TECHNICAL NOTE

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Population Genetics and Forensic Applications Usng Multiplex PCR (CSF1PO, TPOX, and TH01) Loci in the Basque Country*

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ABSTRACT: A population study in a sample of 200 unrelated individuals from the Basque Country (Northern Spain) was carried out using the GenePrint STR Multiplex System. The PCR products were electrophorized on a denaturing polyacrylamide gel and visualized by silver staining. The loci are TH01, TPOX, and CSF1PO. All loci meet Hardy-Weinberg expectations, and independence of alelles at these STR loci was found. A comparison with other population groups appeared to indicate that frequencies are well conserved in Caucasians, but differ from those of other racial groups. We have also calculated Fst as a measure of population subdivision. No appreciable genetic subdivision in the Caucasian populations studied here was found. Some statistical parameters of forensic interest (Pex, PM and PD) were also calculated. No exclusions were found in 100 mother-child and father-child meiosis. To evaluate the applicability of these systems to forensic casework, we studied the minimum quantity of DNA which can be used applying the multiplex methodology, and the minimum quantity that can be typed in a mixed sample. We also examined several samples such as hair roots, semen stains, vaginal swabs, blood stains and temporary teeth, each of these of varying ages.

KEYWORDS: forensic science, multiplex amplification, polymerase chain reaction, short tandem repeat, population genetics, DNA typing, TH01, TPOX, CSF1PO

The STR loci, or DNA microsatellite, may be amplified using two or more primer pairs together (1). This multiplex amplification strategy can be applied to the loci TH01 (2), TPOX (3), and CSF1PO (4).

We have used this procedure; the amplified products were separated on denaturing polyacrylamide gels with a resolution of a single base and silver staining. A population sample from Basque residents was analyzed and then we calculated its genetic-populational parameters (allelic frequencies, heterozygosity, fit to the Hardy-Weinberg equilibrium and non-allelic association). We also compared the gene frequencies obtained in our population sample with those in other populations. Finally, we studied the genetic diversity between populations from the same or different racial group using F statistics, thus contributing to a better understanding of the efficiency of these loci in population genetic profiling.

As for the technical aspects of these loci applied to analysis in forensic casework, we genotype the loci TH01, TPOX and CSF1PO in 13-year-old bloodstains. The similar efficiency of amplification for every allele is also very important when there are mixtures of DNA from two or more individuals, e.g., in vaginal swabs. We also proved the possibility of identifying the mixtures of DNA from different people, one of them being just 5% of the sample. Mixtures were also studied in syringes used by drug-addicted people. The forensic validation was performed in hair roots and temporary dental pulps also.

Materials and Methods

Population Study

Sample—The population sample was of 200 Basque Country resident individuals. These individuals represent a pool of Northern Spain populations, with some components from the West and Southwest Iberian Peninsula populations, but excluding Mediterranean groups.

Multiplex Amplification—Amplifications were carried out preferentially in a Perkin-Elmer 2400 thermalcycler using the CSF1PO, TPOX, and TH01 GenePrint™ multiplex system (5) with 1 μL of BSA (10 μg/μL) per reaction. The quantity of DNA template was 30 ng. The amplification product was checked by electrophoresis in 2% agarose gel with ethidium bromide.

Genetic Typing of Multiplex Amplification Products—Electrophoresis was carried out in denaturing acrylamide gels (4%, 7 M urea and 0.4 mm thick) in a Cambridge electrophoresis unit. Allelic ladders were applied every two lanes. The gels were run at 1500 V. Bands were detected by silver staining as described by Budowle et al. (6).

Statistical Approaches—An unbiased estimate of heterozygosity was obtained following Nei (7). Polymorphism Information Content (PIC) values were obtained as described by Botstein et al. (8). To check possible departures from HWE proportions, three

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different tests were employed: the first, proposed by Odelberg et al. (1989) (9), the second, the exact test G statistic (Sokal and Rohlf) (10), and finally, the Guo and Thompson exact test (11).

Genotypic disequilibrium among pairs of loci, i.e., statistical independence between loci, was tested as described by Ohta (12).

