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Paternity testing with VNTR DNA systems

II. Evaluation of 271 cases of disputed paternity with the VNTR systems D2S44, D5S43, D7S21, D7S22, and D12S11

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Summary. Paternity testing was carried out in 271 cases of disputed paternity using the 5 VNTR systems D2S44 (YNH24), D5S43 (MS8), D7S21 (MS31), D7S22 (g3), and D12S11 (MS43a), and 10–15 conventional marker systems including the HLA-A,B system. By means of the matching criteria for the VNTR systems established elsewhere (Morling & Hansen 1992), all 70 unrelated men who had been excluded by conventional typing were also excluded with 2 or more VNTR systems. Based on the observed exclusion frequencies for the 5 VNTR systems, a theoretical exclusion rate exceeding 0.999 could be obtained. A total of 350 father/child pairs were studied and in 3 paternity cases and one immigrant family, the alleged fathers were excluded solely by one of the 5 VNTR systems possibly reflecting mutations. No mother/child exclusions were observed among 350 mother/child pairs. Linkage analysis between the syntenic systems D7S21 (MS31) and D7S22 (g3) was performed in 29 informative families with 81 children and revealed a recombination distance of about 31 cM. The positive evidence for paternity provided by the 5 VNTR systems in cases with non-exclusions is discussed.

Key words: Paternity cases – DNA – VNTR systems – Single locus probes – Paternity index

Zusammenfassung. Vaterschaftstests wurden in 271 Fällen strittiger Vaterschaft mit Hilfe der 5 VNTR-Systeme: D2S44 (YNH24), D5S43 (MS8), D7S21 (MS31), D7S22 (g3), D12S11 (MS43a) und 12–15 konventionellen Blutgruppensystemen einschließlich des HLA-Systems (HLA A und B) durchgeführt. Mit Hilfe der Matching-Kriterien für VNTR-Systeme, wie sie andernorts (Morling und Hansen 1992) etabliert wurden, wurden alle 70 unverwandten Männer, die durch konventionelle Typisierung ausgeschlossen wurden, auch mit Hilfe von 2 oder mehreren VNTR-Systemen ausgeschlossen. Basierend auf den beobachteten Ausschlußfrequenzen für die 5 VNTR-Systeme könnte eine theoretische Ausschlußwahrscheinlichkeit von mehr als 0,999 erhalten werden. Insgesamt wurden 350 Vater/Kind-Paare untersucht und in 3 Vaterschaftsfällen und einer Emigrantenfamilie wurden isolierte Ausschlüsse der Putativväter ausschließlich in einem der 5 VNTR-Systeme gefunden, was möglicherweise an Mutationen denken läßt. Unter 350 Mutter/Kind-Paaren wurden keine Mutter/Kind-Ausschlüsse gefunden. Linkage-Analysen zwischen den Syntenic-Systemen D7S21 (MS31) und D7S22 (g3) wurden durchgeführt an 29 Familien mit 81 Kindern. Die Analyse ergab eine Rekombinationsdistanz von ungefähr 31 cM. Der positive Vaterschaftsbeweis, wie er durch die 5 VNTR-Systeme in Fällen mit Nicht-Ausschlüssen etabliert wird, wird diskutiert.

Schlüsselwörter: Vaterschaftsfälle – DNA – VNTR-Systeme – Single-Locus-Sonden – Vaterschaftspaternitäts-Index

Introduction

Since 1901 (Landsteiner 1901) when the AB0-systems with a theoretical exclusion of rate of 0.18 could be taken into use, the exclusion power of the systems used for paternity testing has risen drastically. Since around 1970, the HLA-A, B-system with a theoretical exclusion rate of about 0.92 has been the most efficient system for paternity diagnosis, and through selection of a suitable number of systems, a combined exclusion efficiency exeeding 0.999 could be reached. The new techniques making use of DNA probes revealing highly polymorphic loci (Variable Numbers of Tandem Repeats – VNTR-systems) in the human genome have yielded even better chances for exclusion of the biological non-fathers and for obtaining strong positive evidence for paternity of a non-excluded man. One drawback of the VNTR systems is the mutation rates of the VNTR systems currently in use.

