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ORIGINAL ARTICLE

Y CHROMOSOME SINGLE NUCLEOTIDE POLYMORPHISMS TYPING BY SNaPshot MINISEQUENCING

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ABSTRACT

Analysis of Y chromosome haplogroups, defined by single nucleotide polymorphisms (SNPs), is now a standard approach for study of the origin of human populations and measurement of the variability among them. It is also a new forensic tool, because it may allow determination of the origin of any male sample of interest. We have used a strategy for rapid, simple and inexpensive Y chromosome SNP typing of 343 male DNA samples, of which 211 were Macedonians, 111 Albanians and 21 Roma, Serbs or Turks. Using multiplex polymerase chain reaction (mPCR) and a SNaPshot multiplex kit for single nucleotide extension reaction, 28 markers were grouped into five multiplexes. Twenty different Y haplogroups were found in these samples. The most common Y haplogroups in Macedonians were I2a-P37b (27.5%), E1b1b1a-M78 (15.6%), R1a1-SRY1532 (14.2%) and R1b1-P25 (11.4%). In the Albanians E1b1b1a-M78 accounted for 28.8%, R1b1-P25 for 18.0%, J2b2-M241 for 13.5% and R1a1-SRY1532 for 12.6%. We conclude that five haplogroups (E1b1b1a-M78, I2a-P37b, J2b2-M241, R1a1-SRY1532 and R1b1-P25) comprised 72.6% of the Y chromosomes, this being characteristic of the typical European Y chromosome gene pool.

Key words: Y Chromosome, Y Haplogroups, Single nucleotide polymorphism (SNP), SNaPshot, Minisequencing

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INTRODUCTION

The analysis of single nucleotide polymorphisms (SNPs), located within the non recombining region of the Y chromosome (NRY), has been widely accepted in molecular anthropology as a unique tool for evolutionary studies. The low mutation rate and specific distribution of Y-haplogroups in populations, allow for the reconstruction of origin, evolution, and history of groups of humans by tracing male patterns of migration backwards from modern human populations [1]. Y-single nucleotide polymorphisms also constitute forensic tools because they can significantly contribute to forensic analysis by providing information on the ethnic origin of a male DNA sample [2]. Combined with conventional markers, they could be a powerful tool in mass disasters where people from various geographical areas are involved. Recently, Y-SNP typing has been applied in the study of possible association of Y-haplogroups with male-specific (spermatogenic

failure, testes and prostate cancer) and prevalently male-associated (hypertension, autism) diseases [3].

The Y Chromosome Consortium has published a single most parsimonious phylogeny of 153 binary haplogroups based on 243 binary markers and developed a simple set of rules to label unambiguously the different clades nested within this tree [4]. An extensively revised Y chromosome tree containing 311 distinct haplogroups, incorporating approximately 600 binary markers has also been published [5] and a web-based document with a regularly updated version of the Y chromosome tree has also become available [6].

In recent years, many different SNP typing techniques have been developed on the basis of various methods of allelic discrimination and detection platforms [7]. Most of these are based on allele specific hybridization, primer extension, oligonucleotide ligation or invasive cleavage. Detection methods for the products of each type of reaction, include fluorescence, luminescence and mass measurement. We selected the SNaPshot minisequencing approach which consists of single base extension of an unlabeled primer that anneals one base upstream to the relevant SNP. A multiplex minisequencing assay has already been validated for genotyping of the Y chromosome [8,9]. These studies have shown this SNP typing methodology to be robust, reliable and extremely sensitive. We have used a rapid, simple and inexpensive strategy for Y chromosome SNP typing of the multiethnic Macedonian population.

