TECHNICAL NOTE

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Population Studies and Validation of Paternity Determinations by Six Microsatellite Loci

REFERENCE: Ingvarsson S, Finnsdottir V, Sigurdsson A, Geirsson G. Population studies and validation of paternity determinations by six microsatellite loci. J Forensic Sci 2000;45(3):692–695.

ABSTRACT: A single locus system of 6 microsatellite markers was evaluated for paternity testing. A nonradioactive method based on peroxidase labeling of a DNA probe was used to estimate the allele frequency of markers D1S216, D3S1217, D7S480, D9S157, D13S153, and D16S422 by genotyping 1134-1698 chromosomes. The number of detected alleles were 22, 15, 23, 10, 16, and 19, respectively, and the allele frequency varied from 0.001 to 0.317. The genotype of 87 families, consisting of mother, father, and child was determined. The probability that a random individual will give a positive paternity was evaluated. We conclude that the markers can be reliably typed and give sufficient and reliable information for paternity testing.

KEYWORDS: forensic science, paternity, short tandem repeat, dinucleotide, polymerase chain reaction, forensic DNA typing, D1S216, D3S1217, D7S480, D9S157, D13S153, D16S422

Paternity can be tested by using multilocus or single-locus probes (1). Microsatellite markers are widely used in genetic studies, such as human gene mapping, linkage analysis, and identity testing. The alleles of the microsatellite markers are, in general, stably inherited (2). Due to its small size, they are more efficiently amplified using PCR techniques and can easily be resolved using polyacrylamide gel electrophoresis. Microsatellites consist of multiple redundant tandem repeats (mono-, di-, tri-, tetranucleotides etc.). There are over 50,000 short tandem repeats (STR) in the human genome, of which more than 5000 are characterized as genetic markers (3). The most abundant microsatellites in the human genome are the CA repeats. Many of these markers are very informative due to their highly polymorphic nature and are therefore useful in identity and paternity testing. In this study, the use of six microsatellite markers for paternity testing was evaluated.

Materials and Methods

DNA Samples, Primers, and PCR Amplification

Samples were obtained from 849 unrelated individuals and 87 families consisting of mother, father, and child. The preparation of DNA extracts from blood was according to Talmud et al. 1991 (4). To 100 μL of prefrozen (−70°C) blood, 400 μL of freshly prepared 0.17M NH_4Cl was added for 20 min at room temperature. After centrifugation for 30 s in a microcentrifuge, the pellet was washed 3 times in cold 0.9% NaCl by resuspending and additional centrifugation. The pellet was resuspended in 200 μL of 0.05M NaOH and boiled for 10 min. The solution was neutralized with 25 μL of 1M Tris pH8.0. In 9 cases where one or more of the parties in the family were deceased, DNA was extracted from paraffin-embedded blocks of tissue, taken at autopsy or at surgery prior to death. The tissues were treated according to the method described by Smith et al. 192 (5): 300 μL of extraction buffer (10 mM Tris pH8.3, 50 mM KCl, 0.45% Tween-20, 0.45% Nonidet NP40, 100 μg/mL BSA, and 100 μg/mL proteinaseK) were added to a paraffin slice of 10 μm, incubated O/N at 55°C and boiled for 10 min. These extraction methods do not allow precise quantification of the DNA, but 4 μL of sample were used for each PCR reaction and the remaining DNA sample was kept frozen.

The information on the primers for amplification of the D1S216, D3S1217, D7S480, D9S157, D13S153, and D16S422 loci is listed in Table 1. The primers were obtained from Research Genetics (Huntsville, Alabama, USA). The microsatellite allele sizes of cases were determined by including the corresponding PCR product from individual 134702, that serves as a size standard, since allele sizes are well documented (2 and Table 1).

DNA samples were amplified in 25 μL reaction volume using DynaZyme™ polymerase (Finnzymes Oy, Espoo, Finland) following manufacturer's recommendation. After 5 min denaturation at 94°C, samples were subjected to 35 cycles of amplification, consisting of 30 s, at 94°C, 30 s at 55°C, and 1 min at 72°C, followed by a final extension for 10 min at 72°C.

Electrophoresis, Blotting, and Hybridization

PCR products were separated on 6.5% polyacrylamide sequencing gels and transferred for 1 h to a Hybond-N+ nylon membrane (Amersham, Aylesbury, UK). Primers were elongated by a terminal-transferase (TdT) in 100 μL of 40 mM K-HEPES buffer with 1 mM $CoCl_2$ (pH7.2) overnight at 37°C (8). The reactions were stopped with 2 μL of 0.5M EDTA and the probe was purified on sephadex G-25 spin columns. Peroxidase-labeled primer (360 ng) were used to probe the membranes in GOLD buffer for 4 h or overnight at 42°C (Amersham, Aylesbury, UK). The membranes were washed for 20 min in 3X SSC/0.1% SDS at 42°C, for 2 × 15

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Received 12 May 1999; and in revised form 13 July 1999; accepted 16 July 1999.