Since the beginning of 1989, the 5 VNTR systems, D2S44 (probe YNH24), D5S43 (MS8), D7S21 (MS31), D7S22 (g3), and D12S11 (MS43a) have been used for investigations in Danish cases of disputed paternity along with 10–15 conventional marker systems, including HLA-A,B. The matching

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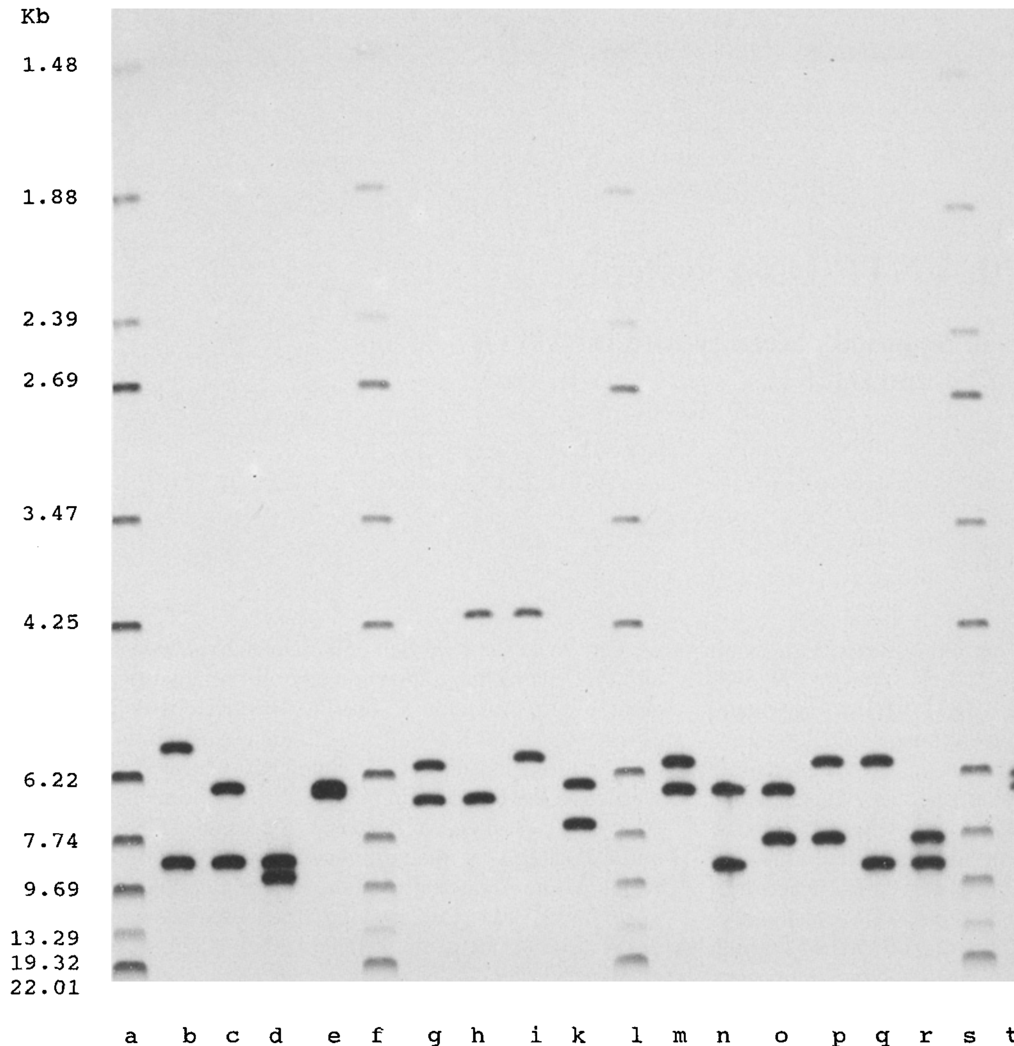

