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DXS6797 contains two STRs which can be easily haplotyped in both sexes

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Abstract DXS6797 is a complex X-chromosomal locus which contains two variable short tandem repeats (STRs) (motif ATCT) separated by 128 non-polymorphic nucleotides. The two STRs can be cleaved apart by *Taq* I digestion. Conventionally, DXS6797 is typed by measuring the overall amplicon length, providing only eight alleles [polymorphism information content (PIC) 0.733, mean exclusion chance (MEC) 0.712]. Separate amplification would increase the discrimination but obscure the haplotype constellation in females. Therefore, we proceed by amplifying the whole sequence containing both repeats (DXS6797 I and DXS6797 II) using a Fam-labelled forward primer and a Tamra-labelled reverse primer. We then measure the length of the entire double-labelled amplicon and a *Taq*-I-digested aliquot to infer, for both males and females, compound haplotypes consisting of DXS6797 I and DXS6797 II repeat length. This procedure has the potential to provide 42 DXS6797 haplotypes. If the crossover rate between both STRs is assumed to be $<1.5\times10^{-6}$, DXS6797 haplotypes could be used for kinship testing like STR alleles. In our German sample (780 X chromosomes), we determined 27 haplotypes (PIC 0.842, MEC 0,834) and in 220 meioses, we found no new mutations.

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Introduction

The hypervariable tetranucleotide short tandem repeat (STR) polymorphism DXS6797 (GDB, G00-365-103), first described by Murray et al. (http://www.gdb.org/), is also known as CHLC.GATA10C11. This marker maps to Xq23.3 and is distanced 112.9 cM from Xp-tel.

The most closely linked forensic markers located in a region of about 18 Mbps are listed in Table 1. Unfortunately, genetic localisation given in centimorgans (cM) cannot be found for all markers in the web gene databases. Hence, the genetic localisation of DXS7424, DXS101 and DXS7133 are given in parenthesis as roughly estimated figures. Haplotyping of the DXS6797 region can be useful for linkage studies in clinical genetics [5] and in kinship testing [15].

To our knowledge, only two population studies concerning DXS6797 have been published by Ying et al. [16] and Shin et al. [12], both investigations using East Asian samples. Conventional DXS6797 typing using template amplification and measurement of amplicon length, however, does not exhaust the full information potential of this locus. As displayed in Fig. 1, DXS6797 contains two variable ATCT repeats (DXS6797 I and DXS6797 II), which are separated by an interspersed non-polymorphic sequence of 128 bps. Separate amplification of both STRs would increase the amount of information compared with investigation of the complex double STR. However, such a strategy cannot elucidate the haplotype constellation in the female sex. Using a Fam-labelled forward primer and a Tamra-labelled reverse primer, we amplified the entire sequence containing both repeats, which can subsequently be separated by a *Taq* I digestion. Length measurement of the whole double-labelled amplicon and a *Taq*-I-digested aliquot provides both the length values of the amplicon and of the restriction fragments each containing one of the STRs. This technique not only enables estimation of the repeat

Table 1 The localisation of DXS6797 and some neighbouring markers within a section of about 18 Mbp at Xq22-23 are indicated as physical distances from the ChrX p-tel in mega base pair (Mbp) according to http://www.ensembl.org/ and in centimorgan (cM) according to http://www.marshfieldclinic.org/, respectively

Marker	Mbps	cM
DXS6789	95.25	103.56
DXS 7424	100.42	(108)
DXS 101	101.21	(109)
DXS 6797	107.28	112.89
DXS 7133	108.84	(113)
GATA172D05	112.97	116.17

numbers of DXS6797 I and DXS6797 II, but permits determination of the haplotypes in both sexes by means of a simple calculation process.

Material and methods

Sample collection

Blood samples for population studies were collected from 532 unrelated Germans (284 males and 248 females) including 110 cases of routine kinship testing concerning female children . For those constellations which have been investigated before in standard STR analysis, the paternity probability of the putative father was >99.99%. Thus, 220 meioses (110 male and 110 female) were checked for regular X-chromosomal inheritance. The parental age structure is shown in Table 7 in the connection with the estimation of the mutation rate.

