DETECTION OF THREE DIFFERENT VNTR'S BY DNA-AMPLIFICATION

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Introduction

The Polymerase Chain Reaction (PCR) can enhance the detection of known VNTR loci, particularly in forensic situations where limited amounts of DNA are available. PCR amplification of several polymorphic VNTR regions has been described. Here, we describe the application of the DNA-amplification technique for the detection of three variable minisatellite loci: the VNTR loci D1S80 (D1S58) and D17S30 (D17S5) and the hypervariable region 3' of the apolipoprotein B gene. Polymorphic PCR-fragments were detected on ethidium-bromide stained polyacrylamide gels.
Table 1 summarizes the characteristics of the three different VNTR-loci involved in this study.

Table 1. Properties of the VNTR's in this study

VNTR	Chromosome Localization	Locus[b]	Repeat Length	% GC[a] content	Ref.
Apo B	2p24-p23		2x15[c]	4	[1,2]
MCT 118	1p	D1S80 (D1S58)	16	58	[3,4]
YNZ 22	17p	D17S30 (D17S5)	70	64	[5]

[a] : GC content of the VNTR repeat units
[b] : Human Gene Mapping Symbols
[c] : The Apo B alleles differ in length from its next neighbouring allele by 30 bp or two 15-bp repeat units.

The primer sequences for the three different VNTR loci were:

Apo B : 5'-primer: 5'-ATGGAAACGGAGAAATTATG-3' (20 mer).
3'-primer: 5'-CCTTCTCACTTGGCAAATAC-3' (20 mer). (Boerwinkle [1]).

pMCT118 : primer 1: 5'-GAAACTGGCCTCCAAACACTGCCCGCCG-3' (28 mer).
primer 2: 5'-GTCTTGTTGGAGATGCACGTGCCCCTTGC-3' (29mer).
(Kasai [4] & Budowle [3]).

pYNZ22 : primer 1: 5'-CGAAGAGTGAAGTGCACAGG-3' (20 mer)
primer 2: 5'-CACAGTCTTTATTCTTCAGCG-3' (21 mer). (Horn [5]).

MATERIALS AND METHODS

1. Isolation of total high molecular weight DNA from liquid blood samples

DNA for PCR analysis was isolated from 1 ml aliquots of blood by standard procedures. DNA concentrations were estimated by minigel electrophoresis in 1% agarose to which 1 µg/ml ethidium bromide was added. Concentration standards used were dilutions of cell-line K562 DNA (Promega).

2. Primer synthesis

Oligonucleotides were synthesized on the Gene Assembler plus (Pharmacia) and were purified by ethanol precipitation.

3. Size fractionation of DNA-amplification products in 6% polyacrylamidegels

PCR products were separated by electrophoresis on a Protean II xi Slab Cell (Biorad) using 6% polyacrylamide (29:1 Acrylamide/Bis mixture; Biorad), in 1x TAE buffer (40 mM Tris-acetate; 1 mM EDTA). Electrophoresis was performed at 200 V (35 mA) for 3-4 hours depending on the genetic system. The fragment sizes were determined by comigration of 500 ng of a 1kb-ladder (BRL) and a 123 bp ladder (BRL).
The amplification products were visualized directly after staining the gel in 1 µg/ml ethidium bromide for 0.5 hours.

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Edited by Ch. Rittner and P. M. Schneider

RESULTS

Amplification of target DNA by the Polymerase Chain Reaction (PCR)

Temperature cycling conditions and optimal reation parameters for each of the three systems are specified in table 2.

Table 2. Optimal reaction parameters and temperature cycling conditions for the three different VNTR-loci

Locus	pMCT118 (D1S80)	ApoB (3' ApoB)	pYNZ22 (D17S30)
[$MgCl_2$]	1.0-1.5 mM	1.0-1.5 mM	1.0-1.5 mM
glycerol	10%	10%	10%
Optimal DNA-input	0-100 ng	5-100 ng	10-200 ng
Minimal DNA-input	<5 ng	<5 ng	<1 ng
3'& 5' primer conc.	0.45 ng	0.30ng	0.30 ng
# cycles	26	26	34
initial denaturation	4' 94°C	4' 94°C	4' 94°C
denaturation	2' 94°C	2' 94°C	2' 94°C
annealing	2' 65°C	6' 58°C	30" 55°C
extension	4' 72°C		4' 72°C
final extension	10' 72°C	10' 72°C	10' 72°C

