to use markers able to identify the haplogroups inside every major clade. Since reactions must be cost- and time-effective, and since in forensic analysis usually only minimal amounts of DNA can be recovered, multiplex PCR with several loci must be developed to analyze as many markers as possible, in order to acquire acceptable results.

The aim of this study was therefore to set up a first multiplex PCR of Y-SNPs able to explore the deepest branches of the phylogenetic tree, employing materials, methods and technologies normally available in forensic laboratories. The multiplex was designed to assay the most frequent haplogroups composing the European major clade in which Italians lie. Therefore, an Italian population sample and subjects from various geographic areas were analyzed to establish the degree of informativeness of this multiplex in assigning subjects to their geographic areas and the haplogroups present in Italians.

Lastly, experiments were also made to verify the effectiveness of this multiplex with degraded DNA.

Materials and Methods

DNA Samples

DNA was extracted from whole blood of healthy men using the QIAamp (Qiagen, Inc, Chatsworth, CA) procedure (6) and quantified by 1% agarose gel electrophoresis. Appropriate informed consent was obtained from all participants. Samples came from 68 Europeans (56 subjects from Central Italy, selected from populations inhabiting the agricultural area of the sub-Apennines, and lacking immigrants; 10 Polish; 1 Greek; 1 Albanian), and also 29 other subjects (17 Asians: 1 Chinese, 10 Taiwanese, 1 Kazak,

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TABLE 1—*SNPs investigated in this study.*

SNP	Polymorphism	Fw/Rv Primer 5′-3′	Amplicon Size
M170	A → C	GTTTTCATATTCTGTGCATTATACAAATTACTAT CATTTTACAGTGAGACACAACCCAC	96 bp
M173	A → C	AAAATTTTCTTACAATTCAAGGGCATT GCTGCAGTTTTCCCAGATCCT	104 bp
M45	G → A	GGTGTGGACTTTACGAACCAACCT CCTGGACCTCAGAAGGAGCTTT	109 bp
M89	C → T	CTGCTCAGCTTCCTGGATTCA CACTTTGGGTCCAGGATCACC	110 bp
M35	G → C	AACTGAGAGGGCATGGTCCC TGAACAACTAATCCATGCAGACTTTC	114 bp
M9	C → G	AGAACTGCAAAGAAACGGCCTA TGCATAATGAAGTAAGCGCTACCT	127 bp
M172	T → G	TTTTATCCCCCAAACCCATTT CATGTTGGTTTGGAACAGTTTATCC	136 bp

1 Kirghiz, 3 Indians, 1 Iraqi; 9 Africans: 3 Kenyans, 2 Magrebinis, 3 Nigerians, 1 Senegalese, and 3 Amerindians from Peru).

SNP Selection

The SNPs M35, M89, M9, M170, M172, M45, M173 loci characterizing European haplogroups which occur in the basal branches of the tree (2) were chosen following a hierarchical strategy based on the known stable phylogeny of the Y chromosome (7). Hierarchical strategy means that selected loci were able to explore the most basal clade F of the tree (M89) and the relevant branches to the tip: K (M9), P (M45), R1 (M173), J2 (M172), I (M170). Moreover the further subclade described in Europeans E3b was explored by M35.

Design and Validation of Amplification Primers

The sequences of the regions spanning the mutations to be examined were drawn from GenBank. Multiplex PCR primer sequences were designed by Primer Express (Applied Biosystems). Table 1 lists the main features of the seven SNPs investigated.

The correct design of primers was checked by singleplex amplification of 10 ng of standard male template, in a final volume of 50 μl, using the following conditions: 1X PCR reaction mix, 800 μM dNTPs, 1.5 mM MgCl2, 1.4 U Taq polymerase, and 0.5 μM each primer. The reaction consisted of denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Singleplex amplification products were checked by PAGE in discontinuous buffer (8) and stained with silver (9).