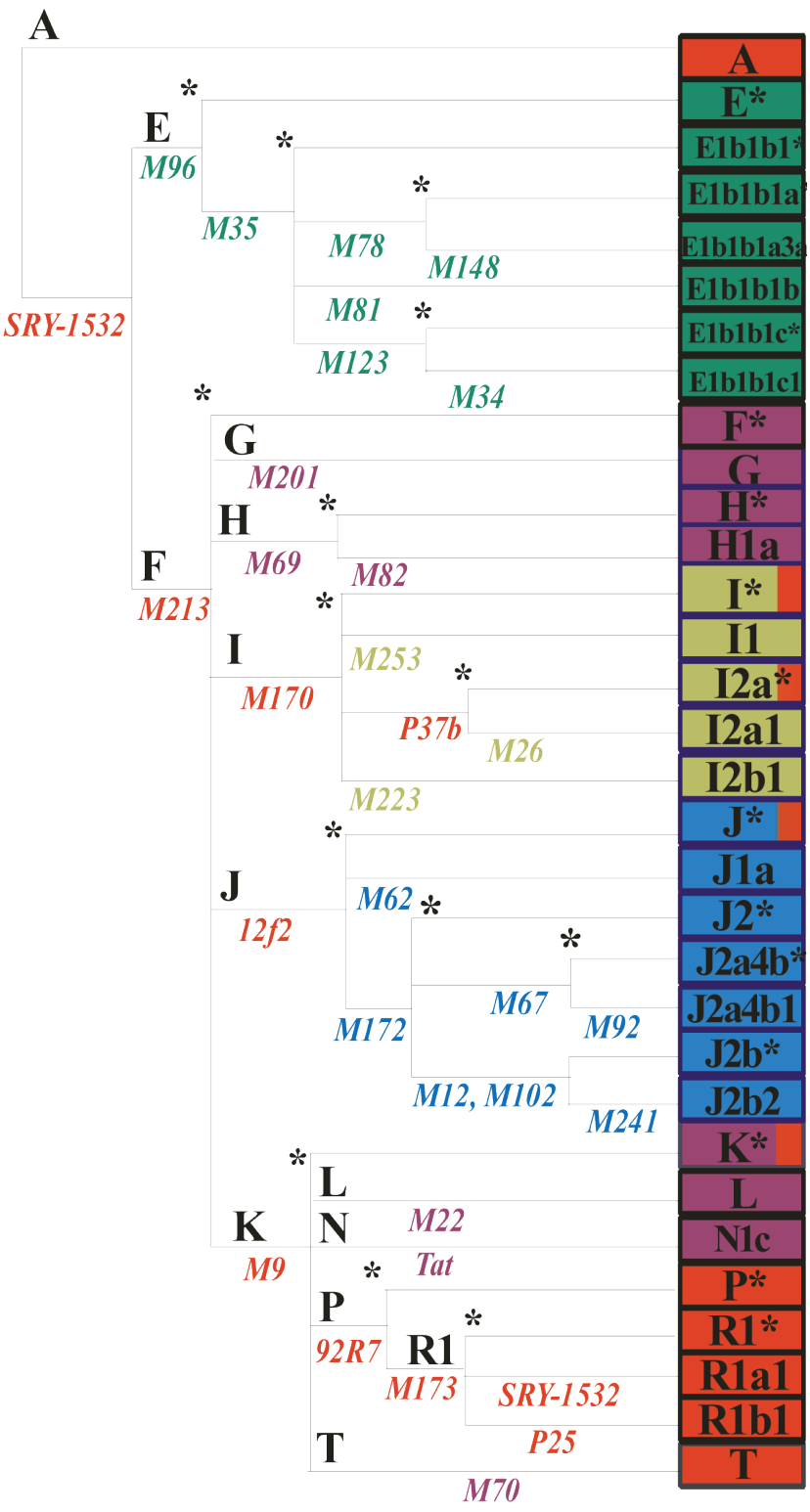


Figure 1. Phylogenetic tree defined with the binary Y-chromosomal polymorphisms analyzed. Marker names are shown above the lines. Colors represent multiplex groups.

Table 1. Polymerase chain reaction primers used for the Y-single nucleotide polymorphism typing.

