Polymerase Chain Reaction Analysis of Allele Polymorphism of Two Hypervariable Loci in a Moscow Russian Population Sample

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PCR analysis of the allele polymorphism of two hypervariable loci D1S111 and RB1-VNTR among 120 unrelated Russians living in Moscow revealed 14 alleles of D1S111 381–861 bp long and 9 alleles of RB1-VNTR 1200–1650 bp long. One of the D1S111 alleles was sequenced, thus permitting allele classification according to the number of tandem repeats. For both loci the observed genotype frequencies were shown to correspond to the Hardy–Weinberg equilibrium. The allele frequency distribution was compared in the Russian and American population samples. These polymorphic loci are proposed for individual identity and paternity tests.

Key words: polymerase chain reaction; allele polymorphism; minisatellite p33.6; RB1-VNTR; Russian population

The class of polymorphic loci known as variable-number tandem repeats (VNTR) in the human genome is marked by high individual polymorphism [1] and is widely used as genetic markers in genome mapping, individual identification, and establishing relatedness.

Intron 1 of the human myoglobin gene was found to contain a sequence composed of four 33-bp tandem repeats [2]. This tandem repeat was cloned and used as a hybridization probe with human DNA [3], which revealed a family of repeats with partly complementary sequences [3, 4]. Among then is the minisatellite p33.6 located in the D1S111 locus (1cen-q24) and composed of tandemly repeated sequences of average length 37 bp [3]. In the same work this locus was shown to be highly polymorphic: eight alleles were found in DNA samples from 14 unrelated Britons.

The hypervariable locus RB1-VNTR is located within intron 16 of the retinoblastoma gene RB1, which is a suppressor gene for eye malignancies in children. The retinoblastoma-associated gene of about 200 kbp resides in chromosome 13q14 [5–7]. A clear-cut correlation was observed between the somatic loss of heterozygosity in RB1 and risk of developing tumors [8]. The extent of polymorphism found for RB1-VNTR allows it to be regarded as an informative marker in studies of disease-linked genes and in individual identification [7, 9].

The lengths of tandem repeats in both RB1-VNTR and D1S111 vary because of internal insertions and deletions. Scharf et al. [9] in their analysis of RB1-VNTR allele polymorphism used a mean repeat size of 50 bp. Variability in unit length has been demonstrated for many VNTR, e.g., COL2A1 and ApoB 3'VNTR [10, 11].

The use of polymerase chain reaction (PCR) [12], in contrast to restriction fragment length polymorphism (RFLP) analysis [13], permits discrimination and exact sizing of alleles differing by a single repeat unit. The possibility of precise genotyping even with minute specimens (5–10 ng) and/or degraded DNA makes the AMP-FLP analysis (PCR-amplified fragment length polymorphism) [14, 15] an efficient tool in population studies and person identification.

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MATERIALS AND METHODS

Restriction endonuclease *Hinc*II, phage T4 DNA ligase, *E. coli* DNA polymerase I Klenow fragment, and Taq^R DNA polymerase were from NPK Biotekh (Moscow), [α-^{32}P]dATP (6000 Ci/mmole) from the Institute of Nuclear Physics (Obninsk); oligonucleotide primers were synthesized in the Institute of Bioorganic Chemistry (Moscow).

Isolation of plasmid DNA, enzyme treatment, and electrophoretic agarose gel resolution were performed by standard protocols [16]. Genomic DNA was isolated from venous blood as described [17]. The population sample of 120 unrelated Russians living in Moscow was composed of specimens collected at traumatological units, Institute of Rheumatology, Bureau of Legal Medicine, and from the staff of GNIIgenetika.

The minisatellite repeat p33.6 was sequenced by the Sanger method [18] using a commercial kit (Fermentas, Vilnius).

The template was ssDNA of recombinant pUC19 [19] with an inserted minisatellite fragment of 566 units (according to 6% PAGE). Prior to cloning, the ends of the amplified DNA fragment were filled using Klenow fragment and four dNTPs. Then the fragment was inserted into *Hinc*II-cleaved plasmid with phage T4 DNA ligase. The products were used to transform *E. coli* JM109 (F′*tra*D36, *lac*I^Q, *lac*ZΔM15, *pro*AB/*rec*A1, *end*A1, *gyr*A96 (Nal^r), *thi*, *hsd*R17, r_k^-, m_k^+, *sup*E44, *rel*A1, Δ(*lac–pro*AB). Recombinant clones were grown on LB agar with 50 μg/ml ampicillin and selected by a PCR assay for the insert.

