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Development of a polymorphic short tandem repeat locus multiplex system for efficient human identification

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ABSTRACT. This study aimed to develop a short tandem repeat (STR) multiplex system, made up of 22 highly informative STR loci, for application in forensic genetics. The system comprised 21 polymorphic autosomal loci (D3S1358, TH01, D21S11, D18S51, Penta E, D5S818,

D13S317, D7S820, D16S539, CSF1PO, Penta D, vWA, D8S1179, TPOX, FGA, D2S441, D17S1301, D19S433, D18S853, D20S482, and D14S1434) and the amelogenin gene locus. Strategies were developed to overcome the challenges involved in creating a multiplex system. Based on the literature and available databases, the STR loci were selected with the aim to obtain discriminatory markers, and followed specific criteria for this purpose. Primers were projected using the Primer3 software, and AutoDimer was used to evaluate possible interactions between them. The 22 selected STR loci were validated individually and jointly, both to assess their sensitivity and to test the efficiency of the multiplex system. Statistical analyses were based on the genetic data of 450 unrelated individuals living in the State of Goiás, thus allowing the establishment of the parameters necessary to use this system. A total of 239 alleles were detected for the 22 loci in the set, allowing for a probability of identity of 4.23×10^{-25} to be obtained. The combined power of discrimination was 0.99999999999999999999999 and the combined power of exclusion was 0.99999. Upon complete validation of the entire system, this multiplex assay was considered to be a powerful tool for application in human identification by DNA analysis.

Key words: Human identification; Microsatellites; Paternity; Multiplex; Allele frequency

INTRODUCTION

The advance of biotechnology-related techniques has allowed the proposal of new strategies for identifying the genetic profile of each person, the tools for which have become quite revolutionary in the practice of individual identification using human molecular genetics (Brevnov et al., 2009; Budowle and van Daal, 2009; Mattana et al., 2012).

According to Sarmento (2006), human identification by DNA analysis may be a helpful tool for the disclosure of guilty criminals and innocent defendants, the identification of bodies and human remains in mass disasters, the determination of parental linkage, and the detection of technical mistakes attributed to labeling errors in clinical pathology laboratories (Ruitberg et al., 2001; Buckleton et al., 2004).

The development of short tandem repeat (STR) multiplex systems with simultaneous amplification has ensured facilities for human identification with an excellent power of discrimination, low consumption of biological samples and inputs, and high practicality in technical routine. As such, the application of multiplex systems to analyze samples has resulted in the acquisition of complete genetic profiles that can be compared and discriminated (Butler, 2006; Zhang et al., 2015).

The ability to analyze multiple-STRs simultaneously, together with multicolor fluorescence detection technologies, has greatly contributed to the consolidation of these important technical principles for the science of determining genetic identity (Buckleton et al., 2004; Hill et al., 2008; Wang et al., 2011). The 13 STR loci that initially comprised the Combined DNA Index System (CODIS) were made extensively available in commercial kits (Opel et al., 2007), and the same panel was soon further expanded to 20 loci (Hares, 2012,

2015). Meanwhile, several other highly polymorphic STR loci, neither a subset of CODIS nor commercially available, were capable of considerably raising the power of discrimination in analysis (Opel et al., 2007; Hill et al., 2009).

According to Wiegand et al. (2006), several real situations of high analytical complexity require a molecular arsenal focused on the power of individual discrimination. In these situations, the application of methods that increase the power of discrimination corroborates the issuance of conclusive opinions. On the other hand, the unavailability of these resources prompts the unfeasibility of analysis (Gill et al., 1991, 2006).

In this sense, Poetsch et al. (2013) concluded that the number of hypervariable loci available for a genetic linkage analysis is directly associated with the percentage of certainty established during the assessment of the analysis (Børsting et al., 2008; Rodovalho et al., 2015; Zhang et al., 2015). Therefore, it is critical that STR panels provide sufficient security, permitting an accurate and unfailing conclusion regarding the genetic linkage analysis. However, both a very close correlation between the quantity and quality of the polymorphic loci present in an STR system, and the degree of accuracy of the methodology, are evident. Thus, the greater the number of STRs analyzed, the greater would be the resolving power of the study (Wiegand et al., 2006).