Multi-plex	PCR Primer	Sequences (5'→3')	PCR Fragment (bp)	Concentration (pmol)
	1. 92R7	F: TGC ATG AAC ACA AAA GAC GTA R: GCA TTG TTA AAT ATG ACC AGC	55	7.5 7.5
	2. 12f2	F: CAC TGA CTG ATC AAA ATG CTT ACA GAT R: GGA TCC CTT CCT TAC ACC TTA TAC A	90	7.5 7.5
	3. P25	F: GGA CCA TCA CCT GGG TAA AGT R: AGT GCT TGT CCA AGG CAG TA	121	3.75 3.75
	4. M170	F: TGC AGC TCT TAT TAA GTT ATG TTT TCA R: CCA ATT ACT TTC AAC ATT TAA GAC C	158	7.5 7.5
1	5. SRY1532	F: TCC TTA GCA ACC ATT AAT CTG G R: AAA TAG CAA AAA CTG ACA CAA GGC	167	3.75 3.75
	6. M173	F: GCA CAG TAC TCA CTT TAG GTT TGC R: GCA GTT TTC CCA GAT CCT GA	172	3.75 3.75
	7. M213	F: GGC CAT ATA AAA ACG CAG CA R: TGA ATG GCA AAT TGA TTC CA	208	10.0 10.0
	8. P37b	F: TTG AAG AAA CCC TGG AGA GG R: CCA ACT CCT CTT CCC ATC AT	248	7.5 7.5
	9. M9	F: GCA GCA TAT AAA ACT TTC AGG R: AAA ACC TAA CTT TGC TCA AGC	340	3.75 3.75
2	1. M223	F: GCT GGA GTC TGC ACA TTG AT R: AGT CGT GGA GGC AAG TAT GC	110	7.5 7.5
	2. M170	F: TGC AGC TCT TAT TAA GTT ATG TTT TCA R: CCA ATT ACT TTC AAC ATT TAA GAC C	158	7.5 7.5
	3. P37b	F: TTG AAG AAA CCC TGG AGA GG R: CCA ACT CCT CTT CCC ATC AT	248	7.5 7.5
	4. M253	F: GAG CAA AGT AGA CAA CAT GGA AA R: CAG CTC CAC CTC TAT GCA GT	305	7.5 7.5
3	1. M96	F: GTG ATG TGT AAC TTG GAA AAC AGG R: GGA CCA TAT ATT TTG CCA TAG GTT	88	7.5 7.5
	2. M34	F: CAG AGT GTT TTC TCA TGT TAA TGC R: GGG GAC CCC AAT AAT CAT AA	92	7.5 7.5
	3. M81	F: TTA TAT TTT CAA TCC CTT AGT AAT TTT R: TGT TTC TTC TTG GTT TGT GTG AGT A	176	7.5 7.5
	4. M35	F: GCA TGG TCC CTT TCT ATG GAT R: GAG AAT GAA TAG GCA TGG GTT C	198	7.5 7.5
	5. M123	F: GCA AGA GCA AGT GAC TCT CAA AG R: TCT TTC CCT CAA CAT AGT TAT CTC A	248	7.5 7.5
	6. M78	F: CTT CAG GCA TTA TTT TTT TTG GT R: ATA GTG TTC CTT CAC CTT TCC TT	301	7.5 7.5
4	1. M67	F: AGG AAG AGT GGA AAG GCT CT R: GTC TTT TCA CTT GTT CGT GGA C	148	7.5 7.5
	2. M172	F: TCC TCA TTC ACC TGC CTC TC R: TCC ATG TTG GTT TGG AAC AG	187	7.5 7.5
	3. M12	F: TGG CAC AAT AAT GGC TCA CT R: TCC ATG TTG GTT TGG AAC AG	197	7.5 7.5
	4. M92	F: GGG TCC ACA AAA AGC TCA AA R: TGG TTT TGT GTC CAG CTC TT	253	7.5 7.5
	5. M62	F: ACT AAA ACA CCA TTA GAA ACA AAG G R: CTG AGC AAC ATA GTG ACC CC	309	7.5 7.5
	6. M241	F: TCT TGA TAA ACC GTG CTG TCT R: CAA TCT CAA TTC ATG CCT CCT	361	7.5 7.5
	7. M102	F: CCA TAT CAA AGC TGT GAA CTG TAT T R: TTC TTA TTC TGG TTT TGG TAG CC	402	7.5 7.5
5	1. M70	F: TCA TAG CCC ACT ATA CTT TGG AC R: CTG AGG GCT GGA CTA TAG GG	81	7.5 7.5
	2. M22	F: GCT GAT AGT CCT GGT TTC CCT A R: TGA GCA TGC CTA CAG CAG AC	106	7.5 7.5
	3. Tat	F: GAC TCT GAG TGT AGA CTT GTG A R: GAA GGT GCC GTA AAA GTG TGA A	112	7.5 7.5
	4. M201	F: TCA AAT TGT GAC ACT GCA ATA GTT R: CAT CCA ACA CTA AGT ACC TAT TAC GAA	144	7.5 7.5
	5. M69	F: TCC CTA TAG TCC AGC CCT CA R: TTC CCT TTG TCT TGC TGA AA	185	7.5 7.5
	6. M82	F: GCC TGT TCA AAT CCA AAA GC R: TGG CAT GAA GCA TGT AAG GA	239	7.5 7.5

