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SHORT COMMUNICATION

SNPSTR *rs59186128*_D7S820 polymorphism distribution in European Caucasoid, Hispanic, and Afro-American populations

A. Odriozola · J. M. Aznar · L. Valverde · S. Cardoso · M. L. Bravo · J. J. Builes · B. Martínez · D. Sanchez · F. González-Andrade · E. Sarasola · M. C. González-Fernández · B. Martínez Jarreta · Marian M. De Pancorbo

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Abstract Single nucleotide polymorphisms (SNPs) in the flanking regions of microsatellite loci (SNPSTRs) help to increase the power of discrimination of short tandem repeat (STR) loci. SNPs are positions in the genome that have been well-conserved over the course of evolution, so analysing them can help distinguish between STR alleles in which the number of repetitions matches due to descent from those which match by chance. This provides support for the determination of biological paternity and other kinship analyses in which mutation needs to be ruled out as grounds for exclusion. Locus D7S820 shows a variable position, SNP *rs59186128,* in the 5′ flanking region. This study is set out (1) to determine the frequencies of SNP *rs59186128* in populations with various geographical origins and (2) to estimate the possible contribution of *rs59186128* to the allele discrimination of locus D7S820. To that end, individuals from European Caucasoid, Hispanic, and Afro-American populations are studied using denaturing high-performance liquid chromatography, which enables locus *rs59186128* to be quickly and highly cost-effectively screened. Moreover, a method is established for determining the haplotypes of SNPSTR *rs59186128*_D7820. The results show that SNP *rs59186128* has a T allele frequency of more than 0.15 in one of the Afro-American populations studied, and the haplotype analysis shows that there is no preferential association between the alleles of SNPSTR *rs59186128*_D7S820, which supports the idea that they could be useful in forensic applications.

Keywords SNPSTR · DHPLC · Paternity testing · SNP · STR · Microsatellite

A. Odriozola · J. M. Aznar · L. Valverde · S. Cardoso · E. Sarasola · M. C. González-Fernández · M. M. De Pancorbo (✉)
BIOMICS Group, Dept. Z. y Biología Celular A., Facultad de Farmacia, Universidad del País Vasco, 01006 Vitoria-Gasteiz, Álava, Spain
e-mail: marian.mdepancorbo@ehu.es

M. L. Bravo · J. J. Builes
Instituto de Biología, Universidad de Antioquia, Medellín, Colombia

B. Martínez
Instituto de Investigaciones Inmunológicas, Universidad de Cartagena, Cartagena, Colombia

D. Sanchez
Laboratorio de Genética Molecular, Metropolitan Hospital, Quito, Ecuador

F. González-Andrade · B. M. Jarreta
Faculty of Medicine, Area of Legal and Forensic Medicine, Universidad de Zaragoza, C/Domingo Miral s/n, 50009 Zaragoza, Spain

Introduction

Single nucleotide polymorphisms (SNPs) are very common throughout the genome. It is estimated that there is one SNP per 1,000 nucleotides [1]. SNPs in the flanking regions of microsatellite loci (STRs) help increase the power of discrimination of those short tandem repeats (STR) loci. Knowledge of these features can reduce the appearance of null alleles due to there being no links between the primers [2], which makes them more interesting from the viewpoint of forensic genetics. This has led to the establishment of a database for this combination of loci under the name "SNPSTR database Release 1.5" [3, 4].

SNPSTRs enable us to distinguish STR alleles that are identical by state from those that are identical by descent [5]. The low rate of mutation in SNPs of around 10^{-8} mutations per generation [6] means that the polymorphisms are circumscribed to certain geographical and population groups. It is possible that these markers can be used in populations where certain SNPs linked to STRs have high heterozygosity levels to distinguish whether STR allele matches are due to chance or descent.

SNPSTRs are haplotypes made up of individual markers that do not recombine with one another. They can be applied to refine biological paternity diagnoses. A variation of one or two units of repetition in an STR locus between parent and descendants raises doubts about the exclusion of paternity [7]. SNPs linked to STR loci can help determine whether that variation is due to nonpaternity or to a mutation caused by polymerase slippage during DNA replication. This mutation often slips through the repair mechanisms [8], so the high number of mitoses that precede the meiotic division that leads to the formation of gametes make mutation in the STR loci highly probable, especially in the generation of male gametes [9, 10]. By contrast, the high level of stability of SNP loci makes them unlikely to mutate [11], so if the SNPSTR haplotypes do not match then biological paternity can be ruled out.

