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Hypervariable regions of DNA for parentage testing and individual identification

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Summary. Four kinds of DNA probes that recognize hypervariable regions (HVR) were studied for parentage testing and individual identification. Allele frequencies and their confidence intervals among unrelated Japanese individuals were obtained. Codominant segregation of the polymorphism was confirmed in family studies. Two a priori probabilities were calculated for each HVR locus: the exclusion probabilities for an alleged father/mother/child trio and for an alleged parent/child duo, and the probabilities of matching of genotypes of two unrelated individuals or two siblings. The ease of availability of the probes and their highly discriminating polymorphic patterns mean they could be very useful for forensic purposes.

Key words: DNA hypervariable polymorphism – Parentage testing, DNA

Zusammenfassung. Es wird eine Analyse der Polymorphismen von 4 hypervariablen Gebieten (HVR, hypervariable region) von 4 verschiedenen DNA-Polymorphismen zur Vaterschaftsfeststellung und zu Identifizierungszwekken vorgenommen. Die Allelfrequenzen und die Konfidenz-Intervalle zwischen unverwandten Japanern werden angegeben. Der co-dominante Erbgang des Polymorphismus wurde durch Familienuntersuchungen bestätigt. Zwei a priori-Wahrscheinlichkeiten wurden für jeden HVR-Locus errechnet: Die Ausschlußwahrscheinlichkeiten für ein Putativ-Terzett „Vater/Mutter/Kind" und für ein Putativ-Duo „Eltern/Kind". Außerdem wurden die Wahrscheinlichkeiten für eine genotypische Übereinstimmung zwischen zwei unverwandten Personen oder zwischen zwei Geschwistern errechnet. Die Zugänglichkeit der Proben und die hochgradig diskriminierenden polymorphen Muster machen die untersuchten Systeme sehr brauchbar für forensische Zwecke.

Schlüsselwörter: DNA-Polymorphismus – Vaterschaftsfeststellung, DNA

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Introduction

Many DNA restriction fragment length polymorphisms (RFLPs) have been reported that recognize the presence or absence of particular restriction enzyme sites giving rise to two possible alleles at each locus [1]. Another class of polymorphisms is that of hypervariable regions (HVRs) of DNA, which result from a variable number of tandem repeats (VNTR). They are valuable genetic markers for human linkage maps [2, 3], parentage testing [4, 5] and individual identification [6–8]. Many of these probes consist of a short core sequence element (VNTR or HVR), which is repeated in tandem along the chromosome and determines the variable polymorphic character of the locus [9]. However, analyses of DNA fingerprints with minisatellite probes involve many difficulties, including that of assessing which bands are allelic. Minisatellite probes provide undefined chromosomal location, which is statistically unsatisfactory. Four types of single-locus HVR probes were chosen for this study. Allele frequencies and their confidence intervals were investigated among the Japanese population with HVR DNA probes located on different chromosomes, which were demonstrated by statistical analyses to be extremely informative for paternity testing and individual identification.

Materials and methods

Samples analyzed. DNA samples from more than 100 unrelated individuals and 20 two-generation Japanese families from north Japan were analyzed in this study. To prepare DNA from peripheral blood, the buffy coat was mixed with several aliquots of 0.2% NaCl containing 10 m*M* ethylenediamine tetraacetic acid (EDTA) at 4°C and the DNA extracted by the standard technique [10]. The amount of DNA was measured by spectrophotometric scanning between 340 nm and 260 nm and calculated using 260 nm absorbance. An optical density (OD) of 1 corresponded to 50 μg/ml DNA. At the same time, the purity of the sample was checked by the ratio between the absorbance at 260 nm and 280 nm (OD_{260}/OD_{280}).

