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ORIGINAL ARTICLE

A GEP-ISFG collaborative study on the optimization of an X-STR decaplex: data on 15 Iberian and Latin American populations

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Abstract In a collaborative work carried out by the Spanish and Portuguese ISFG Working Group (GEP-ISFG), a polymerase chain reaction multiplex was optimized in order to type ten X-chromosome short tandem repeats (STRs) in a single reaction, including: DXS8378, DXS9902, DXS7132, DXS9898, DXS6809, DXS6789, DXS7133, GATA172D05, GATA31E08, and DXS7423. Using this X-decaplex, each 17 of the participating laboratories typed a population sample of approximately 200 unrelated individuals (100 males and 100 females). In this work, we report the allele frequencies for the ten X-STRs in 15 samples from Argentina (Buenos Aires,

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Miscellaneous GEP-ISFG (The Spanish-Portuguese Working Group of the International Society for Forensic Genetics)

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Córdoba, Río Negro, Entre Ríos, and Misiones), Brazil (São Paulo, Rio de Janeiro, Paraná, and Mato Grosso do Sul), Colombia (Antioquia), Costa Rica, Portugal (Northern and Central regions), and Spain (Galicia and Cantabria). Gene diversities were calculated for the ten markers in each population and all values were above 56%. The average diversity per locus varied between 66%, for DXS7133, and 82%, for DXS6809. For this set of STRs, a high discrimination power was obtained in all populations, both in males (≥1 in 5×10^5) and females (≥1 in 3×10^9), as well as high mean exclusion chance in father/daughter duos (≥99.953%) and in father/mother/daughter trios (≥99.999%). Genetic distance analysis showed no significant differences between northern and central Portugal or between the two Spanish samples from Galicia and Cantabria. Inside Brazil, significant differences were found between Rio de Janeiro and the other three populations, as well as between São Paulo and Paraná. For the five Argentinean samples, significant distances were only observed when comparing Misiones with Entre Ríos and with Río Negro, the only two samples that do not differ significantly from Costa Rica. Antioquia differed from all other samples, except the one from Río Negro.

Keywords X-chromosome · STRs · GEP-ISFG · Iberia · Latin America

Introduction

Short tandem repeats (STRs) located on autosomes are the genetic markers of choice in paternity investigation and they are also the most widely used in other cases of kinship analysis. Nevertheless, in some complex cases, independent of the number of polymorphisms being typed, autosomal markers convey very little information. Depending on the parentage constellation available for the analysis, as well as the gender of the subjects, this problem can sometimes be solved by using markers with different modes of transmission. Therefore, most forensic laboratories are nowadays prepared to analyze lineage markers (Y-chromosome and mitochondrial DNA) and many have recently introduced the analysis of X-STR markers in their routine.

Because polymerase chain reaction (PCR) multiplex strategies, population data, mutation rates, and other relevant genetic information concerning X-STRs are still scarce, the GEP-ISFG working group decided to organize a collaborative exercise to optimize an X-STR PCR multiplex strategy including ten markers: DXS8378, DXS9902, DXS7132, DXS9898, DXS6809, DXS6789, DXS7133, GATA172D05, GATA31E08, and DXS7423. After an evaluation by 30 laboratories inside the working group, this newly constructed X-STR decaplex has proven to be technically very robust [12]. Since population data are crucial for forensic

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evaluation of this new multiplex and only a small number of populations have been studied for these markers, we present the results obtained for some Iberian and Latin American populations.

Material and methods

Samples

A total of 1,492 male and 1,467 female samples from unrelated subjects were selected from paternity cases. This sample comprised individuals from six different countries from Iberia and Latin America, residing in 15 different regions. For DNA extraction, each laboratory used their routine methods.

