Int J Legal Med (1998) 111 : 183–187

ORIGINAL ARTICLE

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Subtyping and characterization of D1S80 alleles in a Japanese population using PCR-RFLP

Received: 12 June 1997 / Received in revised form: 8 December 1997

Abstract In a Japanese population study of the D1S80 locus 24 alleles ranging from allele16 to allele43 were analysed using PCR-RFLP. As two repeat units were found to contain the restriction cleavage site (CCAGG) for EcoRII, we digested the alleles with EcoRII, separated the digested fragments on polyacrylamide gels and stained with ethidium bromide. Of the 24 alleles 11 band patterns were identified and tentatively labeled E1 to E11. A total of 42 subtypes were detected in a population group of 111 unrelated individuals. All samples of allele18 were of the E3 type, while about 60% of the allele 24 samples were of the E4 type and about 40% were of the E8 type. The third most frequent allele (allele30) contained four types, E4, E8, E5 and E6. No deviations from Hardy-Weinberg equilibrium were observed. Since this method could differentiate those samples which had the same length but different sequences, it is quite useful for paternity testing and individual identification.

Key words D1S80 · PCR-RFLP · EcoRII · Subtype frequencies · Gene sequencing

Introduction

Amplification of a variable number of tandem repeat (VNTR) sequences by the polymerase chain reaction (PCR) plays an important role in forensic individual identification and paternity testing. The analysis of the VNTR locus D1S80, a system with high discrimination power and exclusion chance, is widely applied in the forensic practice (Kasai et al. 1990; Rand et al. 1992; Kloosterman et al. 1993; Budowle et al. 1995; Sepulchre et al. 1995) and is especially useful for Orientals, since the alleles are more evenly distributed than in Caucasians (Sugiyama et al. 1993; Huang et al. 1994).

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Some interalleles (or variants) which showed different electrophoretic mobility compared with the allelic ladder (Skowasch et al. 1992; Alonso et al. 1993; Nagai et al. 1994) need to be characterized further. Several research studies have been carried out on this polymorphism in the 5′ flanking region after treatment with restriction enzymes HinfI or Tsp509I (Alonso et al. 1995; Duncan et al. 1996) and on the repeat unit variations (Duncan et al. 1997). We have demonstrated three types of variants of allele27 using PCR-RFLP with MspI and EcoRII as restriction enzymes, and have sequenced these variants (Harashima et al. 1997).

In this study, we investigated the electrophoretic band patterns of 24 alleles ranging from alleles 16 to 43 using PCR-RFLP with EcoRII as the restriction enzyme and calculated the frequency distribution of subtypes in a Japanese population.

Materials and methods

DNA was extracted from the blood of unrelated healthy Japanese (n = 111) using the phenol/chloroform method (Inoko et al. 1986).

PCR was performed according to the procedure described by Sugiyama et al. (1993). A 25 µl reaction mixture contained 0.5 µM of each primer, 200 µM of each dNTP, 2.5 U Taq polymerase and the corresponding buffer (TaKaRa, Japan). Amplification was performed at 94 °C – 1 min, 65 °C – 1 min, 72 °C – 2 min for 30 cycles in a DNA Thermal Cycler 480 (Perkin Elmer).

The amplified fragments were separated on native polyacrylamide gels (T 6%, C 2%, 1 mm thick, 30 cm long) in 1 X TBE (pH 8.3) at 350 V, 35 °C for 6 h. A commercially obtained allelic ladder (Perkin Elmer: AmpliFLP™ D1S80 Allelic Ladder) was run on the same gel as a typing standard. The gels were stained with ethidium bromide.

