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D1S80 Locus Typing by Micro Thermal Cycler. Application to Genetic Identity Testing

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ABSTRACT: Based on a micro thermal cycler apparatus, a new protocol for amplification of the D1S80 locus has been developed. The advantages of this system consist of a reduction in time and costs of the amplification process, which greatly facilitates the analysis of the D1S80 locus at the population level and considerably increases its applicability in forensic samples. Using this protocol, an allele distribution study of the D1S80 locus has been carried out in a sample of 257 individuals residing in the Basque Country. In this study, up to 22 different alleles, ranging from 17 to 40 repetitions have been observed; moreover, the 35 and 38-repetition alleles, not reported in the european populations analyzed to date, have been detected.

The sample studied fits Hardy-Weinberg equilibrium, the observed heterozygosity being 0.74, and the expected heterozygosity 0.804 ± 0.012. The Chance of Exclusion and Index of Discrimination are 0.638 and 0.072 respectively.

Moreover, the gene frequency distribution from the Basque resident population does not show significant differences when compared to other European and U.S. Caucasian populations.

KEYWORDS: criminalistics, D1S80, DNA amplification, polymerase chain reaction

The D1S80 locus is a VNTR region, whose moderate repetition (units of 16 bp repeated in tandem 15–40 times) allows its analysis after amplification by the polymerase chain reaction [*1*]. It can be genotyped after separation of resulting fragments in high resolution PAGE followed by silver staining [*2*]. Moreover, the D1S80 locus is highly polymorphic in all the populations studied being the greatest number of different alleles detected in a German sample [*3*]. This locus displays a high frequency of heterozygosity in each population, though the 18 and 24-repetition alleles show high frequencies in all populations studied. However the D1S80 locus proves to be a system of great interest in population genetics.

In addition to this, the possibility of analyzing this locus by PCR makes it a useful tool for the analysis of forensic samples, which usually provide degraded or very low quantities of DNA. The D1S80 locus is also becoming a widely used system in genetic identification testing of blood, blood stains, semen, hair roots, compact bones, body tissues from fire victims and fixed material [*4*]. In order to develop a simple system of amplification of the D1S80 locus, a new protocol has been set up. This system performs the DNA amplification using positive displacement pipette tips, "capillary microsyringes" (Linus), whose high surface-to-volume ratio enables temperature equilibration much faster than in the conventional tube format, as well as significantly reducing volumes employed. So, the advantages of the amplification technique by micro thermal cycler consist of a reduction in time and costs.

The sensitivity of this system is similar to that of the conventional one as was shown by the amplification of decreasing quantities of DNA obtained from different samples. This technique is applicable to DNA samples obtained by different extraction procedures (phenolchloroform, Chelex resin, rapid digestion) as well as from different sources (blood, blood or semen stains, hair roots).

A study of the allele distribution of the D1S80 locus in a sample of 257 individuals residing in the Basque Country has been carried out using a micro thermal cycler, and the results were compared to other European and U.S. Caucasian populations.

Experimental Method

Population Sample

Whole blood samples were obtained by venipuncture from 257 healthy and unrelated individuals resident in the Basque Country and kept in EDTA tubes at −20°C until extraction.

Extraction of DNA from Whole Blood

DNA extraction from 700 μL of whole blood was performed using the phenol-chloroform method and the concentration adjusted to 25 ng/μL.

Extraction of DNA from Blood or Semen Stains

The DNA from blood or semen stains was extracted with Chelex resin [*5*]. With the aim of increasing the concentration of the DNA extracted, the following modifications were made: a 30% stock solution of Chelex resin was prepared and 30 μL of this were added to the sample.

Extraction of DNA from Hair-Roots

The DNA from hair roots was extracted by rapid digestion. The hair root was washed with sterile milli-Ro H_2O and incubated in 200 μL of the digestion buffer (0.01M Tris-HCl pH 8, 0.09% Triton X-100, 35 mM DTT, 0.35 mM NaAc pH 5.2) for 30 min

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at 56°C. After boiling for 10 min, mixing for 10 s and spinning at 12,000 rpm, the supernatant was recovered.