Another point to be taken into account for the application of SNPSTRs is the determination of the gametic phase of heterozygote individuals in both the SNP and STR loci (double heterozygotes). This is necessary to establish that there are no preferential links between SNP alleles and the alleles of physically linked STR loci and for direct application to actual cases of complicated kinship diagnosis. The polymerase chain reaction (PCR)/restricition endonuclease/capillary electrophoresis combination is a useful procedure to determine the gametic phase of double heterozygote individuals.

The variability of SNPs has been widely studied in large population groups. Accordingly, there are databases containing data for a number of validated SNP panels [12], but there is still much to be learned about variability confined to specific populations.

A direct, simple way of studying SNPSTR variability is screening via DHPLC (denaturing high-performance liquid chromatography). Analysing SNPs by this method is fast, cost-effective, and highly reproducible [13], which facilitates the rapid screening of large populations. In this paper, an example is given in the study of SNP *rs59186128* linked to *D7S820* in population samples with various origins: European Caucasoids living in the Basque autonomous community (Spain) and Hispanics and Afro-Americans from Ecuador and Colombia.

Materials and methods

Peripheral blood samples were taken from 483 unrelated healthy individuals. Of 483 individuals, 87 were European residents in the Basque Country, 243 were Hispanic (148 from Ecuador and 95 from Colombia), and 153 were Afro-American (89 from Ecuador and 64 from Colombia). The DNA samples from Caucasoid individuals were extracted via proteolytic lysis with proteinase K, purified with phenol–chloroform–isoamyl alcohol, and precipitated with sodium acetate and ethanol. After precipitation, the DNA was repurified twice with chloroform–isoamyl alcohol to remove any residual phenol that might interfere with the DHPLC analysis. The DNA from the Hispanic and Afro-American population samples was extracted with a QIAamp DNA Micro Kit (Qiagen, Valencia, CA, USA). The DNA from each individual was quantified using PicoGreen® (Invitrogen, Barcelona, Spain).

The 5′ flanking region of microsatellite locus D7S820 which includes SNP *rs59186128* (NCBI, reference sequence NT_007933.14, in position 9,023,702 bp) was amplified with 7S1Forward 5′AACCAGTGTGAACAAGAGTTACACG3′ and 7S1Reverse 5′TGGTGCAATTCTGTCAATGAGG3′ primers to a concentration of 0.1 μM, $MgCl_2$ at 2 mM, buffer 1X, and deoxyribonucleotides at 0.2 mM, in a Bio-Rad™ iCycler (Bio-Rad, Hercules, CA, USA) at 95ºC for 5 min; 30 cycles at 95ºC for 30 s, 57ºC for 30 s, and 72ºC for 30 s.

The amplicons were heteroduplexed by heating them at 96ºC for 5 min with a decrease of 1ºC/min to 25ºC and analysed in a Transgenomic® WAVE® System 4500 with a DNASep Cartridge (Transgenomic, Glasgow, UK). Amplicons were run at 54ºC with a linear gradient of 58.7%–59.2% buffer B (25% acetonitrile and 0.1 M triethylammonium acetate) for 5 min. Chromatograms with two different profiles were obtained, corresponding to homozygote and heterozygote individuals (a and b in Fig. 1, respectively). C/T heterozygote individuals are distinguished from homozygotes because they form a heteroduplex (double helix DNA molecules whose strands are not fully complementary: in this case at position *rs59186128*) whose retention time is lower than that of homoduplex individuals.

After heteroduplexing, the C/C and T/T homozygote individuals form 100% complementary homoduplex molecules, which are detected as a single peak in the chromatogram (Fig. 1a) and do not enable genotype C/C to be distinguished from T/T. The distinction between the two genotypes was made by mixing the amplification product of the homozygote individuals with a known standard C/C. After mixing, C/C homozygotes continued to show the same homoduplex profile while the T/T homozygotes showed a heteroduplex profile identical to that obtained in the analysis of the C/T individuals