Sequencing of the amplified fragments from the singleplex of each locus, to check the specific amplification of the region spanning the selected marker, was accomplished in a GeneAmp® PCR System 2400 thermal cycler using the ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) at a final volume of 20 μL, composed of 4 μL BigDye™ Terminator Ready Reaction mix, 15–30 ng PCR product, 0.25 μM primer, and deionized water. The sequencing reaction was performed for 25 cycles of 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. Extension products were purified with the DyeEx Spin Kit (Qiagen) and dried in a vacuum centrifuge. The vacuum-dried sequencing products were then resuspended in 12 μL of Template Suppressor Reagent (Applied Biosystems), heat-denatured and chilled on ice, and analyzed by capillary electrophoresis on an ABI Prism 310. The capillary was filled with run buffer Performance Optimized Polymer POP 6 (Applied Biosystems). Samples were injected in 30 sec at 2 kv and separated for 25 min at 15 kv and 50°C. The resulting data were analyzed with Sequencing Analysis software, and the sequences were aligned and compared with those present in GenBank by means of Sequence navigator software 1.0.1.

Multiplex PCR

One ng of DNA was submitted to amplification in a GeneAmp® PCR System 9700 Applied Biosystems thermal cycler in a final volume of 50 μL, composed of 1X PCR reaction mix, 800 μM dNTPs, 2.5 mM MgCl2, 2.5 U Hot-start Taq polymerase (Qiagen) and primers (0.07 μM for M45; 0.1 μM for each primer M9, M172, M173; 0.25 μM for M35 and M170; 0.3 μM for M89). Two PCR procedures were tested:

- Hot-start PCR: 1 cycle at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min.
- Touch-down PCR: 1 cycle at 95°C for 15 min, followed by 20 cycles of denaturation at 94°C for 15 sec, annealing at decreasing temperature from 60° to 50°C for 30 sec, extension at 72°C for 20 sec, followed by 28 more cycles under the same conditions with annealing temperature stabilized at 50°C and a final extension at 72°C for 10 min.

PCR products were detected by PAGE (8,9).

Multiplex Primer Single-Base Extensions (Minisequencing)

Inactivation of dNTPs and removal of primers were performed by incubation of 5 μL of amplified products with 2 μL of Exo-Sap (USB) reagent for 15 min at 37°C, followed by enzyme inactivation at 80°C for 15 min. Four microliters of the purified products were submitted to a dideoxy single-base extension of unlabeled oligonucleotide primers using the SnaPshot multiplex kit (Applied Biosystems) following the conditions recommended by the manufacturer. Each minisequencing primer was designed so that its 3′ end was one base upstream from the relevant polymorphism, and some of them were 5′-tailed with poly-T and poly-GACT to produce molecules with sizes in the range from 21 to 50 bases (Table 2). Extension was performed for 25 cycles at 96°C for 10 sec, 50°C for 5 sec, and 60°C for 30 sec in a GeneAmp® PCR System 9700 Applied Biosystems thermal cycler.

TABLE 2—*Extension primers used in this study.*

SNP	Primer Sequence 5′-3′	Length
M173	CAATTCAAGGGCATTTAGAAC	21 nt
M89	(TTTT)CAACTCAGGCAAAGTGAGAGAT	26 nt(rev)
M172	AAGAAAATAATAATTGAAGACCTTTTAAGT	30 nt(rev)
M170	AAATTACTATTTTATTTACTTAAAAATCATTGTTC	35 nt
M35	$(GACT)_5$CGGAGTCTCTGCCTGTGTC	39 nt(rev)
M9	$(GACT)_6$ACGGCCTAAGATGGTTGAAT	44 nt
M45	$(GACT)_6$AAATTGGCAGTGAAAAATTATAGATA	50 nt

Capillary Electrophoresis

A further purification step with spin columns from the DyeEx Spin Kit (Qiagen) was performed to remove unincorporated ddNTPs. The purified product was mixed with 0.3 μL of internal size standard Liz120 (Applied Biosystems), 9 μL of Template Suppressor Reagent (Applied Biosystems), heated for 5 min at 95°C, quenched in an ice bath and injected in an automated ABI 310 5-color sequencer (Applied Biosystems). Electrophoresis was performed for 15 min with an injection time of 5 sec, voltage 15 kv, and temperature 60°C. Fluorescently labeled fragments were sized by the local Southern method implemented in Genescan Analysis software 3.7 (Applied Biosystems).