PCR amplification

Amplification was performed in a single PCR multiplex reaction including the following ten X-STRs: DXS8378, DXS9902, DXS7132, DXS9898, DXS6809, DXS6789, DXS7133, GATA172D05, GATA31E08, and DXS7423. Primer sequences and references are listed in Table 1. For DXS7133, GATA31E08, and DXS9902, new primers were designed using the PRIMER3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer hybridization specificity was checked against the human genome using BLASTN (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Secondary structures and primer dimerization were screened with the help of Autodimer software [22].

PCR amplification was carried out using the QIAGEN Multiplex PCR kit (Qiagen) at 1× Qiagen multiplex PCR master mix and 0.5–5 ng of genomic DNA in a 10-μl final reaction volume. All primers were at 0.2 μM in the PCR reaction. Thermocycling conditions were: pre-incubation for 15 min at 95°C, followed by ten cycles of 30 s at 94°C, 90 s at 60°C, 60 s at 72°C; and 20 cycles of 30 s at 94°C, 90 s at 58°C, and 60 s at 72°C with a final incubation for 60 min at 72°C.

Detection, typing, and analysis of PCR products

Separation and detection were performed in various types of ABI Genetic Analyzers (Applied Biosystems) following manufacturer's instructions. Genotyping was performed through comparison with DNA control reference samples 9947A, 9948, and NA3657 (Table 2) and samples from the proficiency testing of the GEP-ISFG (supplementary Table S1).

This multiplex was optimized for typing with an internal size standard GeneScan-500 LIZ (Applied Biosystems) and filter G5. Nevertheless, for those laboratories with ABI platforms allowing for the detection of dyes with maximum fluorescence at only four different wavelengths, size standard ROX500 and Filter D were recommended.

Allelic ladders

Sequenced ladders were constructed for DXS9898, DXS9902, and DXS7132 in order to allow the unequivocal typing of intermediate alleles detected in most populations.

Table 1 Primer sequences, dye labels, and reference to publication

Locus	Label	Primer sequence (5'–3')	Reference
DXS8378	6-FAM	TTAGGCAACCCGGTGGTCC ACAAGAACGAAACTCCAACTC	[4]
DXS9898	6-FAM	CGAGCACACCTACAAAAGCTG TAGGCTCACCTCACTGAGCA	[8]
DXS7133	6-FAM	CACTTCCAAAAGGGGAAAAA ACTTGTACTTGGTGGGAGGAA	New
GATA31E08	6-FAM	GCAAGGGGAGAAGGCTAGAA TCAGCTGACAGAGCACAGAGA	New
GATA172D05	VIC	TAGTGGTGATGGTTGCACAG ATAATTGAAAGCCCGGATTC	[4]
DXS7423	VIC	GTCTTCCTGTCATCTCCCAAC TAGCTTAGCGCCTGGCACATA	[4]
DXS6809	VIC	TCCATCTTTCTCTGAACCTTCC TGCTTTAGGCTGATGTGAGG	[8]
DXS7132	NED	TCCCCTCTCATCTATCTGACTG CACTCCTGGTGCCAAACTCT	[8]
DXS9902	NED	CTGGGTGAAGAGAAGCAGGA GGCAATACACATTCATATCAGGA	New
DXS6789	NED	CTTCATTATGTGCTGGGGTAAA ACCTCGTGATCATGTAAGTTGG	[8]

Table 2 X-STR profiles of standard DNA samples used as references by the participating laboratories

	9947A	9948	NA3657	K562	References
DXS8378	10/11	11	12	10	[21]
DXS9898	12/15	13	–	12	[8]
DXS7133	9/10	11	9	10	[21]
GATA31E08	13	12	–	13	This work[a]
GATA172D05	10	6	9	12	[21]
DXS7423	14/15	14	13	17	[21]
DXS6809	31/34	31	29	34	[21]
DXS7132	12	13	12	13	[21]
DXS9902	12	13	13	12–13	This work[b]
DXS6789	21/22	20	23	21	[21]

Note that, although we report results for K562 DNA (Promega), this DNA should not be used alone as a standard sample (see Szibor et al. [21] for details)
[a] Genotypes were changed by adding two repeats to the one previously reported by Shin et al. [20] (see nomenclature discussion for details)
[b] Genotypes were changed by adding one repeat to the one previously reported by Szibor et al. [21] (see nomenclature discussion for details)

The construction and reamplification of the ladders followed the same protocol as in Gusmão et al. [10].

