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HUMTH01: Amplification, species specificity, population genetics and forensic applications

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Abstract Length variation at the short tandem repeat (STR) locus HUMTH01 can be reliably detected from small amounts of DNA (0.01–10 ng) extracted from a range of forensic human samples, using the polymerase chain reaction (PCR), horizontal polyacrylamide gels and silver staining. It was shown that the oligonucliotide primers used are specific to humans and some higher primates. Population data bases of Caucasians and Asians living in Victoria (Australia) were constructed and the differences in allele frequencies between Caucasians and Asians confirmed. A new allele provisionally designated HUMTH01*12 was identified. The discrimination power provided by this locus (0.86–0.91) has been used effectively in a range of forensic case studies.

Key words HUMTH01 · Short Tandem Repeat (STR) Population Genetics · Forensic DNA Typing

Zusammenfassung Längenvariabilität am Short Tandem Repeat (STR) Lokus HUMTH01 läßt sich anhand geringer DNA-Mengen (0.01–10 ng), aus einer Reihe forensischer Proben menschlichen Ursprungs extrahiert, mittels Polymerase-Kettenreaktion (PCR), horizontaler Polyacrylamidgele und Silberfärbung verläßlich detektieren. Gezeigt wurde, daß die benutzten Oligonukleotidprimer, mit Ausnahme einiger höherer Primaten, humanspezifisch sind. Es wurden Populationsdatenbanken von Kaukasiern und Asiaten aus Victoria (Australien) erstellt und die Unterschiede in den Allelfrequenzen zwischen Kaukasiern und Asiaten bestätigt. Ein neues, vorläufig als HUMTH01*12 bezeichnetes Allel wurde identifiziert. Das Diskriminationsvermögen, welches dieser Lokus bietet (0.86–0.91), ist in einer Reihe forensischer Fallstudien effectiv eingesetzt worden.

Schlüsselwörter HUMTH01 · Short Tandem Repeat (STR) · Populationsgenetik · Forensische DNA-Typisierung

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Introduction

The amplified fragment length polymorphism (AmpFLP) technique to analyse highly polymorphic variable number tandem repeat (VNTR) regions has been well utilized in recent years for human identification. The loci most commonly used by forensic scientists are D1S80 on chromosome 1 (Kasai et al. 1990; Budowle et al. 1991; Sajantila et al. 1992; Kloosterman et al. 1993), D17S30 on chromosome 17 (Horn et al. 1989; Rand et al. 1992), the hypervariable region 3′ of ApoB on chromosome 2 (Boerwinkle et al. 1989; Ludwig et al. 1989; Rand et al. 1992) and COL2A1 (Rand et al. 1992). These AmpFLPs contain repeats of core units ranging in size from 16 bp (D1S80) to 70 bp (D17S30) and alleles with fragment sizes in excess of 800 bp.

As short tandem repeats (STRs), in comparison to the abovementioned AmpFLPs, are more likely to provide a result with severely degraded DNA, exhibit fewer variants and do not have the problem of unequal amplification among alleles (i.e. drop out of large alleles), it would be useful to identify STRs for use as tools in forensic DNA typing. Edwards et al. (1991), from their analyses of the value of STRs as genetic markers for human identification, hypothesise that there are approximately 400 million trimeric and tetrameric STR loci interspersed throughout the genome of which a high proportion are polymorphic. Approximately half of the 18 STR loci studied by Edwards et al. (1991) were polymorphic.

HUMTH01, a tetranucleotide repeat located on chromosome 11, has been identified as a useful STR marker (Edwards et al. 1991). The amplification product ranges in size from 179 to 203 bp representing a total of 8 different alleles, 7 of which represent different numbers of repeat units ranging from five to eleven. The remaining allele is one bp smaller than the HUMTH01*10 allele (Puers et al. 1993).

In this paper we examine some of the parameters in the amplification of HUMTH01 using the high resolution horizontal polyacrylamide gel electrophoresis (PAGE)

technique followed by silver staining as our method of detecting the amplified product (adapted from Budowle et al. 1991). Such parameters include the minimum amount of DNA template required, the effect of excess DNA template and the ability to amplify DNA from different sources, somatic stability, and species specificity. A new HUMTH01 allele is identified. Two population databases (Caucasians and East and South East Asians from the Australian state of Victoria) are analysed and compared to each other as well as with other published population genetic studies. Finally we demonstrate the usefulness of HUMTH01 in forensic case work.

