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TECHNICAL NOTE

CRIMINALISTICS

María Castañeda,[1] *M.S.; Verónica Mijares,*[1] *José A. Riancho,*[2] *M.D., Ph.D.; and María T. Zarrabeitia,*[1] *M.D., Ph.D.*

Haplotypic Blocks of X-linked STRs for Forensic Cases: Study of Recombination and Mutation Rates*

ABSTRACT: In complex kinship cases, markers situated in haplotypic blocks may provide additional clues to other unlinked markers. We have established a protocol to amplify six X-chromosome microsatellites, located in two haplotype blocks, using PCR with fluorochrome-labeled primers and capillary electrophoresis. The segregation stability was explored in 92 unrelated families with individuals from three generations. Sixty-one different haplotypes were found in the DXS10079-DXS10074-DXS10075 block in the grandfathers and 96 in the mothers, with estimated haplotype diversities of 0.9828 and 0.9842, respectively. Fifty and 73 different haplotypes were found in the DXS6801-DXS6809-DXS6789 block in the grandfathers and the mothers, with estimated haplotype diversities of 0.9711 and 0.9742, respectively. We observed 10 between-cluster and one within-cluster recombinations in 99 female meioses. The overall per-locus mutation rate was 0.0034. This protocol allows for the characterization of the alleles of two sets of linked markers of the X-chromosome that can be useful in complex forensic cases.

KEYWORDS: forensic science, DNA typing, short tandem repeat, X-chromosome, haplotypes, recombination, mutation, kinship

Microsatellites of the X-chromosome have emerged in recent years as a useful tool in kinship analysis and other forensic cases (1,2). As the fathers transmit the same X-chromosome to all their daughters, these markers are particularly useful in deficiency cases, when the child is female and the alleged father is unavailable for testing. For example, it can lead to paternity exclusion if two sisters or half sisters are available because female descendents share the same paternal allele (3–6). Several multiplex protocols have been published to study sets of markers distributed along the chromosome (7,8). In theory, unlinked markers are preferable because the power of discrimination (PD) is usually higher when independent loci are considered. However, in certain complex cases it is more useful to have clusters of linked markers, as haplotype blocks may allow for the reconstruction of the chromosomal structure of the ancestors (9–12). Therefore, the aims of this study were to establish a protocol to amplify six short tandem repeats (STRs), which have been reported to lie within two independent clusters (13,14) and to analyze the mutation rates and the within- and between-cluster recombination rates by studying three-generation families.

Materials and Methods

Individuals

DNA samples were extracted from unrelated volunteer students and their families. They were Caucasians of Spanish ancestry living in Santander, a 200,000-habitant city in Northern Spain. Ninety-two families were included: 85 with a son, five with two sons, one with three sons, and one with four sons. The purposes of the study were explained to potential study subjects. After they gave verbal informed consent, buccal swabs were obtained and labeled by a non-identifiable family-generation code to preserve privacy.

DNA Extraction and Amplification

Genomic DNA was obtained from buccal swabs using a commercial method (Prep Mini Spin kit; GE Healthcare Spain, Barcelona, Spain). The amount and purity of the DNA was checked by light absorbance at 260/280 nm using a Nanodrop-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The sequences of the primers used to amplify DNA by polymerase chain reaction (PCR) are shown in Table 1. The forward primers were 5′-labeled with fluorochromes (Applied Biosystems, Foster City, CA). PCR amplification was carried out in a Gene Amp PCR system 9700 (Applied Biosystems), by mixing 5 μL of Multiplex PCR master mix (Qiagen, Hilden, Germany), 1 μL (10 pmol) of each primer, and 5 ng of genomic DNA in an 18 μL PCR final volume. Cycling conditions consisted of an initial denaturation step for 15 min at 95°C, followed by 10 cycles with denaturation at 94°C for 30 sec, annealing at 60°C for 90 sec, and an extension at 72°C for 60 sec.Then 20 cycles at 94°C for 30 sec, 58°C for 90 sec, and 72°C for 60 sec, followed by a final extension at 60°C for 60 min.