PCR-RFLP was performed according to Harashima et al. (1997). As alleles of different lengths may produce the same sized fragments after digestion with restriction enzyme, the band patterns from the two alleles would overlap making it difficult to distinguish the two alleles from each other. Thus after electrophoresis on polyacrylamide gels, the target alleles were cut from the gels and eluted in 1 ml TE buffer (pH8.0, 10 mM Tris-HCl, 1 mM EDTA) overnight according to the "crush and soak" method (Maniatis et al. 1989). Then 1 µl of the eluted solution was used as template for a second round of PCR using the same conditions. The re-amplified products were treated with EcoRII at 37 °C overnight, and separated on a 12% polyacrylamide gel (30 cm in length) at 350 V for 3 h in 1XTBE. The nine homozygous samples found were analysed

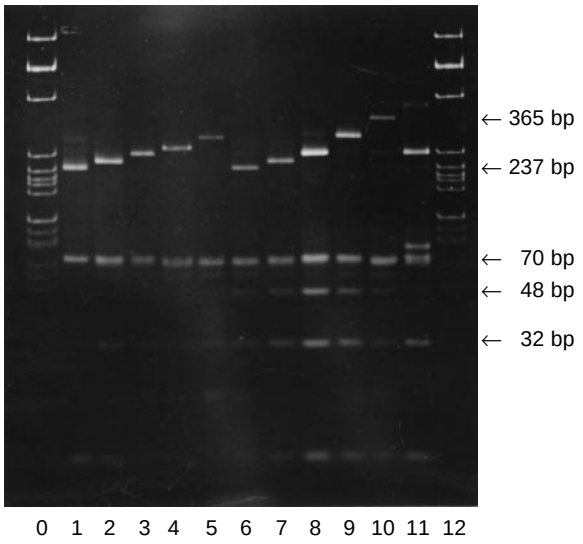

Fig. 1 Electrophoretic band patterns of E1–E11 after treatment with EcoRII. Lane 0, 12: pBR322-HaeIII, lane 1–11: E1-E11

directly using RFLP without re-PCR. The electrophoretic band patterns of the digested fragments were analysed after staining with ethidium bromide using pBR322 DNA digested with HaeIII as a size standard.

The sequencing reaction was performed using the Dyedeoxy Terminator Sequencing Kit (Perkin-Elmer). The re-amplified target alleles were used as templates for the sequencing reaction after purification with Microcon (Amicon, Beverley, USA). The primers for sequencing the forward and reverse strands were the same as those used for PCR. Reactions were carried out at 96 °C – 30 s, 50 °C – 15 s, 60 °C – 4 min for 25 cycles using a DNA Thermal Cycler 480 (Perkin-Elmer). After electrophoresis on an ABI 373 sequencer, the fragments were analysed automatically using Data Collection and SeqEd software (Perkin-Elmer).

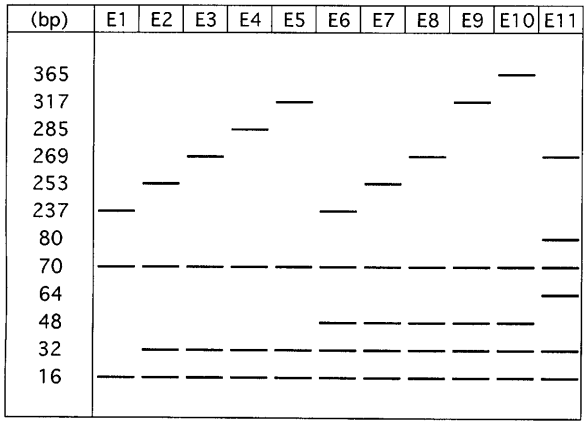

Fig. 2 Predicted fragment sizes following EcoRII digestion

Results and discussion

PCR-RFLP

D1S80 is a highly polymorphic, small-sized (300–900 bp) VNTR locus located on chromosome 1 p applicable to analysis by PCR (Nakamura et al. 1988; Kasai et al. 1990). The repeat unit of D1S80 is 16 bp and contains a cleavage site of the restriction enzyme EcoRII (CCWGG, W = A or G). In our previous report (Harashima et al. 1997), we demonstrated that allele27 could be divided

