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PCR-amplification and detection of the human D1S80 VNTR locus

Amplification conditions, population genetics and application in forensic analysis

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Summary. A series of experiments has been performed to evaluate amplification and typing of the D1S80 VNTR locus. The validation study that has been carried out showed that correct D1S80 typing results can be obtained when a defined amplification protocol and a high-resolution polyacrylamide gel electrophoresis method are used. The use of the Chelex extraction protocol has substantially reduced the processing time. DNA-extraction, amplification and subsequent typing can be performed in one day. The discrimination power of this locus is 0.94 in a Dutch Caucasian population sample. The system is extremely sensitive: 0.1 ng of genomic DNA gave a correct typing result. The test could also detect the correct genotypes in mixed samples containing DNA from different individuals. Even if the major type was in a 20-fold excess, the minority type could still be amplified and typed correctly. We have found no deviation from Hardy-Weinberg equilibrium in a Dutch Caucasian population sample. Evidence for the somatic stability of this locus was obtained from a set of experiments where we compared DNA-profiles from corresponding blood, semen and saliva samples. The results of this study suggest that in the near future analysis of the D1S80 locus by DNA-amplification can be applied in actual forensic case work.

Key words: AMPFLP – D1S80 – Polymerase Chain Reaction (PCR) – Population Genetics – Forensic DNA typing

Zusammenfassung. Eine experimentelle Serie wurde durchgeführt, um die Amplifikations- und Typisierungsbedingungen des VNTR-Locus D1S80 festzulegen. Die Validierungsstudie, welche durchgeführt wurde, zeigte, daß korrekte Typisierungsergebnisse erhalten werden können, wenn ein definiertes Amplifikationsprotokoll und eine hochauflösende polyacrylamidgel-elektrophoretische Methode benutzt werden. Der Gebrauch des Chelex-Extraktionsprotokolls hat die Bearbeitungszeit erheblich reduziert. Die DNA-Extraktion, Amplifikation und anschließende Typisierung können an einem Tag durchgeführt werden. Die Diskriminationskraft dieses Locus ist 0,94 in einer holländischen Populationsstichprobe (Europäer). Das System ist extrem empfindlich: 0,1 ng genomischer DNA ergaben ein korrektes Typisierungsergebnis. Der Test war auch geeignet, korrekte Bestimmung der Genotypen in gemischten Samples durchzuführen, welche DNA von verschiedenen Personen enthielten. Auch wenn der Haupttyp in 20-fachem Überschuß vorhanden war, konnte der Nebentyp noch amplifiziert und korrekt bestimmt werden. In einer holländischen Populationsstichprobe haben wir keine Abweichung vom Hardy-Weinberg-Gleichgewicht gefunden. Evidenz für somatische Stabilität dieses Locus wurde dadurch erhalten, daß ein Satz von Experimenten durchgeführt wurde, in welchen die DNA-Profile korrespondierender Blut-, Samen- und Speichelproben verglichen wurden. Die Ergebnisse dieser Untersuchung legen nahe, daß in der nahen Zukunft die Analyse des D1S80-Locus durch DNA-Amplifikation im aktuellen forensischen Fallmaterial durchgeführt werden kann.

Schlüsselwörter: AMPFLP – D1S80 – Polymerase-Kettenreaktion (PCR) – Populationsgenetik – Forensische DNA-Typisierung

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Introduction

Highly polymorphic Variable Number of Tandem Repeats (VNTR) loci can be used for identity testing [1]. When an appropriate restriction enzyme is chosen for the digestion of genomic DNA these length polymorphisms are usually detected by Southern blot analysis. This conventional typing procedure for forensic samples such as hair roots, bloodstains, semen and saliva stains is often limited by the inherent sensitivity of the Southern

Table 1. Characteristics of the D1S80 VNTR-locus [4, 5]

Locus:	D1S80[a]
Chromosome location:	1p
Repeat length:	16 bp
Number of alleles:	>22[c]
Allele length range[b]:	18–42 repeats (430–814 bp [5, 9])
Most common allele:	24 repeats
Heterozygosity:	79%[d]

[a] Human Gene Mapping Symbol
[b] Allele length range in the Dutch Caucasian population (this study)
[c] The number of 22 alleles refers to the number detected in the Dutch Caucasian population sample (this study)
[d] Observed heterozygosity in the Dutch Caucasian population sample (this study)

blotting procedure. At least 50 ng of undegraded DNA is required for the analysis using a locus specific VNTR probe [2]. In addition, the resolution of alleles that differ by only one repeat unit can be difficult, particularly when the restriction fragments are large and the repeat size is short. Electrophoretic comigration of an actual heterozygote with alleles that differ by only one repeat unit may result in the identification of only a single band pattern.