Experiments on Low Molecular Weight DNA

One mL DNA extracted from whole blood at a concentration of 20 ng/μL was fragmented in a sonicator device (Labsonic, from B Braun) using 4 mm diameter tips, at 50 watt and 0.5-second cycles. Sonicated DNA was submitted to PAGE, hot-start amplification and minisequencing as described in the previous sections. One ng of fragmented DNA was also amplified in a volume of 50 μL with the AmpFlSTR Identifiler PCR Amplification kit (Applied Biosystems), containing 15 autosomal microsatellites (D8S1179, D21S11, D7S820, CSF1P0, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, FGA) and an Amelogenin marker, covering a range of 100 (Amelogenin) to 350 (D2S1338) nucleotides approximately. The amplified products were analyzed on an ABI 310 capillary electrophoresis unit using 1 μL of the PCR product, following the manufacturer's recommendations (ABI Prism 310 Genetic Analyzer Genescan Chemistry Guide).

Results and Discussion

Technologies based on DNA primer extension, such as MALDI-TOF mass spectrometry (10–12) or hybridization, like DHPLC (13) and high-density DNA arrays (14), are available for SNP genotyping. Such technologies are expensive and most suitable for large-scale analysis, require large amounts of DNA, or are not very sensitive and specific because they are designed for genetic screening analysis. Thus, they are not suitable for forensic purposes, in which scientists must always deal with single cases in which minimal amounts of DNA are available.

For SNP genotyping, we used minisequencing, a single-base extension of an unlabeled oligonucleotide primer, which provides the basis for a simple and efficient technique requiring instruments normally available in any forensic laboratory. PAGE of the amplified products from singleplex and multiplex procedures showed amplicons with the expected size (Fig. 1), and sequencing of each singleplex amplification product revealed the expected sequence for all loci (data not shown). Two multiplex amplification procedures were employed in our study, Hot-start PCR and Touch-down PCR in an attempt to achieve positive results with minimal amounts of DNA template. Hot-start PCR yielded more products than Touch-down PCR, but also a related and considerable presence of non-specific amplification products (Fig. 1). Nevertheless, these undesirable artifacts did not interfere with the successive steps of extension, and were not visible in the electropherograms: this means that Hot-start PCR works better because all the loci in the multiplex, starting from only 1 nanogram of DNA in our experimental conditions, could be amplified.

The hierarchical strategy of SNP genotyping suggested by Paracchini et al. (7), based on the known stable phylogeny of the Y chromosome, was chosen in selecting loci for this study. The Y-SNP multiplex, the subject of this paper, was designed to contain markers capable of assigning a subject under investigation to

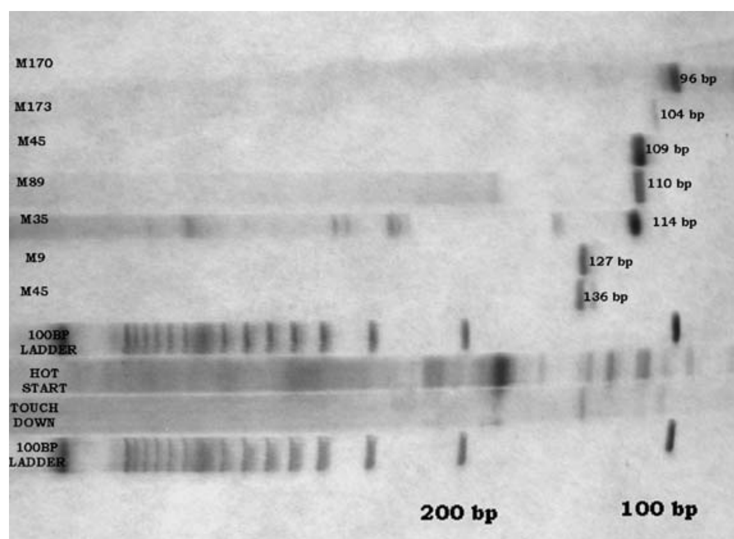


FIG. 1—*Amplified fragments from singleplex and multiplex reactions analyzed by PAGE and stained with silver nitrate. From top to bottom: M170 (96 bp), M173 (104 bp), M45 (109 bp), M89 (110 bp), M35 (114 bp), M9 (127 bp), M172 (136 bp), 100 bp weight marker BRL, multiplex Hot-start PCR, multiplex Touch-down PCR, 100 bp weight marker BRL.*