This study aimed to develop a new STR multiplex system comprising CODIS and non-CODIS highly polymorphic loci for simultaneous analysis, and to assess its efficiency for forensic application. The loci introduced into the system are distinguished from commercial systems, to allow the introduction of new markers in complex genetic analyses that require complementation. Thus, this panel comprised 18 loci commonly present in commercial systems, allowing for the complementary analysis required in situations that include null alleles.

MATERIAL AND METHODS

Selection of the STR loci

This study selected 22 STR loci coupled with the layout of the desired multiplex project, based on literature reviews from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) and STRBase (National Institute of Standards and Technology, Chemical Science & Technology Laboratory; http://www.cstl.nist.gov/strbase/), with the following criteria: location on different chromosomes (physical distance of at least 10 Mb from another locus if on the same chromosome), power of discrimination, allelic diversity, amplicons size, and presence in compatible databases.

Obtaining sequencing primers

The sequencing primers for the selected loci were obtained using the online Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) (Rozen and Skaletsky, 2000), so that the size of the amplified products would be within the range established for the entire planned system. Then, the AutoDimer software (https://www-s.nist.gov/dnaAnalysis/) (Vallone and Butler, 2004) was used to assess sequences that could potentially self-anneal to form primer dimers, an event known as hairpin formation. Genome alignment analysis was performed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), an NCBI tool used to identify locations in the genome where spurious annealing may occur beyond the target DNA.

Obtaining primers and developing the multiplex assay

The allelic variations for each STR locus were analyzed on the basis of literature reviews related to the dimensions that each molecular marker may take because of insertions of rare alleles, which are at the ends of the STR locus. These precautions were taken to avoid possible overlaps between neighboring loci and components of the same fluorescence channels.

The locus organization in the panel was designed by considering the schematic multiplex arrangement with the candidate positions for each locus. The architecture of the system layout allowed for estimation of the spacing between the loci and calculation of the size required for each PCR product according to its reference sequence.

All primers were synthesized by Applied Biosystems (Foster City, CA, USA), and the forward sequence of each STR was labeled with one of the following dye sets: 6-FAM™, VIC®, NED™, and PET® (Applied Biosystems), as shown in Table 1.

Amplification using PCR and detection of genetic profiles

The PCRs for all the developed STRs were performed in singleplex assays to assess the behavior of the primer and ensure that the amplicon corresponded to the expected size. Upon validation of all the individual STRs, amplification in a single-multiplex PCR was carried out. For this, a primer mix was prepared according to the individual concentrations of each primer, established on the basis of the joint amplification signal strength. For the multiplex PCR, a Qiagen® Multiplex PCR Kit (Qiagen, Hilden, Germany) was used together with the primer mix and 5.0 ng genomic DNA, in a final reaction volume of 25 µL. The thermocycling conditions were as follows: an initial incubation at 95°C for 5 min; 30 cycles of 94°C for 30 s, 60°C for 90 s, and 72°C for 60 s; and a final extension at 60°C for 30 min.

Capillary electrophoresis of the PCR products preceded the preparation step with 1 µL of amplified product, 8.5 µL Hi-Di™ formamide (Applied Biosystems), and 0.5 µL GeneScan™ - 600 LIZ® Size Standard v2.0 (Applied Biosystems). The separation and detection of fragments were performed with the ABI 3500 Genetic Analyzer (Applied Biosystems) using POP4 (Applied Biosystems). The samples were injected electrokinetically at 1.2 kV for 15 s. The electrophoresis run time for allele separation was 1210 s at 15 kV and 60°C. The allelic fragments obtained were analyzed with GeneMapper®ID-X version 1.2 (Applied Biosystems). The overall performance of the multiplex system was assessed using the 2800M-positive control DNA (Promega Corporation, Madison, WI, USA).

Sensitivity and concordance test

The sensitivity of the multiplex system was assessed by decreasing the amount (10, 5, 3, 1, and 0.5 ng) of the 2800M-positive control DNA (Promega Corporation) in the PCR. The sensitivity limit was based on the amplification having at least 80 relative fluorescence units (RFU). The DNA from blood samples, buccal swabs, and hair was extracted using the QIAamp® DNA Investigator Kit (Qiagen), and amplified to assess the sensitivity and efficiency of the multiplex for different samples. The direct amplification from blood samples was also assessed for the developed multiplex using the Whatman® FTA® card.