The Polymerase Chain Reaction (PCR) enables the typing of known polymorphic regions with much smaller amounts of human DNA [3], even if the DNA is degraded to some extent. Using appropriate PCR-primers which flank the tandem repeats and thermostable Taq DNA polymerase, typing of VNTR loci can be accomplished on ethidium bromide-stained agarose gels and on ethidium-bromide or silver stained polyacrylamide gels. It is possible to amplify VNTR alleles of up to 5 kbp in length [4], but in general VNTR loci with alleles of 2 kbp or less give more reliable PCR results. PCR amplification of many polymorphic VNTR regions have been described so far [5, 18, 19, 20].

Here, we describe the application of the DNA amplification technique for the detection of the D1S80 locus [5] using a high resolution, horizontal PAGE technique and subsequent silver staining [6, 7].

The characteristics of the D1S80 VNTR-locus are given in Table 1.

In this paper we describe several validation studies for this PCR-based identification system for the forensic practice.

1. The forensic validation of any new genetic marker in identity testing requires the collection of genotype and allele frequency data from the relevant population(s) [8]. To this end these were determined in a Dutch Caucasian population sample of 150 individuals and compared with population genetic studies from Finland [9], USA [9] and Germany [10].

2. The sensitivity of the system was determined with regard to the minimal amount of DNA-template. The ability of the test to detect the correct genotypes in mixed samples containing genomic DNA originating from different individuals was also evaluated.

3. Although highly unlikely, the relatively high degree of mutability of VNTR alleles could pose the problem of mosaicism (somatic instability) which might lead to false exclusions and in very exceptional circumstances to the false inclusion of a suspect. In this study D1S80 genotypes from blood samples were compared with the corresponding semen and saliva samples.

Materials and methods

Nomenclature. The high resolution electrophoresis method makes it possible to assign an allele classification based on the number of repeat units by comparison with an allelic ladder [5, 6]. Alleles were designated as described by Sajantila et al. [9].

Population sample. Blood was obtained from 88 unrelated male and 62 unrelated female Caucasian donors. A sample of 100 µl liquid blood was deposited on clean cotton weave (20 × 20 mm), air dried and stored at room temperature. Matched semen-blood and saliva-blood samples were obtained from the same group of individuals. Liquid semen and saliva samples were also deposited on clean cotton weave and subsequently air dried at room temperature.

DNA extraction and quantification. DNA for PCR analysis was isolated from blood, semen and saliva stains by Chelex extraction using previously described procedures [11, 12]. Chelex-extracted DNA from each sample was quantified using the slot-blot procedure described by Waye et al. [13]. DNA-extracts were normalized to a concentration of 1 ng DNA per µl.

Primer synthesis. The primer sequences for the D1S80-locus (Kasai et al. [5], Budowle et al. [6] and Sajantila et al. [9]) were:
primer 1: 5′-GAA ACT GGC CTC CAA ACA CTG CCC GCC G-3′
(28mer)
primer 2: 5′-GTC TTG TTG GAG ATG CAC GTG CCC CTT GC-3′
(29mer)
Oligonucleotides were obtained from Pharmacia (the Netherlands) or from Operon (CA, USA).

PCR reaction parameters. Reaction mixtures (total volume 50 µl) consisted of

- 5 µl 10 × PCR buffer (GeneAMP, Perkin Elmer Cetus, containing 500 m*M* KCl; 100 m*M* Tris-HCl pH 8.3; 15 m*M* $MgCl_2$ and 0.01% [w/v] gelatin).
- 4 µl 2.5 m*M* dNTP's;
- 1 µl 12.5 µ*M* 3′ primer;
- 1 µl 12.5 µ*M* 5′ primer;
- 2.5 Units AmpliTaq DNA Polymerase (Perkin Elmer Cetus).
- 10 ng genomic DNA
- sterile H_2O added to a final volume of 50 µl.

A 2.5 mM dNTP solution was prepared by mixing equal volumes of 10 mM-solutions of each of dATP, dCTP, dGTP and dTTP (GeneAmp dNTPs, Perkin Elmer Cetus).

No paraffin oil was layered over the reaction mixtures. The tubes were placed in the GeneAmp PCR System 9600 from Perkin Elmer Cetus for amplification.

Temperature cycling conditions for the D1S80-locus were as follows:

- denaturation 10 s, 95°C; annealing 10 s, 67°C; extension 30 s, 70°C; 29 cycles; final extension 5 min, 70°C.

Size fractionation of DNA amplification products. PCR products were separated by electrophoresis in a discontinuous buffer system using ultrathin (400 µm) polyacrylamide gels (7.5% T, 2% C) with piperazine diacrylamide as the crosslinker [6, 7]. Gels contained

60 m*M* Tris-formate buffer, pH = 9.0 and no glycerol. Gels (either 23 cm × 16 cm or 23 cm × 10.5 cm) were cast onto a polyacrylamide gel support medium (Gelbond, FMC).

