Multiplex PCR and Automated Fluorescence Detection of Four Tetrameric STRs in a Western Austrian Population

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Introduction

Short Tandem Repeats (STR) loci, also known as microsatellites, consist of repetitive sequences, generally 2 to 6 nucleotides in lenght. Because of their highly polymorphic nature, STR loci offer high discrimination amongst individuals which can be valuable in forensic matters. The fact that STRs are amplifiable by the Polymerase Chain Reaction (PCR) from small quantities of DNA (about 1 ng or less) and even substantially degraded DNA samples, makes the use of STRs desirable for genetic characterization in a number of forensic cases.
In the study, four STR loci (HUMTH01, HUMvWA31/A, HUMFES/FPS, HUMF13A1) were amplified and typed simultaneously to generate a database of a Western Austrian population.

Material and Methods

Blood was obtained from 382 unrelated individuals living in Western Austria. DNA was isolated from blood samples following digestion in Proteinase K (Boehringer Mannheim) and extraction by phenol/chloroform.
1–3 ng DNA were amplified by multiplex PCR in a total volume of 25 µl consisting of 1x PCR Buffer I, 1.7 mM $MgCl_2$, 250 µM each dNTP and 2 units AmpliTaq®–Polymerase (all Perkin Elmer). Oligonucleotide primer were synthesized commercially and labeled with fluorescent dye markers, either 6–FAM (6–carboxyfluorescein) or HEX (6–carboxy–2´,4´,7´,4, 7–Hexachlorofluorescein) coupled with an aminohexyl linker (Applied Biosystems, Weiterstadt, Germany).
PCR Amplification was carried out on a 9600 GeneAmp PCR Thermocycler (Perkin Elmer): 28 cycles at 94 °C for 45 sec, 54 °C for 45 sec and 72 °C for 90 sec, and a final incubation at 72 °C for 10 min. Prior to PCR all samples were incubated at 95°C for 5 min and hot–started with a deluted Taq–start mix.

Primer

TC11/f	(11p15–15.5)	5´–GTGATTCCCATTGGCCTGTTCCTC–3´
TC11/r		5´–6–FAM–GTGGGCTGAAAAGCTCCCGATTAT–3´
VWA/f	(12p12–pter)	5´–HEX–CCCTAGTGGATGATAAGAATAATCAGTATG–´3´
VWA/r		5´–GGACAGATGATAAATACATAGGATGGATGG–3´
FES/f	(15q25–qter)	5´–GGGATTTCCCTATGGATTGG–3´
FES/r		5´–6–FAM–GCGAAAGAATGAGACTACAT–3´
F13A1/f	(6p24–25)	5´–GAGGTTGCACTCCAGCCTTT–3´
F13A1/r		5´–HEX–ATGCCATGCAGATTAGAAA–3´

1 µl aliquots of the amplification products were combined with 1.2 fmol internal lane standard GeneScan–350 Tamra (Perkin Elmer), prepared by *PstI* digestion of plasmid DNA, subsequently digested by *BstU I* to yield DNA fragments containing a single TAMRA dye to ensure single peaks for unambigious standard calculation. Prior to loading on a denaturating polyacrylamid sequencing gel (6%T, 5%C; 8.3M urea; 1xTBE) the samples were denaturated at 90 °C for 2 min.

Gels were electrophoresed on an 373A Strech DNA Sequencer (ABI) for 5 hours at constant power (30 W), using filterwheel B. Analysis was performed with 672 GeneScan software (ABI) using the local southern method for fragment size estimation.

Statistical analysis

The frequency of each allele for each locus was calculated from the numbers of each genotype in the sample set. Unbiased estimates of expected heterozygosity were computed as described by Edwards, et al. (4). Possible divergence from Hardy–Weinberg expectations (HWE) was determined by calculating the unbiased estimate of the expected homozygote/heterozygote frequencies (3,9,10), the likelyhood ratio test (2,4,11), and the exact test (5).
The precision of band size estimation by the GeneScan 672 software for each allele at a distinct locus was calculated according to Kimpton et al (8). Polymorphic information content (PIC) was calculated using the formula of Botstein et al (1). An interclass correlation criterion (7) was used for detecting disequilibrium between loci.