DNA extraction and template amplification

DNA was extracted from blood specimens using the Invisorb Spin Blood Mini kit (Invitek, Berlin, Germany). Polymerase chain reaction (PCR) amplification was performed using the following primer sequences based on gene bank information (http://www.gdb.org): Primer F: 5′-FAM-TTC CCT CTC TCC CTC TGT CT-3′; Primer R: 5′ TAMRA-TGA CAG AGC AAG ACT CCA TC-3′. Amplifications were carried out in a 12.5-µl PCR reaction volume containing 1–2 ng DNA, 15 mM Tris–HCl, 50 mM KCl, 100 µM each dNTP, 1.5 mM $MgCl_2$ and 1 U HotStart Taq polymerase (Qiagen, Hilden, Germany) using a thermocycler (Biometra, Göttingen, Germany) with a 12-min soak and enzyme activation at 95°C, followed by 30 cycles at 95°C for 1 min, 60°C for 1 min, 72°C for 3 min and 72°C for a 30-min final extension.

To generate sequencing templates, we used the following unlabelled primers: F primer: 5′-TCT CTC CCT TTC TCT TTC CCT-3′; R primer: 5′-AGN CTT GCC ACT GCA CTC CA-3′.

The PCR procedure was carried out in a 25-µl amplification set-up as described for fragment analysis, except that concentrations were 200 µM dNTPs and 10 ng template DNA. The PCR success was checked by polyacrylamide gel electrophoresis as published [3].

STR fragment analysis

An aliquot of 5 µl of the PCR product was digested with 10 U *Taq* I (Roche Applied Science, Mannheim, Germany) at 60°C for 4 h. The high enzyme dosage prevents uncompleted digestions even when crude PCR products are used and the salt concentration is not adjusted to the incubation optimum. One microlitre digested and 1 µl undigested PCR products were mixed with 12 µl formamide and 0.5 µl ROX-400 as internal lane standard and analysed by capillary electrophoresis using the ABI Prism 310 genetic analyser and polymer POP4 (Applied Biosystems, Darmstadt, Germany). Each X chromosome (ChrX) provides one double-labelled amplicon and two restriction fragments labelled with Fam (DXS6797 I) and Tamra (DXS6797 II), respectively. The length of one Tamra-labelled and one Fam-labelled fragment is equal to the length of one double-labelled amplicon. Hence, the complex evaluation of the double-labelled amplicon and the single-labelled restriction fragments allows the assessment of both haplotypes even in females.

Fragment size was determined using the local Southern method implemented in Genescan software. Allele designation was based on sequenced alleles of chosen individuals and of the cell lines NA9947A, NA9948 and NA3657.

Investigation on the allele structure

For analysing the allele structure, we selected 62 hemizygous male DNA samples with regard to their DXS6797 amplicon length. Purification of the sequencing templates was carried out using the MSB Spin PCRapace kit (Invitek). For the cycle sequencing procedure, the BigDye