The trailing ion, which was contained in 2% (w/v) agarose plugs (10 × 15 mm diameter), was 0.28 *M* Tris-borate, pH 9.0. The agarose plugs contained Bromophenol blue which was used as a visual marker for the discontinuous buffer boundary. The distance between the electrodes was 16 cm. A sample of 4 µl of the mixture containing the amplified D1S80-fragments was applied onto the gel surface (1 cm from the cathode) using fiberglass applicator tabs (Pharmacia). Electrophoresis was performed on a 2117 Multiphor II electrophoresis unit (LKB) using the ECPS 3000/150 (Pharmacia) constant power supply and a VH1 Volthour Integrator (Pharmacia). Electrophoresis settings for the 23 × 16 cm gels were: 600 V, 30 mA and 15 W. Gels were run at a constant temperature of 10°C. Electrophoretic separation was stopped when the bromophenol blue front had reached the anodal end of the gel (usually after 850 Vh).

The amplification products were visualized by silver staining of the gel using the method of Budowle et al. [6].

Allelic ladder. Allele determinations were carried out by comparison with an allelic ladder cocktail. In most experiments the allelic ladder cocktail used had been constructed by the FBI [unpublished data]. An infinite supply of this ladder can be obtained by reamplification. We found that suitable reamplification conditions for the FBI ladder were as follows: 20 µl of a 4000 × dilution of the allelic ladder cocktail was amplified in a total volume of 100 µl. Further PCR coditions were:

- 5 min hot start at 95°C;
- 10 Units of Taq-polymerase were added after the hot-start denaturation.

Cycling parameters:

- denaturation 10 s, 95°C; annealing 10 s, 67°C; extension 1 min, 70°C; 15 cycles; final extension 5 min, 70°C.

A sample of 5 µl of the amplified D1S80 ladder fragments was loaded onto the gel.

An alternative allelic ladder was constructed by amplifying DNA from different individuals (see Table 2). PCR products from individual samples were first typed on a polyacrylamide gel, pooled, and concentrated by ethanol precipitation. This allelic mixture also could be reamplified using the conditions described for the FBI ladder. An inherent property of this approach of mixing genomic DNA PCR products instead of individual alleles is that the most common alleles in the population will be in excess and the intensity will therefore be stronger.

Statistics. The frequency of each allele in the population was calculated from the numbers of each genotype that were collected in the sample set. From the allele frequency data the expected number of genotype frequencies was calculated under the assumption of Hardy-Weinberg (H-W) expectations. Possible divergence of genotype frequencies from the (H-W) expectations was determined by calculating the unbiased estimate of the expected heterozygote frequency (h). This frequency is equivalent to the allelic diversity [14].

$$h = n[1 - \Sigma(n_i/n)^2]/(n - 1)$$

where n_1, n_2 ... n_i are the allele counts of the D1S80-alleles in a sample of n chromosomes. In a population following H-W expectations h should be mathematically equivalent to the frequency of observed heterozygotes. The standard error for h was computed as the square root of the variance of a binomial distribution:

$$\sqrt{[h \cdot (1 - h)/N]}$$

where h represents the expected heterozygote frequency and N the number of individuals in the sample.

Table 2. Distribution of D1S80 genotypes in a sample of 150 unrelated Dutch Caucasians

Genotype	Number of genotypes observed (expected)	Frequency	95% UCL
18–18	6 (7.1)	0.040	0.085
18–20	4 (1.5)	0.027	0.067
18–21[a]	2 (0.8)	0.013	0.047
18–22	3 (2.1)	0.020	0.057
18–23	1 (1.1)	0.007	0.037
18–24	23 (24.7)	0.153	0.221
18–25[a]	1 (1.3)	0.007	0.037
18–27[a]	2 (0.5)	0.013	0.047
18–28	4 (3.7)	0.027	0.067
18–29	4 (3.3)	0.027	0.067
18–31	5 (8.0)	0.033	0.077
18–36	2 (0.8)	0.013	0.028
18–37[a]	1 (0.2)	0.007	0.037
18–42[b]	1 (0.2)	0.007	0.037
19–24[a]	1 (0.3)	0.007	0.037
20–24	2 (2.6)	0.013	0.028
20–31[a]	1 (0.8)	0.007	0.037
21–24	2 (1.5)	0.013	0.028
22–24	4 (3.7)	0.027	0.067
22–31[a]	2 (1.2)	0.013	0.028
22–38[b]	1 (0.0)	0.007	0.037
23–28	1 (0.3)	0.007	0.037
23–29[a]	1 (0.3)	0.007	0.037
23–31	2 (0.6)	0.013	0.028
24–24	22 (21.7)	0.147	0.195
24–25	5 (2.3)	0.033	0.077
24–26	1 (0.8)	0.007	0.037
24–28	6 (6.8)	0.040	0.086
24–29	7 (5.7)	0.047	0.094
24–30	1 (1.1)	0.007	0.037
24–31	14 (14.0)	0.093	0.152
24–32[a]	1 (0.3)	0.007	0.037
24–34[a]	1 (0.3)	0.007	0.037
24–36[a]	2 (1.5)	0.013	0.047
26–29[a]	1 (0.1)	0.007	0.037
28–31	4 (2.2)	0.027	0.067
28–33[a]	2 (0.2)	0.013	0.047
29–31	1 (1.8)	0.007	0.037
29–33	1 (0.2)	0.007	0.037
30–31[a]	2 (0.4)	0.013	0.047
31–31	3 (2.3)	0.020	0.057
Homozygosity (n = 31)	= 0.21		
Heterozygosity (n = 119)	= 0.79		
H-W equilibrium expectation ($\chi^2 = 11.62$, $df = 7$, $0.10 < P < 0.25$)			
Power of discrimination (PD) = 0.94			