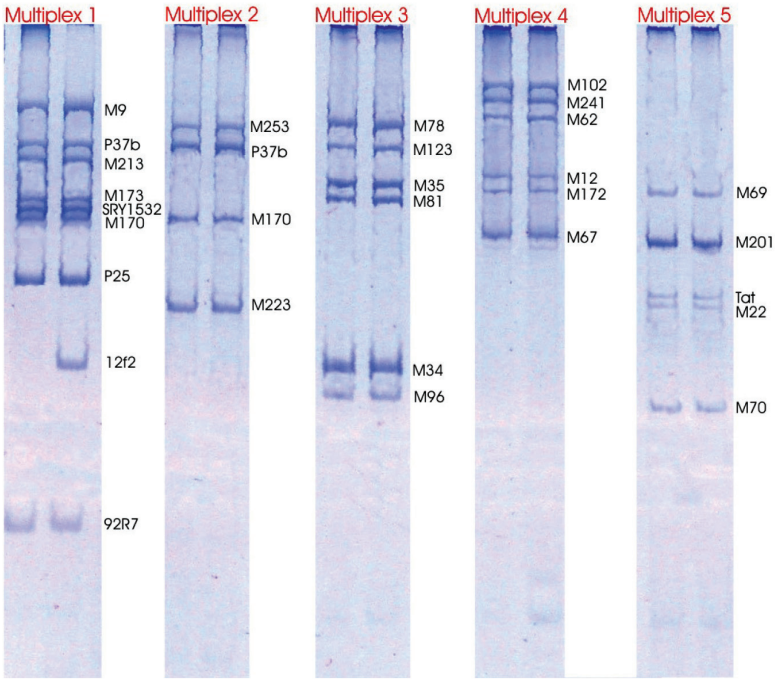


Figure 2. Multiplex PCR products separated by polyacrylamide gel and stained by ethidium bromide.