The PowerPlex® Fusion System (Promega Corporation) was used to perform concordance tests between the common loci in the two systems (D3S1358, TH01, D21S11,

D18S51, Penta E, D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D, amelogenin, vWA, D8S1179, TPOX, FGA, D2S441, and D19S433). The other non-coincident loci (D17S1301, D18S853, D20S482, and D14S1434) were not assessed, because they were not present in any kit currently commercially available in Brazil.

Population analysis of STR polymorphism

Peripheral blood was collected by digital puncture from 450 unrelated individuals living in the State of Goiás, Brazil. The blood was impregnated on Whatman® FTA® cards for further amplification with the newly developed multiplex set and the PowerPlex® Fusion System.

The collection of biological material began after the Research Ethics Committee of the Pontifical Catholic University of Goiás had approved the research (CAAE: 51483415.9.0000.0037 and opinion number 1.721.890).

Statistical analyses

The statistical parameters for the allele frequencies were obtained using Genetix version 4.05 and PowerStats softwares (Tereba, 1999; Belkhir et al., 2004; da Costa Francez et al., 2011). The alleles were tested for Hardy-Weinberg equilibrium using Genepop version 4.1.4 and Arlequin version 3.1 softwares (Excoffier et al., 2007; da Costa Francez et al., 2011). Other statistical parameters associated with the study population, such as the probability of identity, power of discrimination, polymorphism information content, power of exclusion, and typical paternity index, were calculated using PowerStats (Tereba, 1999).

RESULTS AND DISCUSSION

Following the criteria for selecting STRs, 22 polymorphic loci were selected to make up the multiplex panel (Table 1). Of these 22 loci, only TPOX and D2S441 (chromosome 2), D5S818 and CSF1PO (chromosome 5), D18S51 and D18S853 (chromosome 18), and D21S11 and Penta D (chromosome 21) were located on the same chromosome, at a minimum distance of 10 Mb. The developed multiplex set comprised 15 CODIS loci and 6 non-CODIS loci (Penta E, Penta D, D17S1301, D18S853, D20S482, and D14S1434).

To assess the performance of the synthesized primers and identify the annealing temperature common to all of them, the STRs were first assessed individually. Since no locus presented amplification failure and/or artifact excess that could compromise the yield expected for the system, the primers were subsequently combined in a multiplex reaction. As expected, the preliminary tests of the multiplex combination showed a large imbalance between the involved primers, probably due to some factors that interfered with the reaction, such as different annealing temperatures. To balance the combined primers, the amplification intensity was assessed individually to adjust their specific concentrations, assigning higher concentrations to the primers that obtained lower yield and, conversely, lower concentrations for the primers that showed allelic peaks with high RFU. The fluorescence labels, specific concentrations of each primer, and other information pertinent to the loci included in the multiplex are described in Table 1.

After optimization of the PCR in the multiplex system, the set of selected primers was successfully amplified in a single reaction, obtaining a harmonic and noise-free final panel. Figure 1 shows the allelic profile obtained for the 2800M-positive control DNA.

Table 1. Data for the short tandem repeat loci present in the developed multiplex system.

Dye label	Locus	Chromosomal location	Repeat unit	Primer concentration (μM)	Fragment size range (bp)
M™	D3S1358	3p21.31	TCTG/TCTA	0.10	91-143
6-FAM™	TH01	11p15.5	AATG	0.20	156- 205
6-FAM™	D21S11	21q21.1	TCTA/TCTG	0.30	215-271
6-FAM™	D18S51	18q21.33	AGAA	0.20	282-362
6-FAM™	Penta E	15q26.2	AAAGA	0.20	369-494
VIC®	D5S818	5q23.2	AGAT	0.20	111-155
VIC®	D13S317	13q31.1	TATC	0.10	164-204
VIC®	D7S820	7q21.11	GATA	0.20	215-259
VIC®	D16S539	16q24.1	GATA	0.25	268-312
VIC®	CSF1PO	5q33.1	AGAT	0.15	321-369
VIC®	Penta D	21q22.3	AAAGA	0.30	381-489
NED™	Amelogenin	Xp22.1-22.3 and Y	-	0.10	106-112
NED™	vWA	12p13.31	TCTG/TCTA	0.20	115-183
NED™	D8S1179	8q24.13	TCTA/TCTG	0.50	195-251
NED™	TPOX	2p25.3	AATG	0.08	258-302
NED™	FGA	4q31.3	TTTC	0.15	314-444
PET®	D2S441	2p14	TCTA	0.15	78-106
PET®	D17S1301	17q25.1	AGAT	0.50	110-150
PET®	D19S433	19q12	AAGG	0.25	184-212
PET®	D18S853	18p11.31	ATA	0.40	215-242
PET®	D20S482	20p13	AGAT	0.15	259-311
PET®	D14S1434	14q32.13	CTGT/CTAT	0.20	321-369