Fragment Analysis

An aliquot of 1.5 μL of PCR product was mixed with 24.5 μL formamide and 0.5 μL Gene Scan 500 Liz (Applied Biosystems) as an internal size standard. It was heated at 95°C for 5 min and

[1]Unit of Legal Medicine, University of Cantabria-IFIMAV, Av Herrera Oria s/n, Santander 39011, Spain.
[2]Department of Medicine, University of Cantabria-IFIMAV, Av Herrera Oria s/n, Santander 39011, Spain.
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TABLE 1—*Primer sequences (13,17).*

Primer	Sequence	Label
DXS6801		
Forward	CATTTCCTCTAACAAGTCTCC	PET
Reverse	CAGAGAGTCAGAATCAGTAG	–
DXS6809		
Forward	TCCATCTTTCTCTGAACCTTCC	VIC
Reverse	TGCTTTAGGCTGATGTGAGG	–
DXS6789		
Forward	CTTCATTATGTGCTGGGGTAAA	NED
Reverse	ACCTCGTGATCATGTAAGTTGG	–
DXS10079		
Forward	AGATTGTGCCAATGCTCTCC	PET
Reverse	GTTTGCCTGTGTTGTAACATCCTT	–
DXS10074		
Forward	ACTTCCTACTGCCCCACCTT	PET
Reverse	GTTTCCCCTCAGAGAGCTGACACA	–
DXS10075		
Forward	AGGAGGGGCCTAGACAAGTG	PET
Reverse	CAGATTATGCTTGGGCCTGT	–

quenched at 4°C for 5 min. It was injected into an ABI Prism 310 sequencer (Applied Biosystems) to separate and detect the PCR products by capillary electrophoresis with the denaturing polymer POP4 (Applied Biosystems), the electrophoresis voltage was 15 kV, and the injection time was 5 sec. Fragment size was analyzed by the Southern method (15,16) using the GENEMAPPER ID v3.2 software (Applied Biosystems). As controls, we used DNA from the immortalized cell lines K562 and 9947A. The alleles at the six loci were concordant with previous reports (13,17). Allele designation was based on PCR product size and comparison to known samples and control genotypes.

Data Analysis

Allelic frequencies were estimated by counting the female genotypes. The Hardy–Weinberg equilibrium (HWE) was tested with GENALEX software (http://www.anu.edu.au/BoZo/GenAlEx). The forensic efficiency at a single-locus level was calculated with the formulae published by Desmarais (18). In particular, the PD was computed as an index of efficiency in identity cases, and the power of exclusion (PE) as an index in kinship cases. Haplotype frequencies were computed by counting the haplotypes in the grandfathers' and mothers' samples, and the haplotype diversity for each block was estimated as $1 - \sum P_i^2$, where P_i is the individual haplotype frequency. The haplotypes in the mothers were elucidated by comparing the mother's genotypes with the grandfather's haplotype. The existence of recombination in the maternal meioses was analyzed by the comparison of the maternal haplotypes and the haplotypes in the sons. The genetic distance was estimated using Haldane's Mapping Function (19). The possibility of linkage disequilibrium was estimated with GDA software (11). The mutation events were studied in both meioses (grandfather–mother and mother–son). The increase or decrease in one repeat was assumed based on the 4 bp change in the fragment size but not confirmed by sequencing. In some families, additional X-chromosome STRs (DXS8378, DXS9898, DXS7133, GATA31E08, GATA172D05, DXS7423, DXS7132 and DXS9902) (17,20), located outside the two blocks (Fig. 1), had to be studied to elucidate whether a mutational event or a recombination event had occurred.

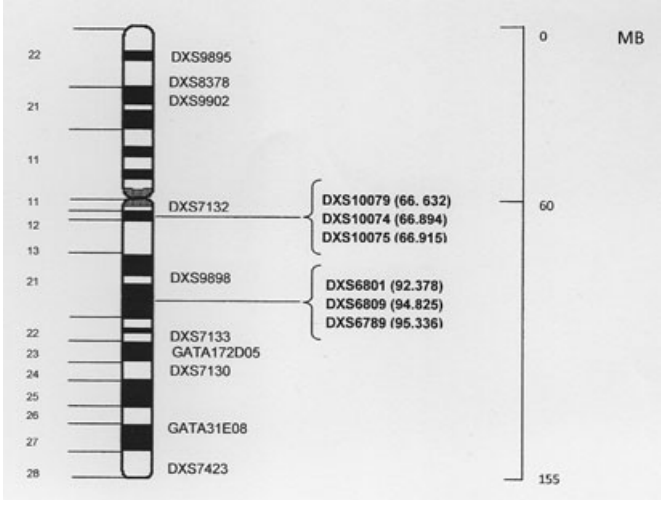

FIG. 1—*Location of the two linked marker groups on the X-chromosome. Other common markers and the cytogenetic bands are also shown. The numbers in parentheses represent the chromosomal position in kilobases (based on references [2] and [3]).*

Results

Sextaplex Performance

The six STRs were successfully amplified in a single reaction, as shown in Fig. 2. Allele designation proposed here is in compliance with the recommendations of the International Society for Forensic Genetics (21).