PCR was performed on Techne PHC-2 (UK) or Polygen (Austria) PolyChain thermal cyclers, in 50 μl of reaction mix containing 67 mM Tris-HCl (pH 8.8), 16.6 mM ammonium sulfate, 0.01% Tween-20, 1 or 2 mM $MgCl_2$ (D1S111 and RB1-VNTR respectively), 0.2 mM of each dNTP, 2.5 un. Taq^R polymerase, and 10–500 pg genomic DNA. Primers TR-15 (5′-ACAATGTGAGTAGAGGAGACC-3′) and TR-16 (5′-ACCACAGAGTGAGGAGCAACC-3′) (66 ng each) were used to amplify p33.6 alleles; they were chosen on the basis of published [3] sequences flanking the p33.6 repeat. The RB1-VNTR alleles were amplified using 99 ng each of primers RB21 (5′-CGTTAATATTTACCTAACGTATGGCCAAGTTTCC-3′) and RB24 (5′-CGAGCCTCGGTCTCATCACCAAGGGGGTGG-3′) [9]. PCR was run in 30 cycles of 94°C/1 min, 55°C/1 min, 72°C/2 min, with first denaturation for 3 min and last synthesis for 7 min.

Amplification products were analyzed by 6% PAG electrophoresis, applying 10–15 μl of the reaction mix per lane. The gels were stained with silver [20].

The observed genotype frequencies were tested for deviation from the Hardy–Weinberg equilibrium using the Rows×Columns program kindly provided by G. Carmody (Carleton University, Ottawa), which was also used to compare the allele frequency distributions in different population samples.

The expected heterozygosity (H_{exp}), probability of random match (pM), power of discrimination (PD), mean exclusion chance (W), and polymorphism information content (PIC) were calculated by a computer program based on the following algorithms:

$$H_{exp} = (1 - Ex_i^2 \times n/(n-1),$$

$$pM = EP_i^2,$$

$$PD = 1 - pM,$$

$$W = \underset{i}{E} x3i(1 - x_i)^2 + \underset{i}{E} x_i(1 - x)^3 + \underset{i<j}{E} x_i x_j(x_i + x_j)(1 - x_i - x_j)^2$$

$$PIC = 1 - \overset{n}{\underset{i}{E}} x_i^2 - \overset{n-1}{\underset{i}{E}} \underset{j=i+1}{E} 2x_i^2 x_j^2$$

where x_i, x_j are allele frequencies, n is allele number, P_i is genotype frequency.

RESULTS AND DISCUSSION

Cloning of the amplified p33.6 allele yielded seven recombinant clones. The nucleotide sequence of the insert in one clone is shown in Fig. 1. It contained 14 repeats mainly of 37 bp in length. These repeats, in their turn, consisted of three subrepeats sometimes differing in size by one or two bp because of internal deletions and insertions. Repeat 14 comprised only two subrepeats. Thus, the 566-bp allele was shown to contain 14 repeats, and alleles were further enumerated according to the repeat number.

Taking a mean tandem repeat length of 37 bp, we see that the smallest allele found in this work (381 bp) is composed of nine repeat units. This is in full accord with the electrophoretic resolution of alleles in 6% PAG. Specifying allele length with 1-bp accuracy, allowance must however be made for small variations within one allele group because of insertions/deletions.

In our opinion, classification of VNTR alleles by the number of repeats is the most correct, since it permits unambiguous interlab collation without confusion in the notation. For instance, in surveys of Caucasoid populations for the D1S80 locus the smallest allele containing 18 repeats [20, 25, 26] was assigned No. 1; however, other studies [27, 28] revealed alleles with a smaller repeat number (13, 17, 17).

