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Paternity testing with VNTR DNA systems

I. Matching criteria and population frequencies of the VNTR systems D2S44, D5S43, D7S21, D7S22, and D12S11 in Danes

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Summary. Paternity testing using DNA polymorphism of variable numbers of tandem repeat (VNTR) regions with restriction fragment length polymorphism (RFLP) was implemented. HinfI-digested DNA was separated by electrophoresis in agarose gels and hybridized with radiolabelled probes detecting the VNTR-systems D2S44 (YNH24), D5S43 (MS8), D7S21 (MS31), D7S22 (g3), and D12S11 (MS43a). The intra gel variability of 970 duplicate investigations on the same gel of DNA from 122 individuals showed no differences exceeding 1.25 mm between the positions of the corresponding DNA fragments. The comparison of 1,624 DNA fragments from 342 mother/child pairs showed only one difference above 1.25 mm which was interpreted as a mutation. Based on these observations, we decided to consider an intra gel difference above 1.25 mm between the non-maternal DNA fragment of the child and the nearest DNA fragment of the putative father as an exclusion in paternity testing. This matching criterion was used for the comparisons of 1,197 DNA fragment differences in 247 pairs of children and putative fathers who had not been excluded by conventional marker systems. In all of these cases, the migration differences between the DNA fragments of non-excluded men and the DNA fragments of the children were less than 1.25 mm except in 6 cases (0.5%). The man/child differences in all of 227 false trios exceeded 1.25 mm in 2 or more of the 5 VNTR systems investigated. Matching criteria for inter gel comparisons in paternity testing were established. The frequency distribution of HinfI digested DNA fragments of the 5 VNTR systems in 650 unrelated Danes is presented and the raw data is available.

Key words: Paternity testing – DNA – VNTR – Single locus DNA probes – Matching criteria – Population frequencies

Zusammenfassung. DNA-Polymorphismen mit einer variablen Anzahl von tandemähnlichen Wiederholungseinheiten (VNTR's), speziell der Typus der Restriktionsfragmentlängenpolymorphismen, wurden in die Vaterschaftsanalyse eingeführt. HinfI-verdaute DNA wurde elektrophoretisch in Agarose-Gelen aufgetrennt und mit radioaktiv markierten Sonden hybridisiert, welche die VNTR-Systeme D2S44 (YNH24), D5S43 (MS8), D7S21 (MS31), D7S22 (G3) und D12S11 (MS43a) detektieren. Die sog. Intra-Gelvariation von 970 Doppeluntersuchungen auf demselben Gel von DNA von 122 Personen zeigte keine Unterschiede, welche größer waren als 1,25 mm – bezogen auf die Positionen der korrespondierenden DNA-Fragmente. Der Vergleich von 1.624 DNA-Fragmenten von 342 Mutter-Kind-Paaren zeigte lediglich einen Unterschied, welcher größer als 1,25 mm war und daher als eine Mutation interpretiert wurde. Hierauf basierend entschlossen wir uns, eine Intra-Geldifferenz größer als 1,25 mm zwischen dem nicht-mütterlichen DNA-Fragment des Kindes und dem nächsten DNA-Fragment des Putativ-Vaters als einen Ausschluß in der Vaterschaftsanalyse zu bewerten. Dieses Match-Kriterium wurde benutzt für die Vergleiche von 1.197 DNA-Fragmentdifferenzen bei 247 Paaren von Kindern und Putativ-Vätern, bei welchen in konventionellen Systemen kein Ausschluß zu beobachten war. In all diesen Fällen waren die Wanderungsunterschiede zwischen DNA-Fragmenten der nicht-ausgeschlossenen Männner und den Fragmenten der Kinder geringer als 1,25 mm mit der Ausnahmen von 6 Fällen (0,5%). Die Mann-Kind-Differenzen in allen der 227 falschen Terzette überschritten 1,25 mm in zwei oder mehr der 5 VNTR-Systeme, welche untersucht wurden. Match-Kriterien für die Inter-Gel-Vergleiche bei Vaterschaftsuntersuchungen wurden etabliert. Die Frequenz-Verteilung von HinfI-verdauten DNA-Fragmenten der 5 VNTR-Systeme bei 650 unverwandten Dänen wird gezeigt und die Rohdaten sind verfügbar.

Schlüsselwörter: Vaterschaftsuntersuchung – DNA-VNTR – Single-Locus-DNA-Sonden, Match-Kriterien – Populationsfrequenzen

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Introduction

The extreme polymorphism of variable numbers of tandem repeat (VNTR) regions and the fact that these regions are in-

herited according to the rules of Mendelian genetics makes the VNTR regions well suited for paternity testing (e.g. Jeffreys et al. 1985). In a population, the variation within a series of alleles of a VNTR region is primarily based on differences in the numbers of the repeat units (Wyman and White 1980), which leads to differences in the total length of the VNTR region, although differences in the sequences of the repeat units exist and can be detected (e.g. Jeffreys et al. 1991).

Different alleles or groups of alleles within a series of highly variable alleles from a VNTR region can be detected by the restriction fragment length polymorphism (RFLP) technique which is based on the separation in an electrophoretic system of DNA fragments including the VNTR region according to the total sizes of the DNA fragments.