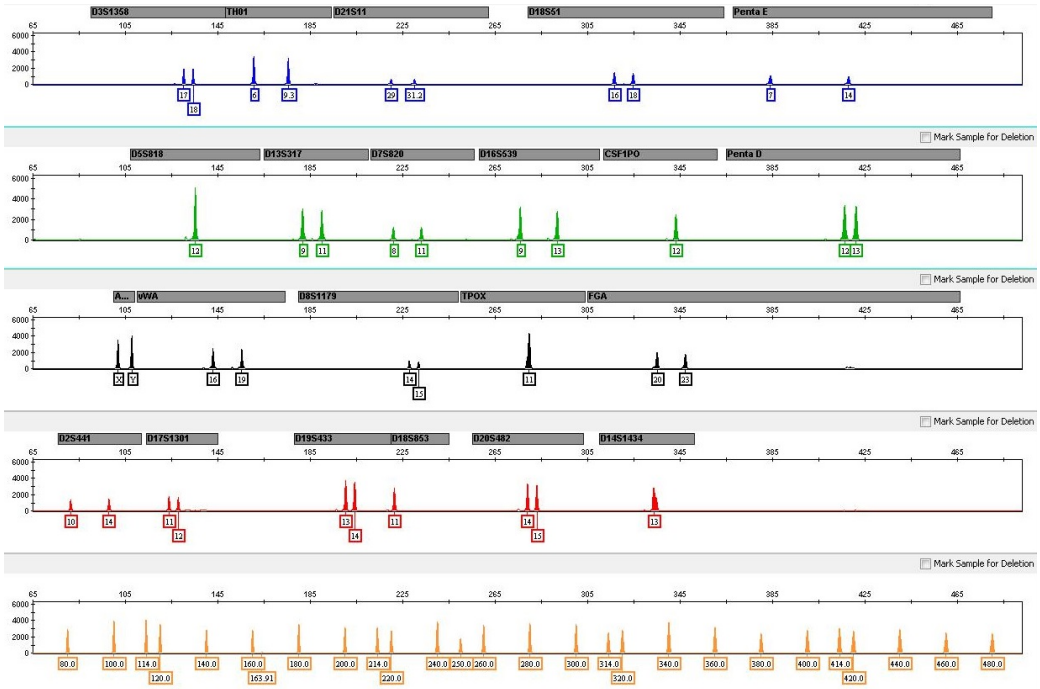

Figure 1. Genetic profile of the 2800M-positive control DNA obtained with the newly developed multiplex system.

The multiplex amplification assessment of the different concentrations of the 2800M-positive control DNA showed 100% allelic identity in the reactions with DNA ranging from 1 to 10 ng. However, DNA at the concentration of 0.5 ng did not present complete amplification for some loci in the system, revealing an incomplete genotypic profile with approximately 90% allelic identity. Direct amplification of blood samples impregnated on FTA® cards and of DNA extracted from peripheral blood, buccal swab, and hair samples showed 100% allelic identity for all samples tested.

A comparison of the allelic profiles obtained by direct amplification of the 450 blood samples impregnated on FTA® cards, using both the newly developed multiplex system and the PowerPlex® Fusion System, showed genotype matching for all loci analyzed.

The allelic frequencies and statistical parameters related to the genetic and forensic linkage analysis, obtained for the 21 autosomal multiplex loci for the population of the State of Goiás, Brazil, are described in **Table S1** and Table 2, respectively.

Table 2. Statistical parameters of autosomal multiplex loci for the population of the State of Goiás, Brazil.