Fig. 1. Two paternity cases and a family investigated with D7S21 (probe MS31) and HinfI. Lanes b–e: paternity case I: mother (b), child (c), excluded man (d), non-excluded man (e). Lanes g–k: paternity case II: mother (g), child (h), non-excluded man (i), excluded man (k). Lanes m–r: Family: mother (m), children (n,o,p,q), father (r). Lanes a,f,l, and s: molecular weight marker (cf. methods)

criteria for possible identity between DNA fragments from 2 individuals and the frequencies of DNA fragments observed among unrelated Danes have been dealt with elsewhere (Morling and Hansen 1992).

The present paper describes the outcome of the analysis of the constellations mother/child/alleged father in 271 cases of disputed paternity and in 39 families with 1–5 children. Estimates of the mutation rates of the VNTR systems and of the linkage between D7S21 (MS31) and D7S22 (g3) are given.

Materials and methods

Material. The present material comprised a) 271 Danish cases of disputed paternity in which "extended investigations", including HLA determinations, had been required, b) 35 immigration cases and c) 4 families of control donors giving a total of 39 families with 91 children.

DNA samples. Three sources of DNA were used: 1) Freshly drawn ACD-blood, 2) isolated live lymphocytes left over from HLA determination, and 3) acetic acid/methanol-fixed chromosome preparations derived from PHA stimulated lymphocyte cultures and stored at –20°C for up to 14 years.

Control of sample identity. To secure the identity of samples through the preparation procedure, DNA was isolated twice from each blood sample: once by means of saturated NaCl solution and once by the phenol-chloroform method.

Isolation of DNA by the NaCl method. Cells from 0.7 ml blood (or an equivalent amount of material) were lysed by freezing at –20°C and digested overnight with proteinase K (Boehringer) according to standard methods. Saturated NaCl solution (6 *M*, 100–120 µl) was added and the mixture was shaken thoroughly for about 15 s (Miller et al. 1988). After centrifugation the supernatant was recovered. DNA was precipitated by adding 2 volumes of 96% ethanol at –20°C.

Isolation of DNA by the phenol-chloroform method. Proteinase K-digested chromosome preparations or leukocytes from 0.7 ml blood was mixed thoroughly with 120 µl of a phenol/chloroform/isoamylalkohol solution (IBI 05154). After centrifugation, the upper phase was recovered. The extraction procedure was repeated once and a third extraction procedure was performed with chloroform alone. DNA was then recovered by adding 2 volumes of 96% ethanol at –20°C.

Restriction enzyme. DNA was digested with the restriction enzyme *Hinf*I (Boehringer) according to the manufacturer's specifications.

Electrophoresis. On each gel the molecular weight ladder (Amersham SJ 5000) was placed in 3–4 lanes. *Hinf*I-digested DNA from one control donor and from the cell line K562 (Promega) was used as controls (Fig. 1). The stock solution of buffer for electrophoresis consisted of 1.34 *M* trizma base, 749 m*M* H_3BO_3, and 25.5 m*M* Na_2EDTA in distilled water, pH = 8.8. Just before use, the stock solution was diluted 1:9 with distilled water, and ethidium bromide (Biorad nr. 161-0430) was added to a final concentration of 0.5 μg/ml. Separation of the DNA fragments was performed by electrophoresis of 1.25 μg DNA per sample in 0.7% agarose gels (20 × 20 cm) (Agarose Type II, Sigma). The gels were run overnight at max 75 mA (50–60 V) until the 2.39 kb band of the molecular weight ladder (Amersham SJ 5000) had migrated 14 cm.

Hybridization. After Southern blotting, hybridization with ^{32}P (Amersham) radiolabeled probes was performed as sequential reprobing on the membrane (Hybond N, Amersham). The probes detected the VNTR systems D2S44 (YNH24) (Wyman and White 1980), D5S43 (MS8), D7S21 (MS31), D7S22 (g3), and D12S11 (MS43a) (Wong et al. 1987). Prehybridization and hybridization were carried out for 18 h in a hybridization oven at 65°C.