General Conditions:
1x PCR buffer: 50 mM KCl; 10 mM Tris pH 8.30; 0.2 mg/ml acetylated BSA; 1.0/1.5 mM $MgCl_2$ dNTP's: 200 µM; Taq Polymerase: 2 Units; Final Reaction-volume: 50 µl.

a. Locus D1S80 (pMCT 118)

Data from the PCR experiments demonstrate that the $MgCl_2$concentration is a most critical parameter. High concentrations of Mg^{2+}generate additional bands and/or reduced amounts of locus specific PCR product. A Mg^{2+}concentration of 1.0 - 1.5 mM was found as an optimum. Standard amounts of primer and Taq polymerase are sufficient for locus specific amplification. It was noted that in our PCR-system some individuals show faint extra bands of high molecular weight. The sensitivity of this system allows detection of alleles of the D1S80 locus without hybridization. However especially with the appearance of shady extra bands in forensic samples it may be desirable to use a DNA-probe to confirm whether or not the fragments are derived from the D1S80 locus. After electroblotting and hybridization we observed that the extra high molecular weight bands did not hybridize with the pMCT118 probe and it may be concluded that these sequences are unrelated to the D1S80 locus.
An example of the allelic variation of the D1S80 locus is given in figure 1. Each lane represents the genomic DNA from a different individual. All alleles could be separated. The resolution obtained could distinguish alleles differing only 16 bp (i.e. one repeat) in length. A suitable marker system was obtained by preparing a cocktail of alleles from different individuals. With this allelic ladder the unknown alleles can be designated directly without measuring relative migration distances. Experiments for a population screening are in progress.

b. Locus ApoB

It was found that amplification of the AopB locus is the most robust system of the three. The system produces easy-to-identify PCR-fragments in a broad Mg^{2+}concentration range. Comparison of annealing at 58°C and extension at 72°C or annealing and extension at 58°C showed that a better amplification was obtained at an annealing & extension temperature of 58°C. This phenomenon is probably due to the very low GC content of the ApoB sequence; which causes the template to denature before complete extension occurs at the standard extension temperature of 72°C. An example of the allelic variation of the apoB locus is given in figure 2. Each lane represents the genomic DNA from a different individual. All alleles could be separated. Experiments for a population screening are in progress.

c. Locus D17S30 (pYNZ22)

Data from the PCR experiments demonstrate that here also the $MgCl_2$concentration is a most critical parameter. The major problem with amplification of the D17S30 locus is that products with fewer repeat units (short alleles) are preferentially amplified over longer alleles. The reason for this phenomenon is that Taq polymerase is more efficient on shorter fragments of this locus. In forensic VNTR-typing this problem can induce ambiguity into the typing results. In order to amplify and directly visualize long D17S30 alleles it was necessary to increase the number of amplification cycles. Optimum amplification was at 34 cycles. We also found that the addition of 10% glycerol to the PCR reaction-mix had both an advantagous effect on the stability of the Taq polymerase during thermocycling and on the sharpness of the bands in the eventual polyacrylamide electophoresis. Because of these findings 10% glycerol was added in all three PCR systems. Decreasing the input of genomic DNA also seemed to increase the efficiency of amplification of the larger alleles. A succesful approach for the suppression of ladder bands in this system was the shortening of the annealing time. Optimum annealing time was found to be 0.5 minute. An example of the allelic variation of the D17S30 locus is given in figure 3.

DISCUSSION AND CONCLUSION

A rapid and relatively simple procedure for the detection of hypervariable regions in the human genome is presented. Making use of oligonucleotide primersets from DNA flanking the minisatelite regions and thermostable Taq Polymerase reproducible and faithful amplification of the loci D1S80 and ApoB has been demonstrated. The electrophoretic separation system used (17 cm vertical 6% PAA gels) appears to be sufficient for separation of most if not all alleles of the VNTR loci analysed. For the D17S5 locus, PCR conditions have to be optimized especially so for the amplification of the longer alleles. The PCR conditions for the three VNTR systems will have to be evaluated through testing in more different biological materials and in real case work. Typing these hypervariable regions by the PCR technique is extremely sensitive. Nanogram amouts of template DNA generated detectable signals in all three systems.