The allele frequency distributions of these loci in the population studied in the present work were compared with those from other populations previously published by the χ^2 test. Those alleles with frequency lower than 5 were discarded.

The neutralist models, Infinite Allele Model (IAM) (13–15) and the one-step Stepwise Mutation Model (SMM) (16), according to Chakraborty and Daiger (17), are useful to test substructuring by comparing the number of rare alleles with the expectations of neutral models. They can therefore serve as an evidence when the observed number of rare alleles is greater than that expected (18).

The population subdivision in the Caucasian group was assessed by the Fst test following Weir and Cockerham (19).

Forensic Validation—The sensitivity studies were performed by analyzing the cell line K562 DNA (Promega) and artificial blood mixtures. The K562 DNA was serially diluted from 10 to 0.02 ng/μL. Artificial blood mixtures were made from heterozygous and homozygous individuals. White cells of each sample were previously counted for mixing the samples in defined ratios prior to the DNA extraction (1:1, 1:4, 1:10, 1:20 and 1:100). DNA extraction of whole blood was performed from 200 μL using the phenol-chloroform method (20). The DNA concentration was adjusted to 30 ng/μL.

Twenty bloodstains had been stored at room temperature for 13 years. A half of each 5-mm-diameter bloodstain was used to extract DNA using Chelex-100 chelating resin (21) with some modifications. The other half of some bloodstains was extracted using the phenol-chloroform method; quanfication of DNA extracted with phenol-chloroform was determined by fluorimetry.

Hairs from 15 individuals were pulled out or shed and were analyzed a month after collection. The DNA was extracted by the rapid lysis procedure. Every hair root was incubated in 200 μL of the PCR buffer with 10 μg Proteinase K, for 30 min at 56°C. After boiling for 10 min it was mixed for 10 s, and spun at 12 000 rpm.

Fifteen semen stains were stored for 6 months before analysis. The DNAs from semen stains were extracted using phenol-chloroform or Chelex resin. The quantities of DNA extracted with Chelex resin were measured by slot-blot using the probe D17Z1 (Gibco-BRL) following the manufacturer's protocol.

Fifteen vaginal swabs were collected after 24 post-coital hours. Differential lysis of spermatic and epithelial cells from vaginal swabs was performed as described by Gill et al. (22).

Teeth were extracted from 15 children with orthodontic problems; a small cotton piece, saturated with blood from the tooth extraction, was also collected in every case. The DNA from the tooth was extracted as described previously by Smith et al. (20), but using only a phenol-chloroform extraction.

Results and Discussion

Population Study

The resolution capacity of the technical approach used is one base pair as shown in an individual with 9.3 and 10 TH01 alleles (Fig. 1*a*).