Materials and methods

DNA samples

The 240 DNA samples used for the population studies were extracted from blood. To assess the typeability of a range of different body samples, a minimum of 5 of each of the following samples were tested: blood stains, seminal stains, vaginal swabs, buccal swabs, hair and semen/vaginal swabs. These included combinations of DNA samples from a total of 7 individuals extracted from blood (5 ×), semen (3 ×), vaginal swab (3 ×), buccal swab (4 ×) and hair (5 ×) for somatic stability studies. Additional samples from various sources were typed as part of case work studies. Non-human DNA samples were all extracted from blood or whole organisms. DNA was extracted by standard proteinase K/SDS digestion followed by phenol/chloroform extraction and either extensive dialysis or ultrafiltration washing (Kanter et al. 1986; Hochmeister et al. 1991a). Quantification of DNA samples was done using 0.8% agarose gels, ethidium bromide staining and comparison to known standards of bacteriophage lambda DNA.

Victorian Caucasian samples were mainly obtained from persons involved in criminal proceedings plus some from the Victorian Red Cross and laboratory volunteers (Gutowski et al. 1994). Victorian Asian samples were mainly from road accident victims selected by name analyses (Gutowski et al. 1994) together with some samples from persons involved in criminal proceedings and laboratory volunteers.

DNA amplification

The primer sequences used were those described by Edwards et al. (1991). The HLPC purified oligonucleotide primers were obtained from Bresatec (Australia). Each 50 µl reaction mixture contained 5 µl 10 × PCR buffer (GeneAmp, Perkin Elmer-Cetus, containing 500 m*M* KCl, 100 m*M* Tris-HCl pH 8.3, 15 m*M* $MgCl_2$ and 0.01% [w/v] gelatin), 4 µl 2.5 m*M* dNTPs, 0 or 5 µl 1.6 µg/µl bovine serum albumin (BSA), 1 µl of 100 ng/µl 3′ primer, 1 µl of 100 ng/µl 5′ primer, 0.4 µl (2 units) AmpliTaq DNA polymerase (Perkin Elmer-Cetus), 1–10 ng genomic DNA, sterile H_2O to a final volume of 50 µl. One drop of mineral oil was added to each reaction prior to amplification. An alternative 10 × PCR buffer, not containing $MgCl_2$ or gelatin (GeneAmp 10 × PCR Buffer II, Perkin Elmer-Cetus) was used in experiments to determine the effect of $MgCl_2$ in the amplification reaction. Volumes ranging from 0 to 10 µl 25 m*M* $MgCl_2$ were added to the amplification reaction mix when using the alternative 10 × PCR buffer. Amplification was carried out over 30 cycles on a Corbett Research FTS-320 or FTS-1 thermal cycler using a programme consisting of denaturation at 94° C for 45 s, annealing at 60° C for 30 s and extension at 72° C for 30 s.

Gel electrophoresis of amplified DNA

Amplified DNA fragments were separated and visualized by polyacrylamide gel electrophoresis and silver staining essentially as described by Budowle et al. (1991). The gels were 7% T, 5% C (Bisacrylamide) containing 60 m*M* Tris-formate buffer (pH 9.0) of the size 23 cm × 11 cm × 0.4 mm. The trailing ion, 0.28 *M* Tris-borate (pH 9.0) with 25 mg/l Bromophenol blue for visualisation, was contained in stacks of 10 strips of Whatman III blotting paper of the size 11.6 cm × 1.5 cm. The standard volume of amplified product added to the sample applicator wicks was 6 µl. The distance from the leading edge of the sample applicator wick to the anodal stack of Whatman III strips was approximately 19.5 cm. The electrophoretic settings were 650 V and 28 mA. The gels were run at a constant temperature of 15–19° C until the Bromophenol blue dye front reached the anodal stack of Whatman III strips (usually after 135–155 min).