Amplification

4 μL of the DNA (100 ng) extracted using the phenol-chloroform method or 4 μL of each sample extracted by the above mentioned methods were amplified with 6 μL of the reaction mix containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM of each dNTP, 1 μM of each primer [2] and 0.2 U of Taq I polymerase (Ampli Taq™ Roche Molecular Systems) without adding mineral oil. Alternatively, 6 μL of D1S80 PCR reaction mix from Roche Molecular Systems were used adding 0.1 U of Taq polymerase.

Amplification was carried out using a micro thermal cycler (Linus Microcycler) with the following cycles: one cycle of 94°C 2 min—65°C 5 s—72°C 35 s, five cycles of 94°C 30 s—65°C 5 s—72°C 35 s, thirty cycles of 94°C 5 s—65°C 5 s—72°C 35 s—30 cycles and one final cycle of 72°C 1 min.

PAGE and Silver Staining

4 μL of the amplified DNA from each sample were loaded onto a 0.4 mm thick polyacrylamide-piperazine horizontal gel (4% T, 3% C), 0.375 M Tris-HCl pH 8.8 buffer, with 3% agarose electrodes in 0.25 M Tris-glicine buffer pH 8.8. A ladder (Roche Molecular Systems) was placed every 4 lanes. The separation distance between electrodes was 10 cm and gels were run at 15 mA, keeping the current constant. Detection of the genotypes was performed by silver staining [2].

Statistical Calculations

Hardy-Weinberg equilibrium was tested using the three-allele model of Rand et al. [6]. The expected number of alleles was estimated as by Chakraborty et al. [7] and the expected heterozygosity as by Nei M. [8]. The comparison of the population sample here studied to other populations was performed through the X^2 test, removing those classes with expected values lower than 5. The parameters of forensic interest here calculated are the Chance of Exclusion (CE) and the Index of Discrimination (ID).

Results

A population sample consisting of 257 unrelated individuals resident in the Basque Country was genotyped for the D1S80 locus after amplification by micro thermal cycler of 100 ng of phenol-chloroform extracted DNA from each individual, PAGE and silver staining.

Up to 22 different alleles were found, ranging in size from 17 to 40 repetitions. The alleles of 34 and 39 repetitions were not observed, while the alleles of 35 and 38 repetitions, not previously reported in the studied European populations, have been detected. The gene frequencies are shown in Table 1. The alleles of 24 and 18 repetitions show the maximum frequencies, 0.346 ± 0.042 and 0.247 ± 0.038 respectively, while the alleles of 35, 37, 38 and 40 repetitions display the minimum frequencies. The number of different genotypes observed is 63, being 18–24, 24–24 and 18–18 the most frequent combinations (0.152, 0.128 and 0.105 respectively).

The Hardy-Weinberg equilibrium test was performed by binning alleles together [9] and using a three-allele model [6] (17–23, 24 and 25–40: $X^2_{\beta\ df} = 6.939$).

The gene frequency distribution of the sample studied was compared to those from the European populations of Galicia and Portugal [10], Germany [6], Finland [4] and to that from the U.S. Caucasian population [11] as shown in Fig. 1. No statistically significant differences were found with the European and U.S. Caucasian populations (Table 2).

The observed heterozygosity (Ho) in the Basque population sample for the D1S80 locus was 0.740. The heterozygosity for this population was lower than that observed in all the populations studied: Galicia (0.878), Portugal (0.793), Germany (0.810), Finland (0.770) and U.S. Caucasians (0.810). The expected heterozygosity (He) being 0.804 ± 0.012 [8]. The Index of Discrimination in the Basque population, calculated as by Wong et al. [12], was 0.117. Bearing in mind the expected heterozygosities of the studied populations, the Index of Discrimination in the Basque population is slightly higher than in other populations, and therefore, this system is equally useful for forensic purposes in our population (Table 3).

The Chance of Exclusion or the "a priori" probability of a falsely accusedly man be excluded with this system, calculated as by Smouse et al. [13] is 0.638, a figure similar to that observed in other populations (Table 3).

In order to estimate the lowest quantity of DNA that can be typed after amplification using a micro thermal cycler, we started with samples of 50, 25, 5 and 2.5 ng of DNA, obtained by dilutions 1:2, 1:4, 1:20 and 1:40 of samples containing 100 ng of DNA both from homozygote and heterozygote individuals. In all the cases it was possible to genotype the amplified 5 ng samples of DNA (Fig. 2), while the resolution was not enough in the case of the 2.5 ng samples. These results show that 5 ng of DNA that can be successfully amplified using this technique when the quantity of DNA available is limiting.