Autoradiography was performed using X-ray films (Fuji, HR-G).

Band sizing. The migration of the bands was measured with a ruler with 0.5 mm resolution, and the kilobase values were calculated by local hyperbolic approximation (Elder and Southern 1987, Morling and Hansen 1992).

Matching criteria. The evaluation of the paternity cases and the family studies was based on side-to-side comparison of the DNA samples. Two DNA fragments were considered as "possibly identical by descent" when the migration distance between the 2 bands was less than 1.25 mm and 2 bands were considered non-identical when their migration difference exceeded 1.25 mm. Thus, exclusion of paternity (maternity) was stated when the migration distance between the paternally (maternally) derived band of the child and the nearest band in the alleged man (mother) exeeded 1.25 mm. The rationale behind the matching criterion has been described in detail by Morling and Hansen (1992).

Calculation of the index of paternity (PI). Using the frequencies of DNA fragments found in a population of unrelated Danes (Morling and Hansen 1992), an index of paternity (Gürtler 1956) could be calculated for each of the 5 VNTR systems. The following symbols were used:

M_a: the chance of the mother giving the DNA fragment "a" to the child,
P_b: the chance of the alleged man giving the DNA fragment "b" to the child, and
f_a & f_b: the frequencies of DNA fragments "a" and "b" in the population.

If the frequency of mutations is ignored, the index of paternity for one system is: $PI = M_a \times P_b / M_a \times f_b$. In most situations, both the mother and the alleged father were heterozygotes and the formula could be reduced to: $PI = 0.5/f_b$. When 5 VNTR systems were used, the combined PI was obtained by multiplying the indices obtained for each of the 5 systems.

Results

In 265 cases, the mother and the alleged father(s) were unrelated, and in 6 cases 2 men were sibs. The majority of the parties were Danes, but in 46 cases one of the parties – mostly the man – came from another ethnic group. Nearly all men from other ethnic groups came from Turkey or the Mediterranean area. In one case, all the parties were Turks (Table 1).

Since no major differences in the VNTR frequencies have been observed between Turks and Danes (Hansen and Morling 1992, in preparation), all cases were pooled for the analysis of the exclusion efficiency. The exclusion efficiencies of each of the 5 VNTR systems are given in Table 2. The least efficient system was D5S43 (MS8) excluding only about 51% of the men excluded by the conventional marker systems. The combined exclusion efficiency observed for the 5 VNTR systems is given in Table 3. In all 70 cases in which the parties were unrelated and in which the men were excluded as fathers by conventional marker systems, the men could also be excluded by 2 or more of the 5 VNTR systems. In another 10 cases in which the men had not been excluded by conventional typing, divergences between the results obtained with the conventional marker systems and with the 5 VNTR systems were observed (Table 4). In cases 1–4, only one alleged man was typed while in cases nos. 5–10, a second non-excluded man was also involved. Three men were excluded as fathers by only one of the systems, namely D12S11, D7S21, and D7S22, respectively. In case 4 (Table 4), the presence of the rare Rhesus combination '-D-' is likely. In cases 5, 7, and 9, the second alleged man was not excluded and had a high index of paternity for the conventional marker systems.

Table 1. Numbers and categories of paternity cases

Category of cases	All parties Danes	Man/mother non-Danes	Total nos. of cases
1 man	168	37	205
2 men	52	7	59
3 men	1	0	1
2 men, sibs	4	2	6
Total	225	46	271

Table 2. Exclusion efficiencies of 5 VNTR systems in 70 cases with exclusion of paternity by one or more conventional genetic marker systems. The parties were unrelated

VNTR system	Probe	Excluded men	
		Number	%
D2S44	YNH24	61	87.1
D5S43	MS8	36	51.4
D7S21	MS31	64	91.4
D7S22	g3	65	92.9
D12S11	MS43a	55	78.6