Segregation Stability

We studied six STRs in three-generation families, including a mother, at least a son and his maternal grandfather. These markers have been previously reported to be located in two clusters in the long arm of the X-chromosome (13,14) (Fig. 1). Some maternal meioses were not informative because women were homozygotes for three markers of the cluster. Therefore, the occurrence of recombination could be studied in 99 meioses. Ten between-cluster recombinations occurred, resulting in an estimated genetic distance of 11.16 cm. Two apparent within-cluster recombinations were found, but after studying additional markers, one of them was identified as a single-locus mutation. Thus, only one recombination event was found within the linked clusters. It occurred between loci DXS6801 and DXS6809 (Table 2).

Overall, four mutations were found: one in DXS10079 from grandfather to mother, one in DXS6789, and two in DXS10074 from mother to son. In two cases, the gain of one repeat unit was observed, whereas a loss of one repeat was found in the other cases. Therefore, the global mutation rate was 0.020, and the average per-locus rate was 0.0034.

Allelic Frequencies and Forensic Efficiency

The allelic frequencies (available from the authors upon request) were similar to those previously published in other populations (13,14). Alleles in female samples did not reveal deviations from HWE (Table 3). DXS10074 and DXS10079 showed the highest forensic efficiency, with a PD of 0.9580 and 0.9467 in women, and 0.8439 and 0.8247 in men, with a PE in trio cases of 0.8263 and 0.8021, respectively, and with a PE in motherless cases of 0.7184 and 0.6856, respectively (Table 3). The pair-wise analysis of linkage disequilibrium revealed only significant p-values for locus DXS10074-DXS10075 (p =0.0037) and DXS10079-DXS6801 (p = 0.0310). The haplotype list is available from the authors

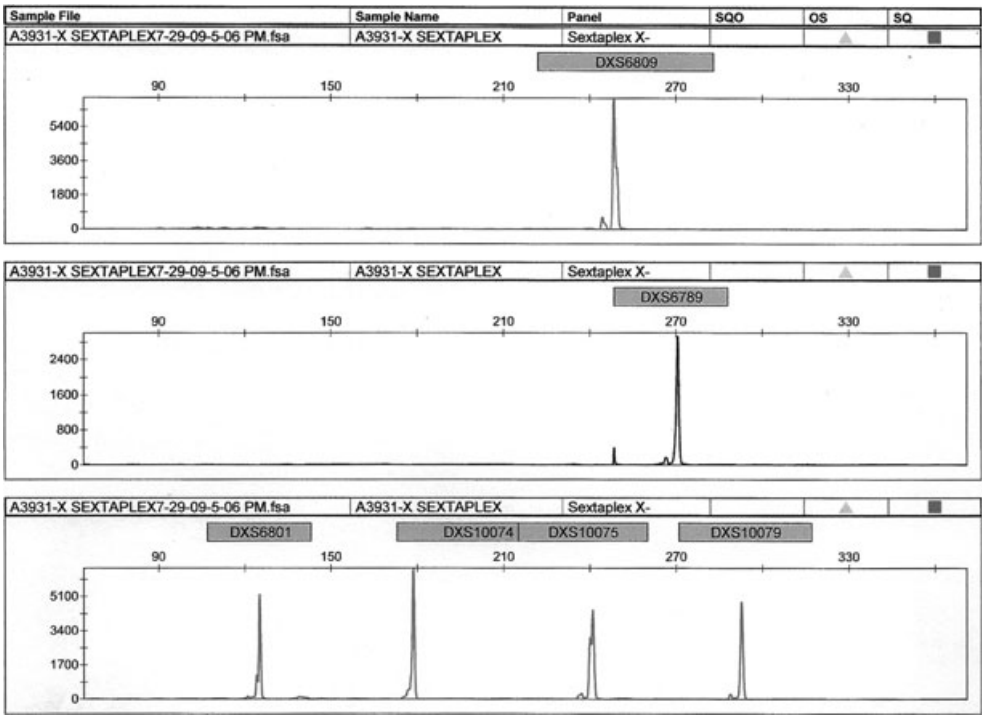

FIG. 2—*Typical electropherogram of the six markers in a male individual, as shown in the ABI 310 sequencer output. The* x*-axis represents the fragment size in pairs of bases and the* y*-axis represents the height of peaks in relative fluorescent units. The small peaks in front of the main peaks represent stutter band peaks.*