DNA probes. All four probes were purchased from Amersham, UK; 1: MR24/1 XY pseudoautosomal HVR-DNA probe (MR24/1) has been assigned to the pseudoautosomal region at the telomers of the X and Y chromosomes; 2: alpha-globin 3′ HVR-DNA probe (3′globin) has been located at 16p12pter and comprises a tandem repeat array of 17 base pairs [11]. The sequence within the core oligonucleotide is (5′-GNGGG(n)ACAG-3′); 3: Ha-ras HVR-DNA probe (Ha-ras) has been located at 11p15.5 [12]; 4: Mucin HVR-DNA probe (Mucin) has been assigned to chromosomal location 1q21 [13].

Labeling of probes. The HVR probes were labeled with ^{32}P-αdCTP (3,000 μCi/mmol) using the Multiprime DNA labeling system (Amersham, $2–3 \times 10^9$ cpm/μg DNA). Two molecular weight standards were also labeled with the same ^{32}P-dCTP (1×10^6 cpm/μg of marker DNA) according to the T4 DNA polymerase labeling method [14].

Southern hybridization. Six restriction enzymes [REs: *Hin*fI, *Alu*I, *Pst*I, *Pvu*II and *Taq*I (Boehringer Mannheim, FRG), *Rsa*I (New England Biolab, Beverly, MA, USA) were used for the digestion of DNA samples (2.5 μg of extracted DNA) and incubated at 37°C for 5–6 h using 10–12 total units of RE per sample. The digested DNA fragments were loaded on a 24-cm-long horizontal 1% agarose gel. Electrophoresis was carried out in 1 × TBE buffer (pH 8.0) at 1 V/cm for approximately 20 h. The sizes of polymorphic restriction fragments hybridizing to the probe were determined by comparison with the mobilities of two DNA size markers. Two kinds of molecular weight standard markers were used for comparison purposes: (1) a 123-base-pair ladder (Bethesda Research Laboratories, Md.) and (2) EcoT fragments of lambda DNA (Takara), which revealed bands migrating at 19.33, 7.74, 6.22, 4.25, 3.47, 2.69, 1.88,

1.49, 0.93, and 0.42 kb, respectively (1 ng DNA/well for the 123-base-pair ladder and 500 ng of DNA/well for lambda DNA). The ladder was loaded 3×; in the *two* outside lanes on each side of the gel and in the *two* center lanes.

The DNA fragments were then transferred to a nitrocellulose membrane (Schleicher and Schuell', Dassel, Federal Republic of Germany). Prehybridization was carried out at 42°C in a solution containing 50% formamide, 5 × SSPE (1 × SSPE = 0.15 *M* sodium chloride, 0.01 *M* sodium dihydrogen phosphate 1 mM EDTA), 0.2 mg/ml salmon sperm DNA, 0.1% SDS, and 5 × Denhardt's solution [1 × Denhardt's solution = 0.2 g/l Ficoll (Sigma, type 400), 0.2 g/l polyvinylpyrrholidone (PVP, Kodak), and 0.2 g/l bovine serum albumin (Sigma, type V)]. The filters were hybridized with the probes at 42°C for 12 h in the same solution as had been used for prehybridization, except that the concentration of salmon sperm DNA was reduced by half. The filters were washed twice at 50°C for 15 min with 0.1 × SSPE and 0.1% SDS followed by a solution of 0.1 × SSPE for few minutes, and then air dried. Autoradiography was carried out at −90°C using Fuji X-ray film with two intensifying screens for half a day.

Standard paternity testing methods. Routine paternity testing methods are taken from standard protocols and include serological analyses of selected red cell antigen systems (ABO, Rh-Hr, MNSs, Kell, P, Duffy and Kidd), red cell enzymes (phosphoglucomutase-1, esterase-D, acid phosphatase, phosphogluconate dehydrogenase) and serum proteins (group-specific component, Km allotype, Gm allotype, alpha$_1$-antitrypsin, transferrin and haptoglobin).