The value of investigations of the polymorphic hypervariable VNTR regions in paternity testing has been documented (e.g. Baird et al. 1986; Balazs et al. 1989; Smith et al. 1990; Henke et al. 1991).

We have implemented paternity testing by RFLP analysis of hypervariable VNTR regions with the single locus probe hybridization technique which we presently use for DNA investigations in crime cases. The technique is essentially the one agreed upon by the European DNA Profiling (EDNAP) Group (Schneider et al. 1991; Gill et al. 1992; Eriksen et al. 1992).

Here, we present the matching criteria for paternity testing by RFLP investigations of HinfI digested DNA using the VNTR systems D2S44 (DNA probe YNH24), D5S43 (MS8), D7S21 (MS31), D7S22 (g3), and D12S11 (MS43a) (Wyman and White 1980; Nakamura et al. 1987; Wong et al. 1986; Wong et al. 1987) and the frequency distributions in 650 random Danes.

Materials and methods

Individuals. A total of 271 Danish paternity cases, 36 immigration cases, 2 Danish families, and 76 random Danes were investigated. A total of 342 mother/child pairs, 247 non-excluded man/child pairs, 52 excluded Danish man/child pairs investigated on the same gels (the men had been excluded from paternity with conventional tests) were investigated. The total group of unrelated, random Danes comprised 650 individuals.

Isolation and digestion of DNA. Blood samples (ACD, EDTA, or clotted blood) or acetic acid-methanol fixed chromosome preparations (48 cases) which had been frozen and stored were digested with Proteinase K (Boehringer). Genomic DNA was isolated with phenol/chloroform or 6 M NaCl solution. The DNA was digested with the restriction enzyme HinfI (Boehringer) according to the manufacturer's specifications.

Electrophoresis. 1.25 µg DNA of each sample was electrophoresed in 0.7% agarose (Sigma type II) gels (20×20×0.7 cm) with TBE pH 8.8 (134 m*M* tris, 75 m*M* boric acid, and 2.5 m*M* Na_2 EDTA) and ethidium bromide (0.5 µg/ml – Biorad no. 161–0430). Electrophoresis was performed at room temperature for approximately 18 h at 60 V (max 75 mA) until the 2.39 kb fragment of the molecular weight marker had migrated 14.0 cm. At least 3 lanes with the Amersham SJ 5000 ^{35}S molecular weight ladder were included on each gel. At least one control sample with known DNA fragment lengths of the VNTR systems investigated was included in each experiment.
The DNA samples from the mother child and the putative father(s) in each case were investigated on the same gel and the investigations were repeated on another day using another DNA preparation.

DNA Probes. DNA single locus probes detecting the VNTR-systems D5S43 (MS8), D7S21 (MS31), D7S22 (g3), D12S11 (MS43a) (Wong et al. 1987; Smith et al. 1990) were from Cellmark and D2S44 (YNH24) (Wyman and White 1980) from Promega. The DNA probes were labeled with a[^{32}P]-dCTP (Amersham) using random priming with hexamers.

Southern blotting. Southern blotting of the DNA onto Hybond N membranes (Amersham) was performed by capillary transfer or vacuum blotting.

Prehybridization. Prehybridization of the membranes was carried out in a 25 ml volume in glass flasks in a rotating hybridization oven (Smith et al. 1990). The membranes were incubated for 30 min at 65°C in 1×–Denhardt's solution (10×Denhardt's solution: 0.02% bovine serum albumin, 0.02 Ficoll 400, 0.02% polyvinylpyrrolidone 44,000) and prehybridized for 1 h at 65°C in hybridization solution (cf. below).

Hybridization. Hybridization was carried out in a 25 ml volume in glass flasks in a rotating hybridization oven. The membranes were hybridized for 18 hours at 65°C in a hybridization solution containing 0.3 ng of the ^{32}P radiolabelled DNA probe per ml, 6.0% polyethylene glycol 6000, 0.1% sodium dodecylsulfate (SDS), 50 µg/ml herring sperm DNA, 1×–Denhardt's solution. After hybridization, the membranes were washed four times for 30 min at 65°C in 1×SSC, 0.1% SDS, 50 mg/ml herring sperm DNA, and once at 65°C for 30 min in 0.1×SSC, 0.01% SDS, 5 µg/ml herring sperm DNA. Sequential reprobing on the membranes was performed with the 5 probes.

Autoradiography. Autoradiography was performed at –80°C on Fuji HR-G X-ray films with intensifying screens (Kyokko Special 195084).

DNA fragment sizing. The migration of a DNA fragment was measured from the point of application to the center of the band with a ruler with 0.5 mm resolution. The measurements were done independently by 2 persons. The DNA fragment lengths in kilobase pairs (kb) were calculated by local hyperbolic approximation (Elder and Southern 1987). The molecular weight marker (Amersham SJ 5000) was used as reference.

Normalized Migration Length (NML). In order to obtain a measure of the differences in migration distances of the DNA fragments when samples were investigated on different gels, the gels were run under standardized conditions. The kb values (KB) can be transformed into normalized migration length (NML) values with the function:

$$NML = \frac{800}{3.8 + KB^{1.5}} + 33.75 \quad (1)$$

as described elsewhere (Eriksen et al. 1992). The function fits the means of normalized migration distance as a function of fragment length. The NML values express the migrations in 'normalized millimeters' on an average gel.