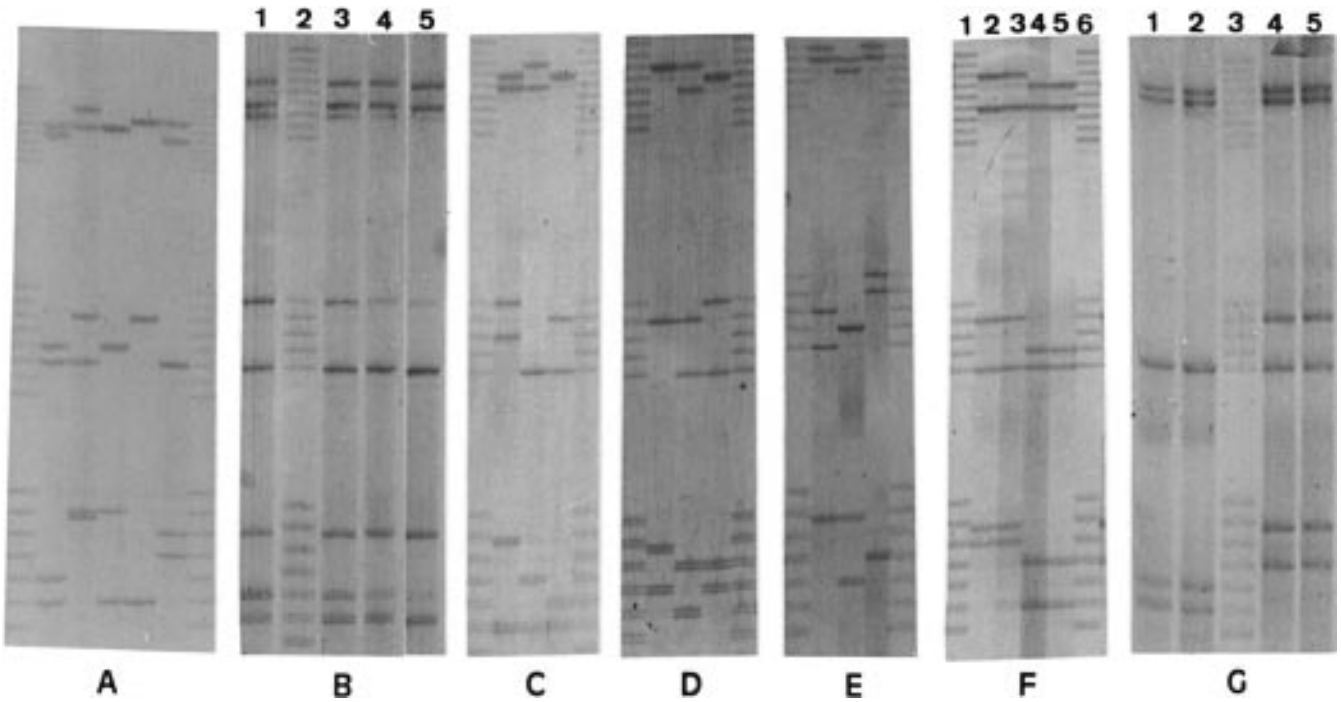

FIG. 1 *Silver-stained DNA profiles of the three simultaneous amplified STR loci: TH01, TPOX and CSF1PO. (*A*) 9.3-10 TH01 genotype; the resolution of denaturing polyacrylamide gels is a single base. (*B*) Sensitivity studies by analyzing mixed DNA samples from two individuals, 1 and 2, 7-7/12-12/9-10 and 6-9.3/8-8/10-12, respectively. Lane 1: 50% each DNA: lane 2: (*L*); lane 3: 75% DNA from the individual 2 and 25% from the individual 1; lane 4: 90% (2) and 10% (1) and lane 5: 95% (2) and 5% (1). (*C*) Blood stains: 13 years old. (*D*) Hair roots: 30 days old. (*E*) Semen stains, stored indoors for up to six months. (*F*) Vaginal swabs showed the male and female profiles. Lane 1: DNA extracted from the male donor blood; lane 2: male DNA extract from a vaginal swab; lane 3: female DNA extract from a vaginal swab; lane 4: DNA from the female donor blood. (*G*) Genetic profiles from temporary teeth and the respective DNAs from blood. Lanes 1–2 and 3–4: DNAs from teeth and blood of two children.*

TABLE 1—*Allele frequencies for the three STR systems in the Basque resident population, three different tests for Hardy-Weinberg equilibrium, heterozygosity, rare alelle test and medico-legal parameters.*

Locus CSF1PO		Locus TPOX		Locus TH01	
Allele	Frequencies	Allele	Frequencies	Allele	Frequencies
9	0.0275 ± 0.008	8	0.5225 ± 0.025	6	0.2300 ± 0.021
10	0.2575 ± 0.022	9	0.0850 ± 0.014	7	0.1600 ± 0.018
11	0.3125 ± 0.023	10	0.0625 ± 0.012	8	0.1225 ± 0.016
12	0.3375 ± 0.024	11	0.2925 ± 0.022	9	0.2100 ± 0.020
13	0.0500 ± 0.011	12	0.0375 ± 0.000	9.3	0.2600 ± 0.022
14	0.0125 ± 0.005			10	0.0175 ± 0.006
15	0.0025 ± 0.002				
Heterozygosity observed 0.70 expected 0.721 ± 0.009		Heterozygosity observed 0.615 expected 0.6305 ± 0.018		Heterozygosity observed 0.82 expected 0.796 ± 0.005	
Odelberg test	$p = 0.479$	Odelberg test	$p = 0.392$	Odelberg test	$p = 0.335$
G test	$p = 0.539$	G test	$p = 0.995$	G test	$p = 1$
Guo and Thompson test	$p = 0.111$	Guo and Thompson test	$p = 0.745$	Guo and Thompson test	$p = 0.250$
Rare allele test	$p = 0.703$	Rare allele test	$p = 0.703$	Rare allele test	$p = 0.412$
PIC	0.665	PIC	0.573	PIC	0.762
Pex	0.446	Pex	0.386	Pex	0.590
PM	0.134	PM	0.218	PM	0.074