Statistical methods. The HVR alleles used in this study were subdivided into 34, 37, 5 and 10 non-overlapping groups of DNA fragments. Therefore, the allele frequency values for HVR were used directly for calculation. The size of the alleles for VNTRs was found to vary by increments that were smaller than the size measurement error. The size of the alleles was measured in 100-bp increments using MR24/1 and 3′globin probes. To evaluate the precision of the estimation, 95% confidence intervals were calculated, taking into consideration that allele frequencies of a locus are multinominally distributed [15]. The distribution of χ^2 determined assuming that the number of homozygotes and heterozygotes in dinominally distributed and the population is in Hardy-Weinberg equilibrium [6].

The Paternity exclusion (alleged father/mother/child) was calculated according to the calculation formula of Ohno et al. [16]. The paternity exclusion (alleged parent/child) and the probability of matching of two random persons and two siblings were calculated according to Selvin [17] and Garber and Morris [18].

Results

We investigated each of the four HVR loci with six different kind of REs (*Hin*fI, *Alu*I, *Pst*I, *Pvu*II, *Taq*I and *Rsa*I). The pattern of polymorphisms after probing

Table 1. Approximate ranges of fragment sizes observed with alternative restriction enzymes

	MR24/1	3′globin	Ha-ras	Mucin
*Alu*I	1.5–5.0	1.0–5.0	0.5– 3.0	3.0–7.0
*Hin*fI	1.5–7.0	0.3–6.0	2.0– 4.0	3.0–7.0
*Pst*I	[a]	1.0–5.0	1.0– 3.0	2.0–7.0
*Pvu*II	6.0–9.0	1.5–8.0	2.0– 4.0	3.0–7.0
*Rsa*I	0.5–4.0	1.0–5.0	5.0–10.0	2.5–7.0
*Taq*I	2.0–6.0	3.0–9.0	1.0– 4.0	3.0–7.0

Data generated by analysis of 30 random individuals. Other alleles may be observed in large population samples

[a] No polymorphic bands were observed

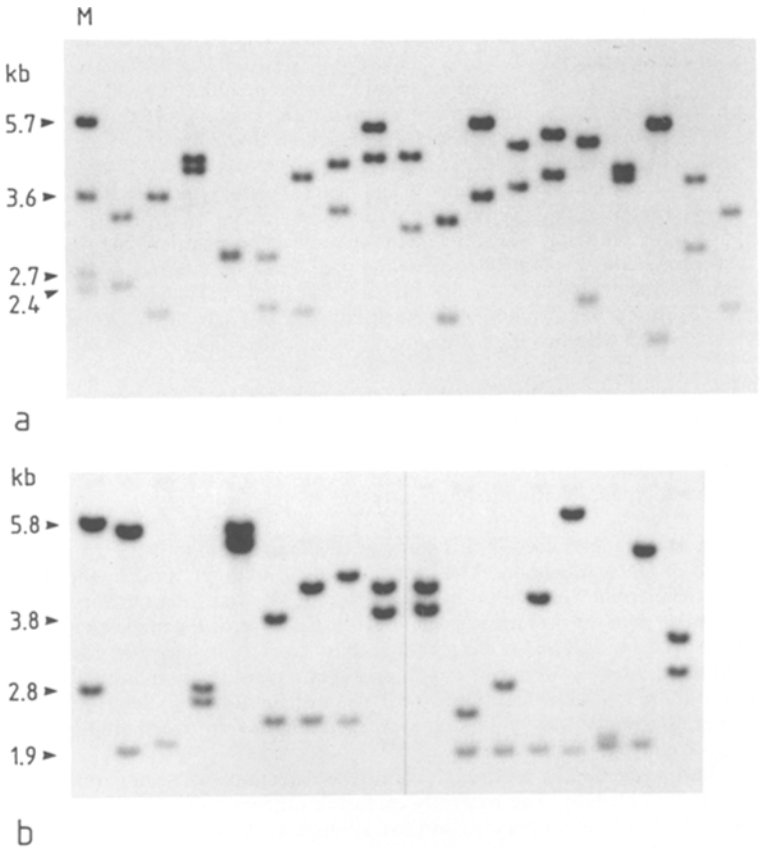

Fig. 1a, b. Autoradiogram obtained from Southern blot analysis. DNA from 19 unrelated individuals, digested with *Hin*fI and probed with MR24/1 (**a**) and from 17 unrelated individuals, digested with *Pvu*II, probed with 3′globin (**b**). The *arrowheads* indicate sizes of marker bands. The sizes of alleles correspond to those in Table 2. Fragment length is given in kilobase pairs. The size was determined according to the method indicated in the text