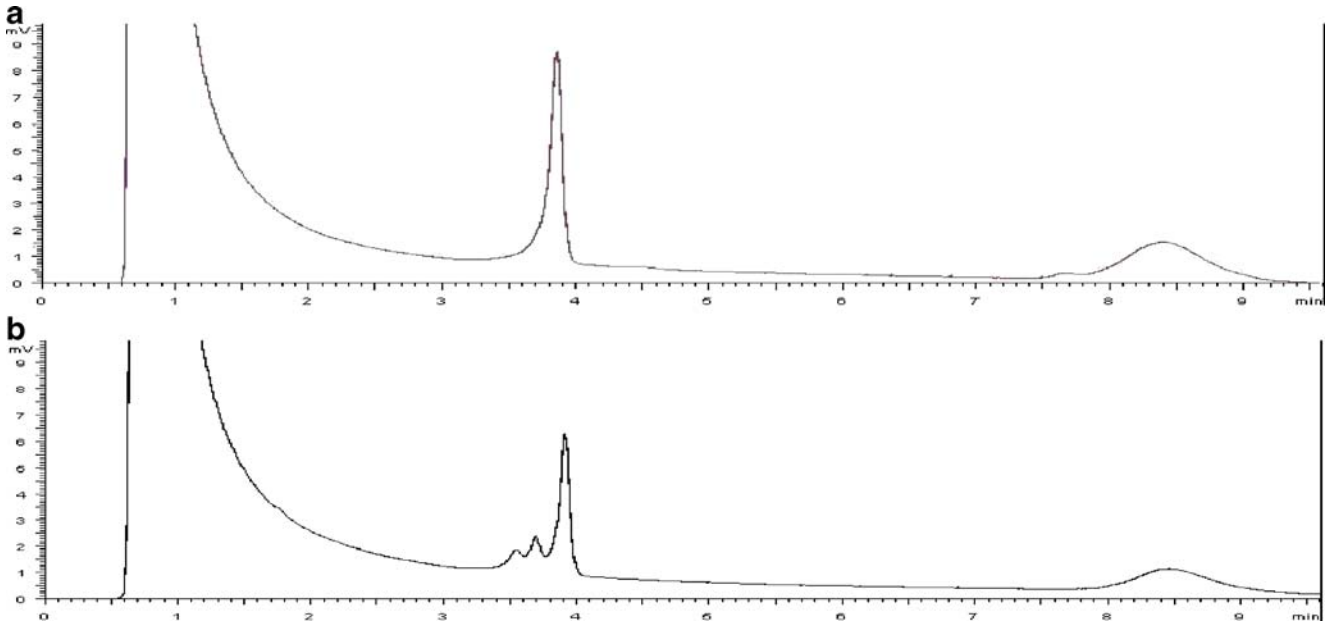

Fig. 1 **a** Chromatogram for DHPLC analysis (gradient of 0.5 units buffer B) of a sample where there is only homoduplex. **b** Chromatogram for DHPLC analysis, via a buffer B percentage gradient of 0.5 units, on an individual with homoduplex and heteroduplex

(Fig. 1b). The chromatograms were analysed using the Navigator Software program by Transgenomic®.

To determine the correspondence between the chromatogram and the base sequence, three samples were selected from each profile, sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and analysed in an AB3130 Genetic Analyzer (Applied Biosystems) using the Sequencing Analysis 5.2 program by Applied Biosystems.

The gametic phase of SNPSTR *rs59186128*_D7S820 and the SNP was determined in the double heterozygote individuals of *rs59186128* and D7S820 loci, amplifying the region that includes the two loci (NCBI, reference sequence NT_007933.14, 9023527-9023959 bp) using 7S1Forward primer and 7MP1Reverse 5′ATGTTGGTCAGGCTGACTATG3′ primer marked with FAM [14] (95ºC for 5 min; 30 cycles 95ºC for 30 s, 62ºC for 30 s, and 72ºC for 30 s; final extension 7 min at 72ºC). All the *rs59186128*_D7S820 double heterozygotes were analysed via digestion with 0.8 units of restriction enzyme *Sml* I (New England Biolabs, Ipswich, MA, USA) at 55ºC for 16 h. The restriction of the C alleles of locus *rs59186128* resulted in two fragments, one with a constant length of 180 bp and the other with variable lengths because it contained the repetition units. The allele of locus D7S820 linked to allele C of locus *rs59186128* was detectable, as its size decreased by 180 bp following enzyme digestion. Fragment sizes were determined by analysing 0.125 µL of denatured digested DNA in an AB 3130 Genetic Analyzer (Applied Biosystems) using GenMapper software by Applied Biosystems (Fig. 2).