Allelic frequencies, expected heterozygosities and PIC for the 3 loci studied here are presented in Table 1.

The studied population sample is in Hardy-Weinberg equilibrium for the three systems as indicated by the results of the Odelberg et al. test and the G and Guo and Thompson test (Table 1). In addition, the rare allele test shows no significant differences between the numbers or rare alleles expected and observed (Table 1), and this is therefore indicative of no population substructure.

It is highly interesting to study the comparison of population databases with forensic genetics. The population resident in the Basque Country is made up of groups from different parts of the Iberian Peninsula, such as Central Spain and Andalusia (Southern Spain). An χ^2 comparison between the Basque Country population and the databases of the aforementioned regions of Spain (23,24) does not yield statistically significant differences.

Other Caucasian populations compared with our population sample were Germany (25), Switzerland (26), and Caucasian-America (5).

As has been observed in studies of other STR loci (27–32), there are practically no statistically significant differences in the allele frequency distributions of the various Caucasian populations studied. However, our population and a German population (33) differ with values of $\chi^2_{4\text{d.f.}} = 12.116$ and $p = 0.016$. On the other hand, the German population sample studied by Wiegand et al. in 1993 (34) was also compared, and no statistically significant difference was found. The observed dissimilitude was due to the 9 repeat alleles from the population sample studied by Puers et al. (33), which shows the lowest frequency (0.114) in the Caucasian populations compared here. This difference may be a causal effect due to the sampling process and therefore does not contradict the overall observation that great similarities exist among the Caucasian populations. Chinese (35), Japanese (36), African-American and Hispanic-American populations (5) were also compared. The differences between inter-racial populations are more accurate. So, the TH01 and TPOX loci show significant differences between our population and Asiatic, African-American and Hispanic-American population samples. The CSF1PO locus also shows differences with Asiatic and African-American samples, but no differences were found with Hispanic-American samples ($\chi^2_{4\text{d.f.}} = 1.297$ and $p = 0.863$). Gill and Evett (37) observed differences between racial groups (Asian, Caucasian and African) in the TH01, VWA, FES and F13AI loci, although the allele frequency distributions were similar within racial groups. The differences in the allele distributions of different races could indicate the potential usefulness of these markers as race markers (38). However, the only locus that probably displays a race-specific allele is TPOX because the 14-repeats allele appears only in Asiatic populations, but it was not found in other population samples such as Caucasians (Galicia (Northern Spain) (39) and Northern Portugal (40)), African-Americans and Hispanic-Americans. Thus, it is necessary to study more African, Asiatic and other populations in order to confirm this hypothesis. The Hispanic-American group shows a intermediate allelic distribution, almost as if it were a mixture of Caucasian, Asiatic and African groups.