with HVR or VNTR probes showed basically no variation with the different restriction enzymes. Each of the probes used in this study recognized a single locus, so that the DNA of an individual had either two polymorphic fragments (heterozygotes) or one (homozygotes). The number and size of the alleles varied widely from locus to locus [9]. The range of approximate allele band sizes observed with alternative restriction enzymes is indicated in Table 1. MR24/1 probing showed no polymorphic bands with *Pst*I, condensed polymorphic bands in a narrow range with *Pvu*II and polymorphic bands over a wide range below 1.0 kb with *Rsa*I. 3′Globin probing showed four polymorphic bands per individual with *Hin*fI, and condensed bands in a narrow range with *Taq*I. Autoradiograms of the 3′globin probe showed variation in the intensity of fragments using six kinds of REs in this experiment. The Ha-ras probing showed intimate bands in a narrow range with *Rsa*I and polymorphic bands below 1.0 kb with *Alu*I. Mucin probing showed almost the same polymorphisms using any REs

Table 2. Allele frequencies and confidence intervals observed in MR24/1-*Hin*fI system

Allele size (kb)	Frequency	95% Confidence interval	
6.5	0.004	0.000	0.012
6.1	0.004	0.000	0.012
5.8	0.046	0.020	0.073
5.4	0.025	0.005	0.045
5.2	0.004	0.000	0.012
5.0	0.025	0.005	0.045
4.9	0.025	0.005	0.045
4.6	0.030	0.008	0.051
4.5	0.042	0.017	0.068
4.4	0.055	0.026	0.083
4.3	0.050	0.023	0.078
4.2	0.068	0.035	0.099
4.1	0.004	0.029	0.089
4.0	0.059	0.029	0.089
3.9	0.034	0.011	0.057
3.8	0.004	0.000	0.012
3.7	0.050	0.023	0.078
3.6	0.046	0.020	0.073
3.4	0.046	0.020	0.073
3.3	0.004	0.000	0.012
3.2	0.017	0.000	0.033
3.0	0.017	0.000	0.033
2.9	0.008	0.000	0.020
2.8	0.034	0.011	0.057
2.7	0.021	0.003	0.039
2.6	0.038	0.013	0.062
2.5	0.038	0.013	0.062
2.4	0.042	0.017	0.067
2.3	0.038	0.014	0.062
2.2	0.034	0.011	0.057
2.1	0.038	0.014	0.062
2.0	0.038	0.014	0.062
1.9	0.004	0.000	0.012
1.6	0.008	0.000	0.020
Total 32 alleles	1.000		

with a small shift of the bands. On the basis of these observations, it was decided to use *Hin*fI for MR24/1 and *Pvu*II for 3′globin, Ha-ras and Mucin, respectively.

In this study, a large number of alleles were detected (Tables 2–5). The size of an allele, which differs by a single repeat of the core sequence, is too small to be distinguished under our southern blotting procedure. The 100-bp increment was adopted to indicate the fragment size in the MR24/1 (*Hin*fI) and 3′globin (*Pvu*II) combination systems (Tables 2, 3). Thirty-four different alleles of the

Table 3. Allele frequencies and confidence intervals observed in 3′globin-*Pvu*II system