Point estimates and 95% Upper Confidence Limits (UCL) for D1S80 genotypes in a population sample of 150 Dutch Caucasian individuals. Expected genotype frequencies (shown in parentheses) were calculated on the basis of H-W equilibrium from the allele frequency data in Table 3

The values of the number of observed heterozygotes and homozygotes and the power of discrimination are also shown

[a] From these genotypes an allelic ladder was constructed using the procedure as described

[b] Precise allele designation could not be performed. The corresponding allele was not present in the allelic ladder cocktail

The 95% confidence intervals for the individual allele and genotype frequency data were taken from statistical tables [15] taking into consideration that allele and genotype frequencies are binomially distributed.

The null hypothesis defines that the allele frequencies in the Caucasian population samples are not significantly different. This hypothesis was tested by pairwise comparisons in the chi-squared test of homogeneity ($R \times C$ contingency table [16]) using a computer program provided by G. Carmody (Carleton University, Canada).

Population homogeneity of particular alleles was examined using a two allele system in 2×2 contingency tables, i.e. the allele of interest in a particular population is category 1, while the sum of all other alleles in that population is category 2.

The power of discrimination (PD) was calculated as

$$1 - \Sigma(P_i)^2,$$

were P_i represents the frequency of each genotype.

Results

Reaction parameters

Data from the PCR experiments demonstrated that the $MgCl_2$ concentration is a critical parameter. High concentrations of Mg^{2+} generate additional bands and reduced amounts of locus-specific PCR products. A Mg^{2+} concentration of 1.5 m*M* was found to be most effective and lower concentrations resulted in reduced or even complete lack of PCR-product. From titration experiments is was evident that a wide range of primer concentrations can be used for D1S80 amplifications; even with different amounts of template DNA there were no noticeable negative effects. For routine amplification we used a 0.25 μ*M* concentration of each primer and 2.5 U Taq-polymerase per 50 μl PCR reaction.

More than 29 cycles of amplification resulted in the appearance of faint additional bands but no increase in the amounts of PCR product. Using the cycling conditions and reaction parameters as described, we found that the larger alleles could be amplified to almost the same extent as smaller alleles with no evidence of preferential amplification.

Sensitivity of the PCR system

From dilution experiments it was shown that a low amount of genomic DNA (0.1 ng) was still sufficient to obtain enough product after 29 cycles. Lower amounts of DNA resulted in reduced amounts of PCR products.

Detection of mixed samples

A series of experiments was performed in order to verify if the correct genotypes could be detected in mixed samples containing genomic DNA from different individuals.

Serial dilutions of quantified DNA samples with different heterozygous D1S80 genotypes were mixed and amplified as described. Without the presence of contaminating DNA we could detect the alleles in samples containing 0.1 ng DNA. In the presence of 100 ng of contaminating DNA we were able to detect the bands when the mixture contained at least 5 ng of the minority type DNA. The difference in intensity of the bands was such that it was possible to determine the genotypes of the contaminating sample and the minority type sample. It was consistently found that if the majority type DNA (contaminating DNA) was present in not more than 20-fold excess, the alleles of the minority type were still detectable.