The average Fst value for the TH01, TPOX and CSF1PO loci is 0.00018, which does not indicate appreciable genetic subdivision in these Caucasian populations (tests combination—Fisher's method—$\chi^2_{6\text{d.f.}} = 6.665$, $p = 0.353$). The average Fst value is two orders of magnitude higher when the different racial groups are tested (Caucasian populations, Asiatic, African-American and Hispanic-American), namely Fst = 0.0199 and $p < 10^{-6}$. This value is similar when Caucasian and African-American or Caucasian and Asiatic populations are pooled (Fst = 0.0131 and Fst = 0.0151; both are highly significant). The previously calculated Fst values in Caucasian populations are variable, ranging between 0.0015 and 0.0048 (37,41). This study presents Fst = 0.00018 for Caucasian loci. The variation of Fst depends on the populations and loci studied. Thus, in our study, Fst calculated for the TH01 locus shows a clear population substructure between racial groups (Fig. 2). However, the CSF1PO locus shows substructure between Caucasian and African-American and Caucasian and Asiatic populations, but no substructure is found between Caucasian and Hispanic-American. Moreover, population substructure is not detected in the TPOX locus between Caucasian-Hispanic American and Caucasian-Asiatic populations. Balding and Nichols (42) proposed that the values of Fst for different loci will tend to be similar because the loci will share the same history of population foundation, growth and immigration. Conversely, Fst may differ among loci due to differences in the mutation process. This cannot be assumed in our study because the mutation rate seems to be similar at the three loci. The most simple explanation of the different Fst

