Pure and Applied Chemistry, *71*: 1683-1690, 1999

Single-tube single-colour multiplex PCR amplification of ten polymorphic microsatellites (ALF10): a new powerful tool for DNA profiling

Sérgio D.J. Pena

GENE - Núcleo de Genética Médica, Av. Afonso Pena 3111/9, Belo Horizonte, Brazil 30130-909.

Abstract: We studied the genomic diversity of a sample of the Brazilian population with ten highly polymorphic autosomal microsatellite loci, each located in a different chromosome. The set of ten microsatellites (ALF10) could be amplified by PCR in a single multiplex reaction and analysed in a single run in a DNA fluorescent automatic sequencer. The discrimination power was very high: the probability of two randomly chosen Brazilian individuals presenting different genetic profiles when typed at the ten loci was 0.9999999999997. Thus, ALF10 was capable of defining genetic individuality. The expected power of exclusion for paternity testing was 0.99998. In 350 paternity trios in which paternity had been previously ruled out by other DNA tests, exclusions at one or more of the microsatellite loci of ALF10 were observed in all cases (100%). This simple single-tube, single-fluorophore, single electrophoretic run multiplex set of ten microsatellites constitutes a new powerful tool for DNA profiling, highly useful for paternity testing, forensic studies and human evolutionary studies.

INTRODUCTION

DNA studies have unravelled a great wealth of genome variation in humans, thus proving unequivocally that each individual is genetically unique (reviewed in ref. 1). The most prevalent type of human genome variation is the single nucleotide polymorphism (SNP), a position at which two alternative bases occur at frequencies >1% in human haploid genomes. When the genome sequences of two random human individuals are compared, they differ at about 1/1000 nucleotides (ref. 2). Since the human genome comprises $3x10^9$ bases, this implies on the existence of millions of SNPs. Other rich sources of genome variability are the variable number of tandem repeat (VNTR) polymorphisms. These are genome segments where a sequence motif is repeated many times, the number of reiterations varying between individuals. If the repeat motif is between 1 and 6 base pairs, these are called microsatellites, while for repeats larger than 6, they are called minisatellites. In the human genome it has been estimated an average tandem repeat polymorphism density of one marker per 16 kilobases (ref. 3). As a percentage of the total repetitive DNA analysed, the distribution of repeat unit sizes is: 50.0% di-, 10.2% tri-, 24.4% tetra-, 5.7% penta-, 2.2% 6 mer-9 mer, and 7.5% 10 mer-229 mer. Altogether tandem repeat polymorphisms represent a total of 0.5% of the GenBank human sequences, microsatellites constituting more than 90% of the total (ref. 3).

The fact that these variable number of tandem repeat polymorphisms are ubiquitous, multiallelic and hypervariable, makes them the most informative polymorphisms in the human genome (ref. 4). Among these VNTRs, microsatellites have the advantage over minisatellites of greater simplicity of study using the polymerase chain reaction (PCR), presence of discrete alleles and applicability to degraded DNA samples (ref. 1). Because of this, they constitute valuable tools in the characterisation of human genetic individuality for forensic purposes (ref. 5) and paternity studies (ref. 6). Microsatellites have also been increasingly used to study the genetic structure of human populations and human evolution (ref. 7,8).

In comparison with minisatellites, the main limitation of microsatellites is their lower informativeness. Thus, batteries of several polymorphic loci typed by PCR are needed to obtain the necessary discrimination

power to replace the more informative minisatellites in practical applications such as paternity testing and forensics (ref. 6). The problem is that undertaking several separate microsatellite PCR amplifications is a cumbersome, time consuming endeavour that significantly increases the chance of laboratory errors. The solution is the utilisation of multiplex procedures based on the simultaneous PCR amplification and electrophoretic analysis of three or more microsatellite loci (ref. 9). The development of highly informative multiplex systems has been greatly facilitated by the availability of fluorescent DNA sequencers that detect allele sizes on real time as they migrate through a laser beam. Microsatellite multiplexes in the range of 7-10 loci have been recently realised (ref. 10) and are already commercially available (PowerPlex 1.1, Promega; Profiler Plus/Cofiler, Perkin-Elmer), but have the incovenience of needing different fluorescent dyes and thus depending on the utilisation of the more complex and expensive DNA sequencers that can handle these multiple fluorophores. We wish to report the development of ALF10, a powerful single-tube multiplex PCR set of ten polymorphic microsatellites that presents the distinct advantage of being single-colour, i.e. utilizing one single fluorophore. We have tested ALF10 in the Brazilian population and have found that it has extremely high discrimination power and is therefore capable of defining genetic individuality. ALF10 thus constitutes a new powerful tool for DNA profiling and should be highly useful for paternity testing, forensic studies and human evolutionary studies.