Table 3. Exclusion efficiencies of the combination of 5 VNTR systems in 70 cases with exclusion of paternity by one or more conventional genetic marker systems. The parties were unrelated

Number of excluding VNTR systems	Observed exclusions	
	Number	%
2	5	7.1
3	12	17.1
4	30	42.9
5	23	32.9

Table 4. Divergences between exclusion of paternity with 10–15 conventional marker systems and 5 VNTR systems

Case no.	Conventional systems			Combined index of paternity
	Exclusion	Index	Excluding VNTR systems	
1	none	2665	D7S22	304,440[b]
2	none	4749	D7S21	50,689[b]
3	none	227	D12S11	21,587[b]
4	Rh-unlikely	210[a]	none	11,298,286
5	none	125	all five	-
6	none	0.04	D2S44, D5S43, D7S22, D12S11	-
7	none	911	D2S44, D12S11	-
8	none	n.c.[c]	D2S44, D5S43, D7S21, D12S11	-
9	none	107	all five	-
10	none	2.2	D7S22, D12S11	-

[a] Index for the Rhesus system not included
[b] Index for the one excluding probe estimated to be 0.01
[c] Index not calculated. The parents of a deceased, alleged father were investigated

Table 5. Calculation of the index of paternity for 5 VNTR systems in the theoretically least informative situation. For each of the systems the child and the alleged father are assumed to possess 2 DNA fragments, and the one inherited by the child is the one represented by the highest frequency in the population

VNTR system	Probe	DNA fragment (kb)	Frequency f_{kb}[a]	Paternity index $PI = 0.5/f_{kb}$
D2S44	YNH24	2.73	0.100	5.0
D5S43	MS8	6.65	0.338	1.5
D7S21	MS31	6.65	0.130	3.8
D7S22	g3	7.01	0.085	5.9
D12S11	MS43a	8.88	0.190	2.6
The five DNA systems combined:				437

[a] The frequencies of DNA fragments in the matching windows are taken from Morling and Hansen 1992

In 6 cases, the 2 alleged men were full sibs. In 4 cases, one of the brothers was excluded by 2 of the 5 VNTR systems used but not by conventional typing. In one case, one of the brothers could be excluded by both conventional typing and by 2 of the VNTR systems. In the last case, one of the brothers was excluded by conventional typing (HLA-A, B) but not by DNA VNTR investigations.

The distribution of DNA fragments in the VNTR systems D2S44 (YNH24), D5S43 (MS8), D7S21 (MS31), and D12S11 (MS43a) among 650 random Danes, and for D7S22 (g3) in 300 mother/child pairs using a matching window of ±1.25 mm is described elsewhere (Morling and Hansen 1992).

A "minimum index of paternity", i.e. the PI value which would give the least positive evidence for paternity, was calculated for the hypothetical situation in which the paternally derived DNA fragment of the child, "b", matches one of the 2 DNA fragments of the man, the child and the alleged man both have 2 DNA fragments in all VNTR systems, and the paternally derived DNA fragment is not present in the mother. For each system, $PI = 0.5/f_b$ (Table 5), where f_b is the maximum frequency observed in the system in the Danish population (Morling and Hansen 1992). The "minimum index of paternity" obtained by multiplication of the PI-values was 437.

Table 6. Observations in the 5 VNTR systems which could be interpreted as mutations

VNTR system	Probe	Origin of germ cell: Maternal	Paternal	Total	Frequency
D2S44	YNH24	0	0	0	0
D5S43	MS8	0	0	0	0
D7S22	g3	0	1	1	0.0014
D7S21	MS31	0	2	2	0.0029
D12S11	MS43a	0	1	1	0.0014
Number of informative meioses:		350	350	700	

Table 7. Linkage analysis between the systems D7S21 (MS31) and D7S22 (g3) in 29 informative families with 81 children

Origin of germ cell	Number of germ cells	Recombinants	
		Number	%
Maternal	73	26	35.6
Paternal	73	19	26.0
Total	146	45	30.8

The occurrence of mutations was investigated in the informative part of the material, namely in 261 trios (mother/-child/non-excluded man) and in 37 families with 89 children (Table 6). No mother/child exclusion was observed among 350 pairs. In 4 cases among 350 father/child pairs in which the men had not been excluded by conventional typing, the men were excluded by only one VNTR system. The paternity indices of all 4 men exceeded 20,000 when the conventional systems and the other 4 VNTR systems were considered.