Calculation of upper and lower limits of matching windows. The actual kb value of a non-maternal DNA fragment of the child matching a DNA fragment in the putative father was transformed into an NML value (NML_0). In intra gel comparisons, the upper (NML_U) and lower (NML_L) values of the window were defined as $NML_U = NML_0 - 1.25$ mm and $NML_L = NML_0 + 1.25$ mm and, in inter gel comparisons, the window was defined as $NML_U = NML_0 - 2.25$ mm and $NML_L = NML_0 + 2.25$ mm. The NML_U and NML_L values were retransformed into upper (KB_U) and lower (KB_L) kb values of the matching window.

Calculation of the frequency of DNA fragments in a matching window. The number of DNA fragments in a matching window (KB_L–KB_U) were counted in 650 random, unrelated Danes and divided by the total number of DNA fragments observed.

Distribution of the frequencies of VNTR DNA fragments. The frequencies of DNA fragments in 112 overlapping 2.5 NML intervals (± 1.25 NML matching windows) starting at 1.4 kb and ending at 21.0 kb were calculated in 650 random Danes for the graphic presentation of the data. The raw data are available.

Typing for classical systems. Typing for the systems ABO, K, MNS, RH, GC, GM, HP, KM, TF, ACP1, ESD, GLO, GPT, PGM1, HLA-A, and HLA-B (Hansen 1989) was performed with standard techniques, and the results were evaluated with standard criteria for exclusion and inclusion in paternity testing (Henningsen 1983).

Calculation of paternity index. The paternity index,

$$PI = \frac{P(\text{actual genetic observations} \mid \text{paternity})}{P(\text{actual genetic observations} \mid \text{non-paternity})}$$

was calculated as described by Gürtler (1956). The value of the denominator was based on direct counting of the number of DNA fragments in the matching window (± 1.25 mm) around the child's kb value.

Results

An example of a typical autoradiogram with 2 paternity cases investigated for the VNTR DNA system D7S22 (g3) is shown in Fig. 1.

Intra gel variation of duplicate investigations

Two DNA preparations were isolated with (i) phenol and (ii) 6 *M* NaCl from a blood sample from each of 122 unrelated individuals. The 2 DNA preparations from each individual were investigated on the same gel and hybridized with 5 probes giving a total of 907 DNA fragments for the analysis (Fig. 2a). The intra gel migration differences (d) were scored in 0.5 mm classes. The frequencies of the differences were: d = 0 mm: 74.7%, d = 0.5 mm: 22.7%, d = 1.0 mm: 2.4%, d = 1.5 mm: 0.1%, and d = 2.0: 0.1%.

Mother/child intra gel migration differences

The intra gel differences between DNA fragments identical by descent in the mothers and their children were analyzed in 342 mother/child pairs investigated with 5 probes giving a total of 1,624 DNA fragments (Fig. 2b). All differences were less than 1.25 mm except in one case with a difference of 2.82 kb (17.5 mm) in D7S21 (MS31) interpreted as a mutation.

Intra gel exclusion criteria in paternity testing

Based on the results of the analysis of the intra gel variation of mother/child pairs, we decided to use a migration difference of 1.25 mm as preliminary exclusion criterion for paternity testing when the investigations of a mother/child/man trio were performed on the same gel.

Man/child intra gel migration differences

The intra gel migration differences between the non-maternal DNA fragments in the children and the nearest DNA fragment in the putative fathers were analyzed in 247 man/child pairs investigated with 5 probes giving a total of 1,197 DNA fragments (Fig. 2c). None of the men had been excluded by investigations in at least 12 classical genetic systems, including HLA-A and -B, with a combined theoretical exclusion efficiency of 0.994. The migration differences were above 1.25 mm in 6 comparisons (0.5%) in 6 different man/child pairs. In the non-excluded man/child group, none of the migration differences exceeded 1.25 mm in more than one VNTR system. The migration differences of 5 of the comparisons were above 1.75 mm.