MATERIAL AND METHODS

Study population

We studied 2000 paternity trios (mother, child, possible father) referred for DNA paternity testing at GENE - Núcleo de Genética Médica de Minas Gerais, Belo Horizonte, Brazil. All these cases were also studied by DNA fingerprinting with multilocus minisatellite probes (ref. 11) and/or by profiling with several other microsatellite loci. Unrelated parental pairs were used for allelic frequency calculations. Most individuals were from the Southeastern portion of Brazil, especially from the state of Minas Gerais. Brazil is a very large country and different native and immigrant populations are distributed unevenly. For instance, European descendants predominate in the South, Amerindian descendants are preponderant in the North and African descendants are a relatively larger proportion of the population in the Northeastern states. Minas Gerais, due to its central position, is the state whose population composition is most representative of Brazil as a whole. Different ethnic groups are also unevenly distributed among the several social strata in Brazil. Since our patients were participants in paternity testing studies in a private laboratory, our sample had a bias in favour of the middle and upper middle class that is mostly Caucasoid. Indeed, the vast majority of individuals were self-defined as “white” in spite of significant variation in skin colour. Although our sample cannot be considered an absolutely random-sample of Caucasoid Brazilians, it is sufficiently representative for the purposes of this study.

Microsatellite loci

The microsatellites used to establish the multiplex set were identified by searches in The Genome Database (http://gdbwww.gdb.org/). Candidates were chosen that had the following characteristics: tetranucleotide (preferred) or trinucleotide repeats, high heterozygosity level, distinct chromosomal locations and different amplicon size. On these bases, and after considerable experimentation, we chose the ten-microsatellite set that is detailed on Table 1. Primer sequences for all these microsatellites can be found at http://gdbwww.gdb.org/.

PCR amplification

PCR amplifications were performed in a final volume of 12.5 μl containing 10-20 ng of human genomic DNA, a mixture of all the 20 primer pairs (see below), 200 μM dNTPs, 10mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.63 units of AmpliTaq Gold (Perkin-Elmer). Primers were custom synthesised with inclusion of a fluorescent Cy5 group in the 5' extremity and were used in final concentrations ranging from 30nM to 65 nM, carefully established to provide even amplification. The thermal cycling program was the following: initial denaturation at 94^{o}C for 1 min, followed by 35 cycles of annealing at 55^{o}C for 1 min, extension at 72^{o}C for 2 min and denaturation at 94^{o}C, followed by a final extension for 10 min a 72^{o}C.

Electrophoretic analysis

Following PCR amplification, 1.5 μl of the mix were transferred to another microtube to which were added 4 μl of a solution of deionized formamide containing 5mg/ml de Dextran Blue 2000 (Pharmacia) and an internal molecular size standard (300 bp). This mix was denatured at 95°C for 3 min and run in an ALF-Express automatic DNA sequencer (Pharmacia) using 0.5mm spacers and standard running conditions. Allele ladders were prepared by amplification of a mixture of DNA from 200 Brazilians and intercalated between every group of three lanes. The electrophoretic runs were visualised and molecular sizes established using the AlleleLinks software (Pharmacia).

Data analysis

Gene count estimates of allele frequencies and estimates of heterozygosity were performed by standard procedures (ref. 12). The discrimination power and the average exclusion power for each locus were calculated according to Aitken (ref. 13) and Garber and Morris (ref. 14) respectively. The conformity of the observed genotype frequencies with Hardy-Weinberg expectations was evaluated by an exact (*E*) test (ref. 15).

RESULTS

Multiplex amplification of the ten loci

We identified a large set of tetranucleotide (preferred) and trinucleotide polymorphic microsatellites in the Genome Database. After experimentation with different loci and different amplification conditions we chose the ten loci listed in Table 1 that were amenable to single-tube multiplex amplification and single-run electrophoretic analysis. These loci are all located in different chromosomes, thus providing assurance that they are genetically independent. As shown in the graph of the electrophoretic run (Fig. 1) they are well spaced and can be easily typed individually without ambiguity. To improve the separation between D5S2501 and D15S657, we added a tail to ten thymidine residues to the 5'-extremity of the upper primer of the latter; these ten extra bases were later subtracted in computing allele sizes. With the use of AmpliTaq Gold, an enzyme ideally suitable for multiplex PCR amplification (ref. 16) the efficiency of the single-tube multiplex amplification was excellent: in more than 95% of amplifications we could reliably type nine or more loci.