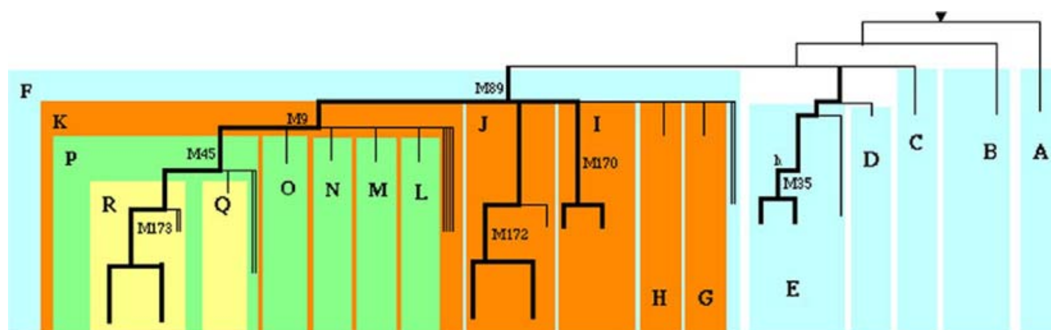


FIG. 2—*Phylogeographic tree (YCC 2002, modified), showing loci investigated in this study: M9, M35, M45, M89, M170, M172 and M173. The different shaded areas represent the major NRY clades.*

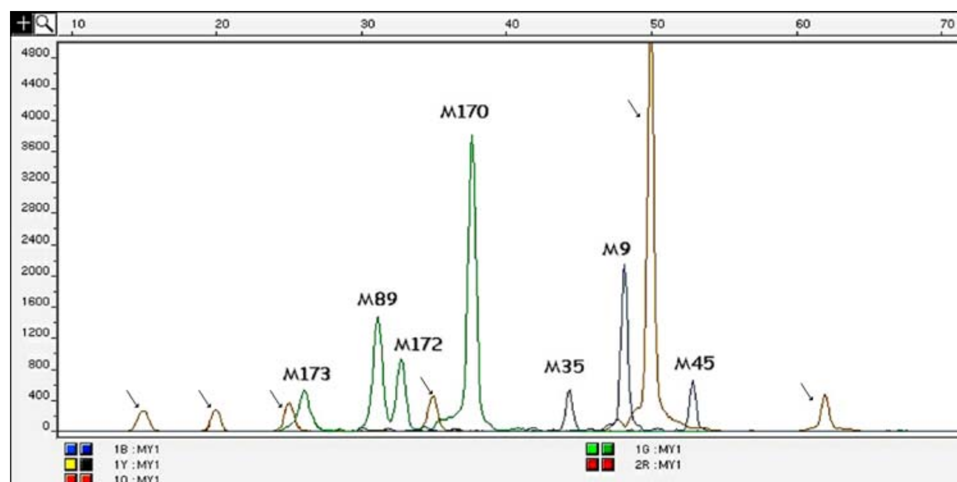


FIG. 3—*Electropherogram showing extension products of seven loci selected in our study. Amplified fragments were analyzed on a 310 CE unit. Arrows indicate the internal size standard peaks (GeneScan 120 LIZ).*

haplogroups found in European populations. According to the new terminology (4), European populations mainly carry haplogroups R1, E3b, J2 and I, with differing frequencies between north and south (2). Although most highly represented in Europe, these haplogroups are not exclusive to Europeans. Group R1 is also found in greater proportions in the Middle East, Central Asia and Northern India-Pakistan and, as a consequence of gene flow during colonial times, in Australia and the Americas; group E3b is also distributed in northeast Africa; group J2 also characterizes central Asia and southern India (2).

The loci selected to investigate European haplogroups were M35, M89, M9, M170, M172, M45, M173, which occur in the basal branches of the tree; the combined presence/absence of markers also assigns haplotypes to other non-European haplogroups (Fig. 2).

All seven extension products were present in the electropherograms, well spaced from each other and with peak heights exceeding 150 rfu for each allele, so that interpretation was easy and reliable (Fig. 3).

The Y-SNP multiplex was tested in a population sample of Europeans and in a small number of subjects from various non-European countries (Table 3). Of the 68 Europeans tested, 57 were assigned to main European haplogroups R1, E3b, J2 and I, because they showed, respectively, the derived state at loci M9, M45, M89 and M173 (haplogroup R1), locus M35 (haplogroup E3b), loci M89 and M172 (haplogroup J2) and loci M89 and M170 (haplogroup I). Eleven Europeans, all from Italy, showed haplogroups less frequent in Europeans, since seven carried out the mutation at locus M89, defining F*(xI,J2,K), and four at loci M9 and M89, defining K*(xP), haplogroups mainly characterizing Asian populations (2,15,16). This genetic admixture was probably caused by recent back-flow to Europe from Asia after the mutation had occurred. The discrepancy with the European representation of haplogroups shown by Underhill et al. (2) may be due either to the small number of subjects sampled by those authors, or to their sampling strategy, not reflecting the actual haplogroups present in all European geographical areas. Subjects from geographical areas outside Europe showed haplogroups common in the region of provenance: haplogroups K*(xP), P*(xR1), R1 and A–E (xE3b) for Asians, A–E (xE3b) and E3b for Africans, and P*(xR1) for Amerindians.