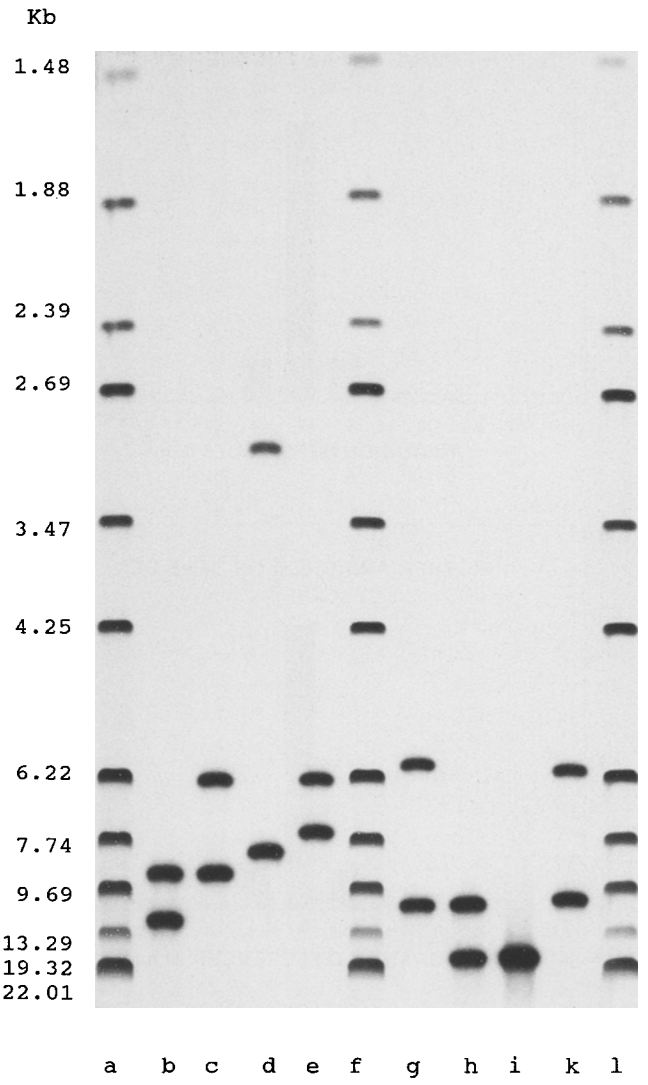


Fig. 1. Autoradiogram of the VNTR DNA system D7S22 (probe g3) in 2 paternity cases. Case 1: Mother (b), child (c), excluded man (d), and non-excluded man (e). Case 2: Mother (g), child (h), non-excluded man (i), and excluded man (k). Molecular weight markers: Lanes a, f, and l

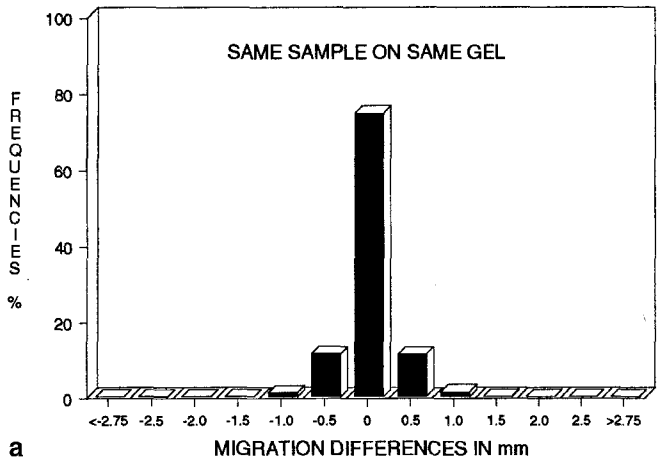
Exclusion power of the VNTR systems

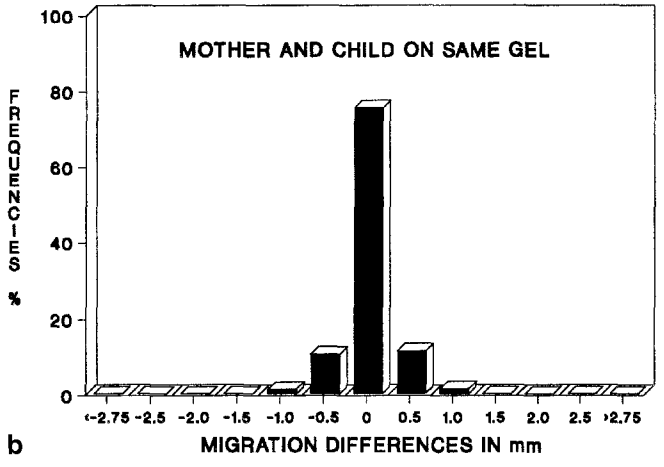
A total of 227 'false' mother/child/man trios investigated on the same gels were created by random reallocation of each of the non-excluded men to another mother/child pair. In the non-father/child group, 70.5% of the migration differences exceeded 1.25 mm (Fig. 3), and in all cases, the migration differences exceeded 1.25 mm in 2 or more probes (Fig. 4).

In all of 52 actual paternity cases in which the men had been excluded by conventional marker systems, the men were also excluded by 2 or more VNTR systems investigated on the same gel.

Inter gel variation of kb values of duplicate investigations

The relative inter gel variability of the kilobase values was investigated by duplicate investigations on different gels of DNA samples from 430 individuals. The relative difference of the kilobases as a percentage of the mean kilobase values was found to increase with increasing mean kb values (Fig. 5). The effect was most pronounced for DNA fragments above 8 kb.