Since the loci D7S21 (MS31) and D7S22 (g3) are both situated on chromosome 7, linkage studies were performed in 29 informative families with 81 children (Table 7). A double intercross was found in 24 families, while only one DNA fragment in either D7S21 or D7S22 was observed for 3 women and for 2 men, respectively. The frequency of recombinations was 45/146, corresponding to a recombination distance between the 2 loci of about 31 cM.

Discussion

A comparatively large number of non-excluded men were found in this study (Table 1): only 83 (25%) out of 326 men were excluded as the biological fathers. The reason was that about 94% of the biological non-fathers had already been excluded by initial investigations comprising 10 or 11 conventional marker systems and, in general, the excluded men were omitted from the extended paternity investigations.

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 when a trio is investigated on the same gel. An empirical data analysis of the intra gel differ-

ences 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 (Morling and Hansen 1992). In our laboratory, 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). Therefore, it is also possible to calculate the upper and lower kb values of the ±1.25 mm matching vindow. We have decided to use the matching criterion ±1.25 mm and to calculate the likelihood quotients (paternity indices) of random matches in accordance with the matching criterion.

When the exclusion efficiencies of the 5 VNTR systems were considered separately (Table 2), the system D7S22 (g3) was the most informative with an exclusion rate of 93%, while D5S43 (MS8) could exclude only 51% of the non-fathers. Henke et al. (1991) obtained similar exclusion efficiencies for the systems D2S44, D7S21, and D7S22, while the exclusion efficiency of about 91% for D12S11 in the German study exceeded the 79% observed in the present study. Other investigators have found similar exclusion efficiencies with D2S44 in Caucasians using other restriction enzymes, e.g. Acton et al. (1990) 94% using *Hae*III and Allen et al. (1990) 88% using *Pvu*II.

When the combined efficiency of the 5 VNTR systems was compared to the combined efficiency of the conventional marker systems including HLA-A, B (Tables 3 and 4), 3 men were excluded as fathers by only one of the DNA systems, namely D7S21 (MS31), D7S22 (g3), and D12S11 (MS43a), respectively, and a fourth similar case of paternity exclusion, also in D7S21, was found in a Turkish immigration family. Since mutations are documented in these systems (e.g. Jeffreys et al. 1988), exclusion of paternity could not be considered conclusive in these 4 cases. If the mutation rate for each of the VNTR systems is estimated to be 1%, which is an overestimate according the observed values (Table 6) as well as to the observations of others (e.g. Jeffreys et al. 1988; Smith et al. 1990; Henke et al. 1991; Brinkman et al. 1991), and a paternity index of 0.01 is then used for the systems D7S21, D7S22, and D12S11, the final indices of paternity in cases 1–3 (Table 4) were 304,440, 50,689, and 21,586, respectively. The possibility of e.g. unknown brothers as the biological fathers of the children could not be ruled out.

In the present material comprising trios of mother/child/non-excluded man and families (Table 6), no mother/child exclusion was observed. The 4 single exclusions of paternity which could indicate mutations led to estimates of mutation frequencies for the systems D7S21, D7S22, and D12S11, which were in agreement with the observations of other authors (Henke et al. 1991; Brinkmann et al. 1991). A mutation rate of 0.46% has been found for D2S44 by Henke et al. (1991), while no observation indicating mutation was seen in the present material. No mutation was observed among about 400 offspring for D5S43 (Brinkmann et al. 1991) and in this material, no indication of mutation was found in 700 informative meioses.