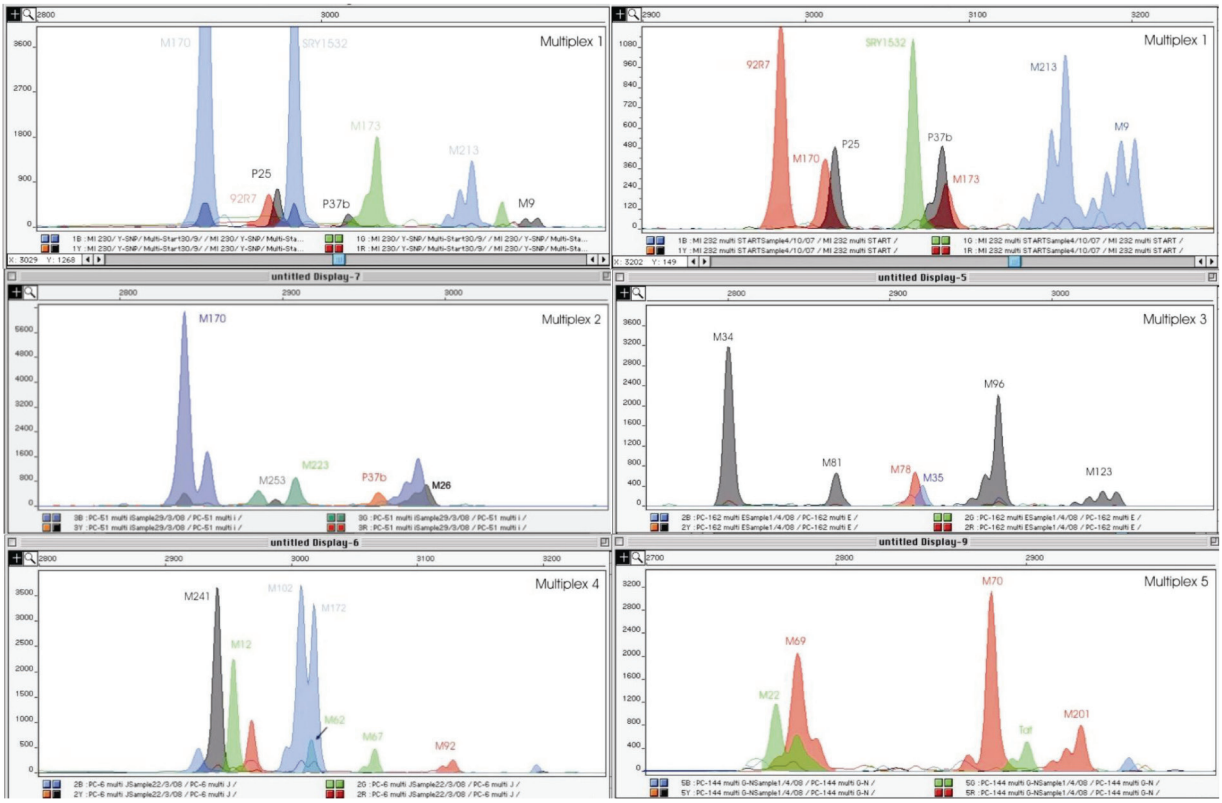


Figure 3. Six SNaPshot (Applied Biosystems) multiplexes from different samples. A) Multiplex 1 from sample assigned to haplogroup I2a-P37b; B) multiplex 1 from sample with R1a1-SRY1532; C) multiplex 2 from sample with I2b1-M223; D) Multiplex 3 from sample with E1b1b1a-M78; E) multiplex 4 from sample with J2b2-M241 and F) multiplex 5 from sample assigned to G-M201.

Table 2. SNaPshot primers used in the study.