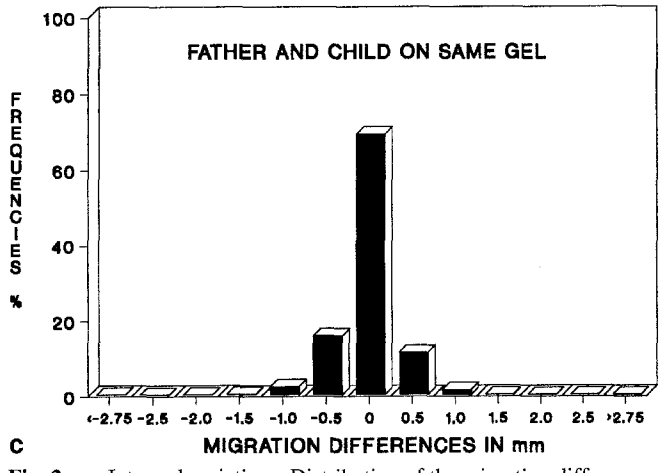


Fig. 2 a-c. Intra gel variations. Distribution of the migration differences between duplicate investigations on the same gels of samples from (a) 122 unrelated individuals (907 bands), (b) 342 mother/child pairs (1,642 bands), and (c) 247 father/child pairs (1,197 bands)

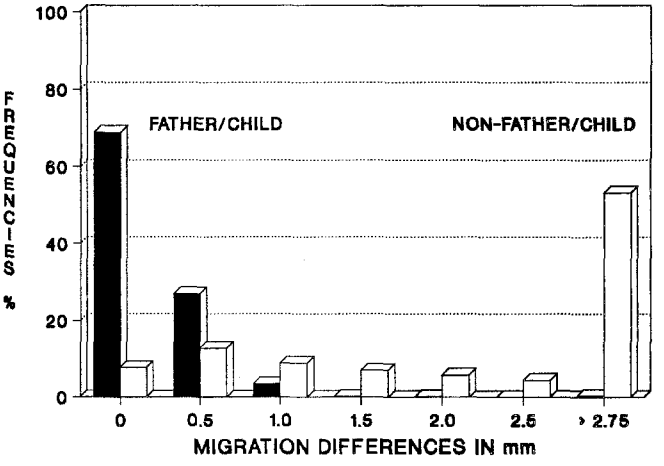


Fig. 3. Migration differences between VNTR DNA fragments of 247 father/child pairs (1,197 bands) and 227 non-father/child pairs (1,135 bands)

Transformation of kb values into normalized migration length (NML)

When electrophoresis was performed under standardized conditions, the kb values could be transformed into normalized migration length (NML) values ('normalized millimeters') by the function (Eq1). The effect of the transformation of different kb values are shown in Fig. 6. Normality of the variance of the differences could not be obtained by standard transformations, including logarithms and hyperbolic transformations.

Inter gel variation of NML values of duplicate investigations

The inter gel differences in NML values were investigated in 706 unrelated individuals. Two DNA preparations were isolated with (i) phenol and (ii) 6 M NaCl from a blood sample from each of the individuals, and investigated on different gels and hybridized with 5 probes giving a total of 6,448 DNA fragments (Fig. 7a).

Inter gel variation of mother/child migration differences

The inter gel differences between the migrations of the DNA fragments identical by descent in the mothers and their children were analyzed in 169 mother/child pairs investigated with 5 probes giving a total of 773 DNA fragments. The mother and the child samples were investigated on separate gels. The distribution of the NML differences (Fig. 7b) were very similar to that of the inter gel NML differences of duplicate investigations (Fig. 7a).

Inter gel exclusion criteria in paternity testing

Based on the results of the inter gel variation between mother/child pairs, we decided to use a migration difference of 2.25 mm as preliminary exclusion criterion for paternity testing if the child and the man were investigated on different gels.

Inter gel variation of man/child migration differences

Samples from the children and men were investigated on separate gels. The inter gel differences between the migrations of the non-maternal DNA fragments of the children and of the nearest DNA fragments of the non-excluded men were analyzed in 90 man/child pairs investigated with 5 probes giving

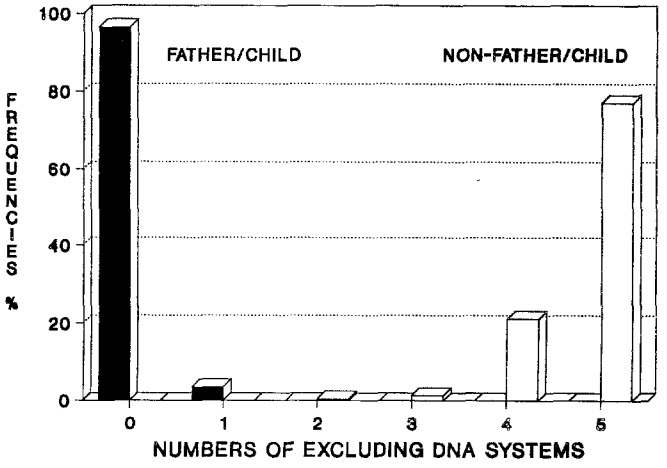


Fig. 4. Numbers of excluding VNTR-systems in 227 father/child pairs and in 227 non-father/child pairs