Allele size distributions

As can be seen in Table 1, nine of the ten loci chosen were tetranucleotide-repeat microsatellites and only one (D2S1353) had trinucleotide repeats. However, three of the tetranucleotide-repeat microsatellites (D3S2387, D4S2431 and D18S1270) presented alleles differing by only two base pairs from other alleles. In the case of D4S2431 this can be understood from the structure of the $(GGAA)_n$ repeat motif. D3S2387, while having primarily a $(GACA)_n(GATA)_n$ microsatellite structure, also contains inside its amplicon a block of $(GA)_n$ repeats that may vary independently of the main tandem repeat region. D18S1270 has a pure $(GATA)_n$ repeat motif and it is not clear why it would have alleles differing by only two bp. We plan to undertake extensive DNA sequencing of alleles of these three loci to establish their detailed structure.

None of the ten loci showed significant departures from Hardy-Weinberg expectations using the exact test of Guo and Thompson (ref. 15). The gene count allele frequency estimates for this sample of the Brazilian population are shown in Table 2. Allele designations are based on amplicon size measured with the AlleleLinks software, except in the case already mentioned of D15S657, where 10 base pairs (corresponding to the poly-T tail) were subtracted. When compared with the allele sequences (data not shown), the amplicons consistently show one base more. This is due to the well-known introduction of an extra base (generally an adenosine) in the 3'-extremity of the PCR product by the terminal transferase activity of *Taq* polymerase. After completing the DNA sequencing of alleles at D3S2387, D4S2431 and D18S1270 we will be able to change the allele nomenclature to repeat numbers.

Genetic Diversity

Heterozygosity and discrimination power

All loci examined showed large levels of average heterozygosity (H), varying from 0.63 for D16S2622 to 0.88 for D3S2387. This shows that the set of loci is very useful to characterise human genetic diversity and genomic individuality. This usefulness was further assessed using two other variability statistics: the probability of discrimination (PD) and the power of exclusion (POE). The probability of discrimination, i.e. the ability of ALF10 of distinguishing the DNA samples of two randomly selected individuals from the Brazilian population, was 0.9999999999997. Its complement, the probability of non-discrimination or match probability (MP), is the probability that two randomly selected individuals present identical profiles. As displayed in Table 3, the decaplex system (ALF10) has a match probability of 3×10^{-13}, three orders of magnitude smaller than the reciprocal of the world population. Thus, it can be used with great advantage in the diagnosis of human individuality, including for forensic purposes.

Paternity testing: power of exclusion

Paternity studies depend on two basic statistics: the power of exclusion (PE) of the loci studied and the paternity index (L), based on the alleles shared between parents and child at each locus. The power of exclusion measures the power of the test battery of excluding the paternity of a falsely accused male individual. The average power of exclusion was calculated for each locus for the Caucasoid Brazilian population (Table 3). The combined average power of exclusion for ALF10 was 0.99998.

We tested empirically the power of exclusion of ALF10 in 350 paternity trios in which the possible father had already been excluded by DNA Fingerprinting with the multilocal probe F10 (ref. 11). Exclusion at one or more microsatellite loci was observed in all cases (100%) and at two or more loci in 349 cases (99.7%). Thus, ALF10 has very high efficiency in paternity testing.

Paternity testing: paternity indices

With the exception of cases in which mutations were observed (see below), ALF10 provided paternity indices above 1,000 in all inclusion paternity trios tested, and above 10,000 in 94% of cases. These paternity indices correspond, respectively, to probabilities of paternity of 0.999 and 0.9999 (calculated with Bayes' theorem and a prior probability of 0.5).

Mutations

The study of paternity trios, especially inclusion cases, has the advantage that it permits the observation of maternal and paternal mutations. A detailed study of these mutations has been undertaken and will be published in detail elsewhere (S.D.J. Pena, manuscript in preparation). From the analysis of these 2,000 paternity trios we could calculate for ALF10 an average paternal mutation rate of approximately 2×10^{-3} per locus per meiosis and an average maternal mutation rate of approximately 2×10^{-4} per locus per meiosis. Effectively, this means that we can expect on average to see one paternal mutation in every 50 inclusion paternity trios studied with ALF10. Brinkmann et al (ref. 17), studying nine other microsatellite loci, encountered the same magnitudes of mutation rates and the same disproportionate concentration of mutation events in the male germ line.