The application of the micro thermal cycler technique to blood stains requires the modification of the DNA extraction protocol with Chelex resin. The usual procedure applied to blood stains of 10 mm^2 does not permit the amplification by micro thermal cycler technique, probably because the concentration of DNA that can be obtained is very low and therefore, the minimum quantity of DNA required for this technique is not always reached. To solve this inconvenience, the concentration of DNA extracted by Chelex resin was increased using a 30% resin stock solution, and adjusting the final volume of extraction to 30 μL which concentrates six times the DNA in the sample. This eliminates the need of further DNA microconcentration. The same modification can be applied to semen stains, which enables stronger signals to be obtained after silver staining. The DNA from semen in swabs was also extracted by Chelex resin following the modified method of Sajantila et al. [14]. The DNA from single hair roots was extracted by the method of rapid digestion. Figure 3 shows the results obtained when using these forensic samples.

Discussion

The micro thermal cycler has two advantages. Firstly, it requires a lower quantity of reagents and the time necessary for amplification is shortened. The reduction in the quantity of the reagents employed occurs because the reaction volume is as low as 10 μL. Therefore, it is possible to reduce the volume of all the reagents as well as the quantity of Taq polymerase. The time required for each amplification cycle is also very short, as the fast temperature equilibration in the capillary microsyringes of the micro thermal cycler results in increased efficiency, the amplification process

TABLE 1—*Distribution of allele and genotype frequencies in the population sample here studied.*

Allele	Absolute frequency	Gene frequency	Genotype	Absolute frequency	Genotype frequency	Genotype	Absolute frequency
17	3	0.006	17/17	1	0.004	22/31	2
18	128	0.249	17/18	1	0.004	23/24	3
19	7	0.014	18/18	25	0.097	23/25	2
20	15	0.029	18/20	4	0.016	23/27	1
21	10	0.019	18/21	1	0.004	23/28	1
22	15	0.029	18/22	4	0.016	23/30	2
23	14	0.027	18/23	3	0.012	24/24	31
24	178	0.346	18/24	43	0.167	24/25	15
25	34	0.066	18/25	7	0.027	24/26	2
26	3	0.006	18/27	1	0.004	24/27	4
27	9	0.018	18/28	5	0.019	24/28	7
28	22	0.043	18/29	5	0.019	24/29	11
29	30	0.058	18/31	3	0.012	24/30	4
30	10	0.019	18/32	1	0.004	24/31	12
31	26	0.051	19/19	1	0.004	24/33	1
32	2	0.004	19/23	1	0.004	24/38	1
33	2	0.004	19/24	2	0.008	25/25	2
34	0	0.000	19/29	1	0.004	25/28	2
35	1	0.002	19/35	1	0.004	25/30	1
36	2	0.004	20/20	3	0.012	25/31	2
37	1	0.002	20/23	1	0.004	27/29	1
38	1	0.002	20/24	3	0.012	27/30	1
39	0	0.000	20/29	1	0.004	28/28	2
40	1	0.002	21/22	1	0.004	28/29	2
	n = 514		21/24	4	0.016	28/30	1
			21/26	1	0.004	29/31	4
			21/29	3	0.012	29/32	1
			22/24	4	0.016	31/31	1
			22/25	1	0.004	31/36	1
			22/27	1	0.004	33/40	1
			22/29	1	0.004	36/37	1
			22/30	1	0.004	N = 257	

taking a total of only 35 min. Secondly, the simplicity in manipulation allows a reduction in time when setting up the samples, so that the binding of Mg^{2+} ions by the dNTPs in the reaction mix is reduced [*15*].

After performing the adequate modifications of the conventional D1S80 amplification protocol to the micro thermal cycler, we have contrasted the results obtained when typing several samples by both amplification systems. The typings matched in all the cases, confirming in this way the applicability of the micro thermal cycler to the analysis of the D1S80 locus.

