TECHNICAL NOTE

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Austrian Caucasian Population Data for the Quadruplex Plus Amelogenin: Refined Mutation Rate for HumvWFA31/A

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ABSTRACT: Human identification of biological specimens has undergone immense change since the development of PCR typing systems for forensic casework. In contrast to RFLP and VNTRs, STRs are the method of choice when the investigated genomic DNA is present in low quantity or in degraded shape. In the current study, the X-Y homologous gene Amelogenin has been added to a widely used multiplex PCR amplification system consisting of four tetrameric STR loci (Quadruplex—HumTH01, HumvWFA31/A, HumFES/FPS, and HumF13A1). The modified Quadruplex was used to type 382 unrelated Caucasians from Western Austria. The population data meet Hardy-Weinberg and linkage equilibrium expectations, and do not show significant deviations from either US, German, and Turkish Caucasian databases. In an investigation of 382 meioses, two mutations were revealed at the HumvWFA31/A locus. Consequently, the data in this paper provide the conditions for adding Amelogenin to the Quadruplex, and suggest that when doing paternity testing, the mutation rate for the HumvWFA31/A locus must be considered.

KEYWORDS: forensic science, HumTH01, HumvWFA31/A, HumFES/FPS, HumF13A1, Multiplex PCR, Amelogenin, population statistics, mutation rates

Short Tandem Repeat (STR) loci, also known as microsatellites, consist of repetitive sequences, generally 2 to 6 nucleotides in length. Because of their highly polymorphic nature, STR loci offer high discrimination among individuals. In addition, STRs are amplifiable by the Polymerase Chain Reaction (PCR) from small quantities of DNA (1 ng or less) and even substantially degraded DNA samples, which make the use of STRs desirable for genetic characterization in forensic cases. In this study, Amelogenin was added to a well described Quadruplex amplification system (HumTH01, HumvWFA31/A, HumFES/FPS, and HumF13A1, (4)). The PCR protocol was optimized and the new PCR conditions are presented. 191 randomly chosen trios, whose paternity had been tested previously by conventional and RFLP analysis, were investigated using the multiplex amplification system. Thus, population data of 382 unrelated individuals living in Western Austria are presented as well as 382 meiotic events between each father-child and mother-child pair.

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Material and Methods

Blood was obtained from 191 families (573 individuals) living in Western Austria. DNA was isolated from blood samples by digestion with Proteinase K (Boehringer Mannheim) and subsequent extraction with phenol/chloroform (1). The extracted DNA was quantified using the slot blot procedure as described by Waye et al. (2). One to five ng of 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, Norwalk, CT). Oligonucleotide primers were obtained 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). The molar concentrations of the primers are given in Table 1.

Prior to PCR, all reaction mixtures except the polymerase were incubated at 95°C for 5 min and subjected to a hot-start with a diluted Taq-start mix (2.6 μL bidistilled water + 0.4 μL AmpliTaq-Polymerase per sample). PCR amplification was carried out in a 9600 GeneAmp PCR Thermocycler (Perkin Elmer) for 28 cycles comprising 94°C for 45 s, 54°C for 45 s, and 72°C for 90 s, and a final incubation at 72°C for 10 min.

Additionally, DNA extracts of 102 unrelated individuals living in Western Austria were typed in HumF13A1 as singleplex reaction in a total PCR volume of 25 μL, consisting of 1X PCR buffer I, 1.5 mM $MgCl_2$, 250 μM each dNTP, 0.25 μM of each primer, and 1.25 Units AmpliTaq-Polymerase (all Perkin Elmer, Norwalk, CT) for 28 cycles comprising 94°C for 30 s, 60°C for 45 s, and 72°C for 60 s, and a final incubation at 72°C for 10 min.