Previous attempts at inferring the regional assignment of an individual from autosomal STR (17–19) have demonstrated that the reliability of conclusion depends on the number of independent markers. Pritchard et al. (20) were able to assign individuals to their population of origin using only a few loci, but other authors have shown that the statistical calculations necessary for assignment are complex and that many variables must be taken into account: e.g., drift, number of loci, allele frequency, and mixed race (21,22). In any case, the final result is only a statistical evaluation based on the allele frequencies of STRs investigated by applying mathematical models. Better results are now achieved with the STR profiles of the Y chromosome, showing some typical but not population-specific haplotypes (exclusive for a certain population) (23,24), and

TABLE 3—*Results of Y-SNP typing of 97 subjects of known geographic provenance.*

Geographic Area of Provenance	M35	M89	M170	M172	M9	M45	M173	Haplogroup*	N
Europe	−	+	−	−	+	+	+	R1	25
	+	−	−	−	−	−	−	E3b	15
	−	+	−	+	−	−	−	J2	14
	−	+	+	−	−	−	−	I	3
	−	+	−	−	−	−	−	F*(xI,J2,K)	7
	−	+	−	−	+	−	−	K*(xP)	4
Asia	−	+	−	−	+	−	−	K*(xP)	10
	−	+	−	−	+	+	−	P*(xR1)	5
	−	+	−	−	+	+	+	R1	1
	−	−	−	−	−	−	−	A–E(xE3b)	1
Africa	−	−	−	−	−	−	−	A–E(xE3b)	6
	+	−	−	−	−	−	−	E3b	3
South America	−	+	−	−	+	+	−	P*(xR1)	3

Plus (+) = derived state, minus (−) = ancestral state.
* The classification of Y Chromosome Consortium (YCC) was followed:

−capital letter (from A to R) indicates a major clade;
−numerals and lower-case letters indicate subclades within each major clade.
−* symbol indicates a paragroup, a lineage not defined by presence of a derived marker, i.e., a chromosome belonging to a clade but not to its subclades.
−x symbol indicates “exclusion.”