TABLE 2—*Example of a family trio that shows a recombination within the linked cluster.*

	DXS10079	DXS10074	DXS10075	DXS6801	DXS6809	DXS6789
Grandfather	19	15	17	12	34	20
Mother	19, 21	8, 15	17	8, 12	31, 34	20, 21
Grandson	21	8	17	8	34	20

Grandfather's alleles are underlined.

TABLE 3—*Forensic efficiency parameters.*

	DXS10079	DXS10074	DXS10075	DXS6801	DXS6809	DXS6789
PD-female	0.9467	0.9580	0.8617	0.7791	0.9324	0.8790
PD-male	0.8247	0.8439	0.6963	0.5945	0.7999	0.7234
PE-trio	0.8021	0.8263	0.6503	0.5380	0.7723	0.6790
PE-mo	0.6856	0.7184	0.5058	0.3914	0.5386	0.6480
p-value	0.5976	0.8767	0.9828	0.4003	0.6590	0.9086
Het male		0.9828			0.9711	
Het female		0.9842			0.9742	

PD-female, single-locus power of discrimination in female cases; PD-male, single-locus power of discrimination in male cases; PE-trio, single-locus power of exclusion in trio cases; PE-mo, single-locus power of exclusion in motherless cases; *p*-value, *p* of the Hardy–Weinberg equilibrium test; Het male, Haplotype heterozygosity in each block (in grandfathers); Het female, Haplotype heterozygosity in each block (in mothers).

upon request. Considering the two blocks independently, 61 different haplotypes were found in the DXS10079-DXS10074-DXS10075 block in the grandfathers and 50 in the DXS6801-DXS6809-DXS6789 block. There were 39 unique haplotypes in the first block and 32 in the second one, with a haplotype diversity of 0.9828 and 0.9711, respectively. Similar results were found in the mothers: 96 haplotypes were found in the first block and 73 in the second block, with estimated haplotype diversities of 0.9842 and 0.9742, respectively.

Discussion

In this study, we found single-locus allelic frequencies similar to those previously reported for these markers in other Caucasian populations (13,14). The study of three-generation families allowed us to scrutinize the stability of the two clusters of linked microsatellites across the meioses, as well as the mutation rates.

We found no within-cluster recombination events at the Xq12 region, which was not unexpected, as those markers (DXS10079,

DXS10074, and DXS10075) are located within a 280 Kb region (13). On the other hand, we found a single recombination event within the three-marker block located in a 3 Mb region at Xq21. Therefore, our data suggest that, from a practical point of view, either of the two clusters can be considered to be inherited as a single haplotypic block, which may be very useful in some deficiency cases. Specifically, the analysis of these markers can help to solve paternity cases if the alleged father is unavailable and two sisters are under analysis because all daughters share their paternal X-chromosome haplotype. The protocol we used provides a convenient method to genotype those markers in a single reaction. It is worth emphasizing that these six markers are tetranucleotide repeats located outside the known coding regions, without known functional consequences, providing only identity information. Thus, they may represent an advantage over trinucleotide repeats, which are more likely to show interpretation difficulties related to stutter peaks. Likewise, they are free of the ethical concerns raised by the markers located in coding regions of the genome.

We found four mutations, all consisting in one-repeat difference, with an average per-locus mutation rate of 0.0034, which is similar to previous reports of STR mutations (13,14,22).

In conclusion, our study suggests that DXS10079-DXS10074-DXS10075 and DXS6801-DXS6809-DXS6789 provide stable haplotype blocks that can help to solve complex kinship cases.

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Additional information reprint requests:
María T. Zarrabeitia, M.D., Ph.D.
Unit of Legal Medicine
University of Cantabria
Av Herrera Oria s/n
39011 Santander
Cantabria
Spain
E-mail: zarrabet@unican.es