Locus	%Hets	MP	PD	PIC	PE	TPI	N
D3S1358	80.9	0.104	0.896	0.73	0.616	2.62	450
TH01	80.2	0.077	0.923	0.76	0.603	2.53	450
D21S11	87.1	0.047	0.953	0.82	0.737	3.88	450
D18S51	87.8	0.026	0.974	0.87	0.750	4.09	450
Penta E	90.7	0.019	0.981	0.90	0.809	5.36	450
D5S818	74.0	0.115	0.885	0.69	0.493	1.92	450
D13S317	76.0	0.075	0.925	0.76	0.527	2.08	450
D7S820	77.8	0.075	0.925	0.76	0.558	2.25	450
D16S539	79.8	0.077	0.923	0.76	0.595	2.47	450
CSF1PO	70.4	0.111	0.889	0.69	0.435	1.69	450
Penta D	85.8	0.035	0.965	0.85	0,710	3.52	450
vWA	77.8	0.072	0.928	0.77	0.558	2.25	450
D8S1179	78.4	0.063	0.937	0.78	0,570	2.32	450
TPOX	67.3	0.143	0.857	0.65	0.388	1.53	450
FGA	88.2	0.028	0.972	0.87	0.759	4.25	450
D2S441	76.4	0.085	0.915	0.74	0.535	2.12	450
D17S1301	66.2	0.166	0.834	0.62	0.372	1.48	450
D19S433	79.3	0.050	0.950	0.80	0.587	2.42	450
D18S853	74.4	0.105	0.895	0.70	0.500	1.96	450
D20S482	69.1	0.146	0.854	0.64	0.415	1.62	450
D14S1434	69.3	0.127	0.873	0.67	0.418	1.63	450

%Hets: percentage heterozygosity; MP: match probability; PD: power of discrimination; PIC: polymorphism information content; PE: power of exclusion; TPI: typical paternity index; N: study population.

In general, the multiplex system showed large allelic variations, ranging from 7 alleles (TH01, D13S317, and D17S1301) to 24 (FGA), and a total of 239 alleles for the whole set. Deviation from Hardy-Weinberg equilibrium was observed for TPOX (P = 0.01016), D17S1301 (P = 0.00319), and D14S1434 (P = 0.03695). According to Hardy (1908), this suggests that the population is under the influence of some factors, such as mutation rate, migration, selective pressure of the environment, or natural selection. By comparing the observed and expected heterozygosities, it was possible to find more homozygotes than expected. As such, amplification failure from a mutational event in the 3' region of the hybridization site of the primers had resulted in null alleles (Gomes et al., 1999; Dakin and Avise, 2004). Leibelt et al. (2003) described a mutation at the hybridization site in the D8S1179 locus in the population of Guam Island. Their study population demonstrated an excess of homozygotes at this locus when genotyped using AmpFlSTR Profiler Plus™. Similar results were found by Budowle

et al. (2000) in the population of Chamorros and Filipinos on the Island of Guam, and by Vanderheyden et al. (2007) in the population in Belga.

The statistical parameters obtained showed high values (Table 2). All loci showed high forensic efficiency, with the power of discrimination values being greater than 0.8, ranging from 0.834 (D17S1301) to 0.981 (Penta E). The most polymorphic locus was Penta E, followed by the FGA and D18S51 loci. The polymorphism information content ranged from 0.62 (D17S1301) to 0.90 (Penta E). The combined power of discrimination for the 21 loci was 0.999999999999999999999999, the combined power of exclusion was 0.99999, and the identity power corresponded to 4.23×10^{-25}.

In conclusion, our STR multiplex system showed a high level of sensitivity, being capable of amplifying 21 autosomal loci and the amelogenin locus from several types of biological samples, including direct amplification on FTA®, which incorporates versatility to the technical routine for obtaining a genetic profile. The high values found in the statistical parameters suggest that the developed multiplex system is highly polymorphic and informative, verifying that the set shows high discriminatory potential and therefore justifying its application in strategies involving human identification and genetic linkage analysis.

Conflicts of interest

The authors declare no conflicts of interest.

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Supplementary material

Table S1. Allelic frequency obtained for the 21 autosomal loci comprising the multiplex system.