A problem with the VNTR loci analysed here is the inability to determine the exact identity of the alleles. Identification of the alleles would greatly simplify the construction of a population database and the exchange of results. The marker systems used in this study (the "1-Kb"- and the "123 bp" ladder) can give only an approximation of the size of the alleles after PCR analysis. An improved marker system was obtained in the VNTR locus D1S80 by preparing a cocktail of alleles from different individuals. A problem here is the availability of individuals with rare alleles.

The VNTR loci used for PCR analysis all have a relatively low level of heterozygosity in common when compared to the most optimal VNTR loci used in Southern blot hybridization analysis. Consequently, these PCR-based DNA-identification systems have a lower discriminating power than the Southern hybridization-based systems such as multi-locus DNA-fingerprinting or locus-specific DNA-profiling. As noted by Sullivan these three VNTR loci combined have a discriminating power of 0.9997. As a result the occurence of inconclusive PCR results in identity disputes will be relatively frequent when compared to traditional methods of DNA-profiling. It is therefore essential to have several more "PCRable" VNTR loci available in order to reach a high level of discrimination power. This, however poses no severe problem in view of the relative abundance of VNTR loci in the human genome. Several other PCRable VNTR systems have been described and they can be included in the set of loci to be typed in forensic material.

Literature

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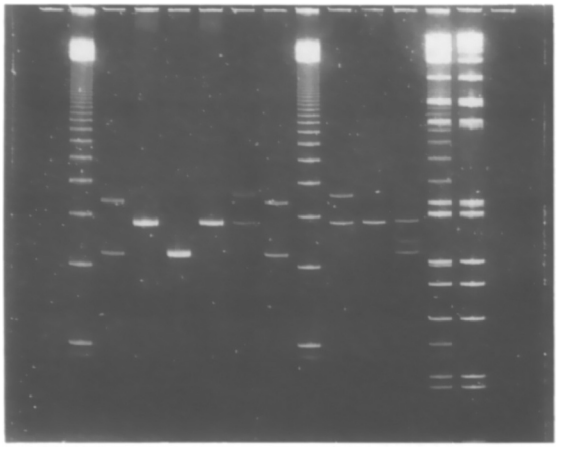

Fig. 1
Amplification of the D1S80 locus for 8 different individuals. The size standards are the 1-kb and the 123-bp ladder (BRL). An example of an allelic ladder is shown in lane 11

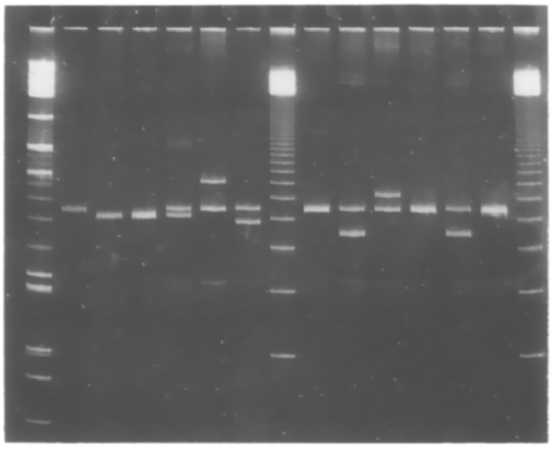

Fig. 2
Amplification of the ApoB locus for 12 different individuals. The size standards are the 1-kb and the 123-bp ladder

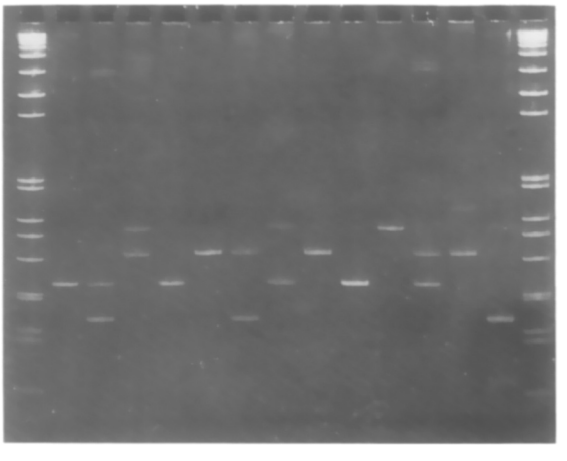

Fig. 3
Amplification of the D17S30 locus for 13 different individuals. The size standard is the 1-kb ladder