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min in 0.2X SSC at 42°C, bathed in a detection reagent containing H_2O_2, luminol and an enhancer (ECL kit, Amersham, Aylesbury, UK) for 1 min and the chemiluminescence was finally detected on a DUPONT Cronex-4 film for 1 min to 1 h. Three markers from separate PCR reactions can be loaded on the same gel and hybridized simultaneously. Analysis of additional markers from the same gel requires stripping of hybridization from the nylon membrane by 0.1% SDS solution at 95°C for 5–10 min, to avoid background signals. The separation power of this electrophoretic system allows separation of alleles that differ by one nucleotide in size.

TABLE 1—*Information regarding the microsatellite markers used in the study. The short tandem repeats are dinucleotides in all markers ((CA)n in addition to (TA)n in D1S216 and D16S422), and D7S480 also includes a mononucleotide repeat ((C)n). The number of alleles and the maximum and minimum allele sizes (size range) have been determined by observation of random unrelated Icelandic individuals. The genotype of individual 134702 was used as a reference for allele size determinations (2).*

Marker	Number of Alleles	Cytological Location	Size range in nt.	Genotype of 134702 (2)	Reference
D1S216	22	1p	228–270	242–242	(6)
D3S1217	15	3p	177–205	197–189	(7)
D7S480	23	7q	183–211	202–201	(6)
D9S157	10	9p	133–151	139–133	(6)
D13S153	16	13q	210–244	222–218	(6)
D16S422	19	16q	176–214	202–200	(6)

NOTE: Further information on the microsatellite markers can be obtained at http://gdbwww.dkfz-heidelberg.de/.

Statistics

The value of q (allele frequency) is obtained from the population database (Table 2). The probability that a random individual will have a particular allele is given by q(2-q). The paternity index is calculated by the following formula: Paternity index (PI) = $1/q_1(2-q_1)q_2(2-q_2)q_3(2-q_3)q_4(2-q_4)q_5(2-q_5)q_6(2-q_6)$. In the cases where the maternal allele could not be identified in the child due to identical alleles in the mother and the child, the frequency of both alleles were summed together for each marker involved.

Results and Discussion

The distribution of the alleles at the particular locus was determined (Table 2). The loci used in this analysis showed skewed unimodal (D13S153), bimodal (D9S157), and multimodal (D1S216, D3S1217, D7S480, and D16S422) distributions. The high informativeness of the microsatellite markers used was demonstrated by the high number of detected alleles (22, 15, 23, 10, 16, and 19, respectively) and 80%–90% heterozygosity (Tables 2 and 3). The

TABLE 2—*Allele frequency of the markers used in the study. The sample material is unrelated, random individuals from Iceland. Total number of chromosomes tested are in parentheses. Allele names are according to the size of the PCR product in nucleotides.*

D1S216 (1346)		D3S217 (1698)		D7S480 (1134)		D9S157 (1436)		D13S153 (1382)		D16S422 (1518)	
Allele	Freq.	Allele	Freq.	Allele	Freq.	Allele	Freq.	Allele	Freq.	Allele	Freq.
228	0.004	177	0.001	183	0.001	133	0.140	210	0.001	176	0.001
230	0.005	179	0.004	184	0.001	135	0.178	212	0.031	178	0.003
232	0.094	181	0.002	185	0.005	137	0.156	214	0.025	182	0.001
234	0.034	183	0.012	187	0.009	139	0.035	216	0.111	184	0.003
236	0.059	185	0.008	189	0.092	141	0.018	218	0.125	186	0.009
238	0.029	187	0.097	191	0.132	143	0.019	220	0.200	188	0.149
240	0.029	189	0.169	192	0.003	145	0.276	222	0.137	190	0.016
242	0.177	191	0.202	193	0.071	147	0.139	224	0.104	192	0.005
244	0.171	193	0.071	195	0.071	149	0.038	226	0.096	194	0.010
246	0.080	195	0.317	196	0.004	151	0.002	228	0.077	196	0.003
248	0.017	197	0.051	197	0.097			230	0.046	198	0.009
250	0.051	199	0.050	198	0.007			232	0.022	200	0.175
252	0.062	201	0.016	199	0.170			234	0.021	202	0.108
254	0.076	203	0.001	200	0.016			240	0.001	204	0.061
256	0.026	205	0.001	201	0.162			242	0.001	206	0.178
258	0.034			202	0.068			244	0.001	208	0.178
260	0.033			203	0.057					210	0.055
262	0.010			204	0.011					212	0.036
264	0.002			205	0.012					214	0.001
266	0.002			206	0.004						
268	0.001			207	0.004						
270	0.003			208	0.001						
				211	0.002						

NOTE: Freq.: Frequency of the given allele.