Genotyping

Allele nomenclature is according to the number of repeat units as described by Edwards et al. (1992) and Puers et al. (1993). A new allele which is referred to by Puers et al. (1993) as HUMTH01*10-1 is referred to here as HUMTH01*9.3 as decided at the 1994 Venice meeting of the International Society for Forensic Haemogenetics (B. Budowle pers. comm.). Allele determination was by fragment size and frequency determination. Genotype assignment was done by side-by-side comparison with an allelic ladder. Phenotypes, including apparent homozygotes, were assumed to be the genotype. Questionable typings were repeated in appropriate mixtures. Allele ladders were constructed by mixing, amplified samples of validated genotype in appropriate ratios.

Statistical analyses

A Chi-square test comparing the observed and the expected genotype frequencies were performed for each population to determine if they were in Hardy-Weinberg equilibrium (genotypes were not grouped for the chi-square test). The G-test (Sokal and Rohlf (1969) was used to examine homogeneity between populations. All tests were carried out using a computer program obtained from G. Morgan (22 Eromba Cres., Ferny Hills, Queensland, Australia 4055). The frequency of heterozygotes and the power of discrimination within each population was provided by the same computer program.

Results

Amplification conditions and sensitivity

Using the conditions described on serial dilutions of quantified genomic DNA, alleles were amplified from as low as 0.05 ng genomic DNA. Amplification of 0.01 ng genomic DNA was achieved with an additional 5 cycles. Additional fragments in the higher molecular weight region of the gel (outside of the HUMTH01 allele region) were observed in some amplifications of 5–10 ng and in most amplifications of more than 20 ng template DNA. These fragments were always weaker than the HUMTH01 allelic bands. Additionally very faint bands of 1, and/or 2, repeat units smaller than the actual allele were occasionally observed when excessive amounts of DNA template (> 10 ng) were used.

Experiments to determine the effect of different concentrations of $MgCl_2$ (0–5 m*M*), dNTPs (50–200 µ*M* of

each dNTP), primers (100–400 ng each) and BSA (0–8 ng) while the rest of the conditions remained as described, demonstrated that the differing concentrations of primers, BSA and dNTPs (at a $MgCl_2$ concentration of 1.5 m*M*) had no obvious effects but that the $MgCl_2$ concentration (at a concentration of 200 μ*M* of each dNTP) was critical. No amplification was observed with 0.5 m*M* or less $MgCl_2$ while 1.5–2.5 m*M* produced the best amplification results when using 2–10 ng genomic DNA.

Investigation of samples from laboratory personnel including whole blood, blood stains, buccal swabs, hair, seminal stains, vaginal swabs and mixed semen/vaginal swabs did not pose a problem. As expected in those instances where the DNA was extracted from different body samples (blood, semen, vaginal epithelial cells, buccal cells, hair) of the same individual (different combinations from 7 individuals), the same genotyping result was obtained from each sample indicating somatic stability.

Population Genetics

Figure 1 displays some of the observed phenotypes for HUMTH01 and the allele ladders used for typing. Each amplified DNA sample was run alongside an allelic ladder for direct comparison and determination of phenotype. Using our horizontal non-denaturing PAGE methods we were able to distinguish a new allele which on the basis of its size has 12 repeat units. This new allele, provisionally designated HUMTH01*12, was found in an individual of Asian ethnicity with the phenotype HUMTH01*9/*12. Both alleles were of equal intensity. This sample was later reamplified (using the primer set used by Gill et al. 1994) and typed using the "Genescan" (Applied Biosystems 373A DNA Sequencer with Genescan 672 software) which sized the amplified products as 171 bp (HUMTH01*9) and 183 bp (12 bp, 3 repeat units, larger than *9) thus further supporting the designation of HUMTH01*12. Further confirmation could be obtained by sequencing. Our detection methods were also capable of distinguishing the recently identified HUMTH01*9.3 allele (Puers et al. 1993) from alleles HUMTH01*9 and *10. As the alleles *9.3 and *10 are close together, mixtures of known and uncertain phenotypes containing either or both of these alleles were frequently run for confirmation.