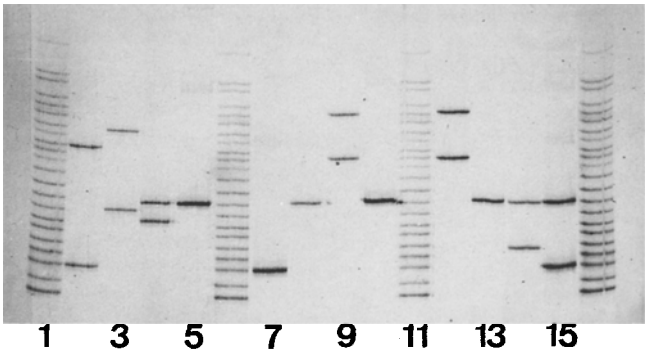

Fig. 1. Silver stained D1S80 polyacrylamide gel diplaying profiles from different unrelated individuals. From left to right: The FBI allelic ladder cocktail is in lanes 1, 6, 11 and 16. The K562 genomic control DNA (genotype 18–29) is in lane 2. D1S80 genotypes: lane 3: 23–31; lane 4: 22[b]–24; lane 5: 24–24; lane 7: 18–18; lane 8: 24–24; lane 9: 28–33; lane 10: 24–24; lane 12: 28–33; lane 13: 24–24; lane 14: 20[a]–24; lane 15: 18–24.

[a] anodic variant of the 20 allele
[b] cathodic variant of the 22 allele

Population genetics

An example of the allelic variation of the D1S80 locus is given in Fig. 1. Each lane represents the genomic DNA of a different individual. The resolution obtained distinguishes alleles differing by only 16 bp (i.e. one repeat) in length. By side-to-side comparison with the allelic ladders the unknown alleles can be designated directly without measuring relative migration distances.

It was noted that in our PCR-system some individuals showed faint extra bands of high molecular weight. These bands were outside the region of the actual D1S80-alleles on the gel and did not interfere with genotype assignment. Genotype assignment by side-to-side comparison with the allelic ladders was facilitated when some of the amplification products in the allelic ladder showed a higher intensity than the remaining alleles. In the Dutch allelic ladder the 3 most common alleles (18, 24 and 31) were more intense (Fig. 4) and function as distinctive markers in the allelic ladder. Alternatively, DNA from a cell line control could serve the same purpose.

All 150 extracted DNA samples could be typed for the D1S80-locus. The distributions of observed genotypes and allele frequencies for the D1S80 locus in the Dutch Caucasian population sample are shown in Tables 2 and 3; 21 different alleles were observed. The most common alleles were 24 ($f = 0.380$) and 18 ($f = 0.217$).

On a number of occasions we observed variant alleles that did not align exactly with the alleles in the ladder.

Table 3. D1S80 Allele frequencies in a sample of 150 unrelated Dutch Caucasians

Allele	Total number (variants)	*f*	95% LCL	95% UCL
16	0	0	0	0.012
17	0	0	0	0.012
18	65	0.217	0.172	0.268
19	1	0.003	0	0.018
20	7 (3)	0.023	0.009	0.048
21	4 (1)	0.013	0.004	0.034
22	10 (3)	0.033	0.016	0.061
23	5 (3)	0.017	0.005	0.039
24	114 (3)	0.380	0.325	0.438
25	6	0.020	0.007	0.043
26	2	0.007	0.001	0.024
27	2	0.007	0.001	0.024
28	17 (1)	0.057	0.033	0.089
29	15 (1)	0.050	0.028	0.081
30	3	0.010	0.002	0.029
31	37	0.123	0.088	0.166
32	1	0.003	0	0.018
33	3 (1)	0.010	0.002	0.029
34	1	0.003	0	0.018
35	0	0	0	0.012
36	4	0.013	0.004	0.034
37	1	0.003	0	0.018
38[a]	1	0.003	0	0.018
42[a]	1	0.003	0	0.018

Expected heterozygosity (= allelic diversity) = 0.787 ± 0.033

Point estimate frequencies (*f*) and 95% confidence limits for D1S80 allele frequencies in the Dutch Causasian population (n = 300 chromosomes). The number of observed variants for each allele are shown in parentheses

The value for the allelic diversity is also shown

[a] Precise allele designation could not be performed. The corresponding allele was not present in the allelic ladder cocktail

These variants represent minor allele length or sequence differences. Variant alleles were classified according to their electrophoretical behavior (anodal or cathodal relative to the closest allele in the ladder). All variants were pooled with the main allele for statistical analyses.

The observed number of genotypes as well as the expected number of genotypes based on the assumption of H-W expectations for this population are shown in Table 2. In this population sample 41 genotypes out of 231 possible genotypes were encountered with the genotype 18–24 being the most frequent ($f = 0.153$). Because of the population sample size and the large number of different genotypes, any genotype class with less than 3 observations was pooled for determining whether or not this population meets H-W expectations. The Dutch population sample meets H-W expectations for D1S80 ($\chi^2 = 11.62$, $df = 7$, $0.10 < P < 0.25$). We also tested for H-W equilibrium by calculating the unbiased estimate of the expected heterozygote frequency (see Table 3). Observed ($h^{obs} = 0.79$) and expected ($h^{exp} = 0.787 \pm 0.033$) heterozygote frequencies did not deviate significantly and on the basis of both tests it was concluded that the population sample was in agreement with the expectations of H-W equilibrium.