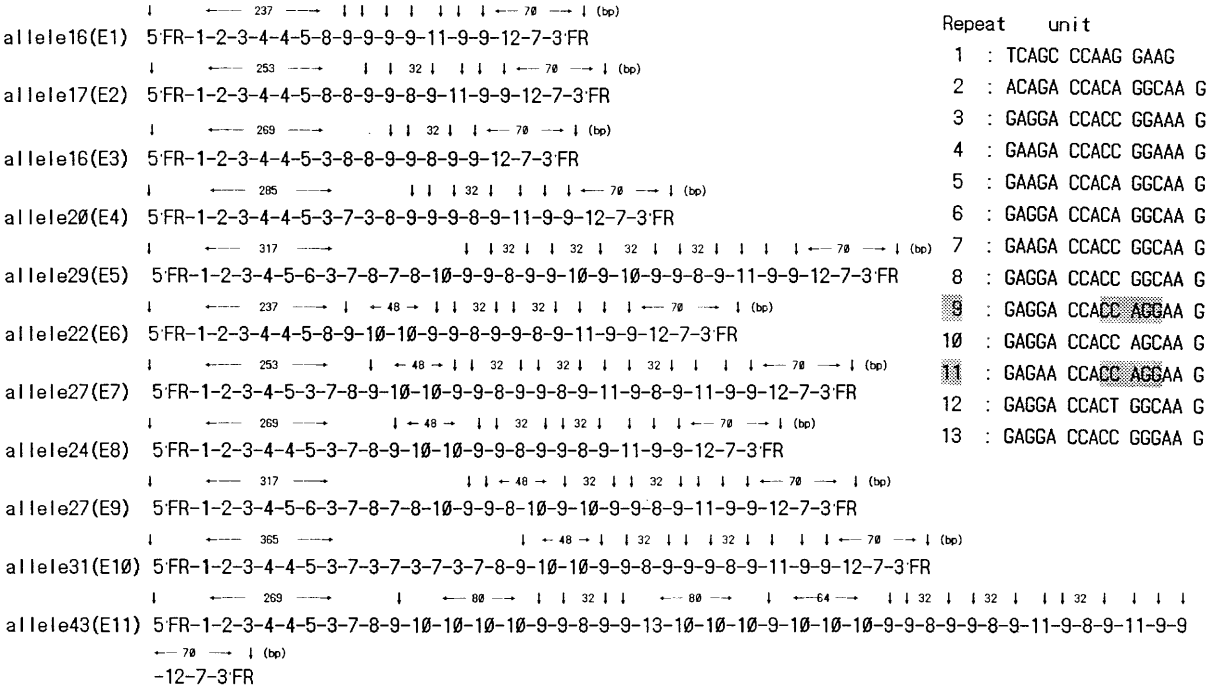

Fig. 3 Sequence structures of 11 kinds of D1S80 alleles ranging from E1 to E11, and 13 kinds of 16 bp repeat unit. E1: allele16 from AmpliFLP™ D1S80 Allelic Ladder, E2: allele17, E3: allele16, E4: allele20, E5: allele29, E6: allele22, E7: allele27, E8: allele24, E9: allele27, E10: allele31, E11: allele43. The arrow (↓) shows EcoRII restriction site

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allele16  5'FR-1-2-3-4-4-5-3-8-8-9-9-8-9-9-12-7-3'FR
allele18  5'FR-1-2-3-4-4-5-3-8-8-9-9-8-9-11-9-9-12-7-3'FR
allele19  5'FR-1-2-3-4-4-5-3-8-8-9-9-8-9-9-11-9-9-12-7-3'FR
allele20  5'FR-1-2-3-4-4-5-3-8-8-9-9-8-9-11-9-9-9-12-7-3'FR
allele21  5'FR-1-2-3-4-4-5-3-7-8-9-9-8-9-9-8-9-11-9-9-12-7-3'FR
allele27  5'FR-1-2-3-4-4-5-3-8-8-9-9-10-9-10-9-10-9-10-9-9-8-9-11-9-9-12-7-3'FR
allele29  5'FR-1-2-3-4-5-6-3-7-8-9-10-9-8-9-9-10-9-10-9-10-9-9-8-9-11-9-9-12-7-3'FR
allele34  5'FR-1-2-3-4-5-6-3-7-8-9-10-9-8-9-8-9-9-10-9-9-10-9-10-9-10-9-9-8-9-11-9-9-12-7-3'FR
allele36  5'FR-1-2-3-4-4-5-6-3-7-9-9-9-8-9-8-9-9-8-9-8-9-9-10-9-10-9-10-9-9-8-9-11-9-9-12-7-3'FR
allele39  5'FR-1-2-3-4-5-6-3-7-8-9-10-9-9-8-9-9-10-9-10-9-9-8-9-11-9-9-9-10-9-10-9-9-8-9-11-9-9-12-7-3'FR
allele41  5'FR-1-2-3-4-5-6-3-7-8-9-10-9-11-9-9-9-9-8-9-9-9-8-9-10-9-9-8-9-9-10-9-10-9-9-8-9-11-9-9-12-7-3'FR
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Fig. 4 Sequence structures of 11 alleles of E3 type. The arrow (↓) shows EcoRII restriction site