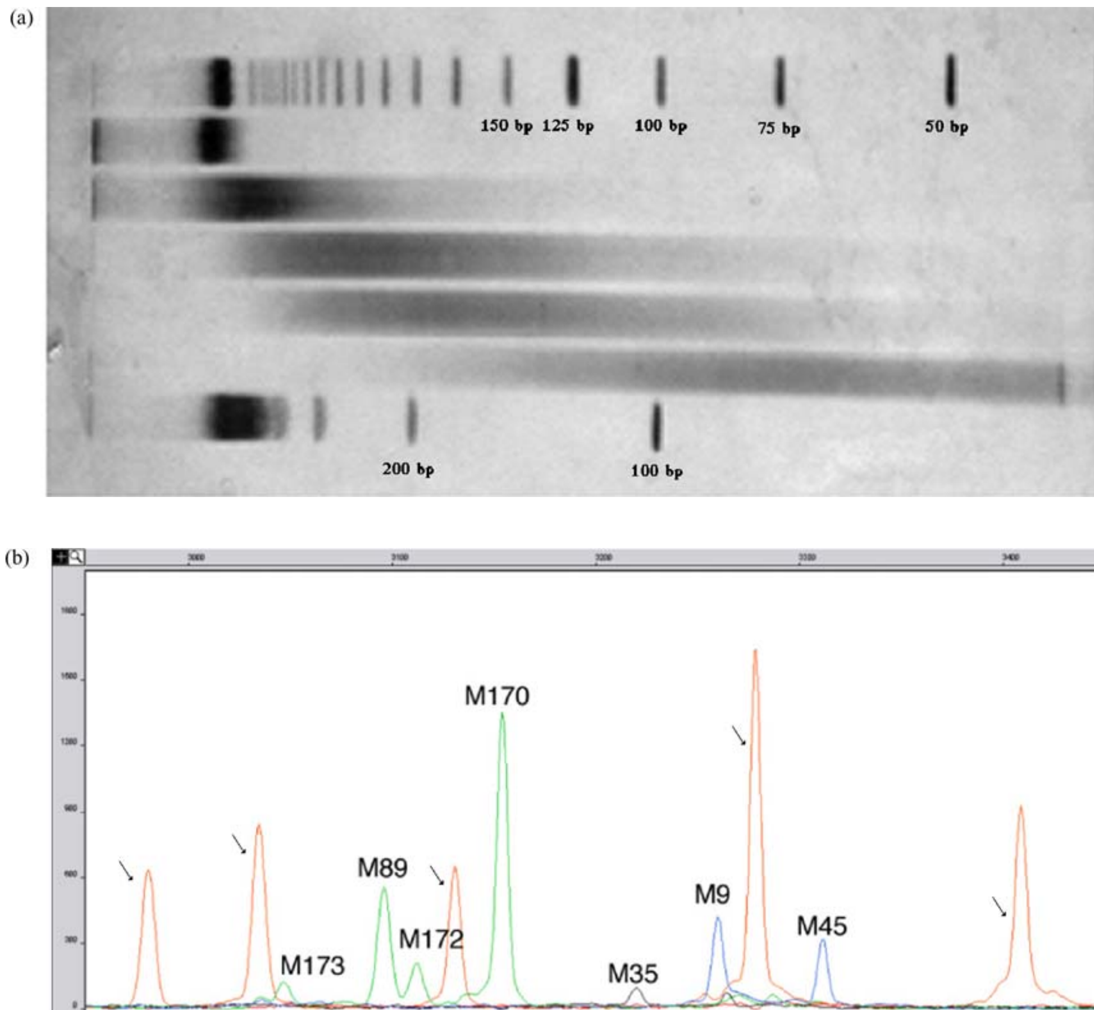
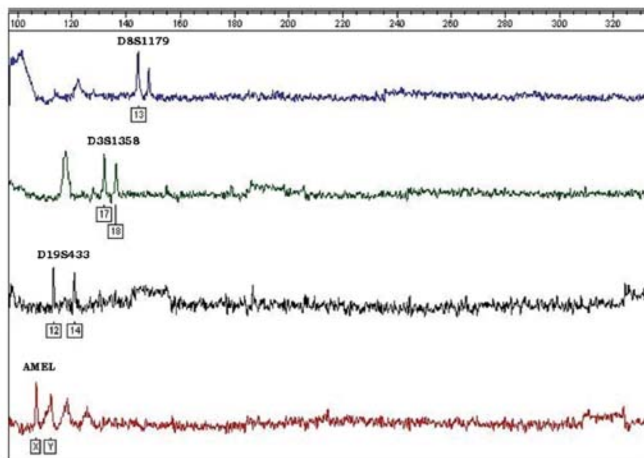


FIG. 4—*Y-SNP-multiplex and microsatellite amplification from low molecular weight DNA fragmented by sonication.*
a) PAGE and silver staining of sonicated DNA. From top to bottom: 25 bp ladder (Invitrogen), high molecular weight DNA, DNA sonicated at 5, 50, 100 and 200 min, 100 bp ladder (New England Biolabs).
b) Electropherogram of Y-SNP multiplex obtained by amplification of 1 ng DNA sonicated for 200 min. and run on a 310 CE unit. All 7 extension products are present together. GeneScan 120 LIZ size standard (arrows). Typing results: K(xP).*
c) Microsatellite typing with AmpFlSTR Identifiler PCR Amplification Kit (AB) of 1 ng high molecular weight DNA (left) and 1 ng of fragmented DNA by sonication for 200 min (right) from same sample.