Sequencing analysis

Before sequencing, 1 μl of the PCR product was purified with 0.5 μl of ExoSAP-IT (USB Corporation) and incubated at 37°C for 15 min followed by 85°C for 15 min for enzyme inactivation. Sequencing reaction was performed using the ABI Big Dye Terminator Cycle Sequencing Ready Reaction kit v3.1 (Applied Biosystems) and post-purification was achieved with Sephadex G-50 columns (GE Healthcare). Products were visualized in an ABI PRISM 3130 Genetic Analyzer electrophoresis system and analyzed with Sequencing Analysis 3.7 software (Applied Biosystems).

Statistical analysis

Allelic frequencies and gene diversities were calculated using software ARLEQUIN ver 3.0 [6]. The same software was used to test Hardy–Weinberg equilibrium in female samples, linkage disequilibrium in male samples, and genetic distance estimations based on the number of different alleles (F_{ST}). Unweighted pair group method with arithmetic mean tree was built from the distance matrix (F_{ST}) using the option neighbor and drawtree in the PHYLIP software package [7] and visualized with the Treeview software [16].

Statistics for forensic efficiency evaluation of each locus, namely MEC in trios involving daughters (MEC_T) as well as in father/daughter duos (MEC_D), and power of discrimination in females (PD_F) and in males (PD_M) were calculated using the formulae according to Desmarais et al. [2].

Results and discussion

In the annual GEP-ISFG quality control exercise (www.gep-isfg.org), an increasing number of participating laboratories are reporting results based upon the analyses of X-STRs. Nevertheless, there is still a large diversity of the markers and nomenclatures that are being used by the different GEP laboratories. For instance, in 2006, of the 19 different markers reported, only six of them were typed by, at least, five laboratories (the minimum specified by the GEP for a marker to be evaluated); and nomenclature discrepancies were detected in two loci: HPRTB and DXS8377. In order to overcome these problems, the GEP decided to organize a collaborative exercise on the optimization of an X-STR multiplex [12]. In 2007 GEP-ISFG quality control, 18 out of the 24 laboratories that reported results on the analyses of X-STRs used this X-decaplex. The number of laboratories typing X-chromosome markers increased from 11 to 24 and, from the 20 different markers reported, 11 were typed by more than five laboratories.

Multiplex optimization

Markers were selected taking into account the gene diversity values reported in different populations (e.g., [3, 5, 8, 13, 14, 17–20, 23]); and the potential for multiplexing. Preference was given to simple rather than complex STRs, following the ISFG recommendations concerning locus selection for forensic applications [11]. In order to reduce the potential PCR-generated slippage artifacts, loci comprising trinucleotide repeats were avoided.

To evaluate the optimized multiplex, primer mix stock solutions were sent to the participating laboratories which were asked to analyze two female bloodstains, using the

protocol described in "Materials and methods." This newly constructed decaplex has proven to be technically very robust since most laboratories successfully typed the distributed samples [12].

Multiplex performance

Laboratories that correctly typed the test samples were asked to collaborate in data collection by typing approximately 200 unrelated individuals (100 males and 100 females).

After a first analysis of the genotype results, unexpected differences between laboratories were obtained in the frequency of intermediate alleles, namely at DXS9902, DXS9898, and DXS7132.

DXS9902 was first described by Edelmann et al. [3] as a simple GATA repeat, with no intermediate alleles detected in a German sample nor in a Korean sample studied by Shin et al. [19]. However, in the last proficiency test organized by the GEP, one sample was typed as DXS9902*11 by eight laboratories and as DXS9902*11.1 by the other eight laboratories. After sequence analysis, this sample was found to carry an intermediate allele 11.1 due to a T insertion in a tract of nine Ts ending five bases upstream of the GATA repeat.