Multi-plex	SNP Primer	Polymor-phism	Hgr	Sequence (5'>3')	Size (bp)	Conc. (pmol)
1	M170	A>G	I	R: ACA CAA CCC ACA CTG AAA AAA A	22	2.25
	P25	C>A	R1b1	F: CCC CCC CTC TGC CRG AAA CCT GCC TG	26	0.75
	92R7	G>A	P	R: CCC CGC ATG AAC ACA AAA GAC GTA GAA G	28	1.00
	SRY1532	A>G	R1a1	F: CCC CCC TTG TAT CTG ACT TTT TCA CAC AGT	30	1.00
	M173	A>G	R1	F: CCC CCC CCC CTT ACA ATT CAA GGG CAT TTA GAA C	34	1.00
	P37b	T>C	I2a	F: CCC CCC CCC CCC CCC CCC CTG ATA GGG TGG GAT TGG TTC A	40	1.00
	M213	T>C	F	R: CCC CCC CCC CCC CCC CCC CCC TCA GAA CATT AAA ACA TCT CGT TAC	45	1.25
	M9	C>G	K	F: CCC CCC CCC CCC CCC CCC CCC CCC CGA AAC GGC CTA AGA TGG TTG AAT	48	1.00
2	M170	A>G	I	R: ACA CAA CCC ACA CTG AAA AAA A	22	2.25
	M223	G>A	I2b1	R: CCC GCT GAA GAT GAT GCA ATT TAT TTA C	28	1.25
	M253	C>T	I1	F: AGT ATT GTT GAT AGA TAG CAA GTT GA	26	1.25
	P37b	T>C	I2a	F: CCC CCC CCC CCC CCC CCC CTG ATA GGG TGG GAT TGG TTC A	40	1.25
3	M34	G>T	E1b1b1c1	R: TTG CAG ACA CAC CAC ATG TG	20	1.50
	M81	C>T	E1b1b1b	F: CCC CCC TAA ATT TTG TCC TTT TTT GAA	27	1.50
	M78	C>T	E1b1b1a	F: CCC CCC CCC CAC ACT TAA CAA AGA TAC TTC TTT C	34	1.75
	M35	G>C	E1b1b1	R: CCC CCC CCC CCC CCC CCC CCA GTC TCT GCC TGT GTC	36	1.50
	M96	G>C	E	F: CCC CCC CCC TAA CTT GGA AAA CAG GTC TCT CAT AAT A	40	0.25
	M123	G>A	E1b1b1c	R: CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CTT CTA GGT ATT CAG GCG ATG	51	1.75
4	M12	C>T	J2b	R: GAG CAA CAT AGT GAC CCC CAT	21	1.00
	M241	A>G	J2b2	R: CTA CTG TAC TTA AAA ATC TTG CCA	24	1.00
	M62	T>C	J1a	R: CCC CCC CCC ATG TTT GTT GGC CAT GGA	27	2.50
	M102	C>G	J2b	R: GTT TAT TCT TAT TGT CTT TTC ACA TCT TA	29	1.00
	M172	T>G	J2	F: CCC CCC CCC CCC CCA AAC CCA TTT TGA TGC TT	32	0.50
	M67	A>T	J2a4b	F: CCC CCC CCC CCT CAA AAT ATG TGT AAT TCA AAA AAC A	37	1.00
5	M22	A>G	L	F: CCG CCA TTC CTG GTG GCT CT	20	0.50
	M69	T>C	H	F: GAG GCT GTT TAC ACT CCT GAA A	22	1.00
	M70	A>C	T	R: CCC CCC CCT AGG GAT TCT GTT GTG GTA GTC TTA G	34	0.75
	Tat	T>C	N1c	R: CCC CCC CCC CCC CCC CCC TCT GAA ATA TTA AAT TAA AAC AAC	42	1.00
	M201	G>T	G	F: CCC CCC CCC CCC CCC GAT CTA ATA ATC CAG TAT CAA CTG AGG	42	0.25

MATERIALS AND METHODS

Materials. We have studied 343 DNA male DNA samples from the DNA bank at our institution, of which 211 were Macedonians, 111 Albanians and 21 Roma, Serbs or Turks. The study was approved by the Ethics Committee of the Macedonian Academy of Sciences and Arts, Skopje, Republic of Macedonia (R. Macedonia).

Methods. The set of 28 Y-SNP markers was grouped hierarchically into five multiplex polymerase chain reaction (mPCR)/primer extension reactions, so as to determine the most frequent haplogroups using one or two multiplexes (Figure 1). Most of the PCR and minisequencing primers have already been described [8], the remainder were designed by us.

Multiplex Polymerase Chain Reaction. The PCR primers were designed to give a variety of PCR fragment sizes, that allow their separation by polyacrylamide gel electrophoresis (Table 1 and Figure 2). The PCR multiplexes were performed in 25 μL final volume, with 1X Reaction buffer, 300 μM of dNTPs, 2 mM of $MgCl_2$, 2U of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, USA) and 10 ng of genomic DNA. Cycling conditions were 10 min. at 95°C, then 35 cycles of 1 min. at 95°C, 1 min. 15 s. at 58°C, 3 min. at 72°C and a final extension at 65°C for 15 min.

Multiplex Minisequencing. Before a single base extension (SBE), 1 μL of PCR product was cleaned up with 0.5 μL of ExoSAP-IT (USB Corporation, Clevelend, OH, USA) and incubated at 37°C

Table 3. Y Haplogroup distribution in the population of R. Macedonia.