Genotyping of 120 unrelated individuals revealed 14 alleles of the D1S111 locus, ranging from 381 to 862 bp (Fig. 2, Table 1). The most frequent were alleles 14, 12 and 17 (Table 1). Among the genotypes observed (29%

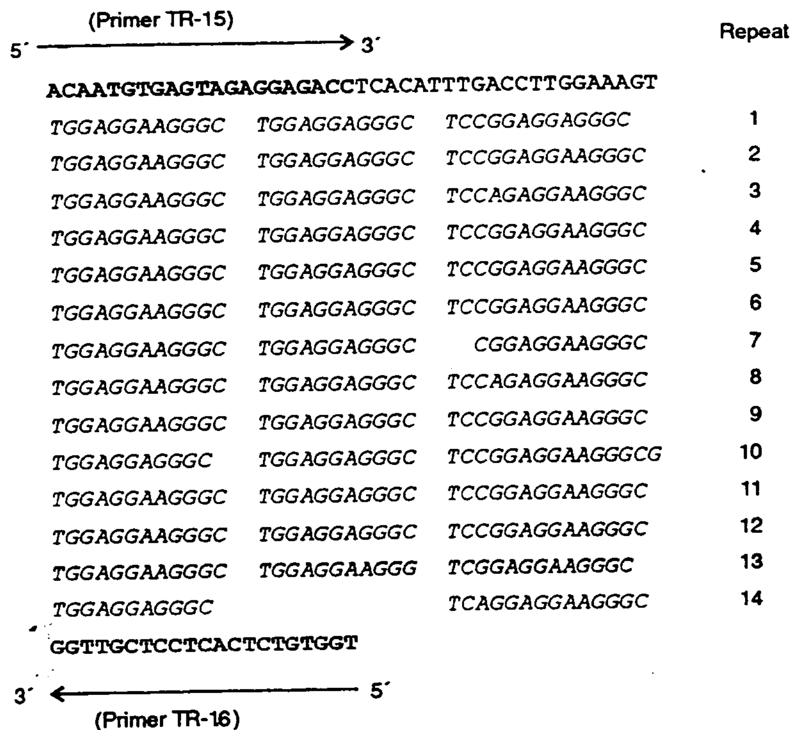

Fig. 1. Nucleotide sequence of the amplified 566-bp allele of D1S111; repeats are in italics, primer-complementary parts in boldface.

of total possible), the most common were 14/14 and 14/17 (Table 2).

To amplify the RB1-VNTR alleles, we used the longer primer pair of the two proposed [9]; hence the resulting alleles were extended by 50 bp. For example, our 1250-bp allele corresponded to the 1200-bp one in [9] and, judging from the sequence reported therein, contained 22 repeat units. According to the above convention, we designated it No. 22.

Our population sample proved to carry nine RB1-VNTR alleles of 1200–1650 bp (Fig. 3, Table 1; 1300-bp allele 23 was not found). The most frequent alleles were 27 and 29; the most common genotypes among those observed (30% of total possible) were 27/27, 27/29, and 22/27 (Table 3).

For both loci, the observed genotype frequencies fit the Hardy–Weinberg equilibrium by the χ^2 and G-statistic tests (Table 4), as evaluated with the R×C program based on the Roff–Bentzen algorithm [29] which permits statistical assessment when the number of observations in many classes is below five and the standard χ^2 test is inapplicable.

Further, we used the criterion [30] for isolating "modes" in an allele frequency distribution, whereby a

TABLE 1. Frequencies of D1S111 and RB1-VNTR Alleles Among 120 Unrelated Russian Muscovites

D1S111				RB1-VNTR			
Allele No.	Length, bp	Occurrence	Frequency	Allele No.	Length, bp	Occurrence	Frequency
9	381	1	0.004	21	1200	1	0.004
10	418	8	0.033	22	1250	34	0.142
11	455	4	0.017	23	1300	–	–
12	492	37	0.154	24	1350	9	0.038
13	529	7	0.029	25	1400	2	0.008
14	566	101	0.421	26	1450	3	0.013
15	603	9	0.038	27	1500	135	0.562
16	640	23	0.096	28	1550	5	0.021
17	677	37	0.154	29	1600	49	0.204
18	714	4	0.017	30	1650	2	0.008
19	751	3	0.013				
20	788	2	0.008				
21	825	2	0.008				
22	862	2	0.008				

TABLE 2. Expected and Observed D1S111 Genotype Frequencies Among 120 Unrelated Russian Muscovites