Results and Discussion

We typed four tetrameric Strs in a multiplex reaction optimizing amplification parameters in order to yield a maximum signal–to–noise ratio. Firstly, we put a 2 min cooling ramp between denaturation and annealing step to get clear peaks with a weak background, which was rather time–consuming. This procedure also resulted in a preferential amplification of systems with higher Tm–values (i.e. HUMTH01, VWA). As we dropped the ramp, denaturation was exceeded up to 90 sec which led to a well–balanced result.
The distribution of observed allele frequencies for the four loci in a population sample of 382 unrelated individuals living in Western Austria are shown in Table 1.

Table 1: Allele frequencies (N=382).

Allele	HUMTH01	VWA	F13A1	FES
3.2			0.086	
4			0.045	
5			0.190	
6	0.229		0.285	
7	0.157		0.346	
8	0.122		0.003	0.004
9	0.152			
9.3	0.331			
10	0.009			0.298
11			0.005	0.450
12			0.003	0.200
13			0.003	0.046
14		0.102	0.005	0.001
15		0.124	0.020	
16		0.206	0.008	
17		0.246	0.003	
18		0.213		
19		0.090		
20		0.016		
21		0.003		
% precision[a]	99,93	99,88	99,92	99,94
SD	0,02	0,11	0,04	0,04
range[b]	0,41–0,85	0,62–0,98	0,01–1,1	0,06–0,99
PIC	0,74	0,79	0,71	0,61

a) across gels
b) fragment size estimated by the GeneScan 672 software for a given allele across gels

The genotype frequency distributions for HUMTH01, VWA and FES do not deviate from HWE based on the homozygosity test, likelyhood ratio test, and the exact test (Table 2). The F13A1 locus does depart from Hardy–Weinberg expectations.

The chi–square test for total homozygotes and heterozygotes does not detect departures from expectations for F13A1 (p=0,347). This is misleading and demonstrates the limited power of the particular test. The number of 5–5, 6–6, and 7–7 observed F13A1 homozygotes in our sample population was 22, 19, and 43, respectively. However, the expected number of homozygotes is 13.8, 31, and 45.7, respectively. Obviously, only the 7–7 observed and expected number of homozygotes are similar. The 5–5 and 6–6 homozygote situation tends to cancel one another.

The more powerful tests, such as the exact test, readily detect the departure (p=0,002). The apparent departure should not raise substantial concern for the use of the F13A1 locus and the product rule in human identity testing. The counting method (i.e., the frequency of an observed genotype) could be used for estimating an F13A1 type.

However, one should not confuse statistical significance with practical or *forensic significance*. When comparing the frequency of the F13A1 alleles in Western Austrians with other studies, such as that described by Hammond, et al. (6) for United States Caucasians, there is very little difference. For example, the frequencies of F13A1 alleles 3.2, 4, 5, 6, and 7 in West Austrians is 0.086, 0.045, 0.190, 0.285, and 0.346, respectively, and in United States Caucasians the frequencies are 0.083, 0.020, 0.192, 0.345, and 0.325, respectively. Under the assumption of independence using either database would yield similar estimates of the frequency of F13A1 types. The data suggest that it might be considered more appropriate to assume independence at the F13A1 locus in the Western Austrian sample population to obtain a valid estimate of the rarity of F13A1 types.

An analysis was performed to determine whether or not there were any detectable associations between any pair–wise comparisons of the four STR loci. An inter–class correlation test analysis demonstrated that there were no departures from expectation (Table 3).

Table 2:Tests for independence on STR loci.

	HUMTH01	VWA	F13A1	FES
Obs. Homozygosity	23.3%	16.8%	22.5%	34.6%
Exp. Homozygosity[a]	22.4%	18.2%	24.6%	33.3%
Homozygosity Test[b]	0.665	0.478	0.347	0.607
LikelyhoodRatio Test[b]	0.268	0.972	0.003	0.153
Exact Test[b]	0.459	0.956	0.002	0.218

Table 3: Test for independence between loci

Loci	Probability
HUMTH01/VWA	0.585
HUMTH01/F13A1	0.476
HUMTH01/FES	0.677
VWA/F13A1	0.974
VWA/FES	0.262
F13A1/FES	0.867

a) Expected homozygosity is an unbiased estimate.
b) These values are probability values.

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