Genotype and allele frequency calculations, heterozygosity, the exact test of Hardy–Weinberg, and tests for differentiation between population samples were carried out with GENEPOP version 4.0 [15]. The test of linkage disequilibrium was performed with Arlequin 3.01[16].

Results

DHPLC analysis revealed 438 C/C and T/T homozygote individuals (Fig. 1a) and 45 C/T heterozygote individuals (Fig. 1b). The allele frequencies are shown in Table 1. The C allele was more frequent than the T allele in all the population samples analysed. The highest frequency of the T allele found was in the population sample of Afro-Americans from Colombia (0.156), and the lowest was in Hispanics from Ecuador (0.027). Corresponding to the lower frequency of the T allele, the T/T genotype was detected only in Afro-Americans and Hispanics from northern Colombia. The genotype distributions of all the individuals analysed fitted the Hardy–Weinberg equilibrium.

The heterozygosity observed in SNP *rs59186128* was between 0.054 and 0.095 in all the populations studied except the sample of Afro-Americans from Colombia, where it was 0.219. The distribution of the allele frequencies of SNP *rs59186128* between pairs of populations was compared using Fisher's exact test (Table 2). Only the Afro-American population from Colombia showed significant differences from the rest of the population samples analysed.

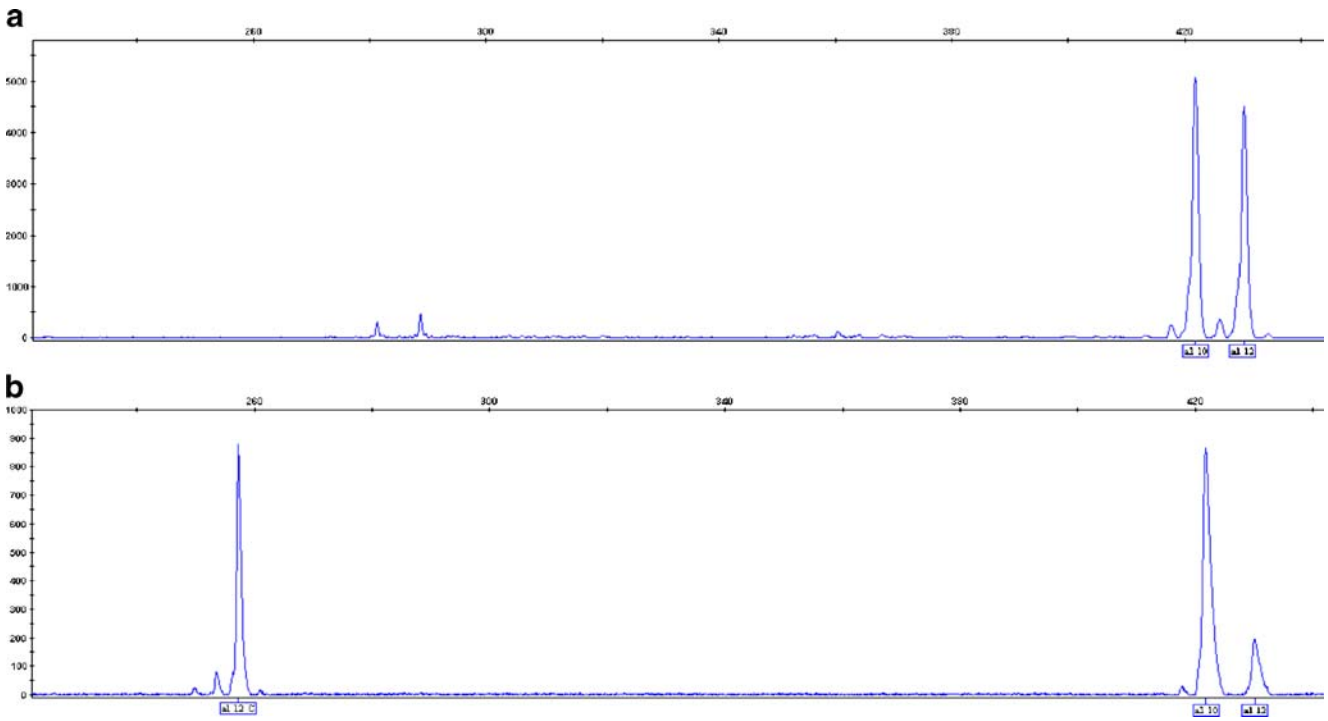

Fig. 2 **a** Electropherogram of a heterozygote individual for microsatellite locus D7S820, which carries alleles 10 (423 bp) and 12 (431 bp). **b** Electropherogram of the same individual, showing how the fragment corresponding to allele 12 of locus D7S820 associated with variant C of SNP *rs59186128* decreases to 251 bp following digestion with *Sml* I, which enables the linkage phase to be determined, as shown in this example, where allele C is linked to the allele with 12 repeats