Allele size (kb)	Frequency	95% Confidence interval	
8.7	0.004	0.000	0.010
7.8	0.011	0.000	0.023
7.0	0.011	0.000	0.023
6.7	0.011	0.000	0.023
6.4	0.011	0.000	0.023
6.2	0.007	0.000	0.017
6.0	0.018	0.002	0.033
5.8	0.018	0.002	0.033
5.6	0.014	0.004	0.028
5.4	0.025	0.007	0.043
5.2	0.039	0.016	0.062
5.0	0.011	0.000	0.023
4.8	0.011	0.000	0.023
4.6	0.011	0.000	0.023
4.4	0.039	0.016	0.062
4.2	0.021	0.004	0.038
4.1	0.007	0.000	0.017
4.0	0.021	0.004	0.004
3.8	0.039	0.016	0.061
3.7	0.011	0.000	0.023
3.6	0.028	0.009	0.048
3.4	0.028	0.009	0.048
3.3	0.004	0.000	0.010
3.2	0.039	0.016	0.061
3.1	0.004	0.000	0.010
3.0	0.021	0.004	0.038
2.9	0.011	0.000	0.023
2.8	0.028	0.009	0.048
2.7	0.004	0.000	0.010
2.6	0.021	0.004	0.038
2.5	0.014	0.000	0.028
2.4	0.060	0.033	0.088
2.3	0.034	0.014	0.057
2.2	0.021	0.004	0.038
2.1	0.060	0.033	0.088
2.0	0.244	0.195	0.295
1.9	0.039	0.016	0.062
Total 37 alleles	1.000		

Table 4. Allele frequencies and confidence intervals observed in Ha-ras – *Pvu*II system

Allele size (kb)	Frequency	95% Confidence interval	
4.4	0.047	0.020	0.074
3.8	0.098	0.060	0.136
3.2	0.009	0.000	0.020
2.7	0.748	0.692	0.804
2.6	0.098	0.060	0.136
Total 5 alleles	1.000		

Table 5. Allele frequencies and confidence intervals observed in Mucin probe – *Pvu*II system

Allele size (kb)	Frequency	95% Confidence interval	
6.4	0.007	0.000	0.017
6.1	0.078	0.047	0.109
5.6	0.014	0.000	0.028
5.1	0.032	0.011	0.052
4.9	0.028	0.009	0.048
3.9	0.011	0.000	0.022
3.8	0.716	0.664	0.769
3.7	0.096	0.061	0.130
3.5	0.011	0.000	0.023
3.3	0.007	0.000	0.017
Total 10 alleles	1.000		

MR24/1 probe were found in a sample of 119 unrelated Japanese (Table 2) digested with *Hin*fI. Allele sizes were between 1.6 and 6.5 kb. The 3′globin probe showed 37 different alleles in a sample of 141 unrelated Japanese (Table 3) following digestion with *Pvu*II. As shown in Tables 4 and 5, Ha-ras and Mucin probes showed 5 and 10 different alleles digested with *Pvu*II in 117 and 141 unrelated Japanese, respectively.

Relative allele frequencies (%) for these three VNTR probes are shown schematically in Fig. 2. A relatively small range was observed for MR24/1 (0.004–0.068) and 3′globin (0.004–0.244), but for the Ha-ras and Mucin probes, the range was much higher. Confidence intervals (95%) for each allele frequency are also indicated in Tables 2–5. The heterozygosity rates for MR24/1, 3′globin, Ha-ras and Mucin were calculated to be 0.959, 0.917, 0.419 and 0.469, respectively.

The HVR probes used in this study were located on different chromosomes as mentioned previously. The expected and observed frequencies of homozygotes and heterozygotes are summarized in Table 6. Fairly close similarities were found between the observed and expected frequencies, and chi-square value was between 0.313 and 2.91. In Table 7, the exclusion probability is shown when both the parents and the child are typed (AF/M/C) or when one parent and the child (P/C) are typed. The high scores of exclusion probability (MR24/1;