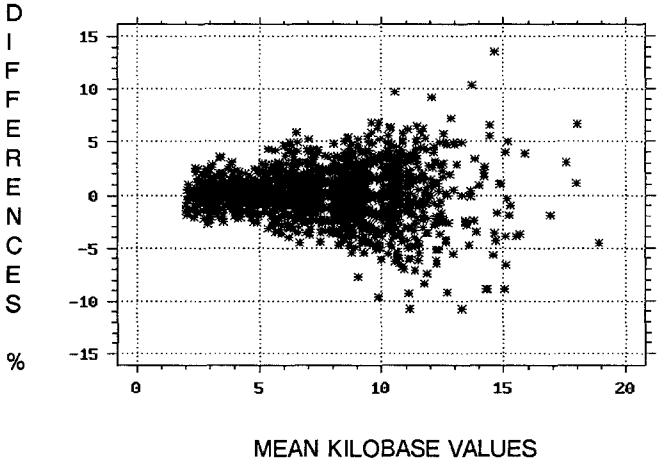


Fig. 5. Relative inter gel variation. Distribution of 4,000 inter gel differences in per cent of kilobase values of duplicate investigations on different gels of DNA-samples from 430 unrelated individuals

a total of 405 DNA fragments (Fig. 7c). None of the men had been excluded by investigations in at least 12 classical genetic systems, including HLA-A and -B, with a combined theoretical exclusion efficiency of 0.994. The migration differences were above 2.25 mm in 2 comparisons (0.5%) in 2 different man/child pairs. These 2 man/child comparisons also exceeded 1.25 mm in the intra gel analysis (cf. above).

Distributions of the DNA fragments of the VNTR systems

Figure 8 shows the frequency distributions of DNA fragments of the D5S43 (MS8), D7S21 (MS31), D12S11 (MS43a), and D2S44 (YNH24) VNTR systems in 650 random Danes. The allele distribution of D7S22 (g3) was based on the analysis of 300 maternal alleles in mother/child pairs. This approach was chosen for D7S22 because, in some cases, fragments were lost due to the low molecular weights of the DNA fragments or due to weak hybridization with the g3 DNA probe. The frequencies of the DNA fragments were calculated in consecutive windows (± 1.25 mm) corresponding to the matching/exclusion criterion.

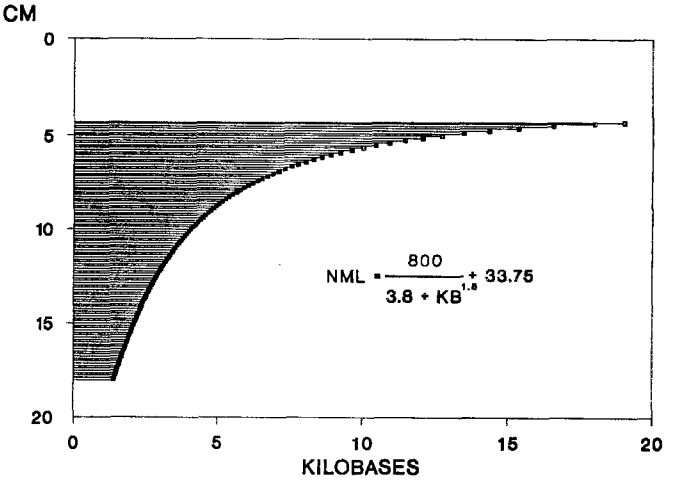


Fig. 6. The effect of transformation of kilobase values into Normalized Migration Length values ('normalized millimeters')

Discussion

The purpose of the present study was to establish simple exclusion/inclusion (matching) criteria for paternity testing by analysis of hypervariable VNTR regions with single locus probes and to validate the methods in actual paternity cases. We use the same basic technique for DNA investigations in paternity cases and in crime cases, i.e. the technique agreed upon by the European DNA Profiling (EDNAP) Group (Schneider et al. 1991; Gill et al. 1992; Eriksen et al. 1992). We found it important that the matching criteria for intra gel investigations of mother/child/putative father(s) could be easily documented and understood.

The repeat unit sizes of the highly polymorphic VNTR systems used are far less than the total length of the VNTR region, and single repeat differences cannot be detected by conventional electrophoretic systems. Therefore, the length of a DNA fragment including a hypervariable VNTR region is usually expressed as an estimate of the length in basepairs. This estimate is based on the relative migration of the DNA fragment in an electrophoresis gel compared to the migration of molecular weight marker DNA fragments of known molecular weight. Thus, the results of the analysis of hypervariable VNTR-regions represent themselves as traits with a quasi continous distribution in the population and not as discrete alleles.

The length of a DNA fragment is usually measured in basepairs with a certain measurement error due to e.g. the experimental variation and the simple fact that DNA fragments are visualized as bands with shapes and width. The error associated with the estimation of the DNA fragment size increases with increasing kb values (Gill et al. 1990; Eriksen et al. 1992). For small DNA fragments in the range 1.5–6 kb, a mean coefficient of variation may be acceptable (e.g. Baird et al. 1986), but for larger fragment sizes (> 8 kb) such an approach will lead to fallacies. Gill et al. (1990) found that the logarithm for the standard deviation of fragment size was directly proportional to the molecular size, while Eriksen et al. (1992) found that the data fitted a more complex function (Eq 1). It should be noticed that this phenomenom is of very