exclusion power of each locus is: 98% (D1S216, D7S480, D13S153), 97% (D16S422), and 96% (D3S1217, D9S157), respectively, and in combination of the systems, more than 99.99%. All loci in each of the six systems were observed to be in Hardy-Weinberg equilibrium and transmitted in a Mendelian fashion. In all 87 family cases analyzed in this study, the maternal and paternal alleles of the child were found in the mother and the father, respectively, and the sharing of alleles between parents and child was compatible with a first degree genetic relationship and no mother-child or father-child exclusions were observed in any of the used markers. In all, mutations were not detected in 224 meioses for marker D1S216, 384 meioses for marker D3S1217, 174 meioses for marker D7S480, 252 meioses for marker D9S157, 216 meioses for marker D13S153, and 322 meioses for marker D16S422. In a larger study, a total of 305 mutations were observed in 278,338 genotypings, partly due to the use of DNA from continuous lymphoblastoid cell lines (2). In general, we conclude that the PCR primers used in this study are locus-specific, unlinked and have a good balance between variability and stability. We also conclude that the method is highly sensitive and the assay can be used to analyzed low amounts of genomic DNA and even degraded DNA samples, as from paraffin-embedded blocks of tissue.

Replication slippage is detected as one to three "shadow" or stutter bands of lower intensity, smaller than the actual allele size, as a multiple of 1–2 nt depending on the type of the repeat (mono- or dinucleotide). This stutter band problem is the major disadvantage of using microsatellite markers in forensic work due to risk of wrong allele typing. Strand mispairing by forward slippage is thought to be the main mechanism responsible for producing stutter bands at tandem dinucleotide repeats (9). Also, alleles of smaller size are more efficiently amplified in the PCR, and, therefore exhibit stronger signal than alleles of larger size. Due to this, the general rule is to read the band showing the strongest signal and the band of the slowest electrophoretic mobility. By using this criteria, we conclude that the stutter bands do not interfere with the correct reading of alleles due to the high resolution power of the electrophoresis system and since they have signals at a lower intensity than the band representing the actual allele.

The calculated PI varied from $2.17 \times 10^2 - 2.26 \times 10^7$ in the 87 cases tested with the standard 6 microsatellite markers. The sharing of alleles of the casework child and random individuals of unknown relative status in the database was done in order to check whether this system of 6 microsatellites can result in false positive paternity (Table 4). In this database, the confirmed mother and father of the casework child was omitted. There was no match detected between the paternal alleles of all 6 markers in 73 casework children and alleles in the database from 174 randomly selected individuals. Only 67 out of 12,702 comparisons matched for 5 out of 6 markers tested (Table 4). This observation is in line with the calculated paternity index and indicates that a false positive inclusion rate is very low. The observed frequencies of genetic inconsistencies between the children and the random individuals in the six systems are: 78% (D1S216), 67% (D3S1217), 78% (D7S480), 69% (D9S157), 73% (D13S153), and 71% (D16S422), respectively.

TABLE 3—*Information regarding observed heterozygosity. The calculation of observed frequencies of heterozygosity was based on genotyping of 567-849 individuals.*

Marker	Number Tested	Observed Heterozygosity Frequency
D1S216	673	0.900
D3S1217	849	0.796
D7S480	567	0.892
D9S157	718	0.836
D13S153	691	0.889
D16S422	759	0.862

TABLE 4—*Number of paternity exclusions by comparing 73 child genotypes with genotypes from 174 random individuals that are neither mother nor father of the child in question. The non-maternal allele (both alleles were used in cases were child and mother have identical alleles) in the child was used for comparison with the alleles in the 174 individuals. The total number of comparisons is 12702.*

Number of Markers Excluding Paternity	Exclusions Number	Frequency
6/6	1904	0.150
5/6	4421	0.348
4/6	4000	0.315
3/6	1835	0.144
2/6	475	0.037
1/6	67	0.005
0/6	0	0

Non-paternity should not be diagnosed solely on the basis of an exclusion at a single locus and additional markers can be used in these particular cases. The cases with only one marker indicating exclusion of paternity were analyzed with additional microsatellite markers (D2S378, D6S292, D8S553, and INT2) to confirm exclusion (information on the markers can be obtained at http://gdb www.dkfz-heidelberg.de/). Furthermore, the same additional microsatellite markers were used in the 3 cases showing a PI lower than 500 to offer further support of paternity. Detection of paternal alleles of high frequency, same pattern of alleles in mother and child or mother-less cases can result in low PI. Due to the high number of well characterized microsatellite markers, it is relatively easy to include additional markers in the system of analysis, when required, for example, if the paternity index is considered to be too low or if exclusion is based on one marker only.

We conclude that this battery of 6 microsatellite markers is capable of resolving all standard paternity cases.

Acknowledgments

The authors thank Gunnar Ingimundarson for help with the statistical analysis and Jean Weissenbach for providing genomic DNA from individual 134702 for use as size standard.

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