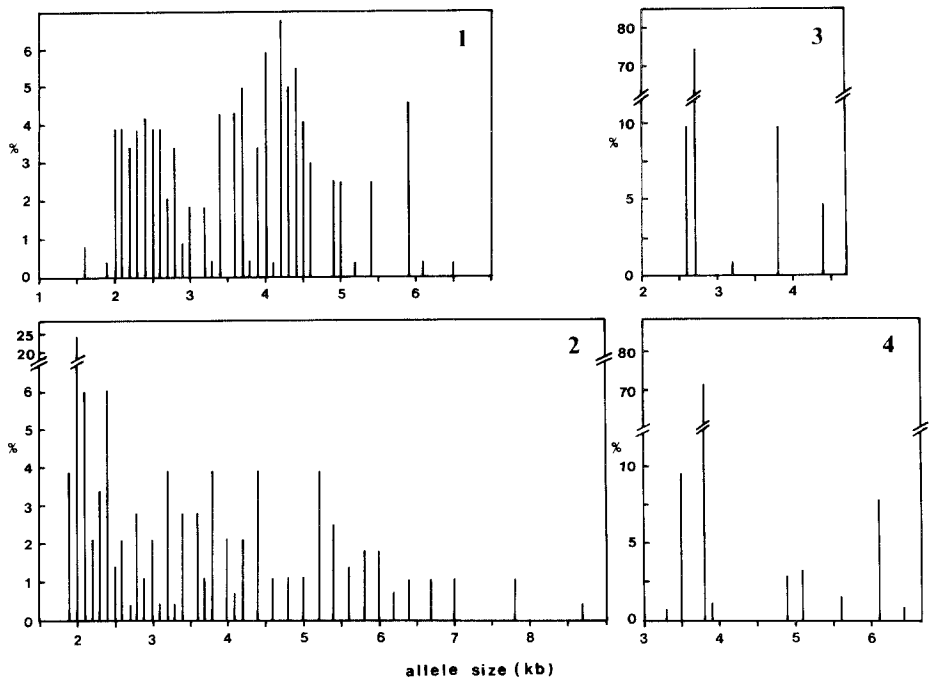

Fig. 2. Relative frequency (percent) of alleles according to sizes (kb) for the four HVR probes analyzed (*1*, MR24/1-*Hin*fI; *2*, 3′globin-*Pvu*II; *3*, Ha-ras-*Pvu*II; *4*, Mucin-*Pvu*II)

Table 6. Expected (Exp.) and observed (Obs.) frequencies of homozygotes and heterozygotes

Probe	Enzyme	Number of alleles	Number of individuals	Homozygotes		Heterozygotes		χ^2	$P(d.f. = 1)$
				Obs.	Exp.	Obs.	Exp.		
MR24/1	*Hin*fI	34	119	6	4.8	113	114.2	0.313	$0.50 < P < 0.75$
3′Globin	*Pvu*II	37	139	17	11.8	124	129.2	2.50	$0.10 < P < 0.25$
Ha-ras	*Pvu*II	5	117	72	68.0	45	49.0	0.562	$0.25 < P < 0.50$
Mucin	*Pvu*II	10	141	85	74.9	56	66.1	2.911	$0.10 < P < 0.25$

Significant deviation of observed frequencies from expected frequencies

0.918, 3′globin; 0.843) indicate a high degree of usefulness in parentage tests. Twenty cases of two-generation families were investigated with HVR probes, whose paternity relations were confirmed with the standard paternity testing systems. Every band of a child can be traced to either of the parents. No mutations were observed. The cumulative exclusion probability was estimated to be 0.993 with the assumption that there is no linkage disequilibrium between the bands.

In Table 7, the probability of matching was summarized indicating the usefulness for forensic individualization. From these four analyses, the probability of two randomly selected individuals having the same genotype was found to be

Table 7. Exclusion probabilities in parentage testing and probability of matching of four HVR loci

Probe	Enzyme	Alleles	Exclusion probability		Probability of matching	
			AF/M/C	P/C	2 Random persons	2 Siblings
MR24	*Hin*fI	34	0.918	0.848	3.23×10^{-2}	0.271
3′Globin	*Pvu*II	37	0.843	0.733	1.02×10^{-2}	0.294
Ha-ras	*Pvu*II	5	0.240	0.093	0.362	0.631
Mucin	*Pvu*II	10	0.293	0.126	0.300	0.590
Cumulative 4 systems			0.993	0.968	3.58×10^{-6}	2.97×10^{-2}

AF, Alleged father; M, mother; C, child; P, parent

3.58×10^{-6}. On the other hand, the probability of two randomly chosen siblings having the same genotype was 2.97×10^{-2}.