Y Haplogroup	Macedonians *n* (%)	Albanians *n* (%)	Others *n* (%)	Total *n* (%)
E1b1b1a-M78	33 (15.6)	32 (28.8)	3 (14.3)	68 (19.8)
E1b1b1b-M81	–	2 (1.8)	–	2 (0.6)
E1b1b1c1-M34	5 (2.4)	2 (1.8)	–	7 (2.0)
G-M201	8 (3.8)	3 (2.7)	1 (4.8)	12 (3.5)
H-M69	3 (1.4)	2 (1.8)	3 (14.3)	8 (2.3)
1*(xI1,I2a,I2b1)-M170	–	2 (1.8)	1 (4.8)	3 (0.9)
I1-M253	4 (1.9)	7 (6.3)	–	11 (3,2(
I2a-P37b	58 (27.5)	2 (1.8)	–	60 (17.5)
I2b1-M223	4 (1.9)	2 (1.8)	1 (4.8)	7 (2.0)
J*(xJ1a,J2)-12f2	7 (3.3)	2 (1.8)	–	9 (2.6)
J2*(xJ2a4b,J2b)-M172	10 (4.7)	3 (2.7)	2 (9.5)	15 (4.4)
J2a4b-M67	6 (2.8)	3 (2.7)	2 (9.5)	11 (3.2)
2b2-M241	11 (5.2)	15 (13.5)	1 (4.8)	27 (7.9)
L-M22	1 (0.5)	–	–	1 (0.3)
N1c-Tat	1 (0.5)	–	–	1 (0.3)
P*(xR1)-92R7	1 (0.5)	–	1 (4.8)	2 (0.6)
R1*-M173	1 (0.5)	–	–	1 (0.3)
R1a1-SRY1532	30 (14.2)	14 (12.6)	1 (4.8)	45 (13.1)
R1b1-P25	24 (11.4)	20 (18.0)	5 (23.8)	49 (14.3)
T-M70	4 (1.9)	–	–	4 (1.2)
TOTAL	211 (100.0)	111 (100.0)	21 (100.0)	343 (100.0)

for 60 min. followed by 15 min. at 85°C to inactivate the enzyme.

Multiplex single base extension reactions were performed in a 5 μL final volume, combining 2 μL of SNaPshot ready reaction mix (Applied Biosystems), 1.5 μL of cleaned PCR product and 1.5 μL extension primers. The minisequencing primers were 5' tailed with a poly-C sequence to produce extension products 21 to 51 nucleotides long to allow separation by capillary electrophoresis (Table 2). The cycling conditions were 10 s. at 96°C, 10 s. at 50°C and 30 s. at 60°C, for 25 cycles.

To remove unincorporated ddNTPs, the final products were incubated with 1U of shrimp alkaline phosphatase (USB Corporation) for 1 hour at 37°C, and 15 min. at 85°C to inactivate the enzyme.

Capillary Electrophoresis. The SNaPshot (Applied Biosystems) products were separated by capillary electrophoreis on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). Analysis of electropherograms was performed using the GeneScan 3.1 software (Applied Biosystems) and the size of fragments was determined on the basis of GeneScan-120 LIZ size standard (Applied Biosystems) (Figure 3).

RESULTS

Multiplex 1 consists of eight SNPs and allows detection of the more frequent major clades in Europe. It also includes the 12f2 deletion, which determines haplogroup J and was typed by presence/absence in the PCR amplification (Figure 2). Multiplex 1 determines haplogroups R1b1-P25, R1a1-SRY1532 and I2a-P37b. We performed multiplex 1 in all samples, and depending on the results, multiplex 2, 3, 4 or 5 was performed in order to define the haplogroup more precisely.

Multiplex 2 comprises four SNPs, multiplex 3 and 4 comprise six SNPs each, while multiplex 5 is a combination of five SNPs. Multiplex 2 subdivides haplogroup I, multiplex 3 haplogroup E, multiplex 4 haplogroup J, while haplogroup 5 determines haplogroups G-M201, H-M69, L-M22, N1c-Tat and T-M70.

Among the DNA samples we studied, we detected 20 different Y haplogroups, of which five haplogroups (E1b1b1a-M78, I2a-P37b, J2b2-M241, R1a1-SRY1532 and R1b1-P25) comprised 72.6% of the studied Y chromosomes (Table 3). The distribution of the Y haplogroups in Macedonians, Albanians and males of other ethnic origin (Roma, Serbs and Turks) is given in Table 3. The most common Y haplogroup in Macedonians was I2a-P37b (27.5%), which was followed by E1b1b1a-M78 (15.6%), R1a1-SRY1532 (14.2%) and R1b1-P25 (11.4%). In the Albanians, E1b1b1a-M78 was found in 28.8%, R1b1-P25 in 18.0%, J2b2-M241 in 13.5% and R1a1-SRY1532 in 12.6%. In the small group of Roma, Serbs or Turks, R1b1-P25 was found in 23.8% and E1b1b1a-M78 and H-M69 in 14,3% each. All three males with haplogroup H-M69 were of Roma ethnic origin.