Table 1 Genotype frequencies, allele frequencies, and Hardy–Weinberg equilibrium of SNP *rs59186128* in the population samples studied

Population sample	Number	Genotypic frequencies			Allelic frequencies		Hardy–Weinberg
		CC	CT	TT	C	T	*p*
European Caucasoid	87	0.931	0.069	–	0.966	0.034	1.000
Ecuadorian Hispanics	148	0.946	0.054	–	0.973	0.027	1.000
Colombian Hispanics	95	0.895	0.095	0.010	0.942	0.058	0.264
Afro-Americans from Ecuador	89	0.910	0.090	–	0.955	0.045	1.000
Afro-Americans from Colombia	64	0.734	0. 219	0.047	0.844	0.156	0.163

Table 2 Comparison of allele distributions of SNP *rs59186128* between the populations studied using Fisher's exact test (*p* values)

Population sample	European Caucasoid	Ecuadorian Hispanics	Colombian Hispanics	Afro-Americans from Ecuador	Afro-Americans from Colombia
European Caucasoid	–				
Ecuadorian Hispanics	0.774996	–			
Colombian Hispanics	0.349490	0.163514	–		
Afro-Americans from Ecuador	0.592156	0.202808	0.825478	–	
Afro-Americans from Colombia	0.000562**	0.000030**	0.011606*	0.004182*	–

*$p<0.05$ (statistically significant difference); **$p<0.001$ (statistically significant difference)

A haplotype analysis of SNPSTR *rs59186128*_D7S820 was conducted, but the haplotype of homozygote individuals showed no ambiguity in at least one of the two loci. This was not, however, the case for the double heterozygotes. To learn the haplotype of the double heterozygote individuals, the gametic phase of the alleles of the *rs59186128*_D7S820 loci was determined by amplifying DNA fragment of 403–443 bp which contained the position *rs59186128* and the repetition region of the locus D7S820 (5–15 TCTA repetitions) jointly. The PCR product was digested with the restriction enzyme *Sml* I, and the lengths of the restriction products were analysed. Figure 2 shows an example of the results for the determination of the gametic phases of a C-12/T-10 double heterozygote.

Once the gametic phases of all the double heterozygotes had been determined, it was possible to establish the haplotypes; the frequencies of which are shown in Table 3. Linkage disequilibrium analysis between these loci in each population revealed that the alleles of SNP *rs59186128* showed no preferential linkages with any allele of locus D7S820 except in four individuals in the sample of Hispanics from Ecuador, where allele 11 was observed in association with the T allele.

Discussion

The study of STR loci is currently the best established method for determining biological kinship [17]. However, these loci have a high mutation rate, and mutations are sometimes observed that can make determination difficult.

SNP loci do not suffer from this problem because single base mutation repair mechanism and excision and mismatching repair mechanisms, among others [18], are highly effective. Combining STRs with SNPs in sets known as SNPSTRs provides a highly useful way of determining cases of kinship. They are particularly useful in determining biological kinship in the absence of the mother, with possibly related parents. Conventional analysis using STRs may prove insufficient, as the possibility of kinship obtained from uncle/nephew and father/son may be very similar when the number of exclusions observed is low [19]. In such cases, SNPSTR analysis would help to determine the type of kinship.