No.	Genotype	Expected		Observed	
		Frequency	Occurrence	Frequency	Occurrence
1	9/15	0.000	0.038	0.008	1
2	10/10	0.001	0.133	0.017	2
3	10/14	0.028	3.367	0.017	2
4	10/16	0.006	0.767	0.017	2
5	11/11	0.000	0.033	0.008	1
6	11/12	0.005	0.617	0.008	1
7	11/15	0.001	0.150	0.008	1
8	12/12	0.024	2.852	0.075	9
9	12/14	0.130	15.571	0.100	12
10	12/15	0.012	1.388	0.008	1
11	12/16	0.030	3.546	0.017	2
12	12/17	0.048	5.704	0.025	3
13	13/13	0.001	0.102	0.008	1
14	13/14	0.025	2.946	0.033	4
15	13/15	0.002	0.262	0.008	1
16	14/14	0.177	21.252	0.183	22
17	14/15	0.032	3.787	0.033	4
18	14/16	0.081	9.679	0.100	12
19	14/17	0.130	15.571	0.142	17
20	14/18	0.014	1.683	0.017	2
21	14/19	0.011	1.263	0.008	1
22	14/20	0.007	0.842	0.017	2
23	14/22	0.007	0.842	0.008	1
24	15/17	0.012	1.388	0.008	1
25	16/16	0.009	1.102	0.017	2
26	16/17	0.030	3.546	0.008	1
27	16/19	0.002	0.287	0.017	2
28	17/17	0.024	2.852	0.042	5
29	17/18	0.005	0.617	0.017	2
30	17/21	0.003	0.308	0.017	2
31	17/22	0.003	0.308	0.008	1

mode is the allele with a frequency exceeding the sum of those for alleles differing from it by a single repeat unit. Thus D1S111 exhibited a tetramodal allele distribution: 14 (0.421), 12 (0.154), 17 (0.154), and 10 (0.033); for RB1-VNTR the distribution was also tetramodal: 27 (0.528), 29 (0.204), 22 (0.142), and 24 (0.038).

The RB1-VNTR allele frequency distribution found for our Russian population sample was similar to those for US Caucasians, US Hispanics, and Mexican Hispanics (in decreasing order) (Table 5). This was manifest in the prevalence of mode 27. Noteworthy in the Russian sample is allele 21 (in heterozygous state) which was not reported in the American samples.

For Afro-Americans the pattern was quite different, with moderate prevalence of mode 28 and more homogeneous allele frequency distribution, resulting in higher heterozygosity as compared with other populations (Table 5).

Comparison of the RB1-VNTR allele frequency distributions in different populations with the use of the R×C program revealed no statistically significant differences between Russians, US Caucasians, and Mexican Hispanics (data not presented). However, Russians differed from Mexicans more than US Caucasians; this appears to reflect gene exchange between American populations.

The observed heterozygosity in RB1-VNTR (Table 5) for the Russian sample was lower than in others except Mexicans. The *PD* values were virtually the same for Russians and the US Caucasians or Hispanics. On the whole, the RB1-VNTR allele frequency distribution and information content are much the same for Russians and North-American Caucasians. Compared with Afro-Americans, the value of this locus for individual identification in other populations is considerably lower.

A family study of allele distribution (Fig. 4) testifies to independent inheritance of RB1-VNTR and D1S111. These loci were used to examine 17 cases of disputed paternity (maternity undebatable, one supposed father examined, prior typing in four other loci: D1S80, ApoB 3′VNTR, IgH 5′VNTR, D17S5 [26]). In all cases paternity was not excluded. No mutant alleles were found (about 40 meioses). Inclusion of these two loci added two decimal digits on average to the reliability of pater-