To check amplification, 4-μL aliquots of the PCR products were loaded on an 8% non-denaturing polyacrylamide gel, subjected to electrophoresis (1X TBE, pH 8.3) for 30 min at constant power (5 W) and visualized by silver staining (5). The intensities of the bands were compared to the intensities of the bands of 0.13 μg of the 123 bp DNA ladder (Gibco BRL, Life Technologies, Gaithersburg, MD) to estimate the amount of amplification product to be loaded on a denaturing polyacrylamide sequencing gel (6%T, 5%C; 8.3 M urea; 1X TBE, pH 8.3). 0.2 μL up to 2 μL of the

TABLE 1—*Primer sequences and molar concentrations of the primers in the multiplex PCR assay: Amelogenin (3), HumTH01, HumvWFA/31A, HumFES/FPS, and HumF13A1 (4).*

Primer and Size Range	Chromosomal Location	Primer Sequence	Molar Concentration
HumAMEL/f 106 bp	Xp22.1–p22.3	5′-6-FAM-CCCTGGGCTCTGTAAAGAATAGTG-3′	0.16 μM
HumAMEL/r 112 bp	Yp11.2	5′-ATCAGAGCTTAAACTGGGAAGCTG-3′	0.16 μM
HumTH01/f 154–174 bp	11p15–15.5	5′-GTGATTCCCATTGGCCTGTTCCTC-3′	0.16 μM
HumTH01/r		5′-6-FAM-GTGGGCTGAAAAGCTCCCGATTAT-3′	0.16 μM
HumvWFA/f 127–167 bp	12p12-pter	5′-HEX-CCCTAGTGGATGATAAGAATAATCAGTATG-3′	0.4 μM
HumvWFA/r		5′-GGACAGATGATAAATACATAGGATGGATGG-3′	0.4 μM
HumFES/FPS/f 206–234 bp	15q25-qter	5′-GGGATTTCCCTATGGATTGG-3′	0.4 μM
HumFES/FPS/r		5′-6-FAM-GCGAAAGAATGAGACTACAT-3′	0.4 μM
HumF13A1/f 180–247 bp	(6p24–25)	5′-GAGGTTGCACTCCAGCCTTT-3′	0.52 μM
HumF13A1/r		5′-HEX-ATGCCATGCAGATTAGAAA-3′	0.52 μM

undiluted PCR product were combined with 3.5 μL deionized formamide and 1.2 fmol internal lane standard GeneScan-350 Tamra (Perkin Elmer, Norwalk, CT). The samples were denatured at 90°C for 2 min and snap cooled on ice prior to loading on the 373A Stretch DNA Sequencer and electrophoresed for 7 h at constant power (30 W), using filterwheel B. Analysis was performed with 672 GeneScan software (PE, ABD) using the Local Southern method for fragment size estimation.

Cloning Reactions

Five ng of DNA were amplified using the same procedure as described for the HumF13A1 singleplex reactions, except for applying the HumvWFA31/A primers in a concentration of 0.5 μM including a final 30 min extension step at 72°C for non-template dATP addition. PCR products were run on a 0.8% agarose gel (40 mM Tris-acetate, pH 8.5; 1 mM EDTA, (1)) including 5-μL ethidium bromide (10 mg/mL) for 1 h at 90 V. Bands were visualized with UV light prior to excising them from the gel using autoclaved pipette tips, cut at the tip. DNA was extracted from the gel using GenElute Agarose Spin Columns (Supelco, Bellefonte, PA). Ligation and cloning were performed with the TA Cloning Kit (Invitrogen, San Diego, CA) following the manufacturers instructions. DNA from the positive clones was extracted using the Quantum Prep Plasmid Miniprep Kit (Biorad, Hercules, CA).

Sequencing Reactions

Sequencing reactions were performed on a 9600 GeneAmp PCR System using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (AmpliTaq DNA Polymerase FS, Perkin Elmer) and about 500 ng of the plasmid DNA as template in a total assay of 20 μL: 96°C for 15 s, 50°C for 15 s, and 60°C for 4 min for 25 cycles. The insert was sequenced from both directions using the universal primers in a concentration of 3.2 μM: −21M13 (5′-TGT AAA ACG ACG GCC AGT-3′) and M13Rev (5′-CAG GAA ACA GCT ATG ACC-3′).

The sequencing products were extracted in $^1/_{10}$ volume 3 M sodium acetate (pH 4.6) and 2.5 volumes 96% ethanol followed by a washing step with 70% ethanol. DNA was solubilized in 4-μL deionized formamide and 1-μL 25 mM EDTA (pH 8.0), denatured at 95°C for 2 min, snap cooled on ice, and loaded on a denaturing polyacrylamide sequencing gel (T = 5%, C = 3%, 8 M urea, 1X TBE, 34 cm Well-to-Read). Electrophoresis was carried out on a 373A Stretch Sequencer at constant power (26 W) for 10 h. Analysis was performed using ABI Prism Sequencing Analysis Software (Version 2.1), sequences were aligned with Sequence Navigator Software (Version 1.0.1, both Perkin Elmer).