This also suggests that there is no evidence for mistyping due to the allelic dropout phenomenon (i.e. failure to amplify larger alleles from a heterozygote individual). Consequently, it can be concluded that PCR-analysis of the D1S80 locus generally permits correct genotyping.

Male and female Dutch Caucasians showed a similar distribution for their D1S80 allele frequency data (using a R × C contingency table ($\chi^2 = 11.84$, $0.6 < P < 0.7$).

A comparison of our data with a German [10], an American [9] and a Finnish [9] population sample was made (Fig. 2). Possible heterogeneity in D1S80 allele frequencies between the Dutch, German, US and Finnish population samples was assessed using a R × C contingency table. Deviation from population homogeneity was observed in this comparison ($P < 0.001$). However, the US sample was not statistically different from the Finnish sample ($0.2 < P < 0.3$) or the German sample ($0.1 < P < 0.2$). All other pairwise comparisons were found to be statistically different (Table 4). On closer examination of the allele frequency distributions among the 4 Caucasian population samples, it appeared that the differences in the frequency of the 31 allele may be the major cause of the deviation. Furthermore, the 18 and the 25 allele also deviate from population homogeneity. When a R × C contingency table test was performed comparing allele 31 and the sum of all other alleles among the 4 population groups, there was a significant difference ($P < 0.002$); however there was no difference among the German, US and Finnish samples ($0.1 < P < 0.2$) for this allele.

Additionally, comparing the 18 allele using 2 × 2 contingency tables, it was found that the Dutch and the Finnish samples ($P < 0.02$) deviated significantly for this allele. The 24, 28 and 29 D1S80 allele counts were not significantly different among the 4 population samples. Other alleles were too small in sample size for comparison.

The following two examples may illustrate the effects of the D1S80 allele frequency differences among the Caucasian population samples.

1. The maximum possible discrepancy among the 4 population groups for the expected most common D1S80 genotype frequency (18–24) is observed between the US ($f = 0.205$; 1 in 4.9) and the Dutch ($f = 0.165$; 1 in 6.1) population samples.

2. The highest discrepancy among the 4 population groups was observed when the expected 24–31 genotype frequencies were compared and occurred between the Dutch ($f = 0.094$; 1 in 10.6) and the US ($f = 0.029$; 1 in 34.5) population sample. It should be noted however that the numbers of individuals included in the population sample sets are too small for a great deal of confidence to be placed on the interpretation of these data.

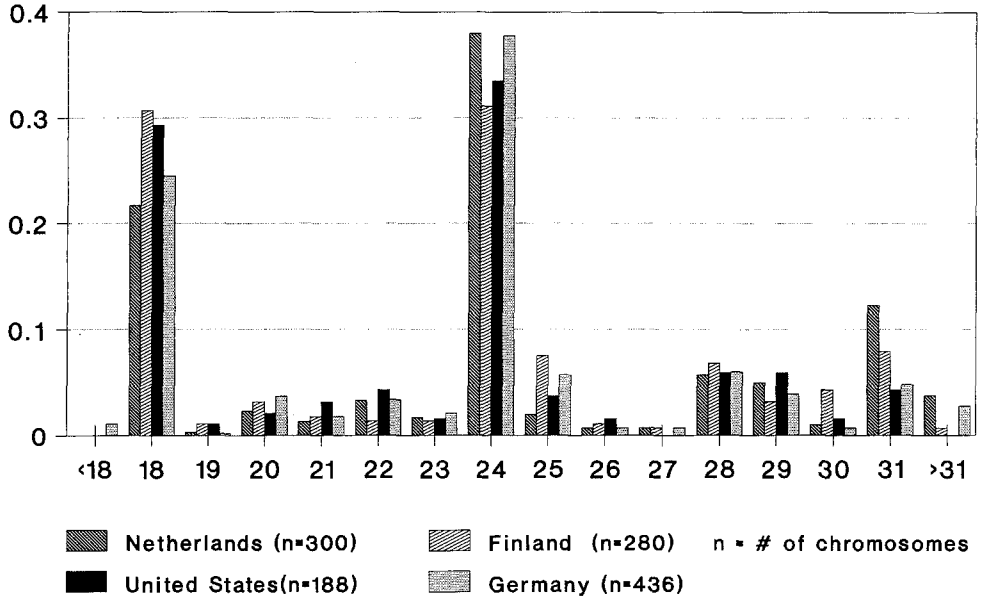

Fig. 2. D1S80 allele frequencies in four Caucasian population samples i.e. The Netherlands ($N = 150$), Germany ($N = 218$), Finland ($N = 140$), United States ($N = 99$). N refers to the number of individuals typed. *Bars* represent the point estimate frequency data

Table 4. P values for pairwise comparisons of D1S80 allele frequencies among 4 Caucasian population samples. The particular alleles that deviate significantly from population homogeneity are given in the last column between parentheses