Our definition of mutations is based on a pragmatic data analysis of the mother/child migration distances (Morling and Hansen 1992). We cannot exclude that differences below 1.25 mm represent mutations and, thus, our estimates are probably lower than the true mutation rates. We observed mutations only in males. This may reflect (i) the fact that the men were not the true fathers, (ii) chance deviations, or (iii) a possible difference between the mutation frequencies of men and women in the VNTR systems.

In case 4 (Table 4), the presence of the rare Rhesus combination '-D-' is likely and the paternity index including the VNTR systems was 11,298,286. In cases 5, 7, and 9, two unrelated men were involved and, in each case, one of them was excluded by the VNTR systems in spite of high PI value for the conventional systems including HLA.

When considering the risk of mutations, silent alleles etc. at least 2 independent VNTR exclusions are normally required before the exclusion of an alleged man can be established with a likelihood quotient exceeding 10,000:1. Out of 76 non-fathers [70 unrelated men, Table 3, and 6 men from the cases including sibs], 10 men were excluded with only 2 of the VNTR systems and, consequently, the use of 5 marker systems seems necessary.

Studies of 146 informative meioses demonstrated genetic linkage between D7S21 and D7S22 with a recombination distance of about 31 cM (Table 7). No obvious linkage disequilibrium has been observed between defined DNA fragments of the D7S21 and D7S22 systems, and linkage equilibrium between DNA fragments of the 2 syntenic systems can so far be assumed. The question of linkage disequilibrium between certain DNA fragments of the D7S21 and D7S22 systems cannot, however, be answered with certainty on the basis of this small sample size.

The combined efficiency of exclusion for the 5 VNTR systems used in this study exceeded 99.9% (Table 3). The calculation of the paternity index was based on the population frequencies of the DNA fragments observed in the matching window. Thus, the positive evidence for paternity (PI) is expected on average to exceed 1,000. In Table 5, the least informative situation in the present system is demonstrated. For each of the 5 VNTR systems, the most commonly observed DNA fragment (Morling and Hansen 1992) was used as the paternally derived DNA fragment in a case in which both the child and the man are heterozygotes. The combined paternity index in such a case was 437. On the other hand, based on the size of the population, a "minimum frequency" of 0.005 for rare or unobserved alleles was used in the systems D2S44, D5S43, D7S21, and D12S11, and 0.01 in D7S22. Using this "minimum frequency" for the calculation of an index (PI) in a similar way as demonstrated in Table 5, a combined "maximum index" of 5×10^9 would be obtained. For comparisons, the most frequent haplotype in the HLA-system, HLA-A1, B8, leads to a paternity index of around 5, while an HLA index of $PI_{HLA} = 8{,}501$ has been found in a case in which a putative father of the type HLA-A3,A28;B27,B41 had passed the haplotype *HLA-A28,B41* to the child (Hansen 1989). When the paternity indices calculated for the 5 VNTR systems were combined with the indices obtained by investigations with the conventional systems including HLA, the combined paternity index in each case exceeded 10,000.

Hitherto, Danish paternity investigations have been based on investigations of 10–15 conventional marker systems, when necessary including the HLA-A,B system and with an overall exclusion efficiency of 99.4% (Hansen 1989). The use

of the 5 VNTR systems D2S44, D5S43, D7S21, D7S22, and D12S11 gave a combined exclusion efficiency of more than 99.9% and, thus, a paternity investigation comprising the 5 DNA systems alone seems to be superior to the previous investigations when dealing with unrelated men. It should, however, be noted that if paternity testing is based on the investigation of only 5 genetic systems, the likelihood quotient for non-exclusion of e.g. a possible brother of the biological father is at least $1:2^5$ (approximately 0.03). In the present material, 6 cases involving brothers were investigated and in one of the cases, none of the brothers were excluded by any of the 5 VNTR systems.

A small number of men will appear to be excluded by only one VNTR system: biological fathers excluded due to a mutation and non-fathers excluded by only one VNTR system due to chance (e.g. due to the fact that a brother of the man is the biological father of the child). In such cases, either some additional VNTR systems or conventional marker systems, e.g. the HLA system, could be used to obtain further information.

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