The analysis of Basque population sample has shown the existence of 22 different alleles, this figure being among the highest found within the populations studied. Only the German population [*6*] shows a higher number of alleles. This difference in the number of alleles between our population and the German one, is due to the intermediate alleles detected (17M, 22M, 23M, 24M and 25M) in the latter; these alleles are not observed in our sample, probably because different ladders were employed in both studies. With regard to the conventional classes, the number of different alleles in both the German and Basque populations is the same.

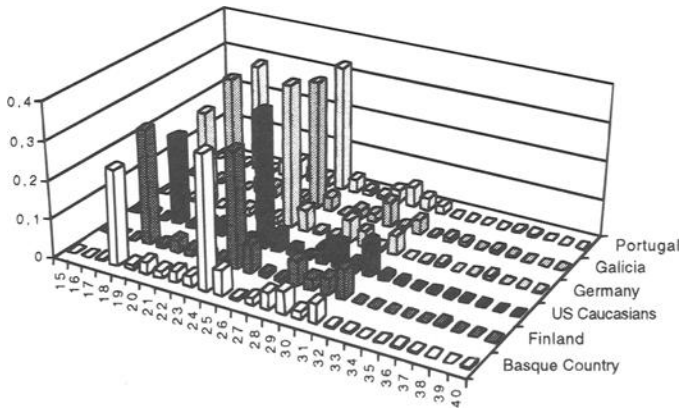

FIG. 1—*Gene frequency distributions of several Caucasian populations.*

TABLE 2—X^2 *comparison of the D1S80 allele distributions from the Basque Country population and those from the Galicia, Portugal, Germany, Finland and U.S. Caucasian populations.*

	Absolute allele frequencies				
Alleles	Galicia	Portugal	Germany	Finland	U.S. Caucasian
18	65	64	107	86	94
20	8	8	16	9	10
21	—	11	8	5	13
22	9	8	13	4	8
23	4	5	7	4	4
24	74	74	160	87	142
25	8	8	24	21	19
27	...	—	3	—	3
28	5	8	26	19	17
29	14	12	17	9	25
30	—	7	3	12	9
31	8	5	21	22	35
X^2	7.583	12.970	10.160	16.217	14.425
P	>0.05	>0.05	>0.05	>0.05	>0.05

TABLE 3—*Heterozygosities (Hobs, Hexp), chance of exclusion (CE) and index of discrimination (ID) of D1S80 locus in several populations.*

Population	*N*	*n*	Hobs	Hexp	CE	ID
Basque Country (this study)	257	22	0.74	0.80	0.636	0.070(Hexp)
Galicia (Lareu et al., in press)	109	18	—	0.79	0.604	0.080(Hexp)
Portugal (Lareu et al., in press)	110	18	—	0.79	0.613	0.093(Hexp)
Germany (Skowasch et al., 1992)	218	27	0.81	0.79[a]	0.620	0.065(Hobs)
Finland (Sajantila et al., 1992)	140	20	0.77	0.79	0.615[a]	0.080(Hexp)
U.S. Caucasian (Eisenberg et al., 1991)	200	21	—	0.80[a]	0.635[a]	0.070(Hexp)[a]

N = number of individuals; *n* = number of different alleles; [a]data not reported by the authors.

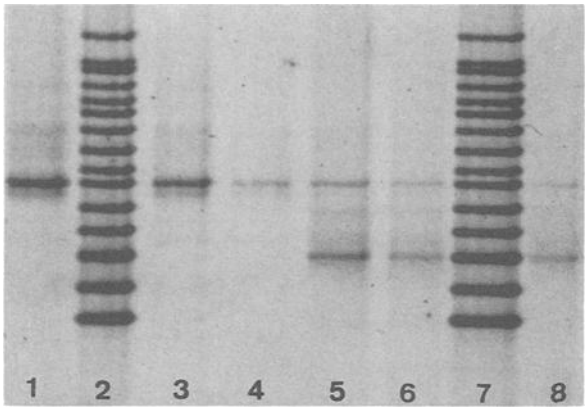

FIG. 2—*D1S80 typing of a serial dilution of DNA. Allelic ladder: lanes 2 and 7. DNA from individual 1 (24,24): lane 1 (50 ng), lane 3 (25 ng), lane 4 (5 ng). DNA from individual 2 (18,24): lane 5 (50 ng), lane 6 (25 ng), lane 8 (5 ng).*