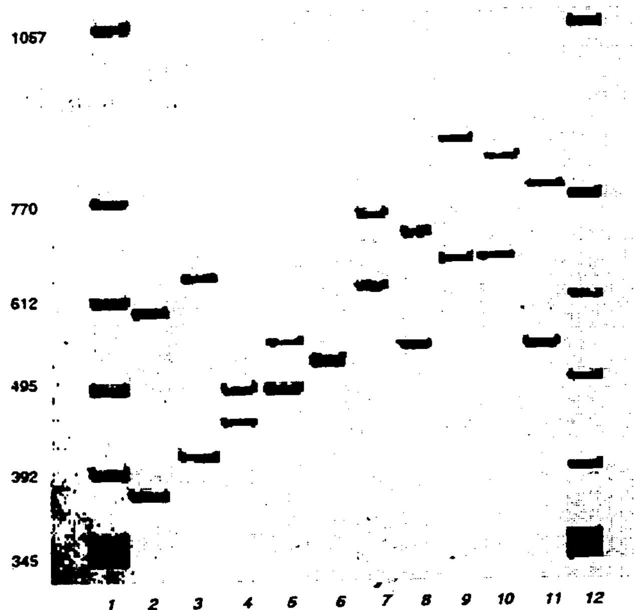

Fig. 2. Electrophoretic resolution of amplified p33.6 alleles (computer scan): *1,12*) ϕX174/*Hinc*II markers; *2–11*) complete set of D1S111 alleles obtained upon genotyping of 10 unrelated individuals with genotypes *2*) 9/15, *3*) 10/16, *4*) 11/12, *5*) 12/14, *6*) 13/13, *7*) 16/19, *8*) 14/18, *9*) 17/22, *10*) 17/21, *11*) 14/20.

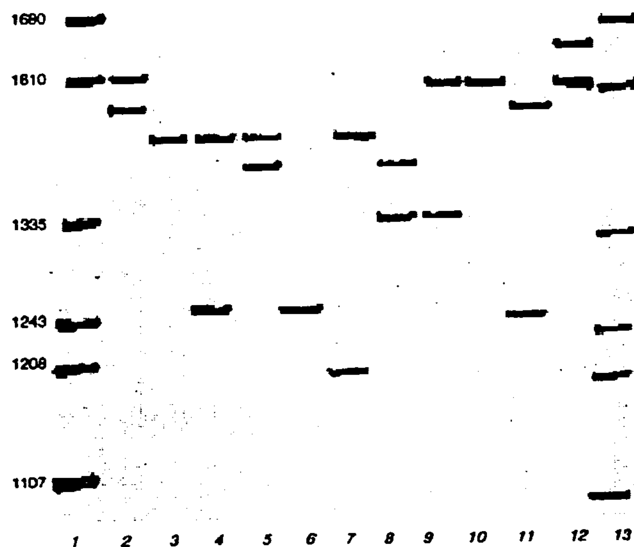

Fig. 3. Electrophoretic resolution of amplified RB1-VNTR alleles (computer scan): *1,13*) λ/*Hinc*II markers; *2–12*) complete set of RB1-VNTR alleles obtained upon genotyping of 11 unrelated individuals with genotypes *2*) 28/29, *3*) 27/27, *4*) 22/27, *5*) 26/27, *6*) 22/22, *7*) 21/27, *8*) 24/26, *9*) 24/29, *10*) 29/29, *11*) 22/28, *12*) 29/30.

nity as compared with the four-VNTR analysis. Thus the minimal probability of paternity was 99.99% (Bayes algorithm) for a panel of six loci.

Since the p33.6 minisatellite and the polymorphic locus D1S80 studied earlier reside in the same chromosome 1, their possible linkage was tested in a separate experiment. Analysis of large families with three–four children suggests independent inheritance. It should, however, be admitted that studying the association of alleles from different loci and the mutation process requires much more data, and our conclusions are only preliminary.

The data in Table 4 indicate the appreciably higher information content and hence the higher practical value

TABLE 3

No.	Genotype	Expected		Observed	
		Frequency	Occurrence	Frequency	Occurrence
1	9/15	0.000	0.038	0.008	1
1	21/27	0.005	0.562	0.008	1
2	22/22	0.020	2.408	0.017	2
3	22/27	0.159	19.125	0.150	18
4	22/28	0.006	0.708	0.008	1
5	22/29	0.058	6.942	0.092	11
6	24/26	0.001	0.113	0.017	2
7	24/27	0.042	5.062	0.050	6
8	24/29	0.015	1.838	0.008	1
9	25/29	0.003	0.408	0.017	2
10	26/27	0.014	1.688	0.008	1
11	27/27	0.316	37.969	0.350	42
12	27/28	0.023	2.812	0.025	3
13	27/29	0.230	27.562	0.175	21
14	27/30	0.009	1.125	0.008	1
15	28/29	0.009	1.021	0.008	1
16	29/29	0.042	5.002	0.050	6
17	29/30	0.003	0.408	0.008	1

