HIGH RESOLUTION ANALYSIS BY PCR ON AN AUTOMATED DNA SEQUENCER OF INTERNAL VARIATION AT A PSEUDOAUTOSOMAL VNTR (DXYS17)

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INTRODUCTION

PCR-based DNA typing has become a valid alternative for the time-consuming and laborious typing of VNTR loci by Southern blot analysis. It has not only increased the sensitivity of DNA typing to levels of a few nanogram of DNA but it allows also to have a higher resolution for small size differences. Genotyping becomes possible which is in contrast to conventional Southern blot methods where the low resolution for small size differences, sometimes, lead to 'pseudo-homozygosity' (1).
At the present time, PCR analysis of only a few VNTR loci has become applied in identity testing and paternity determinations (e.g. ApoB, D17S5, COL2A1 ..). In order to increase the discriminatory power of PCR based DNA typing, we investigated the pseudoautosomal VNTR DXYS17 as a candidate marker in combination with a fluorescent approach for detection of the alleles on an automated DNA sequencer.

MATERIALS AND METHODS

DNA was extracted according to standard procedures from venous blood samples of 394 random Caucasian individuals of Belgian origin.
Amplifications were carried out in a Perkin-Elmer 9600 with 25 cycles consisting of 25 sec. at 94°C for denaturation, 25 sec. at 55.9°C for annealing and 2 min. 20 sec. at 68°C for extension. All reactions were done in a volume of 15 μl containing a modified Taq-buffer (2), 2 mM $MgCl_2$, 0.2 mM dNTP, 1 μM of each oligonucleotide (602-A 5'-ACTCACTAGCACATGCTGTG and 602-B 5'-GCGCAAAGAAATTCTGGTGA) and 250 ng of DNA.
10 μl of the amplified products was used for a quality control on 6% polyacrylamide (PAGE) gels. Positive amplifications were diluted 16-fold and 5 μl was loaded on a 6% Hydrolink Long Ranger gel and run on the A.L.F. DNA Sequencer. Analysis of the runs was done with the A.L.F. Fragment Manager Version 1.0.

RESULTS AND DISCUSSION

Conventionally, sizing of amplified VNTR alleles is performed either on high concentration composite-agarose gels or on PAGE gels with visualisation of the alleles by ethidium bromide. These gels can not be re-used and do not allow for standardization of the electrophoresis conditions other than the gel matrix. Therefore, we used an automated DNA sequencer to do a population survey for the alleles of the DXYS17 locus. The A.L.F. DNA sequencer was chosen for this purpose and this system showed to give highly reproducible runs: the maximum difference in mobility between two identical alleles on the same gel was 0.56% and between the transmitted alleles from parent to child was 0.09%. Moreover, fragments between 500 and 1100 bp could be separated in less than 4 hours and the gel could be reloaded with other samples. Preliminary experiments with shorter gel plates, where the well-to-laser distance was reduced from 24 cm to 12 cm, showed that the length of the run could be reduced to 1 hour with only a slight decrease in the difference in mobility between identical alleles (maximum 0.88%). Sizing of the amplified fragments on the A.L.F. System was initially done in denaturing (urea) conditions. However, when comparing the resolution for separation of the different alleles with non-denaturing conditions, we observed that two different alleles migrated with the same speed in urea gels and with different mobility in native gels (Fig. 1A and B). This observation was reproducible and we hypothesized

that these two alleles were of the same length but had a different internal structure. Sequence analyses proofed this hypothesis and showed that both alleles were almost identical at the 5' and the 3' end but with a different structure of the internal repeats. The DXYS17 consists of two different repeats: one of 28 bp and one of 33 bp. The 28-bp unit is similar to the 33-bp unit but shows a 5 bp deletion in the middle.

The observation that two non-identical VNTR alleles migrate with the same mobility on agarose gels or urea-PAGE gels and different on native PAGE gels has some important implications in forensics:

1. A genotype of the two alleles (9 and 10) would be identified as heterozygous on native PAGE gels and homozygous on agarose gels. This would lead to a false inclusion in case of two homozygotes (9-9 and 10-10) or a homozygote (9-9 or 10-10) and a heterozygote (9-10) when sizing of the amplified alleles would be done on agarose gels or denaturing PAGE gels.
2. The frequency of the two alleles is 9.1% (9) and 14.6% (10). A conservative frequency estimate (2p) for a homozygous typing is 47.4% while for a heterozygous typing a figure of 2.7% is obtained.