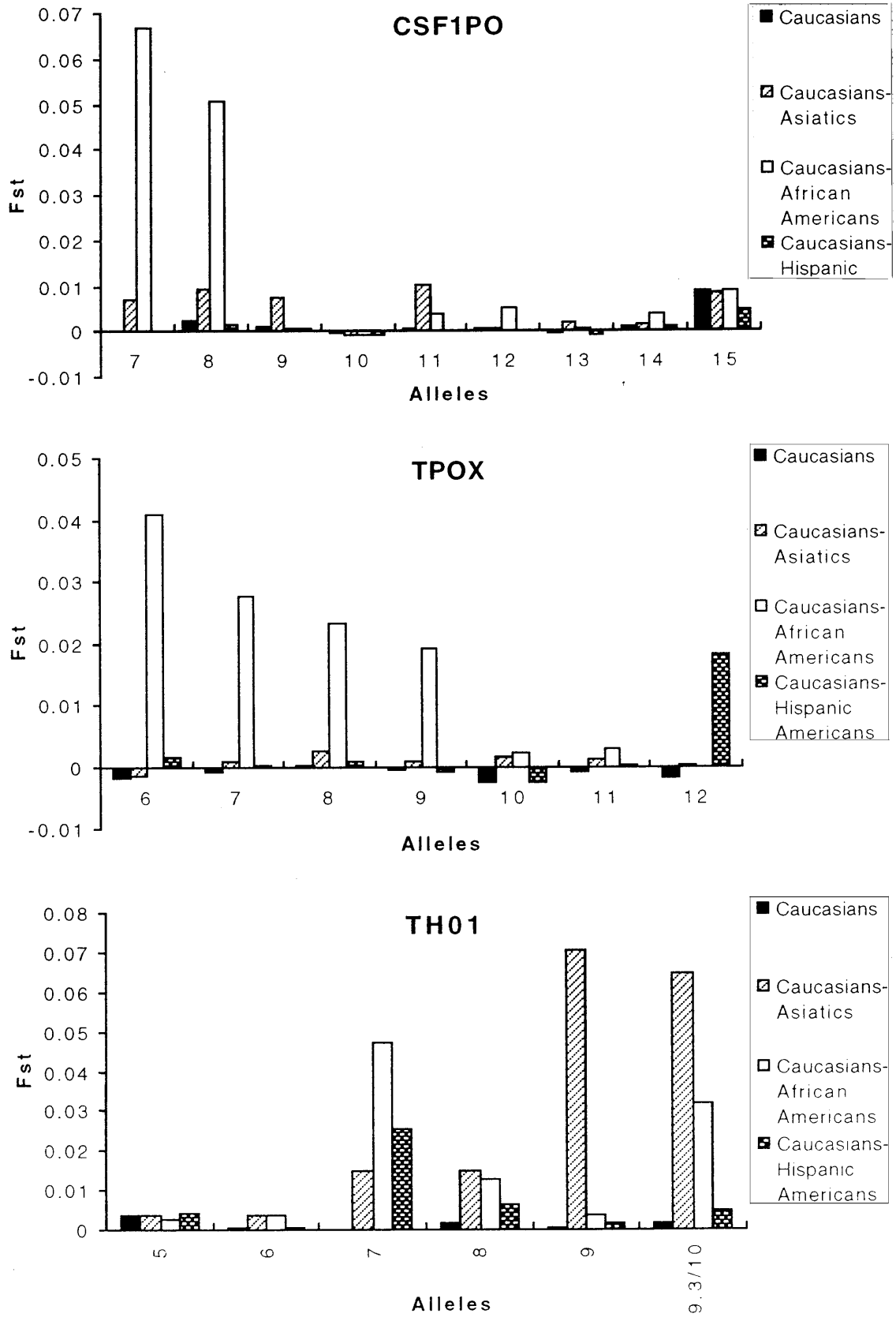

FIG. 2—*Fst values between Caucasians and other racial groups for the three STR systems.*

values per locus may be offered by the intrinsic nature of F statistics. These statistics refer to the extent to which different populations within species have differentiated over the time since the ancestral population, by the process of genetic sampling, or drift, between successive generations (19). Due to the random fluctuation introduced by the drift factor in genetic frequencies, the Fst values vary in the different loci when they are studied within the same population pool, as can be seen in the results of our work.

Parameters of forensic interest are given in Table 1. The loci studied here fit to Hardy-Weinberg equilibrium based on the Odelberg, Guo and Thompson and G tests, so this state of equilibrium can be used to predict the frequency of a determined genotype of each locus in the Basque Country population. On the other hand, because no evidence of allelic association between these loci was obtained, it is then possible to apply the product rule in order to estimate the probability of having a combined genotype for the three loci. Based on the Hardy-Weinberg equilibrium and non-allelic association between loci, we have calculated the combined medico-legal parameters: Pex, PM and ID. The combined values are Pex = 0.864, PM = 0.002 and PD = 0.998. As can be expected from the similarity of gene frequencies distributions within Caucasian populations, our Pex and ID values show no differences from other European and Caucasian American populations. In all cases, these values were approximately three times lower than those calculated in African-Americans. In conclusion, the databases have been established for CSF1PO, TPOX and TH01 loci in the Basque Country population, and so it could be used there for human identity testing.

Family Studies

One hundred mother-child and father-child meiosis were investigated. No mother-child exclusions were found, nor were exclusions detected in those cases where paternity had previously been confirmed. The inheritance analysis of the STR loci alleles in 100 meiosis showed that no mutations affecting fragment length or mobility occurred. The employment of denaturing polyacrylamide gel electrophoresis provides an extraordinary degree of precision (33). Using allelic ladders, it is even possible to identify single base variants (33). On the other hand, the system of analysis which we used does not allow us to rule out the existence of mutation due to base changes. However, according to Urquhart et al. (43,44) and Möller and Brinkmann (45), the STR loci do not seem to have significant microvariation in their sequences. So, the mutation rate could be reasonably low, and the probable reduced microvariation does not prevent the use of this multiplex analysis in paternity casework.

Forensic Validation

Sensitivity Studies—Two approaches were used to establish the multiplex typing sensitivity of the 3 STR loci: (*a*) to determine the minimum amount of the K562 cell line DNA that may be typed after amplification, and (*b*) to study the minimum amount of DNA from an individual to be detected if a mixture of DNAs from two different people has been amplified. The minimum amount of typed K562 DNA was 0.1 ng and 5% was the lowest percentage of a DNA in a 100 ng mixture, which yields a clear typing of the 3 loci simultaneously (Fig. 1*b*). But this result indicates that the minimum DNA quantity required from the less-represented DNA is 5 ng. This is very high compared with the expected amount (50 times higher) and it demonstrates that the simultaneous presence of two different templates of DNA gives rise to a competition between them, where the most represented individual has an advantage due to the exponential nature of the PCR progress.

Artificial Forensic Samples—We have studied some experimental samples such as bloodstains, hair roots, semen stains, vaginal swabs and temporal teeth from individuals whose genetic profile had previously been determined, except in the case of bloodstains.