Population samples compared	P	Individual allele deviation
NL × Ge	0.036 ± 0.006	(25*; 31**)
NL × USA	0.007 ± 0.003	(31**)
NL × Fi	<0.001	(18*; 25***)
Ge × USA	0.196 ± 0.013	
Ge × Fi	0.004 ± 0.002	(18*)
Fi × USA	0.218 ± 0.019	

Test of homogeneity between all 4 Caucasian sample sets: $P = <0.001$ (18*; 25*; 31***)

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$

NL = The Netherlands ($N = 150$); Ge = Germany ($N = 218$); Fi = Finland ($N = 140$); USA = United States ($N = 99$)

Somatic stability of the D1S80 locus

Pairwise comparisons were made between 21 stains of blood and semen and 20 stains of blood and saliva and no evidence for somatic instability of the D1S80 locus was found. All semen and saliva samples tested matched the corresponding blood samples for the D1S80 locus. An example of D1S80 profiles from different tissue sources is shown in Fig. 3.

Casework example

DNA recovered from soft tissue samples of decomposed human remains is often degraded [17]. In this case example we performed an identity test on a decomposed and distintegrated human male body. DNA for PCR analysis was isolated from the muscle and the skin tissues by standard procedures using proteinase K digestion and chloroform/phenol extraction [2]. The DNA concentration was estimated by minigel electrophoresis in 1% agarose NA (Pharmacia AB, Molecular Biology, Sweden) to which 1 µg/ml ethidium bromide was added. Concentration standards used were dilutions of genomic DNA from K562 cell-line (Promega).

High quantities of partly degraded DNA were obtained from the tissue samples, however the majority of the DNA that was recovered was less than 2000 bp in length. D1S80 profiles were obtained from the degraded DNA which originated from the muscle and the skin tissue samples. Subsequently the profiles were compared with DNA from the blood samples of the putative mother, father and sister (Fig. 4). In this example we could demonstrate mendelian inheritance of the parental alleles via the daughter and the victim could not be excluded as potentially being a member of this family. However in this case the statistical weight on the possible family association was not strong and on the basis of Single Locus Probe profiling of a tissue sample with less degraded DNA the possible relationship between both the putative parents and the victim was excluded.

Discussion

Several validation studies were done using a rapid and relatively simple procedure for the detection of the D1S80 variable region in the human genome. Making use of oligonucleotide primer sets and thermostable Taq Polymerase, reproducible and reliable typing has been demonstrated. The applied electrophoretic separation system appears to be sufficient for separation of the alleles. The PCR conditions for this system were evaluated by varying the concentrations of magnesium, Taq-polymerase and primers. The D1S80 typing system was also performed by testing DNA from different biological materials and by typing in actual case work. PCR analysis and subsequent high resolution polyacrylamide gel electrophoresis permits correct genotyping of an indi-

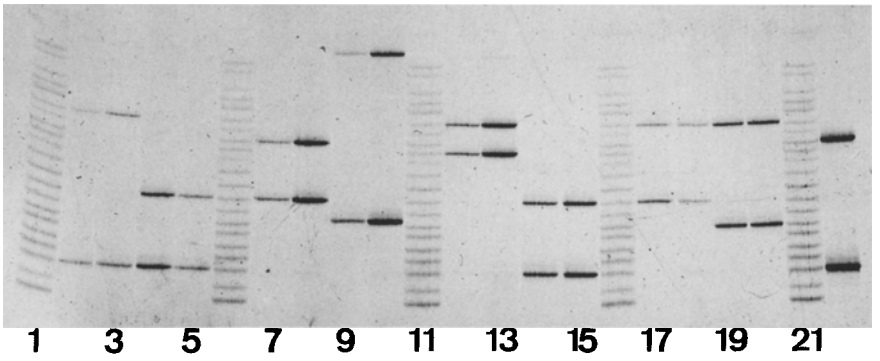

Fig. 3. Somatic stability of the D1S80 locus. Pairwise comparison between corresponding blood × semen samples. Lane 1, 6, 11, 16 & lane 21 are the FBI allelic ladder cocktail. The K562 genomic control DNA (genotype 18–29) is in lane 22. The blank (no template DNA) is not shown. Lane 2, 3 (genotype 18–31); lane 4, 5 (genotype 18–24); lane 7, 8 (genotype 24–29); lane 9, 10 (genotype 22^a–$39^?$); lane 12, 13 (genotype 28–31); lane 14, 15 (genotype 18–24); lane 17, 18 (genotype 24–31) and lane 19, 20 (genotype 22–31) represent pairwise comparisons of blood and semen from the same individual