A population survey in 394 random Caucasians revealed in total 17 different alleles with a minimum frequency of 0.13% and a maximum frequency of 21.9%. The genotype distribution of the different alleles were in Hardy-Weinberg equilibrium as could be shown by different tests. The observed homozygosity (85.8% ± 1.8%) did not differ significantly from the expected (83.7% ± 0.5%). Also no

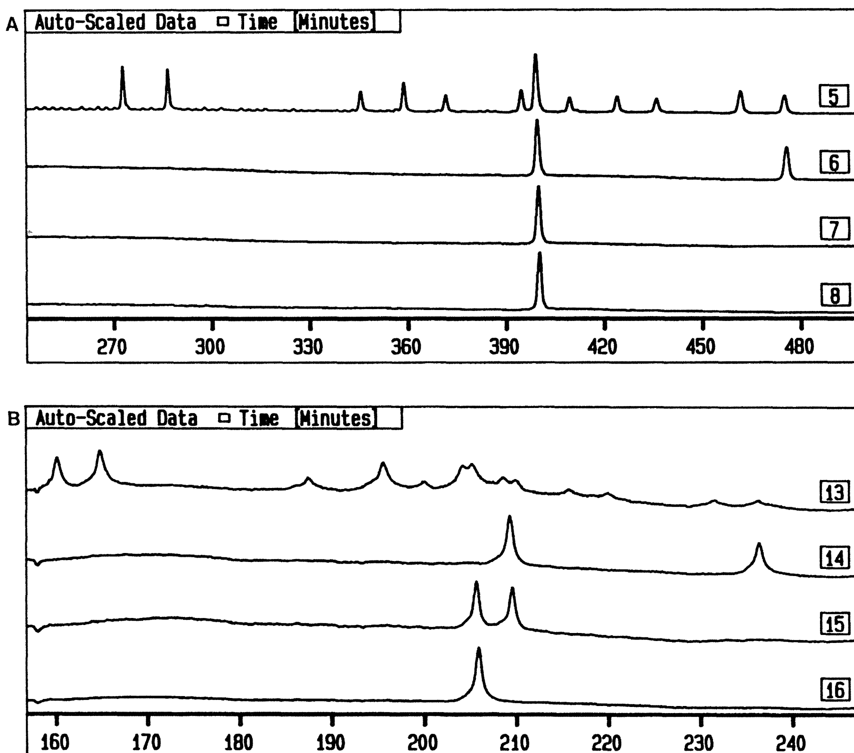

FIGURE 1: Analysis of the amplified DXYS17 alleles in a nuclear family (father: lanes 6 and 14; child: lanes 7 and 15; mother: lanes 8 and 16) on the A.L.F. DNA Sequencer in 6% Hydrolink Long Ranger gel with urea (A) and without urea (B). Lanes 5 and 13 represent an allelic marker with 13 of the 17 observed alleles in the Belgian population.

significant difference was observed between the number of observed and expected homozygous (8 vs. 6.0 ± 0.8) genotypes and heterozygous (49 vs. 49.5 ± 3.5) genotypes (1). However, when the allele frequencies were compared between females and males a significant difference was observed for one allele (no. 12; Fig. 2). Similarly, a significant difference was observed between males and females for two genotypes (3-11 and 11-12). The basis for these differences could be found by a segregation analyses and showed that there was a significant difference in the distribution of some alleles over the sex chromosomes: allele 12 was present on 72.7% of the X chromosomes while allele 11 was present on 31.2% of the X chromosomes. For allele 3 an equal representation was found on both sex chromosomes. Another explanation, heterogeneity of the population sample, could be rejected as there was no difference in allele or genotype distributions between females and males for several autosomal loci (D17S5, ApoB and IGHJ).

The DXYS17 locus is one of the most polymorphic VNTR loci typeable by PCR. The probability of identity between two non-related individuals is 0.048 and the probability for paternity exclusion is 0.673. In over 170 meioses, no evidence was found for any mutation and there were no alleles who failed to be amplified which would result in exclusion of maternity.

The amplification and analysis of the DXYS17 locus is very robust and has proven to be successful in over 100 forensic cases. The use of the A.L.F. DNA Sequencer for sizing of amplified VNTR allele is highly accurate and allows to compare two samples on two different gels (4). This is certainly an advantage in the analysis off forensic DNA samples where it is possible that some samples have to be compared with others typed several months before.

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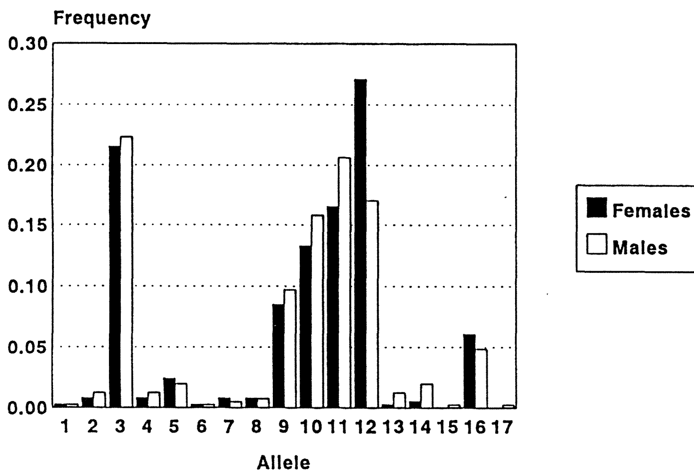

FIGURE 2: Distribution of DXYS17 alleles in the Belgian population classified to sex (188 females and 206 males). Alleles are ranked in descending order from the largest (lowest mobility) to the smallest (highest mobility).