(c)



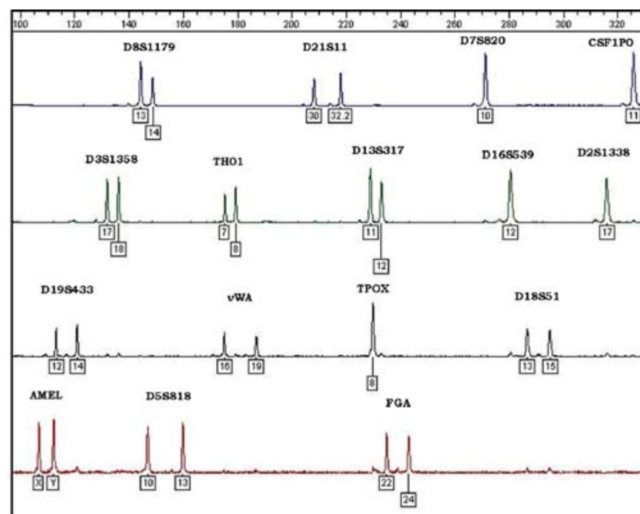


FIG. 4—*Continued.*

with much more closely linked Y-SNPs from the geographical viewpoint.

Although the small number of people examined here does not allow conclusive results, the present work demonstrates the possibility of identifying the geographical area of provenance of a subject by studying the biallelic markers of the Y chromosome located in the deepest branches of the phylogenetic tree. Despite acting as a single locus, Y provides powerful information. For markers specific to a single geographical area, affiliation is near certain, whereas for those shared by different areas it is necessary to go more shallowly into the phylogenetic tree and examine further specific markers chosen according to results achieved from this multiplex.

In this preliminary approach, most of our time was spent in perfecting a robust, efficient multiplex, capable of working with minimal amounts of DNA, such as are normally available in forensics. Further work is still necessary, to increase the number of European subjects analyzed, to obtain more precise SNP frequencies, and to assess possible gene back-flow,which is able to create a more complex gene admixture than that already in place—after mutations had occurred. It is also necessary to increase the number of SNPs in the multiplex and to create further multiplexes, to encompass mutations necessary to distinguish all haplogroups. For this aim, studies are in progress in our laboratory on further multiplexes capable of differentiating among all haplogroups and exploring the loci inside European haplogroups.

Experiments to explore the suitability of the Y-SNP multiplex with low molecular weight DNA were performed by amplifying DNA fragments sized less than approximately 150 bp, produced by sonication. To avoid interference by inhibitors of the polymerase chain reaction present in old samples from casework, which might have prevented amplification independently of template size, only DNA from fresh blood samples was used. Positive amplifications were obtained from 1 ng of DNA for all seven SNPs included in our multiplex, whereas only profiles with high noise background and questionable peaks below the threshold value of 50 RFU were obtained from the few microsatellites sized below 150 nucleotides: D8S1179 (128–168 bp), D19S433 (106–140 bp), D3S1358 (114–142 bp) and Amelogenin (Fig. 4). No improvement of results was obtained by adding more DNA template, up to 200 ng in our experiments (data not shown).

In conclusion, the main achievement of this work is the development of an effective, robust multiplex PCR of seven Y-chromosome SNPs starting from 1 ng of DNA template, exploring polymorphisms on fragments between 96 and 136 nucleotides in size, and also yielding positive results with degraded DNA. The multiplex includes binary polymorphisms that are hierarchically organized, capable of allowing inferences on the geographical area of origin of a subject, and guiding the choice of further polymorphisms to be analyzed for evolutionary or forensic purposes. This hierarchical strategy is time- and cost-effective and saves material, and this is an important feature of forensic work when only small amounts of DNA are available.

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References

1. Jobling MA, Tyler-Smith C. Fathers and sons: the Y chromosome and human evolution. Trends Genet 1995;11:449–56. [PubMed]
2. Underhill PA, Passarino G, Lin AA, Shen P, Mirazón Lahr M, Foley RA, et al. The Phylogeography of Y chromosome binary haplotypes and the origins of modern human populations. Ann Hum Genet 2001;65:43–62. [PubMed]
3. Underhill PA, Shen P, Lin AA, Jin L, Passarino G, Yang WH, et al. Y chromosome sequence variation and the history of human populations. Nat Genet 2000;26(3):358–61. [PubMed]
4. The Y chromosome consortium. A nomenclature system for the tree of human Y-chromosomal binary haplogroups. Genome Res 2002;12(2):339–48. [PubMed]
5. Jobling MA. Y-chromosomal SNP haplotype diversity in forensic analysis. Forensic Sci Int 2001;118:158–62. [PubMed]
6. Scherczinger CA, Bourke MT, Ladd C, Lee HC. DNA extraction from liquid blood using QIAamp. J Forensic Sci 1997;42:893–96. [PubMed]
7. Paracchini S, Arredi B, Chalk R, Tyler-Smith C. Hierarchical high-throughput SNP genotyping of the human Y chromosome using MALDI-TOF mass spectrometry. Nucleic Acids Res 2002;30(6):e27. [PubMed]
8. Allen RC, Graves G, Budowle B. Polymerase chain reaction amplification products separated on rehydratable polyacrylamide gels and stained with silver. BioTechniques 1989;7:736–44. [PubMed]