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1   ctaacaacta tctctccctt tctctTTCCC  TCTCTCCCTC   TGTCTCtatc atctatctat
61  ctatctatct atctatctat ctatctatct atctatgtat catttatcta tctaaaatct
121 atctctatca tcaatcTCGA tgtgtctctg ttatctatca tctgtctatc ttctatctct
181 atgtgtctct atctatctgt catctaccta tcacctctct atatctatct atctatctat
241 ctatctatct atctatctat ctatctggtt ttgggtgtgt gtgtgtgtgt gttttntttt
301 tttgaGATGG  AGTCTTGCTC  GTCAcccag  gctggagtgc agtggcaaga nctcgggtca
361 cngnaaccg
```

Fig. 1 DXS6797 sequence: The repeat regions DXS6797 I and DXS6797 II are written in *bold italics*. Primer binding sequences for routine amplification primers are printed in *capitals*. The *Taq* I site is in *bold capital letters*. Binding sites for primers used for the sequencing procedure are *underlined*

Table 2 Allele frequencies for DXS6797 (complex marker)

Allele	Length (bp)	Females				Males			Total		
		Number	Frequency	95% CIs	Homozygote	Number	Frequency	95% CIs	Number	Frequency	95% CIs
19	284	1	0.002	0.000–0.011	0	0	0.000	0.000–0.013	1	0.001	0.00 –0.007
20	288	5	0.010	0.003–0.023	0	2	0.007	0.001–0.025	7	0.009	0.004–0.018
21	292	93	0.188	0.156–0.228	8	64	0.225	0.178–0.278	157	0.201	0.175–0.233
22	296	161	0.325	0.284–0.368	23	95	0.335	0.280–0.393	256	0.328	0.295–0.362
23	300	147	0.296	0.257–0.339	24	77	0.271	0.220–0.327	224	0.287	0.256–0.320
24	304	60	0.121	0.094–0.153	5	34	0.120	0.084–0.163	94	0.121	0.099–0.145
25	308	19	0.038	0.023–0.059	2	9	0.032	0.015–0.059	28	0.036	0.024–0.052
26	312	10	0.020	0.010–0.037	0	3	0.011	0.002–0.031	13	0.017	0.009–0.028
n		496			62	284			780		

Table 3 Allele frequencies for DXS6797 I

Allele	Length (bp)	Females				Males			Total		
		Number	Frequency	95% CIs	Homozygote	Number	Frequency	95% CIs	Number	Frequency	95% CIs
8	100	1	0.002	0.000–0.011	0	1	0.0035	0.000–0.020	2	0.003	0.000–0.011
9	104	4	0.008	0.002–0.021	0	7	0.0246	0.010–0.050	11	0.014	0.007–0.025
10	108	260	0.524	0.479–0.569	70	148	0.5211	0.461–0.581	408	0.523	0.487–0.559
11	112	192	0.387	0.344–0.432	38	111	0.3908	0.334–0.450	303	0.389	0.354–0.424
12	116	30	0.061	0.041–0.085	1	15	0.0528	0.030–0.086	45	0.058	0.043–0.076
13	120	9	0.018	0.008–0.034	1	2	0.0070	0.001–0.025	11	0.014	0.007–0.025
	n	496			110	284			780		

Table 4 Allele frequencies for DXS6797 II

Allele	Length (bp)	Females				Males			Total		
		Number	Frequency	95% CIs	Homozygote	Number	Frequency	95% CIs	Number	Frequency	95% CIs
8	170	1	0.002	0.000–0.011	0	0	0.000	0.000–0.013	1	0.001	0.000–0.007
10	178	11	0.022	0.011–0.039	0	8	0.028	0.012–0.055	19	0.024	0.015–0.038
11	182	135	0.272	0.234–0.314	18	77	0.271	0.220–0.327	212	0.272	0.241–0.305
12	186	233	0.470	0.425–0.515	57	139	0.489	0.430–0.549	372	0.477	0.441–0.513
13	190	88	0.177	0.145–0.214	7	50	0.176	0.134–0.225	138	0.177	0.151–0.206
14	194	25	0.040	0.033–0.074	1	9	0.032	0.015–0.059	34	0.044	0.030–0.060
15	198	3	0.006	0.001–0.018	0	1	0.004	0.000–0.020	4	0.005	0.001–0.013
	n	496			83	284			780		

Ready Reaction kit (Applied Biosystems) was employed according to the manufacturer's instructions. The primers described for the template generation were also used as sequencing primers; 25 cycles were carried out as follows: 95°C for 30 s and 60°C for 4 min. The PCR products were sequenced with the ABI Prism 310 genetic analyser.

This paper follows the guidelines for allele nomenclature as published [1].

Statistical analysis

Frequencies of alleles, haplotypes and mutation rates are indicated with exact 95% confidence intervals (CIs) according to Clopper and Pearson (StatXact-4, Cytel Software Corporation, Cambridge, MA, USA). Formulae for calculation of parameters of forensic interest such as polymorphism information content (PIC), mean exclusion chance (MEC), etc. were used as reviewed earlier [15].

Statistical analysis of the DXS6797 population data to check for Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) in 780 ChrX haplotypes of males and females was performed using exact tests (SAS-Genetics, SAS Institute Inc., Cary, NC, USA).

Results

The German population sample investigated here exhibited eight alleles of the complex DXS6797 marker when the alleles are defined by amplicon length, not considering the internal structure (Table 2). As displayed in Tables 3

Table 5 Haplotype frequencies for DXS6797

DXS6797		Females				Males			Total			