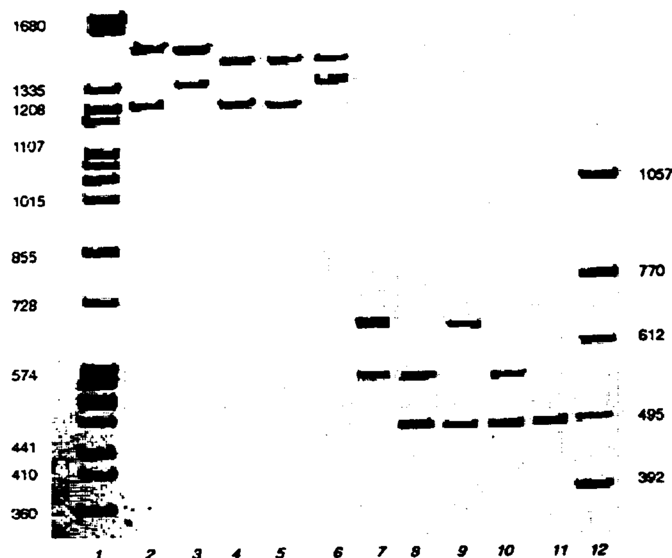

Fig. 4. Electrophoretic resolution of amplified D1S111 (*2–6*) and RB1-VNTR (*7–11*) alleles (computer scan); a family study: *1,12*) λ/*Hinc*II and ϕX174/*Hinc*II markers; genotypes *2*(father) 22/27, *3*(child 1) 24/27, *4*(child 2) 22/26, *5*(child 3) 22/26, *6*(mother) 24/26; *7*(father) 14/17, *8*(child 1) 12/14, *9*(child 2) 12/17, *10*(child 3) 12/14, *11*(mother) 12/12.

TABLE 4. Goodness-of-Fit Tests and Information Parameters for Loci D1S111 and RB1-VNTR

	D1S111	RB1-VNTR
χ^2	32.3579	10.9484
Probability	0.9370±0.0077	0.9880±0.0034
G-Statistic	41.5625	14.0621
Probability	0.9350±0.0078	0.9840±0.0040
H_{obs}	0.650	0.583
H_{exp}	0.765	0.622
pM	0.081	0.187
PD	0.919	0.813
W	0.935	0.503
PIC	0.547	0.417

of D1S111 versus RB1-VNTR for individual identification in the Russian population. The combined *pM* for the two loci is 0.015.

The AMP-FLP analysis of six hypervariable loci in the Russian population (this work and [26]) is comparable in resolving power (*pM* = 3.68) to individual typing with three–five monolocus probes [31]. At the same time this technique is rapid, less dependent on the quantity and quality of material, and obviates the use of radioactive compounds. A weighty advantage is also the discrete spectrum of AMP-FLP alleles, which permits one to deal with genotypes rather than phenotypes.

Judging by the *pM* value, polymorphism of the six loci studied fails to distinguish on average only four individuals in a population of 10 million. Addition of every next variable locus decreases the *pM* 5–15 times on average, yielding a value about 10^{-12} for 10 loci. This is commensurate with the *pM* for multilocus probes (e.g., for 33.6 $pM \approx 10^{-10}$ [4]). It appears preferable to increase the number of AMP-FLP loci with short tandem repeats: since these are shorter (120–350 bp [32,

TABLE 5. Allele Frequencies, Sample Volume, and Distribution Parameters for Locus RB1-VNTR in Different Populations

Allele No.	Frequency				
	Russians	US Caucasians	Afro-Americans	US Hispanics	Mexican Hispanics
	0	0	0	0	*0.0074*
21	0.004	0	0	0	0
22	*0.142*	*0.1016*	*0.0508*	*0.0508*	*0.0508*
24	*0.038*	*0.0156*	0.0593	0.0169	0.0074
25	0.008	0.0078	*0.1271*	0.0254	0.0147
26	0.013	0.0703	0	0.0169	0.0074
27	*0.562*	*0.5859*	0.2542	*0.5932*	*0.6912*
28	0.021	0.0547	*0.3983*	0.0932	0.0662
29	*0.204*	*0.1406*	0.0593	0.0847	*0.1397*
30	0.008	0.0234	0.0254	*0.1186*	0.0074
31	0	0	0.0169	0	0
34	0	0	*0.0085*	0	0
Number of individuals	120	64	59	59	68
H_{obs}	0.583	0.618	0.750	0.613	0.494
PD	0.813	0.79	0.92	0.79	0.68
Number of modes	4	4	4	3	4

Note: American data from [9]. "Modal" allele frequencies italicized.

33]) than VNTR, they will more probably by preserved in degraded DNA specimens. The narrow discrete spectrum of STR alleles permits co-amplification of several loci with subsequent precise determination of amplified allele lengths in genotyping.

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