9. Budowle B, Chakraborty R, Giusti AM, Eisenberg AJ, Allen RC. Analysis of the VNTR locus D1S80 by the PCR followed by high-resolution PAGE. Am J Hum Genet 1991;48:137–44. [PubMed]
10. Haff LA, Smirnov IP. Multiplex genotyping of PCR products with MassTag-labeled primers. Nucleic Acids Res 1997;25:3749–50. [PubMed]
11. Fei Z, Ono T, Smith LM. MALDI-TOF mass spectrometric typing of single nucleotide polymorphisms with mass-tagged ddNTPs. Nucleic Acids Res 1998;26:2827–28. [PubMed]
12. Buetow KH, Edmonson M, MacDonald R, Clifford R, Yip P, Kelley J, et al. High-throughput development and characterization of a genomewide collection of gene-based single nucleotide polymorphism markers by chip-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Proc Natl Acad Sci USA 2001;98:581–84. [PubMed]
13. Underhill PA, Jin L, Lin AA, Mehdi SQ, Jenkins T, Vollrath D, et al. Detection of numerous Y chromosome biallelic polymorphisms by denaturing high-performance liquid chromatography. Genome Res 1997;7:996–1005. [PubMed]
14. Wang Y, Hung SC, Linn JF, Steiner G, Glazer AN, Sidransky D, et al. Microsatellite-based cancer detection using capillary array electrophoresis and energy-transfer fluorescent primers. Electrophoresis. 1997;18:1742–9. [PubMed]
15. Semino O, Passarino G, Oefner PJ, Lin AA, Arbuzova S, Beckman LE, et al. The genetic legacy of Paleolithic Homo sapiens sapiens in extant Europeans: a Y chromosome perspective. Science 2000;290:1155–59. [PubMed]
16. Rosser ZH, Zerjal T, Hurles ME, Adojaan M, Alavantic D, Amorim A, et al. Y-chromosomal diversity in Europe is clinal and influenced primarily by geography, rather than by language. Am J Hum Genet 2000;67(6):1526–43. [PubMed]
17. Bowcock AM, Kidd JR, Mountain JL, Hebert JM, Carotenuto L, Kidd KK, et al. Drift, admixture, and selection in human evolution: a study with DNA polymorphisms. Proc Natl Acad Sci USA 1991;88:839–43. [PubMed]
18. Pamilo P, Nei M. Relationships between gene trees and species trees. Mol Biol Evol 1988;5:568–83. [PubMed]
19. Rosenberg NA, Pritchard JK, Weber JL, Cann HM, Kidd KK, Zhivotovsky LA, et al. Genetic Structure of Human Population. Sciente 2002;298:2381–5.
20. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. Genetics 2000;155:945–59. [PubMed]
21. Lowe AL, Urquhart A, Foreman LA, Evett IW. Inferring ethnic origin by means of an STR profile. Forensic Sci Int 2001;119:17–22. [PubMed]
22. Brenner CH. Difficulties in the estimation of ethnic affiliation. Am J Hum Genet 1998;62:1558–60; author reply 62:1560–1. [PubMed]
23. Forster P, Rohl A, Lunnemann P, Brinkmann C, Zerjal T, Tyler-Smith C, et al. A short tandem repeat-based phylogeny for the human Y chromosome. Am J Hum Genet. 2000;67(1):182–96. [PubMed]
24. Bosch E, Calafell F, Santos FR, Perez-Lezaun A, Comas D, Benchemsi N, et al. Variation in short tandem repeats is deeply structured by genetic background on the human Y chromosome. Am J Hum Genet. 1999;65(6):1623–38. [PubMed]

Additional information and reprint requests:
Adriano Tagliabracci, M.D.
Istituto di Medicina Legale
Policlinico Torrette
I-60020 Ancona (Italy)
E-mail: a.tagliabracci@univpm.it