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. (6). Possible divergence from Hardy-Weinberg Expectations (HWE) was determined by calculating the unbiased estimate of the expected homozygote/heterozygote frequencies (7–9), and the exact test (10). An interclass correlation criterion (11) was used for detecting disequilibrium between loci.

Results

Optimization of the Multiplex Reaction

Amplification of the X-Y homologous gene Amelogenin was combined with a well investigated Quadruplex PCR system (4). Amplification parameters were optimized in order to yield a maximum signal-to-noise ratio. First, we placed a 2 min cooling ramp between the denaturation and annealing steps to obtain clear peaks with low background signal, which led to a rather time-consuming PCR protocol. This step also resulted in a preferential amplification of systems with higher T_m-values. Hot-start PCR, quick cooling from denaturation to annealing temperatures, and extension of elongation time up to 90 s led to a more balanced result. Amplification products were separated on non-denaturing polyacrylamide gels and visualized by silver staining. The intensity of the amplicons was compared with the 123 base pair DNA ladder (Life Technologies, Gaithersburg, MD) to estimate the amount of amplification product to be loaded on the 373A Sequencer.

STR Polymorphism

The distribution of observed allele frequencies for the four loci in a population sample of 382 unrelated Caucasian individuals living in Western Austria is shown in Table 2. Additionally, 102 unrelated Western-Austrians (for a total of 484 individuals) were typed at the HumF13A1 locus. The genotype frequency distributions for all four STRs do not deviate from HWE based on the homozygosity test and the exact test (Table 3). An analysis was performed to determine whether or not there were any detectable associations between any pair-wise comparisons of the four STR loci. Interclass correlation test analysis demonstrated that there were no significant departures from expectation (Table 4).

TABLE 2—*Allele frequencies for 382 (or 484) unrelated individuals living in Western Austria. The allele designation is according to the number of repeats.*

Allele	HUMTH01 $N = 382$	HUMvWA31/A $N = 382$	HUMF13A1 $N = 484$	HUMFES/FPS $N = 382$
3.2	—	—	0.089	—
4	—	—	0.043	—
5	—	—	0.177	—
6	0.229	—	0.308	—
7	0.157	—	0.341	—
8	0.122	—	0.004	0.004
9	0.152	—	—	—
9.3	0.331	—	—	—
10	0.009	—	—	0.298
11	—	—	0.005	0.450
12	—	—	0.002	0.200
13	—	—	—	0.046
14	—	0.102	0.004	0.001
15	—	0.124	0.019	—
16	—	0.206	0.006	—
17	—	0.246	0.002	—
18	—	0.213	—	—
19	—	0.090	—	—
20	—	0.016	—	—
21	—	0.003	—	—

TABLE 5—*HumvWFA31/A-Mutations in two families.*

Families	HUMvWFA31/A Alleles
Case 1	
Putative Father	18/18
Mother	14/18
Child	18/19
Case 2	
Putative Father	16/20
Mother	18/19
Child	18/19

TABLE 6—*Nucleotide sequences of the observed HumvWFA31/A alleles 18 and 19 in Case 1 and 2.*

HUMvWFA31/A Alleles	Sequence Structure
Allele 18	5′-flanking region-TCTA $(TCTG)_4$ $(TCTA)_{13}$-flanking region-3′
Allele 19	5′-flanking region-TCTA $(TCTG)_4$ $(TCTA)_{14}$-flanking region-3′

TABLE 3—*Tests for independence on STR loci.*

	HUMTH01 $N = 382$	HUMvWA31/A $N = 382$	HUMF13A1 $N = 484$‡	HUMFES/FPS $N = 382$
Obs. Homozygosity	0.233	0.168	0.231	0.346
Exp Homozygosity*	0.224	0.182	0.252	0.333
Homozygosity Test†	0.665	0.478	0.305	0.607
Exact Test†	0.451	0.945	0.101	0.218

*Expected homozygosity is an unbiased estimate.
†These values are probability values.
‡102 additional unrelated individuals were typed in a singleplex reaction for HumF13A1. The 382 HumF13A1 genotypes amplified in the multiplex reaction also meet HWE (data not shown).