To ensure the correct typing of these alleles during this study, the most frequent DXS9902 alleles were sequenced and a ladder was constructed, including intermediate alleles 12.1 and 13.1 (supplementary Fig. S1), and sent to all laboratories.

Allele 8.3 at DXS9898 is described as having a high frequency in all European populations studied until now (e.g., [1, 13, 17, 18, 24]). During the present study, when using the conditions described in "Materials and methods," it was observed, by some laboratories, that allele 8.3 differed in approximately 6 bp from allele 10, and allele 7 differed in approximately 4 bp from allele 8.3, which can lead to genotyping errors if sequenced allelic ladders are not used. The same kind of typing problem was observed for some DXS7132 alleles, in the same size range of DXS9898 alleles 7 and 8.3. Therefore, to ensure the correct typing of these two STRs, the most frequent alleles were sequenced and ladders were constructed and sent to all laboratories (Fig. S1).

Because of the coincidence of the size range in which this problem was detected, we compared the results obtained when running the DXS9898 and DXS7132 sequenced ladders with two different size standards, GeneScan-500 LIZ and GeneScan-600 LIZ (Applied Biosystems), and using two different polymers (POP4, in an ABI310, and POP7, in an ABI3130).

In all cases, the use of GeneScan-600 LIZ corrected this problem by allowing the detection of the real size differences between alleles. Moreover, when using GeneScan-500 LIZ, size differences were overestimated in the range between the 139- and 150-bp size standard fragments (supplementary Fig. S2).

In conclusion, the difficult size assignment experienced by some laboratories during this study was not due to a distortion in the mobility of the involved alleles caused by the electrophoresis' conditions but to a sizing problem when using GeneScan-500 LIZ size standard for fragments in the range of 139–150 bp.

Nomenclature

Although there are no specific recommendations on X-STR allele designation, ISFG recommendations for autosomal and Y-STRs were followed [11, 15]. Therefore, no nomenclature changes were introduced for the STRs to which sequence information is available, and a nomenclature based on the recommendations of the DNA Commission of the ISFG has already been published. Thus, DXS8378, DXS7133, DXS7132, and GATA172D05 alleles were named according to Edelmann et al. [4]; DXS9898 according to Hering and Szibor [13]; DXS7423 according to Zarrabeitia et al. [23]; DXS6809 according to Edelmann et al. [5]; and DXS6789 was named according to Hering et al. [14].

The already existing allele nomenclature for DXS 9902 is based on the number of GATA repeats [3]. However, the use of TAGA designation results in one additional repeat. In conformity to the ISFG guidelines, in the present study, we used this last nomenclature and, consequently, the reference samples genotypes were changed by adding one repeat (see Table 2). Therefore, our population data differ by one repeat compared to those obtained by using previously published reference DNA information [21].

GATA31E08 has been previously described by Shin et al. [20] and alleles were named according to the total number of AGAT repeats. However, upstream to this STR, there is an AGGG unit that can be present in two or three copies [9]. Therefore, the repeat structure should be changed to $(AGGG)_{2–3}(AGAT)_n$ and reference samples genotypes should be changed by adding two additional repeats (see Table 2).

Population sample profiles

Allele frequencies were calculated for the ten X-STRs in the 15 studied populations, namely Argentina (Buenos Aires, Córdoba, Río Negro, Entre Ríos, and Misiones), Brazil (São Paulo, Rio de Janeiro, Paraná, and Mato Grosso do Sul), Colombia (Antioquia), Costa Rica, Portugal (Northern and Central regions), and Spain (Galicia and Cantabria) (Table S2). Exact test of differentiation did not

reveal significant differences for any loci between male and female subgroups; therefore, samples were pooled and allele frequencies were those in supplementary Table S3. Gene diversities were calculated for the ten markers in each population and all values were above 56%. The average diversity per loci varied between 66%, for DXS7133, and 82%, for DXS6809.