I	II	Number	Frequency	95% CIs	Homozygote	Number	Frequency	95% confidence internal	Number	Frequency	95% CIs	Number of sequenced haplotypes
8	12	0	0.000	0.000–0.007	0	1	0.004	0.000–0.020	1	0.001	0.000–0.007	1
8	13	1	0.002	0.000–0.011	0	0	0.000	0.000–0.013	1	0.001	0.000–0.007	0
9	11	1	0.002	0.000–0.011	0	1	0.004	0.000–0.020	2	0.003	0.000–0.009	1
9	12	1	0.002	0.000–0.011	0	4	0.014	0.004–0.036	5	0.006	0.002–0.015	2
9	13	1	0.002	0.000–0.011	0	2	0.007	0.001–0.025	3	0.004	0.001–0.011	1
9	15	1	0.002	0.000–0.011	0	0	0.000	0.000–0.012	1	0.001	0.000–0.009	0
10	10	4	0.008	0.002–0.021	0	0	0.000	0.000–0.013	4	0.005	0.001–0.013	1
10	11	85	0.171	0.139–0.208	7	53	0.187	0.143–0.237	138	0.177	0.151–0.206	8
10	12	121	0.244	0.207–0.284	13	71	0.250	0.201–0.305	192	0.246	0.216–0.278	10
10	13	41	0.083	0.060–0.111	1	22	0.078	0.049–0.115	63	0.081	0.063–0.102	7
10	14	9	0.018	0.008–0.034	0	2	0.007	0.001–0.025	11	0.014	0.007–0.025	3
11	8	1	0.002	0.000–0.011	0	0	0.000	0.000–0.013	1	0.001	0.000–0.007	0
11	10	6	0.012	0.005–0.026	0	7	0.025	0.010–0.050	13	0.017	0.009–0.028	2
11	11	39	0.079	0.057–0.106	2	21	0.074	0.046–0.111	60	0.077	0.059–0.098	5
11	12	97	0.196	0.162–0.233	10	54	0.1901	0.146–0.241	151	0.194	0.166–0.223	5
11	13	38	0.077	0.055–0.104	2	23	0.0810	0.052–0.119	61	0.078	0.060–0.099	5
11	14	9	0.018	0.008–0.034	1	5	0.0176	0.006–0.041	14	0.0179	0.010–0.030	2
11	15	2	0.0040	0.001–0.015	0	1	0.0035	0.000–0.020	3	0.0038	0.001–0.011	1
12	10	0	0.0000	0.000–0.007	0	1	0.0035	0.000–0.020	1	0.0013	0.000–0.007	1
12	11	8	0.0161	0.007–0.032	0	1	0.0035	0.000–0.020	9	0.012	0.005–0.022	1
12	12	10	0.0202	0.010–0.037	0	8	0.0282	0.012–0.055	18	0.023	0.014–0.036	2
12	13	6	0.0121	0.005–0.026	0	3	0.0106	0.002–0.031	9	0.0115	0.005–0.022	1
12	14	6	0.012	0.005–0.026	0	2	0.007	0.001–0.025	8	0.010	0.004–0.020	1
13	10	1	0.002	0.000–0.011	0	0	0.000	0.000–0.013	1	0.001	0.000–0.007	0
13	11	2	0.004	0.001–0.015	0	1	0.004	0.000–0.020	3	0.004	0.001–0.011	1

Table 5 (continued)

DXS6797		Females				Males			Total			
I	II	Number	Frequency	95% CIs	Homozygote	Number	Frequency	95% confidence internal	Number	Frequency	95% CIs	Number of sequenced haplotypes
13	12	4	0.008	0.002–0.021	1	1	0.004	0.000–0.020	5	0.006	0.002–0.015	1
13	13	2	0.004	0.001–0.015	0	0	0.000	0.000–0.013	2	0.003	0.000–0.009	0
n		496			37	284			780			

Table 6 Statistical parameters of forensic interest

	DXS6797 (complex marker)	DXS6797 I	DXS6797 II	DXS6797 haplotypes
PIC	0.733	0.529	0.637	0.842
MEC I	0.526	0.289	0.411	0.706
MEC II	0.712	0.486	0.61	0.834
MEC III	0.575	0.346	0.463	0.729
HET	0.754	0.573	0.665	0.851
PD_f	0.898	0.731	0.833	0.961
PD_m	0.753	0.572	0.665	0.85

PIC Polymorphism information content, *MEC* mean exclusion chance (*I* for autosomal markers after Krüger et al. [9], *II* for X-chromosomal markers in trios after Kishida and Tamaki [8], *III* for X-chromosomal markers in father/daughter pairs after Desmarais et al. [6]), *HET* observed heterozygosity, PD_f power of discrimination in females, PD_m power of discrimination in males

Table 7 The parental age structure at birth and DXS6797 mutation rates with the exact CIs according to Clopper–Pearson [4]

Age (years)	Mothers	Fathers
15–20	40	20
21–25	45	36
26–30	11	24
31–35	8	14
36–40	6	9
41–45	0	2
>45	0	5
Mutations/meioses	0/110	0/110
Exact 95% CIs	0.000–0.030	0.000–0.030
Exact 95% CIs (cumulative)	0.000–0.015	

and 4, DXS6797 I and DXS6797 II exhibit six and seven alleles, respectively, hence, DXS6797 has the potential to provide 42 haplotypes, although only 27 haplotypes could be found in the 780 ChrX of this study. Of the observed haplotypes, 24 occur with a frequency <0.1. The different haplotypes and the frequencies found in our population sample are listed in Table 5.

In Table 6, a comparison of statistical parameters of forensic interest between the analysis of the complex DXS6797 marker, the single STRs and the haplotyping procedure is given. The gain of the information power by haplotyping can be clearly recognised.

To facilitate an inter-laboratory standardisation, we used a set of established cell line DNAs [14] to provide a tool for allele ladder calibration. The alleles and haplotypes of DXS6797 I–DXS6797 II for standard DNA are as follows: NA9947A (female) 10–11, 12–11; NA9948 (male) 10–11; NA3657 (male) 11–12. Summarising the repeat counts of the haplotypes, the complex STR of the control DNAs NA9947A, NA9948 and NA3657 exhibits the genotypes; 21–23, 21 and 23, respectively.