DISCUSSION

The R. Macedonia has a multiethnic population consisting of Macedonians (64.2%), Albanians (25.2%) and Roma, Serbs, Turks, Aromuns and others (10.6%) (2002 census). The most common Y haplogroups in this population are E1b1b1a-M78, I2a-P37b, J2b2-M241, R1a1-SRY1532 and R1b1-P25. Y haplogroup composition of the population conforms with previous findings in the Southeast European (SEE) population [10].

The Y haplogroup E1b1b1a-M78 was the most frequent haplogroup in Albanians and the Roma, Serb or Turk group, but was second in frequency in Macedonians. It is the most common haplogroup E lineage in Europe with a frequency peak centered in the Balkans [11,12]. It is also found in the Middle East and in eastern and northern Africa. Its frequency in Kosovar Albanians (46%) and Macedonian Roma (30%) is most likely a result of genetic drift [10].

The most frequent haplogroup in Macedonian males is I2a-P37b (27.5%), which has maximum frequency in Herzegovinians (64%) and Bosnians (52%) and substantial frequencies in all SEE populations except for two reproductively isolated and non slavic speaking populations, Kosovar Albanians and Macedonian Romani [10]. In the Albanians in Macedonia, I2a-P37b had a very low frequency (1.8%) (Table 3), similar to Albanians from Kosovo (2.7%) and different from Albanians in Albania (17%) [10].

The R1b1-P25 haplogroup showed an overall frequency of 14.3%, being 18.0% in Albanian and 11.4% in Macedonian subjects. This lineage shows a frequency peak (40-80%) in western Europe and decreases in eastern (with the exception of 43% in the Ossetians) and southern Europe [13,14]. However, it shows two intermediate local peaks in mainland Croatians and Serbians, and among Kosovar Albanians, Albanians and Greeks [10].

The R1a1-SRY1532 haplogroup showed similar frequencies in Macedonians and Albanians and was less frequent in the Roma, Serb or Turk groups. The frequency in Macedonia agrees with that of 16% determined in SEE [10]. Its distribution increases from west to east with peaks in Finno-Ugric and Slavic populations. R1a1-SRY1532 frequency decreases slowly to the south of Europe.

Haplogroup J is defined by a 12f2 polymorphism and has two major subclades, J1-M267 and J2-M172, of which the latter is more prevalent in Europe [15]. The J2b-M102 lineages are more frequent in SEE comprising 5% of all chromosomes, with a peak in Kosovar Albanians [10]. The J2a4b-M67 cluster is predominant in Greeks and Italians [16]. The J2b2-M241 was the fifth most frequent Y haplogroup in the populations of R. Macedonia due to its high prevalence in Albanians (13.5%).

The most significant difference (p <0.0001) was found in the case of Hgr I2a-P37b, which was prevalent (27.5%) in Macedonians and infrequent in Albanians (1.8%). Although present at a relatively low frequency in both groups, I1-M253 was significantly more frequent in Albanians (6.3 *vs.* 1.9% in Macedonians, $p = 0.0383$). The E1b1b1a-M78 haplogroup was statistically more frequent in Albanians (28.8 *vs.* 15.6% in Macedonians, $p = 0.0050$). The same applies to J2b2-M241 (13.5 *vs.* 5.2%), being more common in Albanians ($p = 0.0093$). The R1a1 and R1b1 haplogroups were present with similar frequencies in both populations.

In conclusion, we have developed a simple, robust and efficient Y-SNP typing assay that can find application in evolutionary and forensic studies in the major ethnic groups of R. Macedonia. The hierarchical strategy using the SNaPshot multiplex kit (Applied Biosystems) made Y chromosome SNP typing rapid and inexpensive.

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