Hardy–Weinberg equilibrium was tested in female samples. For a significance level of 0.005 (Bonferroni's correction assuming ten tests per population), departures from Hardy–Weinberg expectations were only significant in two cases: DXS7423 in Galicia and DXS6809 in Rio de Janeiro. This cannot be justified by the presence of null alleles since: (a) no nulls were detected in male samples; (b) there is no excess of homozygotes for DXS7423 in Galicia; and (c) these systems were found to be in equilibrium in closely related populations.

Unusual allele patterns and null alleles

Out of the 4,426 studied chromosomes, triplet-allele patterns were detected in two females; one from Northern Portugal at GATA31E08 and the other from Costa Rica at DXS6809 locus. Additionally, a male sample from Costa Rica showed a double-allele pattern at DXS6809. These rare patterns could be explained either by locus duplication or due to an extra X-chromosome. The latter explanation is less plausible since no other loci, in the same samples, show these unusual patterns.

In the case of GATA31E08, a female was genotyped as 9/12/13. A daughter and her father were also typed for this marker and genotyped as 9/13 and 9, respectively. In the daughter, the two alleles (9 and 13) presented the same amplification intensity, implying that allele 9 was transmitted by the father and a single allele 13 by the mother. The genotype of the mother is, therefore, GATA31E08*(9–12)/13.

At DXS6809, one sample belongs to a male with a 32/33 genotype. A second sample was from a female and, after the analysis of a daughter (DXS6809*34) and her father (DXS6809*34), it was possible to deduce her genotype as DXS6809*(32–33)/34.

Null alleles were detected in three male samples, two at DXS7133, in Northern Portugal and São Paulo, and one at

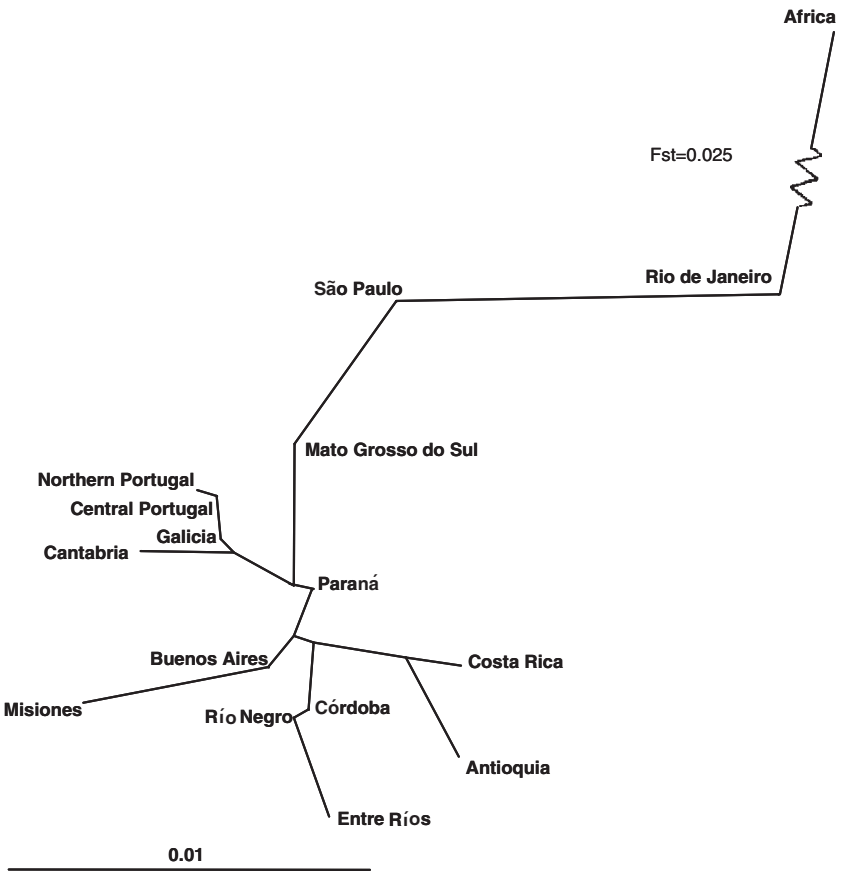

Fig. 1 Neighbour-joining tree based on pairwise F_{ST} genetic distances calculated between the 15 populations from the present study. A sample from sub-Saharan Africa (unpublished data from Uganda) was also included for comparison purposes

GATA172D05, in Buenos Aires. A null allele at GATA 172D05 has been previously described in a Hispanic male from US population [8], when using the same primers.