Twenty 13-year-old blood stains were analyzed. Their DNAs were highly degraded (the largest fragments were less than 2000 bp). It was possible to know if the typing was correct because every stain was cut through the middle, then two small specimens from each half were separately analyzed (Fig. 1*c*). The same combined genotype was obtained in 19 pairs of samples, but one pair could not be related because one sample did not yield a clear profile.

Hair roots, both recently pulled out and 30 days after pulling out, yielded good results (Fig. 1*d*). The exposure to indoor conditions for a month did not affect the usefulness of the hair vestiges. However, no results were observed from shed hairs. The poor results are probably due to the more accurate effect of degradation when the number of attached cells to the hair root is low, as this reduces the possibility of finding DNA fragments suitable for analysis and, on the other hand, most of the hairs probably died before shedding. This factor also reduces the possibility of finding intact DNA fragments to be used by PCR. So, this methodology is not appropriate to individual identification of shed hairs; instead, the study of mtDNA as proposed by Wilson et al. (46) is a reliable means of forensic identification of shed hairs.

The semen stains, stored indoors for six months, showed exact genetic typing when compared with the genetic profile obtained from the blood (Fig. 1*e*) in 90% of the cases; in the remaining 10%, partial types were observed. These results were similar when phenol-chloroform or Chelex DNA extraction procedures were used.

The DNA from vaginal swabs was quantified both from vaginal and spermatic phases. The human DNA quantification (D17Z1 probe) from vaginal phase showed values ranging from 8 to 1600 ng and the quantification of spermatic phase from 0 to 400 ng. The difference between the total DNA quantified by fluorimetric and D17Z1 probe was particularly high. DNA quantification performed by fluorimetric methods is not a reliable estimate of human DNA concentration, as was established by Prinz et al. (48). The microscopic slides of swabs show high microorganism contamination and, so, the number of epithelial female and spermatic cells is very reduced in comparison to the bacterial and yeast cells. Therefore, the concentration of vaginal swab DNAs should be measured using the human DNA concentration when samples are highly degraded (47). Prinz et al. (48), studying urine samples, found that excess amounts of co-extracted non-human DNA can inhibit the specific amplification of human target sequences. In contrast, the human DNA from swabs studied here could be amplified in every case.

The vaginal swabs showed the male and female profiles after differential lysis of the samples (Fig. 1*f*). The genetic typing of spermatic phase DNA from swabs collected 24 hours post-coitus was obtained in 85% of the cases. However, when the vaginal swabs were collected 1 hour post-coitus, the genetic typing was obtained in 100% of the cases.

Another practical approach to detection of DNA mixtures was performed by the analysis of syringes used by drug-addicted people. The fact that there was very little blood remaining in the

syringes yielded a total amount of extracted DNA of less than 5 ng in most of the samples. However, this was not a problem because two different DNA profiles were observed in some samples from syringes without a visible/evident amount of remaining blood. These results confirm that the quantity of DNA in mixed samples is a minor problem when the proportion between the different DNAs is not very disequilibrated.

Dental pulps from temporary teeth were also typed. Figure 1*g* shows the same genetic profiles from temporal teeth as those displayed by the respective DNAs from blood. Dental and roentgenographic records allow a rapid personal identification in major disasters, traffic accidents, fires, etc. In addition, recent studies have been focused on DNA analysis of dental pulp. Such a procedure can be successfully applied to sex determination, VNTR (pYNH24, Apo 3′, D1S80), HLA-DQA1 polymorphisms (49,50) and STR polymorphisms determined from dental DNA (51). On the other hand, most children have no dental records, so it is not possible to perform a morphologically based identification. Therefore, we were especially interested in temporary teeth because this is a molecular genetic approach to childhood identification. We have studied DNA only from recently extracted temporary teeth. The cellularity of these pulps is very high, so it is very easy to obtain the genetic profile from childhood teeth.

Finally, we have been able to observe the somatic stability of these loci, as the analysis of the DNA extracted from semen, vaginal mucous, hair and temporary teeth showed—in all the cases—identical DNA profiles to those of the DNA from blood samples of the corresponding individuals.

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