Microsatellite instability (MSI) is highest in cancerous tissue, e.g. in colon carcinoma, where 12–16% of sporadic neoplasia shows high MSI [20]. These tissues are sometimes the only biological samples available for paternity testing or genetic identification (to correct errors in the identification of biological samples at a hospital, to determine pedigrees in the investigation of hereditary diseases, or to conduct studies into predisposition) [21]. In

Table 3 Gametic phases of SNPSTR *rs59186128*_D7S820 in double heterozygote individuals from the population samples analysed

Population sample	Double heterozygote	Locus D7S820											
	*rs59186128*_D7S820	8 *rs59186128* T allele	9	10	11	12	13	8 *rs59186128* C allele	9	10	11	12	13
European Caucasoid	12		0.083	0.333		0.083		0.083	0.083	0.083	0.083	0.167	
Ecuadorian Hispanics	16	0.063		0.125	0.250	0.063		0.125	0.063	0.125		0.188	
Colombian Hispanics	18	0.056	0.167	0.167	0.056	0.056		0.111	0.056	0.056	0.222		0.056
Afro-Americans from Ecuador	16	0.250		0.188	0.063			0.125		0.250	0.125		
Afro-Americans from Colombia	28		0.107	0.321	0.071			0.107		0.286	0.107		

such cases, conventional analysis may give rise to incorrect genotyping in some STR loci. Once again, SNPSTR analysis could help to correct those errors.

To discriminate between mutation and exclusion of kinship requires the right combination of SNPSTRs, where (1) the SNPs are highly polymorphic and (2) the haplotype distribution shows no preferential allele associations. The first of these conditions requires a search for and screening of SNPs very close to STR loci in order to select those SNPs with the highest heterozygosity, which are the most effective. The heterozygosity of SNPs varies from one group of humans to another [22], so the best suited SNPSTR combinations may also differ. A detailed study of populations is, therefore, needed to identify the SNPSTR combinations that provide most information in each specific population.

Once the informational capabilities of the SNP loci of interest are established, a haplotype analysis of SNPSTRs is required to cheque that there are no preferential allele associations. There are two ways of doing this: (1) cloning the PCR products of the SNPSTR loci and sequencing and (2) enzyme digestion of the SNPSTR PCR products and analysis of the resulting restriction slices. The second method can be applied, thanks to the restriction enzyme *Cel*-1, which specifically cleaves DNA to any single base pair mismatches that are present in heteroduplexes [23] or whenever the SNP alleles create or eliminate restriction enzyme targets.

When the gametic phases of the double heterozygotes found in this study were determined, no preferential linkages were observed between the alleles of locus *rs59186128* and those of locus D7S820. This finding confirms expectations as D7S820 is microsatellite locus and is of particular interest in determining whether a match in the number of repetitions is due to descent or to chance, regardless of what allele of D7S820 is affected by changes in the number of repetitions.

One factor which limits the usefulness of this SNP is the frequency of the least common allele. SNP locus *rs59186128* is registered in the GenBank, but there are no population data for its allele frequencies. The least common allele (T) has low frequencies in most of the population sample analysed here, except for the Afro-American population sample from Colombia, so the combination of SNP *rs59186128*/STR D7820 is forensically useful in that population.

The Afro-American populations from Ecuador and Colombia studied here show similar allele frequency distributions for numerous STR loci [24], which could led to their being interpreted as populations with a high level of mixing. However, an analysis of SNP locus *rs59186128*, whose mutation rate is far lower than that of STR loci, shows significant differences between the two groups. The differences observed are, therefore, probably due to their different demographic histories, perhaps with different founder effects in their formation, as previously observed by Salas et al. [25] in studies of several African populations in Central and South America.

Increasing interest in SNPSTRs requires a search for and characterisation of SNP loci close to STR markers with forensic applications. In this respect, the combination of DHPLC and capillary electrophoresis permits the analysis of the SNPs described previously and the discovery of new ones in a short time. Due to the possibility of automatic analysis, DHPLC technology enables large numbers of individuals from different populations to be analysed quickly and conveniently. It, therefore, provides a suitable, cost-efficient alternative to other techniques such as direct sequencing [26], whose application to SNP analysis in large numbers of individuals makes studies substantially more expensive. Moreover, the real-time PCR technique is highly expensive and is useful only for genotyping SNPs which are already known, so it cannot be used to search for new SNPs. However, the SNPs revealed by DHPLC could be analysed using snapshot technology, which permits multiplex analysis of SNPs, as in the case of mitochondrial DNA, for which as many as 22 SNPs are analysed in a single reaction [27].

In short, the findings of this study indicate that a combination of DHPLC and capillary electrophoresis implemented via a digestion with restriction endonucleases in double heterozygotes is an excellent tool for integrated analysis of SNPSTRs, which may be extremely useful in determining complex cases of kinship.

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