into four types, a main type and three variants, using PCR-RFLP with EcoRII as the restriction enzyme and a sequencing method. In order to obtain detailed information about the D1S80 alleles, in this study 24 alleles from 111 samples ranging from allele16 to allele43 were treated with EcoRII. The electrophoretic band patterns of the digested fragments were compared. The band patterns of the 24 alleles could be summarized into 11 types, as shown in Fig. 1 and 10 types were distinguished by the position of the longest band between 237 bp and 365 bp and the presence or absence of the 48 bp band. One type could be characterized by the presence of 64 bp and 80 bp bands. Variations in band patterns reflected structural variations in the amplified fragments.

Figure 2 illustrates the 11 types of band pattern variations and shows the predicted fragment sizes. The types that had no 48 bp band were labeled in increasing order from E1 to E5 in order to match the size increments of the longest fragments. Similarly, the types that possessed a 48 bp fragment were labeled from E6 to E10. The type that had no 48 bp fragment but did have 64 bp and 80 bp fragments was labeled E11. According to these band pattern types, the alleles were thus subtyped, for example, alleles 24 were subtyped into two types: allele24(E4) and allele24(E8). The nine PCR samples that showed single bands using 6% polyacrylamide gel electrophoresis could be further differentiated by PCR-RFLP into seven homozygotes and two heterozygotes according to their band