DISCUSSION

The PCR-based utilisation of polymorphic microsatellites for human individuality testing presents several advantages over minisatellite analysis that depends on restriction endonuclease digestion, Southern blotting and DNA probes. First and most important, the procedure is rapid, simple and non-isotopic. Second, alleles are discrete and can be typed unambiguously. Third, being PCR-based, microsatellite analysis is extremely sensitive and samples with as little as 3 pg of DNA (equivalent to one cell) can be reliably typed. Lastly, since most microsatellites have amplicon sizes below 500 bp (and often below 200 bp), typing is possible even when the sample DNA is seriously degraded, as frequently is the case with forensic evidence. However, microsatellites have average heterozygosity levels almost always below 90%, being considerably less informative than minisatellites. This inconvenience can be overcome by

simultaneous typing of large number of microsatellite loci using multiplex PCR amplification and analysis in fluorescent DNA sequencers, such as reported in the present article.

ALF10, the set of ten polymorphic autosomal microsatellite loci described herein, has the important quality of allowing single-tube, single-electrophoretic run, single-colour multiplexing. The fact that all microsatellites are located in different chromosomes affords the genetic independence necessary to apply the product rule for calculation of the match probability of the whole DNA profile and joint paternity indices. In consequence, typing of the ten microsatellite loci in ALF10 provides the high levels of genetic discrimination needed for forensic or paternity testing applications. For instance, in reference to paternity testing, ALF10, with a single PCR reaction, provides similar levels of exclusion power and paternity indices as a combination (two PCR reactions) of the Second Generation Multiplex (SGM) developed by the UK Forensic Service and the Powerplex 1 multiplex commercially available from Promega (ref. 18).

ALF10 presents the enormous convenience of being a single-colour multiplex system. Thus, in contrast with other multiplex systems, it can be resolved in single-fluorophore DNA sequencers such as the ALF and the ALF-Express (Pharmacia). However, it can also be used to great advantage with multiple-fluorophore machines such as the Applied Biosystems Model 373 or Prism 377 DNA Sequencers (Perkin-Elmer). With these machines we could, for instance, label mother, child, possible father and the allelic ladder with different fluorophores and run the whole paternity trio in the same lane of the gel, a particularly efficient and reliable set-up for paternity testing laboratories. Likewise, evidence and suspect DNA samples could be run in the same lane in forensic studies.

In conclusion, this simple single-tube single-fluorophore multiplex set of ten microsatellites, constitutes a new powerful tool for DNA profiling and should be highly useful for paternity testing, forensic studies and human evolutionary studies.

ACKNOWLEDGEMENTS

I am grateful to Kátia Torres de Souza, Cristiane Lommez de Oliveira, Cláudia Benedetto, Valéria Matarelli Pereira Calijorne and Márcia Caldeira Brant Neves Campos, all from GENE - Núcleo de Gneética Médica, for their invaluable and expert help in these studies.

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Table 1 - Basic characteristics of the ten microsatellite loci in the ALF10 multiplex set. The loci are ordered by increasing amplicon size.

Locus	Chromosome	Allele size range	Repeat motif
D16S2622	16	68-96 bp	TAGA
D1S1612	1	82-138 bp	AAGG
D2S1353	2	126-168 bp	ATA
D3S2387	3	172-212 bp	GACA/GATA
D4S2431	4	221-265 bp	GGAA
D18S1270	18	270-300 bp	GATA
D5S2501	5	306-330 bp	GATA
D15S657	15	332-368 bp	GATA
D10S1237	10	388-436 bp	GATA
IFNAR-ALU	21	456-484 bp	TAAA

Table 2 - Allele frequencies for the ten microsatellite loci of ALF10 in the Brazilian Caucasoid population. Alleles are denominated according to the molecular size of the amplified product. N is the number of unrelated individuals in the sample.