Sequencing of 62 male DNA samples revealed regular ATCT repeats for DXS6797 I and DXS6797 II in all cases. In all cases, the sequences were found in agreement with the results of the length measurements. Comparison of our sequencing results with the DXS6797 sequence published in the database (GDB, G00-365-103) showed no differences with regard to the repeat flanking regions. Investigation of the X-chromosomal mode of inheritance in 110 family trios with female children provided the expected result in each case. Hence, DXS6797 is characterised by a low mutation rate (Table 7). Significant deviations from the HWE were not established; the exact permutation test yielded values are p=0.2346 and p=0.6707 for DXS6797 I and DXS6797 II, respectively.

Test for association did not indicate an LD between both DXS6797 STRs (p=0.1662).

Discussion

Several complex STRs comprising two or more variable repeat components are established in forensic science. VWA, D3S1358, D21S11, ACTBP2, DXS6809, DXS101 and DXS8377 may be mentioned as some examples in which standard analysis focuses the attention on the measurement of the complete amplicon length only. To our knowledge, very few attempts have been published to make use of the hidden information based on internal sequence variability [13].

An exceptional situation in DXS6797, however, facilitates this attempt: a block of 128 bps inserted between two

repeats with a Taq I restriction site gives the opportunity for a special sophisticated approach to employ this locus in kinship testing. Strictly speaking, DXS6797 I and DXS6797 II are two distinct markers which can be haplotyped. Haplotyping of closely linked loci provides powerful tools to solve complicated deficiency kinship cases as has been demonstrated earlier [7, 15]. However, in most cases, a limitation for a haplotyping approach occurs in a diploid genotype. This applies generally to autosomal loci and to ChrX markers in the female sex. In such cases, the phases (that means the combination in cis- or trans- position) are not easily recognisable. Only under lucky circumstances haplophases can be deduced from the pedigree.

In contrast, our approach to analyse DXS6797 detects the haplotype not only in males but also in females. Unfortunately, both DXS6797 I and DXS6797 II show only a low variability. Nevertheless, haplotyping bears the potential to provide 42 haplotypes. Only three haplotypes show a frequency >0.10, and 14% of all haplotypes are rarer than 0.02. Since no hints for the existence of an LD between both closely linked loci DXS6797 were found, we can calculate haplotype frequencies from the allele frequencies of the single STRs. However, when dealing with haplotypes of closely linked loci, the fact that LD may vary in different populations has to be taken into account. Whilst our group observed a strong LD between DXS101 and DXS7424 [7], other authors [10] failed to indicate the same phenomenon in a Korean population.

The mutation rates of both linked STRs seem to be low. This is in agreement with the observation that short STR stretches are not prone to high mutation rates [2]. However, with regard to DXS6797, more investigations are needed to give definite statements. It is a commonly accepted thumb rule that the physical distance of 1 Mb equals a recombination rate of 1% (1 cM) [11]. The distance between DXS6797 I and DXS6797 II is only 128 bps. Hence, we expect a crossover rate within DXS6797 lower than 1 in 7,000 meioses, which is much less than the expectation for STR mutability.

The investigation of DXS6797 in a Chinese population [16] can hardly be compared with our results, since no details are communicated with regard to the DXS6797 structure investigated. The investigation of the complex DXS6797 locus in a Korean sample [12] seems to display no gross differences to the European allele distribution.

By including DXS6797, we have established a closely linked panel for ChrX, which can be useful in difficult deficiency kinship cases. Owing to the close linkage to the DXS7424–DXS101 cluster and DXS7133, this new marker may contribute to establish a ChrX haplotyping procedure as an efficient tool in human kinship testing.

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