TABLE 4—*Interclass correlation test.*

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

Family Analyses

In 382 meioses, two mutations affecting fragment length occurred in HumvWFA31/A (Table 5).

In the first case, allele 19 of the child is, under the assumption of a new mutation, due to an insertion of one repeat unit. In the second case mother and child possess the same alleles, 18 and 19.

These data were confirmed by sequencing the alleles which were previously cloned. Each inserted fragment was sequenced from both directions, using universal primers (−21M13 and M13Rev), the consensus sequences of the inserts are reported in Table 6. The sequences of all # 18 alleles in the two cases showed no difference, as well as alleles # 19, except of having one more TCTA-repeat.

Discussion

Four STRs have been typed simultaneously in combination with the X-Y homologous gene Amelogenin in an Austrian Caucasian population sample consisting of 191 trios, whose paternity had been confirmed previously by conventional and RFLP analysis. This multiplex PCR protocol has been used in the laboratory for two years and has produced reliable results from template concentrations with as little as 100 pg of DNA. The HumvWFA31/A locus is the least sensitive marker in the multiplex. Thus, when amplifying 50 pg template DNA or less, HumvWFA31/A peaks are too low to be called automatically by the GeneScan Analysis software (using a minimum peak height value of 50), whereas the Amelogenin and HumTH01 loci still produced good results.

The addition of the Amelogenin locus to the multiplex reaction can offer the possibility to assign gender and to detect mixtures of male and female samples, even when the male portion is small. In addition, the power of discrimination of the multiplex increases by a factor of two, when Amelogenin is included. Further investigations on the ratio of the components and the amenability to amplify both components are under investigation in our laboratory.

The distributions of alleles of all four STRs in the Austrian population sample meet Hardy-Weinberg Expectations and are similar to other reported British Caucasian, US Caucasian, and

German Caucasian data (4,6,13,14). The presented population sample is mainly composed by white Caucasians living in Western-Austria. However, a minor component are descendants of Turkish foreign workers. Alper et al. (15,16) investigated the distributions of the HumFES/FPS, HumTH01, and HumvWFA31/A loci in Turkish Caucasians. The authors concluded that neither of the Turkish sample populations showed significant differences in their allele frequencies compared with a German sample population. In the present study, there was no way to determine which samples were of Turkish origin and which samples were Austrian, because they were randomly chosen from paternity cases. Regardless, the aim of this study was to investigate whether there is substantial evidence for substructuring due to the presence of individuals in the database of Turkish descent, or not. The data suggest that these populations are similar to other Caucasian populations.

The investigation of 382 meioses did not detect any mutations concerning variation in fragment length, except at the HumvWFA31/A locus, where two independent mutations were observed.

In the first case, the child received alleles 18 and 19. Allele 19 of the child might be due to an insertion of one repeat relative to allele 18. Since both parents possess allele 18, and since there are no nucleotide sequence differences between these alleles, it was not possible to determine which allele mutated.

In the second case, the child also carries alleles 18 and 19. The mother possesses the same alleles. Without further investigations it can be suggested that allele 18 was inherited from the mother and that allele 19 mutated from allele 20 from the father. However, in both cases the mutations occurred in alleles with a high number of repeats, which was also reported by Brinkmann et al. (17). These authors found one HumvWFA31/A mutation in 453 meioses, which equals 0.2%. In our study two mutations in 382 meiosis were observed, which is a rate of 0.5%. Thus, the refined mutation rate would be three in 853 meiosis, or approximately 0.36%. Although this mutation rate is higher than it is in the other markers in this multiplex, a rate of 0.4% will not hinder the use of HumvWFA31/A in paternity testing.

In conclusion, the multiplex method described here enables amplification and typing of four STR loci in combination with the X-Y homologous gene Amelogenin. This system produced reliable results in casework and paternity cases. The population data meet Hardy-Weinberg and linkage equilibrium expectations and are similar to other reported Caucasian data. Two independent mutations were observed in the HumvWFA31/A locus, providing a refined mutation rate for this marker, which can be used in assessing paternity data.

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