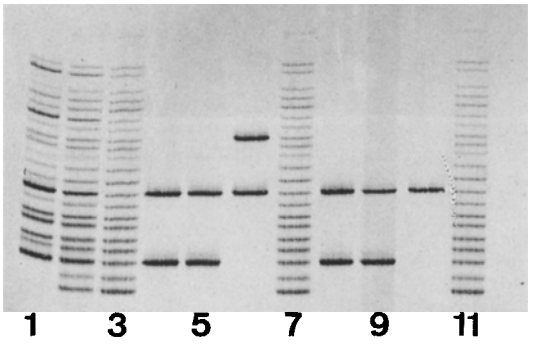

Fig. 4. Casework example. Identity testing of a disintegrated human body. D1S80 profiles from degraded DNA of different body tissues (muscle and skin) were compared with DNA from blood samples of the putative mother, father and sister. Lane 1, 2, 3, 7 and 11 are different allelic ladder cocktails. Lane 1 contains the Dutch allelic ladder cocktail. Lane 2 contains a 1:1 (v/v) mixture of the Dutch and the FBI alllelic ladder cocktail. Lane 3, 7 and 11 are the FBI allelic ladder cocktail. The blank (no template DNA) and the K562 genomic control are not shown. Lane 4, 8: represent DNA from the putative mother (genotype 18–24); lane 5: represents DNA from a skin sample of the victim (genotype 18–24); lane 6: represents DNA from the putative father (genotype 24–29); lane 9: represents DNA from a muscle sample of the victim (genotype 18–24); lane 10: represents DNA from the putative sister (genotype 24–24)

vidual for the D1S80 locus. The Dutch Caucasian population sample complied with H-W equilibrium expectations and can be used as a basis for estimating genotype frequencies for the general Dutch Caucasian population.

The sample set was subdivided according to sex and examined for differences in allele frequencies. No deviation was found between the male and the female frequency distribution. Deviation from population homogeneity was found in the comparison between the Dutch and the German, U.S. and Finnish population samples. The main cause for this deviation appeared to be the difference in the allele 31 frequencies. It must be taken into account however that the sample sizes are relatively small to make a significant comparison possible. This applies especially when comparing less common alleles for population homogeneity. Perhaps with a larger data sample it can be determined if the observed deviatons are real.

Typing of the D1S80 locus by the PCR technique is extremely sensitive. Subnanogram amounts of template DNA generated good signals. Additionally, the typing results from samples containing two different sources of DNA demonstrate the possibility of typing mixed body fluid samples. D1S80 types were consistent in the different tissues tested within an individual.

This D1S80 locus used for PCR analysis has a relatively low level of heterozygosity ($h^{obs} = 0.79$) when compared with the presently used VNTR loci typed by Southern blot hybridization analysis. This is due to the presence of two frequently occurring alleles (i.e. 18 and 24). Consequently, this PCR-based DNA-identification system has a lower discriminating power (DP = 0.94 in the Dutch population sample) than the Southern hybridization-based systems such as multi-locus DNA-typing or locus-specific DNA-profiling. Therefore, the occurrence of less informative PCR results in identity disputes will be relatively more frequent when compared with traditional methods of DNA-profiling. As shown in this report a victim was included as potentially being a son of a family by D1S80 typing. However Single Locus Probe profiling excluded this possibility. This observation can be anticipated because of the lower discrimination power of the D1S80 locus.

Thus, it is therefore desirable to have several more VNTR loci amenable to PCR availabe, in order to reach a high level of discrimination power. This, however poses no severe problem in view of the relative abundance of VNTR loci in the human genome. Several other VNTR systems that can be amplified by PCR have been described and they could potentially be included in the set of loci to be typed in forensic material. These include a VNTR-region at the 3′ end of the Apo B locus [18]; the D17S30 locus [19] and a highly polymorphic region 3′ to the human Type II alpha 1 collagen gene [20]. In addition, dimeric, trimeric and tetrameric short tandem repeats have a high degree of polymorphism which could lend themselves to forensic identity testing

[21]. Sextyping also can be included in a PCR-based identity test by amplification of the repetitive DYZ1 sequence in the distal Y chromosome long arm [22] or by amplification of the single copy X-Y homologous amelogenin gene [23].

In conclusion, using a defined amplification protocol and a high-resolution polyacrylamide gel electrophoresis method we obtained correct D1S80 typing results. DNA extraction, amplification and subsequent typing can be performed in one day. Furthermore the system is extremely sensitive. As little as 0.1 ng input of genomic DNA resulted in a visible typing result. Detection of the correct genotypes in mixed samples containing DNA from different individuals proved to be possible. Even if the majority type was in 20-fold excess, the minority type could still be amplified and typed correctly.

We have found no deviation from Hardy-Weinberg expectation in a Dutch Caucasian population sample.

Also, we have found no evidence for somatic instability of this locus.

It would appear that the analysis of the D1S80 locus by DNA-amplification is a viable candidate for application in forensic case work analyses.

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