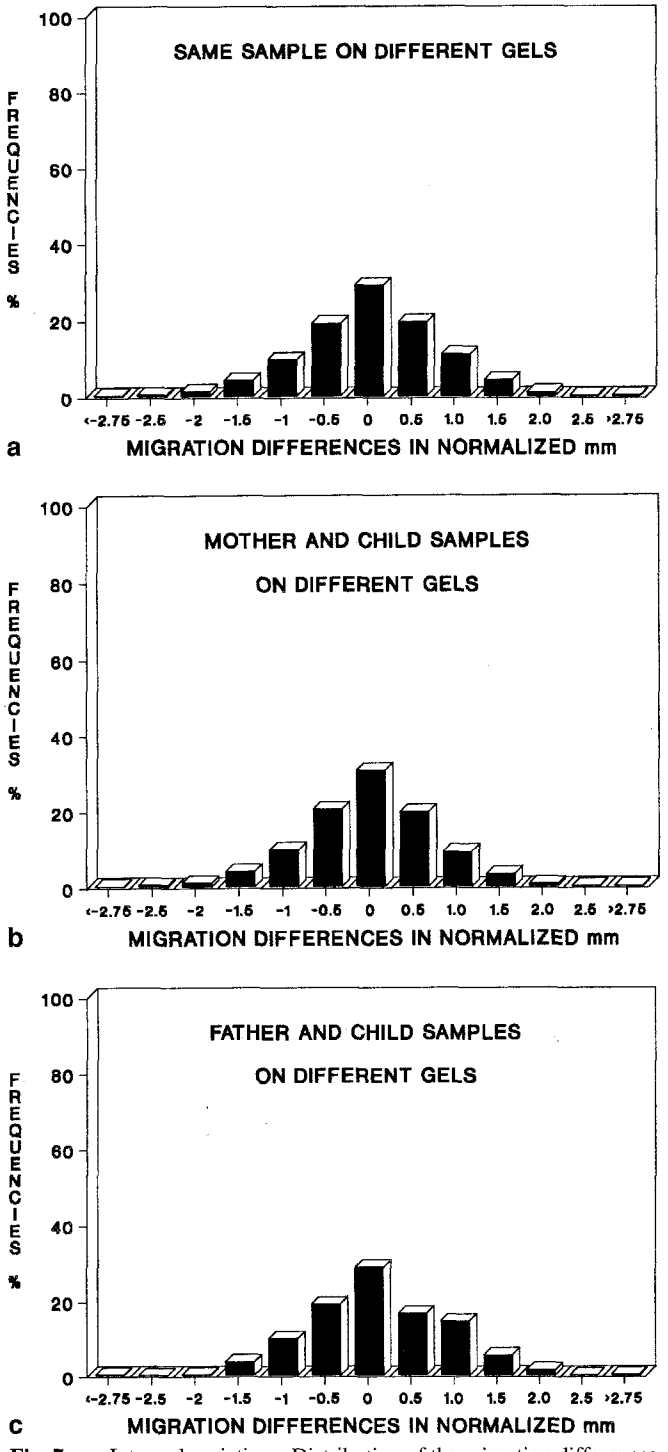


Fig. 7 a-c. Inter gel variations. Distribution of the migration differences between duplicate investigations on different gels of samples from (a) 706 unrelated individuals (6,448 bands), (b) 169 mother/child pairs (773 bands), and (c) 90 father/child pairs (405 bands). The migration differences were calculated from Normalized Migration Lengths (normalized millimeters) which were obtained by transformation of the kb values into NML-values

little importance in systems where almost all fragments are in the 1.5–6 kb range (Baird et al. 1986; Gjertson et al. 1988; Balazs et al. 1989; Morris and Glassberg 1989).

Several methods have been suggested for the analysis of VNTR data for paternity testing (see e.g. Gjertsson et al. 1988; Morris and Glassberg 1989). It is usually possible to investigate DNA from the mother child and the putative father on the same electrophoresis gel. Thus, the relevant test in a paternity case can be reduced to a simple side-to-side comparison of the DNA fragments of the child and the putative father(s).

We have established simple exclusion/inclusion criteria which are based solely on the physical distances between the DNA fragments in the gel when a trio is investigated on the same gel. In our hands, the difference in the physical positions in the gel of DNA fragments of duplicate investigations is the test value with the lowest variability, and the variability between duplicate investigations is constant and normally distributed in the fragment size interval 1.5–20 kb (Eriksen et al. 1992).

An empirical data analysis of the intra gel differences between duplicate investigations of the same blood sample and of intra gel differences of mother and child samples suggested that, for practical purposes, the maximal difference was less than 1.25 mm. The results support the assumption that, within the error of measurement, the lengths of the VNTR regions investigated are inherited as Mendelian traits. Thus, we decided on a minimal relevant difference of 1.25 mm for the definition of exclusions of putative fathers in paternity testing performed with DNA from the mother, child, and putative father on the same gel.

In the comparison between children and putative fathers who had not been excluded by conventional testing, 6 (0.5%) discrepancies above 1.25 mm were found. One of the discrepancies was observed in a case with a suboptimal sample. The remaining 5 discrepancies could be due to either single exclusions or mutations (Hansen and Morling 1992).

The analysis of the exclusion efficiency of the VNTR systems showed that 70% of the migration differences between DNA fragments of the non-fathers and the paternal DNA fragments of the children were above 1.25 mm and that all of 227 men in non-father/child trios were excluded by 2 or more systems. A more extensive evaluation of the power of exclusion of the VNTR systems investigated is presented elsewhere (Hansen and Morling 1992).

In rare cases, it may not be possible to investigate all parties in a paternity case on the same gel. For the comparison of investigations on different gels, we have chosen to work with data which have been transformed into normalized migration length values – NML values ('normalized millimeters'). The transformation, in our hands, leads to low and constant variance of the fragment sizes throughout the 1.5–20 kb range.