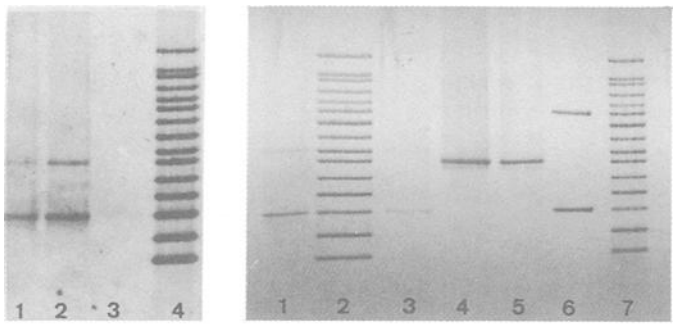

FIG. 3—*D1S80 typing of different forensic samples.* A) *Allelic ladder: lane 4. Negative control: lane 3. Individual (18,24): lane 1, DNA from a 9 mm² blood stain extracted with 30% Chelex resin; lane 2, DNA from whole blood extracted by the phenol-chloroform method.* B) *Allelic ladder: lanes 2 and 7. Positive control (18,31): lane 6. Individual 1 (18,25): lane 1, DNA from whole blood extracted by the phenol-chloroform method; lane 3, DNA from a hair root extracted by rapid digestion. Individual 2 (24,24): lane 4, DNA from a semen stain extracted with 30% Chelex resin; lane 5, DNA from whole blood extracted by the phenol-chloroform method.*

The following three alleles have not been detected in the population sample from the Basque Country, the 16-repetition allele, observed in the populations of Portugal [*10*] and Germany [*6*], the 34-repetition allele observed in Portugal and Finland [*4*] and the 39-repetition allele present in all the populations excluding Germany. On the contrary, in the sample from the Basque Country, it was possible to detect the 35 and 38-repetition alleles, not previously reported in Caucasian populations.

The frequent alleles in our population are those having a moderate number of repetitions, as happens in all the populations studied to date.

The number of possible genotypes, 253 versus the 63 observed in the population sample studied here, does not enable the direct application of the X^2 test to confirm Hardy-Weinberg equilibrium. By forming groups of alleles (binning) as [*9*], a less sensitive but informative approach to test for deviations between the expected and observed values, can be performed. The allele distribution in our population sample shows that it is possible to establish a 3-allele model whose corresponding genotype distribution confirms the Hardy-Weinberg expectations.

The comparison of the population sample studied here to other populations has been carried out by the X^2 test. To ensure the necessary reliability, those classes showing expected values lower than 5, were excluded. No significant differences were found in all comparisons. These results are similar to those obtained in enzyme and protein polymorphisms when comparing the Basque Country resident population to other Spanish and European ones [*16*]. Likewise, the comparisons of the Northern Spanish populations with others from Europe, demonstrate that in most of the cases the X^2 test does not show significant differences [*17, 18*]. Taking into account that the Basque Country resident population is located among the Northern Spanish populations, the results obtained in the comparison of the D1S80 locus agree with the previous results obtained using conventional polymorphisms [*17*].

The observed heterozygosity (Ho) in the studied population sample is lower than expected, however, the population does conform with Hardy-Weinberg equilibrium.

The high number of alleles detected in this population as well as the high heterozygosity expected (He), determine that the Chance of Exclusion of the D1S80 system is slightly higher than that obtained in other populations already studied. This indicates that this locus is of great interest for the Biological Paternity Testing in the Basque Country. On the other hand, Table 4 shows the values for the Index of Discrimination, the minimum value being that the German population. However, when calculating the Index of Discrimination for the German population as by Wong et al. [*12*] the formula used for the other populations this parameter rises to 0.079. For this reason, the Index of Discrimination for our population (0.072) is slightly lower than that obtained in the European populations. Nevertheless the D1S80 locus is of unquestionable interest in the criminalistic field in the Basque Country.

To summarize, the technique of amplification of the D1S80 locus by micro thermal cycler here described, greatly facilitates the use of this locus for population studies, paternity testing and forensic purposes, as it considerably reduces the costs and time required. On the other hand, though no statistically significant differences in the allele distribution of this locus among the European populations are found, it is important to take into consideration the need for data bases from each European population group because of the slight variations in the values of Chance of Exclusion and Index of Discrimination shown by the different populations reported.

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