Fig. 5 D1S80 allele frequencies and subtype frequencies in a Japanese population

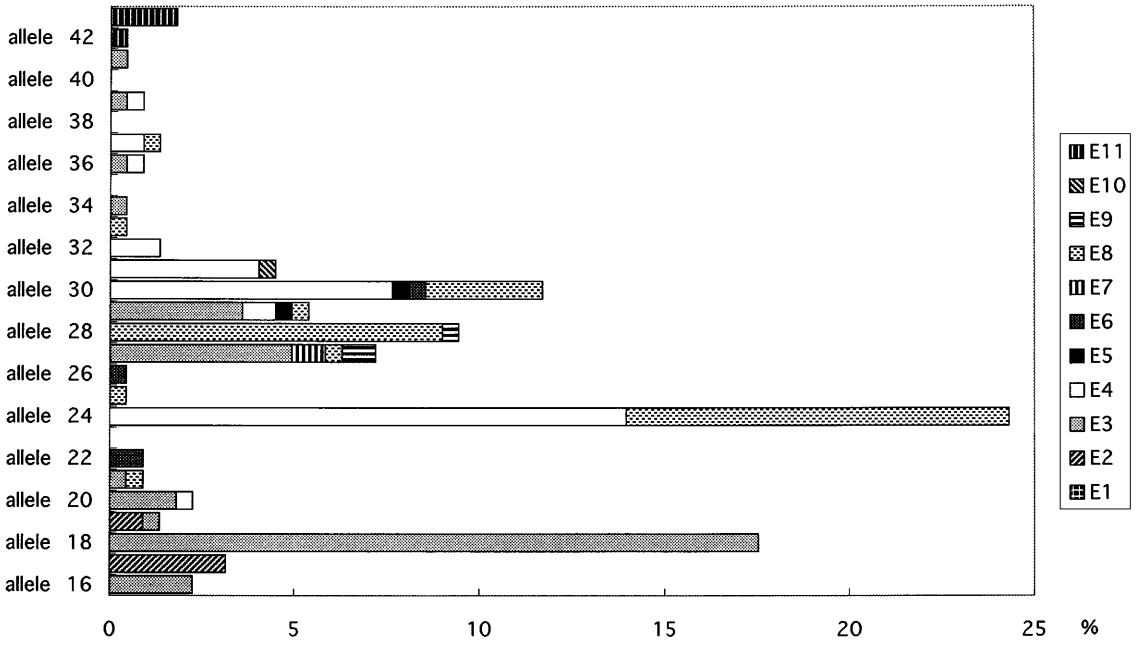

Table 1 Frequency distribution of D1S80 subtypes in Japanese

	Subtype	N	Freq
1	allele16 (E3)	5	0.0225
2	allele17 (E2)	7	0.0315
3	allele18 (E3)	39	0.1757
4	allele19 (E2)	2	0.009
5	allele19 (E3)	1	0.0045
6	allele20 (E3)	4	0.018
7	allele20 (E4)	1	0.0045
8	allele21 (E3)	1	0.0045
9	allele21 (E8)	1	0.0045
10	allele22 (E6)	2	0.009
11	allele24 (E4)	31	0.1396
12	allele24 (E8)	23	0.1036
13	allele25 (E8)	1	0.0045
14	allele26 (E6)	1	0.0045
15	allele27 (E3)	11	0.0495
16	allele27 (E7)	1	0.0045
17	allele27 (E8)	2	0.009
18	allele27 (E9)	2	0.009
19	allele28 (E8)	20	0.0901
20	allele28 (E9)	1	0.0045
21	allele29 (E3)	8	0.036
22	allele29 (E4)	2	0.009
23	allele29 (E5)	1	0.0045
24	allele29 (E8)	1	0.0045
25	allele30 (E4)	17	0.0766
26	allele30 (E5)	1	0.0045
27	allele30 (E6)	1	0.0045
28	allele30 (E8)	7	0.0315
29	allele31 (E4)	9	0.0405
30	allele31 (E10)	1	0.0045
31	allele32 (E4)	3	0.0135
32	allele33 (E8)	1	0.0045
33	allele34 (E3)	1	0.0045
34	allele36 (E3)	1	0.0045
35	allele36 (E4)	1	0.0045
36	allele37 (E4)	2	0.009
37	allele37 (E8)	1	0.0045
38	allele39 (E3)	1	0.0045
39	allele39 (E4)	1	0.0045
40	allele41 (E3)	1	0.0045
41	allele42 (E11)	1	0.0045
42	allele43 (E11)	4	0.018
	Total	222	1

patterns. The seven homozygotes included allele18 (E3)/allele18 (E3), allele24 (E4)/allele24 (E4), allele27 (E3)/allele27 (E3), allele31(E4)/allele31 (E4), and three allele28 (E4)/allele28 (E4). The two heterozygotes were allele24 (E4)/allele24 (E8).

Sequencing

In our previous report we found 12 types of 16 bp repeat units (Harashima et al. 1997). In this study, we sequenced one sample from each of the 11 band pattern types and discovered a new type of repeat unit (GAGGACCAC-CGGGAAG). Thus, a total of 13 types of 16 bp repeat units was found in D1S80 alleles.

Figure 3 shows alleles with different structures, i.e. different arrangements of the 13 kinds of repeat units. The differences in RFLP band patterns were considered to be caused by the different arrangements of these repeat units. As the cleavage site of EcoRII could be seen in units 9 and 11, the alleles could be digested differently relative to the different positions in which they were found in unit 9 and unit 11 (Fig. 2). The sizes of the fragments after EcoRII digestion, shown in Fig. 1, were exactly as predicted.

Figure 4 showed the structures of the 11 kinds of alleles typed as E3 which was the most frequent type. The electrophoretic band patterns of E3 showed 269 bp, 76 bp 32 bp, and 16 bp bands which matched the predicted fragment sizes shown here. This illustrated that alleles of different lengths could show the same band pattern after digestion using EcoRII.