Discussion

Hypervariable DNA polymorphisms are now starting to be widely applied especially in forensic individualization [6, 7, 19] and in paternity suits [5, 20]. Almost all of them are multi-locus probes which recognize several HVR or VNTR loci under conditions of reduced stringency. Single-locus probes can easily be handled under high stringency conditions and are more sensitive and reproducible than multi-locus probes. The traditional statistical analyses of paternity and matching probabilities are not suitable for application to the results of multi-locus probes because of the undefined chromosome location. As all four probes in this study are located different chromosomes they may be assumed to be statistically independent.

Since each of the probes used recognized a single locus, the DNA of an individual has either two polymorphic fragment sizes (heterozygote) or one (homozygotes). We preliminarily tested six kinds of RE on extracted DNA from 30 unrelated Japanese. According to the results indicated in Table 1, MR24/1 showed relatively condensed polymorphic bands in a narrow range with *Pvu*II. The probe 3′globin showed a total of four fragment sizes (inheritance of two sets of two polymorphic bands) with *Hin*fI. Under these conditions, the most reasonable combination of probe and RE was selected as follows; *Pvu*II for 3′globin and Ha-ras or Mucin, *Hin*fI for MR24/1 and Ha-ras or Mucin.

In the cases of MR24/1 and 3′globin fragment sizes were calculated in 100-bp increments. The alleles detected by MR24/1 and 3′globin probes in Japanese population were grouped into 34 and 37 size classes, but these categories do not represent individual alleles. The recognition of the single repeat sequence of 3′globin (17 bp [21]) might be beyond the discrimination capabilities of agarose gel electrophoresis. This might be one of the reasons for the relatively high number of homozygote individuals compared with the number expected (Table 6). This probe has theoretically over 300 repeat units between 1.90 and 8.00 kb, but the use of 100-bp increments might be a conservative approach for estimation of the fragment size. Allen et al. [22] reported that one of the four HVR probes, 3′globin, was used for paternity testing among the white and black

populations in the United States. In this report, variation in intensity of alleles in autoradiograms was simply explained as being relative to the allele size, i.e. a 4-kb allele would be expected to bind about twice as much probe as 2-kb allele. Such variations in intensity were not observed in HVR or VNTR probes [23] other than the 3′globin probe. Not only the allele size but also some factors affecting the Tm (melting temperature of duplex DNA [10], i.e. changes in GC content due to the allele size) should be taken into consideration. The heterozygosity frequency of the Mucin probe in a Caucasian population has been reported to be over 85% (product specification, Amersham) but in this study on Japanese was below 50% (46.9%). A different distribution of allele sizes with some VNTR probes among races was reported by Balazs et al. [24]. The heterozygosity frequencies of the other three probes were almost the same in Caucasians [11, 12, 22] and in the Japanese in this study.

Jeffreys et al. [25] reported that the mutation rate among some 'fingerprinting' probes was 5.2% per gamete. On the other hand, Reeders et al. [21] found the mutation rate of 3′globin to be 1 in 1500 meioses. In more than 200 cases tested with 3′globin probe, no mutations have been observed [22]. This kind of mutation is negligible for individual identification. The single-locus HVR loci showed a more stable inheritance. In the case of parental exclusion with HVR probes, however, it should be confirmed with other HVR probes located on other chromosomes.

The probes are available from Amersham UK, and can be used without the constraint of a patent. The exclusion probability (AF/M/C) was over 99%, and the probability of identity was calculated to be 3.58×10^{-6}. The combination of single-locus DNA polymorphisms from HVR loci presents very powerful information for parentage testing and individual identification, as pointed out by Wainscoat et al. [26].

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