D2S1353 (N = 6481)		D3S2387 (N = 7810)		D4S2431 (N = 7804)		D10S1237 (N = 7596)		D18S1270 (N = 7386)	
Allele	Freq. (%)	Allele	Freq. (%)	Allele	Freq. (%)	Allele	Freq. (%)	Allele	Freq. (%)
126	0.05	160	0.10	211	0,05	356	0.03	258	0.01
129	0.03	164	0.01	217	0,45	360	0.04	260	0.01
132	0.03	168	0.33	219	1,17	364	0.04	264	0.01
135	0.05	170	0.08	221	0,69	368	0.11	266	0.11
138	0.22	172	1.05	223	1,13	372	0.17	268	0.22
141	7.75	174	0.01	225	0,22	376	0.08	270	0.61
144	3.67	176	5.72	227	3,53	380	0.04	272	2.63
147	22.62	178	0.01	229	0,6	384	0.18	274	11.28
150	21.37	180	5.74	231	4,93	388	0.57	278	10.83
153	19.18	182	0.22	235	8,34	392	5.62	280	4.40
156	13.76	184	9.46	237	2,73	396	8.56	282	6.46
159	7.02	186	0.06	239	2,86	400	19.12	284	22.64
162	4.09	188	10.20	241	19,24	404	27.15	286	0.56
165	0.15	190	0.74	243	0,82	408	13.73	288	24.55
168	0.02	192	14.43	245	29,3	412	9.23	290	0.07
		194	0.95	249	15,73	416	5.95	292	13.54
		196	19.97	253	6,62	420	4.74	296	1.85
		198	0.78	257	1,2	424	4.09	298	0.03
		200	16.79	261	0,24	428	0.46	300	0.16
		202	0.28	265	0,11	432	0.04	304	0.03
		204	8.53	269	0,03	436	0.04	308	0.01
		206	0.04	271	0,01	444	0.01		
		208	3.23			448	0.01		
		210	0.01						
		212	1.02						
		216	0.15						
		220	0.06						

D1S1612 (N = 6057)		D5S2501 (N = 6344)		D15S657 (N = 7388)		D16S2622 (N = 7320)		IFNAR-ALU (N = 5232)	
Allele	Freq. (%)	Allele	Freq. (%)	Allele	Freq. (%)	Allele	Freq. (%)	Allele	Freq. (%)
82	0.05	302	0.24	318	0.05	68	0.07	448	0.04
90	0.15	306	4.82	322	0.05	72	1.97	452	0.04
94	3.57	310	9.39	326	0.60	76	6.60	456	0.11
98	27.18	314	41.90	330	15.92	80	31.82	460	19.74
102	7.12	318	22.64	334	28.33	84	50.74	464	22.06
106	4.28	322	14.45	338	17.07	88	8.37	468	8.52
110	8.98	326	5.74	342	23.01	92	0.44	472	17.66
114	14.43	330	0.80	346	10.49			476	10.45
118	12.83	334	0.02	350	3.79			480	21.29
122	11.16	338	0.02	354	0.61			484	0.08
126	6.60			358	0.07				
130	2.97			368	0.01				
134	0.63								
138	0.07								

Table 3 - Average heterozygosity (H), probability of discrimination (PD), match probability (MP), expected exclusion power [PE(e)] and observed exclusion power [PE(o)] for the ten microsatellite loci in the ALF10 multiplex set. The observed exclusion power was based on 350 paternity trios in which the possible father had already been excluded by DNA Fingerprinting. The loci are ordered by increasing chromosome number.

Locus	H	PD	MP	PE(e)	PE(o)
D1S1612	0.85	0.96	0.04	0.72	0.70
D2S1353	0.83	0.95	0.05	0.68	0.62
D3S2387	0.88	0.98	0.02	0.75	0.72
D4S2431	0.84	0.96	0.04	0.69	0.55
D5S2501	0.74	0.90	0.10	0.54	0.49
D10S1237	0.84	0.96	0.04	0.71	0.66
D15S657	0.80	0.93	0.07	0.62	0.54
D16S2622	0.63	0.98	0.20	0.44	0.34
D18S1270	0.84	0.95	0.05	0.68	0.60
IFNAR-ALU	0.82	0.94	0.06	0.64	0.62
TOTAL			3×10^{-13}	0.99998	1.00

Figure 1 - Graph of alleles of all ten loci in ALF 10 displayed by the AlleleLinks software after electrophoretic analysis in the ALF-Express automatic fluorescent DNA sequencer. The abscissa is size of the PCR products in base pairs and the ordinate is fluorescent intensity measured by the laser densitometer. Vertical lines that are not part of the original AlleleLinks graph indicate the territories of the ten loci. Three first three lanes from top to bottom display the results of ALF10 results in an inclusion paternity trio constituted by, respectively, mother (M), child (C) and father (F). The bottom lane shows allelic ladders constructed by amplification of a pool of DNA from 200 Brazilians. The arrow indicates the internal molecular size standard.