RFLP type frequencies

Figure 5 and Table 1 show the frequency distribution of subtypes of in 24 alleles from 111 Japanese samples. All of the allele18 samples were of the E3 type, while about 60% of the allele24 samples were of the E4 type and about 40% were of E8 type. The third most frequent allele (allele30) consisted of 65% E4 type, 27% E8 type and 4% each of E5 type and E6 type.

The E1 type was found only in the alleles cut from the commercially obtained AmpliFLP™ D1S80 Allelic Ladder and was not found in the 111 Japanese samples.

The E2 type was found mainly in alleles 17 and 19, E3 mainly in alleles 16, 18, 20, 27 and 29, E8 mainly in alleles 24, 28, and 30 and E11 was found only in alleles 42 and 43. In general, E2 and E3 were usually found in short alleles, while E4 and E8 were usually found in long alleles.

In this study on a Japanese population sample of 111 individuals the mean exclusion chance (MEC) and discrimination index (DI) were calculated to be 0.75 and 0.83 after genotyping using PCR. After PCR-RFLP the MEC and DI were calculated to be 0.91 and 0.94.

Because of the relatively small population samples, a reliable estimation of deviation from Hardy-Weinberg equilibrium is not possible using each separated allele. A less sensitive but informative approach for testing for deviation for preliminary data might be by binning alleles together and then by retesting with reshuffled allele categories. Calculations have been carried out using a 5-allele model. The combined χ^2 value showed no significant deviation between expected and observed values (Table 2).

Figure 6 shows the result of a case of paternity testing using D1S80 PCR-RFLP. Alleles 24 were separated from the mother (allele24/allele30), the child (allele24/allele24), and the putative father (allele24/allele28) respectively and digested with EcoRII. In lane 2 allele24 from the mother produced a 285 bp fragment but lacked the 48 bp fragment, in lane 4 allele24 from the man showed 269 bp and 48 bp fragments, while allele24 from the child

Table 2 Check for Hardy-Weinberg equilibrium

	Subtype	*n*	Freq
A	1– 3	51	0.22973
B	4–11	43	0.19369
C	12–18	41	0.18468
D	19–25	50	0.22523
E	26–42	37	0.16667
Genotype	*n*	ob. N	ex. N
AA	1	5.86	4.029
AB	9	9.88	0.078
AC	12	9.42	0.707
DA	13	11.49	0.199
EA	15	8.50	4.971
BB	4	4.16	0.006
BC	6	7.94	0.475
BD	10	9.68	0.010
BE	10	7.17	1.120
CC	4	3.79	0.012
CD	12	9.23	0.828
CE	3	6.83	2.150
DD	5	5.63	0.071
DE	5	8.33	1.333
EE	2	3.08	0.381
			χ^2 = 16.371
			p = 0.291
			(df = 14)

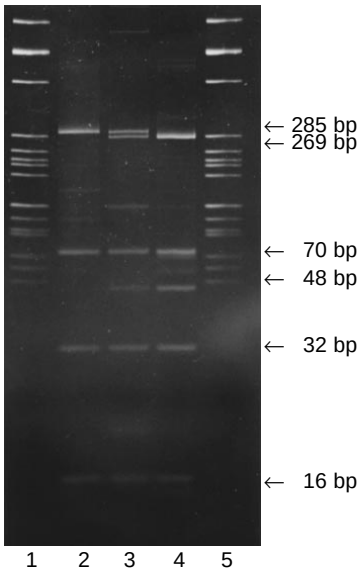

Fig. 6 Electrophoretic band patterns of PCR-RFLP in a case of paternity testing. The alleles 24 of a triplet were digested with EcoRII. Lane 1, 5: pBR322-HaeIII; lane 2: E4, mother; lane 3: E4/E8, child; lane 5: E8, putative father

in lane 3 had all three fragments. The result that the subtypes of alleles 24 of the triplet (mother-child-man) were E4, E4/E8, and E8 respectively demonstrated that the man was the biological father of the child.

Therefore, PCR-RFLP is a simple, economic and efficient subtyping method for D1S80, since it can differentiate between samples of the same length that have different sequences. If the number of population samples is increased, more subtypes may be detected. Thus, it is quite useful not only for paternity testing and individual identification, but also in the study of human evolution.

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