It is worth mentioning that the frequencies of null alleles (Table S3) are subject to large confidence intervals since they could only be detected in male samples (where no allele amplification is observed). Null alleles in females can only be searched by using a different primer set or by transmission studies. In the case of DXS7133 in Northern Portugal, since one null allele was found in 212 males, in the sample of 424 females, two of them are expected to be heterozygous for the null alleles, raising its frequency to 0.6%. The same holds true for DXS7133 in São Paulo and GATA172D05 in Buenos Aires, where the expected frequencies of null alleles are 0.9% and 0.6%, respectively.

Linkage disequilibrium analysis

Gametic association was tested for all pairs of loci in the male sample. For a significance level of 0.0011 (obtained after Bonferroni correction, for 45 comparisons inside each population), a single significant p value was found out of the 675 pairwise comparisons, between DXS7133 and DXS6789 loci (Supplementary Table S4). This lack of LD suggests an absence of linkage or significant associations due to genetic substructure in any of the studied populations (particularly, in those known to be admixed) for the ten markers in the multiplex. These results are in accordance with the distances between the selected X-chromosome loci (>5 Mb), except for DXS6809 and DXS6789. These two markers are closely located at the X-chromosome and in kinship analysis they should be treated as haplotypes. Nevertheless, the absence of any signs of association between alleles of these two STRs allows the use of single-locus allele frequencies to predict the haplotype frequencies in the studied populations.

Forensic efficiency parameters

Based on allele frequency distributions, forensic efficiency statistical parameters were calculated for each locus in the 15 populations (supplementary Table S3). On average, DXS7133 was the least polymorphic marker whereas the most diverse was DXS6809. The least diverse population was Entre Ríos; and Rio de Janeiro presented the highest diversity values. In all populations, the values obtained for power of discrimination were high in females (between 0.9999999992 and 0.99999999998) and males (between 0.999997 and 0.9999996). The combined mean exclusion chance values varied between 0.999990 and 0.999998 in trios and between 0.9995 and 0.99991 in duos.

Comparisons between populations

Pairwise genetic distance analysis was performed for all population samples included in this work (see supplementary Table S5). Because of the presence of intermediate alleles at four loci, genetic distance estimations were based on the number of different alleles (F_{ST}) rather than on sum of squared size differences (R_{ST}), in accordance with Pereira et al. [17].

When comparing the Iberian samples, no significant genetic distances were found between Portugal and Galicia and neither between Cantabria and Galicia. However, although the F_{ST}s were low, significant p values were obtained between Cantabria and the two Portuguese samples.

Inside Brazil, significant differences were found between Rio de Janeiro and the other three populations as well as between São Paulo and Paraná.

Between the five Argentinean samples, significant distances were only observed when comparing Misiones with Entre Ríos and with Río Negro, the only two samples that are not significantly different from Costa Rica.

Significant F_{ST} values were obtained between Antioquia and all the other samples, except the one from Río Negro.

Figure 1 shows the tree built from the F_{ST} distance matrix between the 15 studied populations and a sub-Saharan African sample (unpublished data). The Iberian samples group together and all Latin American samples form a second more diverse group, excluding the Brazilians. The Brazilian samples are those standing closer to the African sample, except for Paraná that stands between the Iberian and Latin American clusters.

In conclusion, the X-chromosome decaplex under evaluation proved to be extremely robust and informative. It is likely to be quite useful in forensic practice, particularly in "deficient paternity" and other kinship cases.

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