Based on a pragmatic, empirical data analysis similar to the one described for intra gel comparisons, a minimal relevant difference of 2.25 'normalized millimeters' was chosen as the exclusion criterion in inter gel father/child comparisons (Fig. 7).

In non-exclusion cases, the paternity index which is a true likelihood quotient can be calculated (Gürtler 1956). For this purpose, we have investigated the sizes in kilobase values of DNA fragments of the VNTR regions investigated in 650 random Danes. The raw data, on which the size distribution of HinfI digested DNA fragments of the VNTR regions D2S44 (YNH24), D5S43 (MS8), D7S21 (MS31), D7S22 (g3), and D12S11 (MS43a) (Fig. 8) is based, is available from the authors.

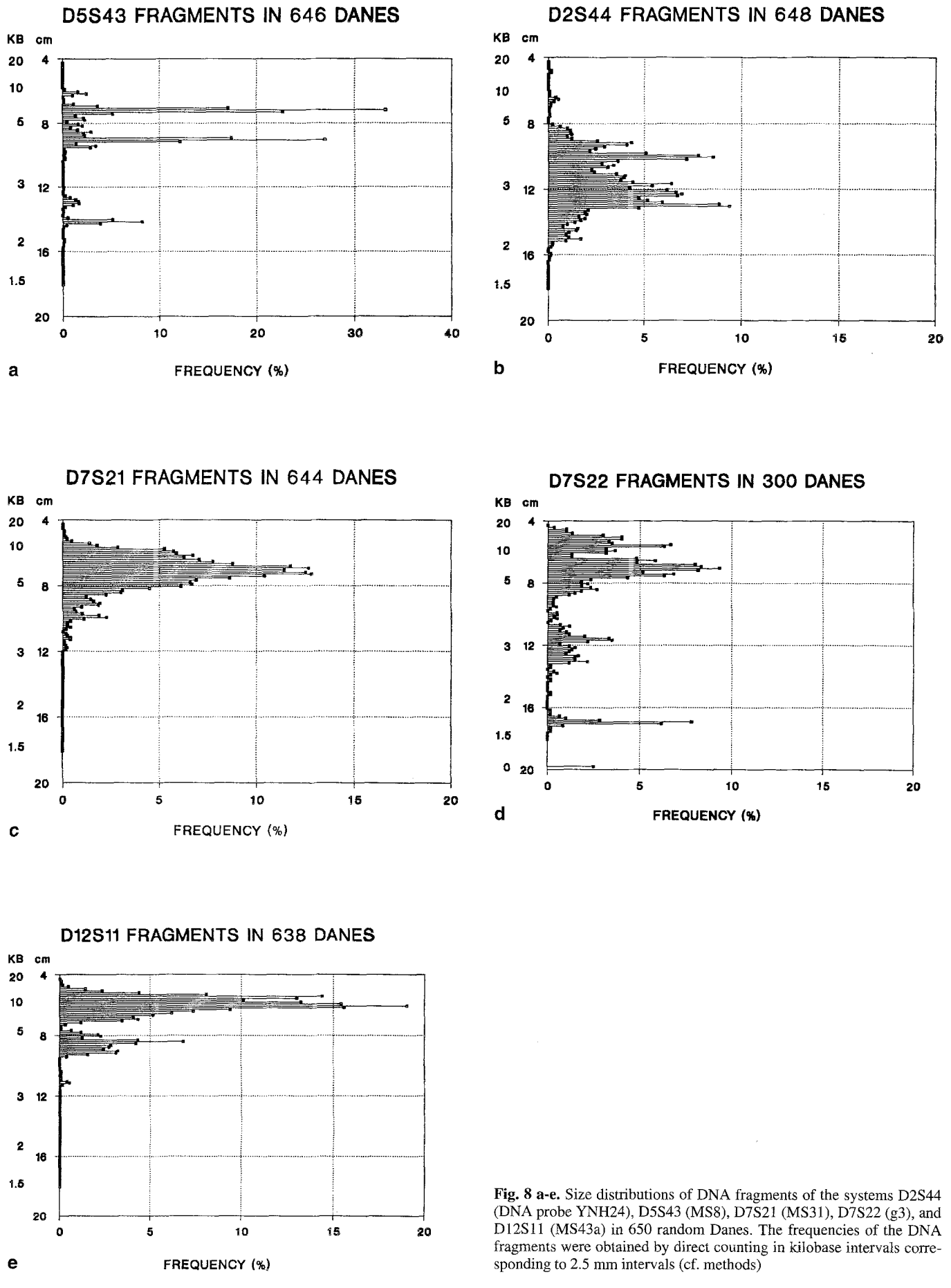


Fig. 8 a-e. Size distributions of DNA fragments of the systems D2S44 (DNA probe YNH24), D5S43 (MS8), D7S21 (MS31), D7S22 (g3), and D12S11 (MS43a) in 650 random Danes. The frequencies of the DNA fragments were obtained by direct counting in kilobase intervals corresponding to 2.5 mm intervals (cf. methods)

The efficiency of the investigation of the DNA systems in actual Danish paternity cases is presented elsewhere (Hansen and Morling 1992).

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