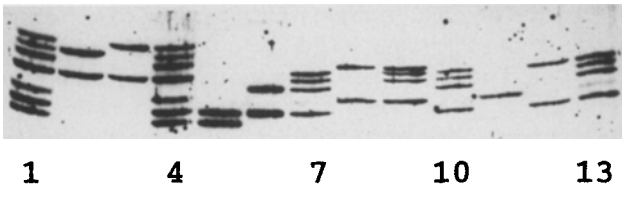

Fig. 1 Some HUMTH01 phenotypes and allele ladders. Lanes 1 and 4: allele ladder exhibiting alleles 5, 6, 7, 9, 10, 11, 12; Lanes 7 and 10: allele ladder exhibiting alleles 6, 8, 9, 9.3; Lane 13: allele ladder exhibiting alleles 6, 7 (weak), 8, 9, 9.3; Lane 2: 9/11; lane 3: 9/12; lane 5: 5/6; lane 6: 6/8; lane 8: 7/10; lane 9: mix of 7/10 and 9/9.3; lane 11: 7/7; lane 12: 6/9.3

Table 1 Frequencies of HUMTH01 alleles in the Victorian Caucasian and Asian populations

Allele	Observed frequency (%)	
	Victorian Caucasians	Victorian Asians
5	0.3	0
6	21.5	14.0
7	17.2	24.7
8	9.6	4.5
9	18.2	48.3
9.3	31.8	2.2
10	1.3	5.1
11	0	0.6
12	0	0.6
n[a]	151	89

[a] Number of individuals typed

Table 2 Frequency of HUMTH01 genotypes in the Victorian Caucasian and Asian populations

Genotype	Observed frequency (%)	
	Victorian Caucasians	Victorian Asiens
5-6	0.7	0
6-6	5.3	0
6-7	6.6	10.1
6-8	3.3	1.1
6-9	9.9	14.6
6-9.3	11.3	2.2
6-10	0.7	0
7-7	2.0	5.6
7-8	2.6	3.4
7-9	4.6	21.3
7-9.3	15.9	1.1
7-10	0.7	2.2
8-8	1.3	0
8-9	6.6	4.5
8-9.3	4.0	0
9-9	2.0	22.5
9-9.3	10.6	1.1
9-10	0.7	7.9
9-11	0	1.1
9-12	0	1.1
9.3-9.3	10.6	0
9.3-10	0.7	0
n[a]	151	89

[a] Number of individuals typed

A total of 9 alleles were identified in the 240 samples examined (151 from Victorian Caucasians and 89 from Victorian Asians); HUMTH01*5, *6, *7, *8, *9, *9.3, *10, *11 and *12. Table 1 displays the distribution of HUMTH01 allele frequencies in the Victorian Caucasian and Asian populations. Alleles HUMTH01*5, *11 and *12 were rare in the populations they were observed in (allele HUMTH01*5 in the Victorian Caucasian population and alleles HUMTH01*11 and *12 in the Victorian

Asian population). The most common allele in the Caucasian and Asian populations was HUMTH01*9.3 and *9 respectively.

Table 2 displays the distribution of HUMTH01 genotypes in the 2 populations studied. Both populations appear to be in Hardy-Weinberg Equilibrium as determined from observed and expected number of genotypes, Caucasians: $X^2 = 17.67$, df = 21 $0.5 < P < 0.7$, Asians: $X^2 = 14.37$, df = 28 $0.975 < P < 0.99$. Out of a possible 45 genotypes 22 were observed in this study. The frequency of heterozygotes was found to be 0.78 and 0.68 in Victorian Caucasians and Asians respectively. The most common genotype was HUMTH01*7/*9.3 in Victorian Caucasians at a frequency of 0.159 and HUMTH01*9/*9 in Victorian Asians at a frequency of 0.225. This translates to any phenotype not being observed in more than 1 in 6.3 and 4.4 randomly chosen Victorian Caucasian and Asian individuals respectively. The power of discrimination of HUMTH01 was calculated to be 0.91 and 0.86 in the Victorian Caucasians and Asian populations respectively.

A comparison of allele frequencies between the Victorian Caucasian and Asian populations showed that there is a significant difference between these 2 populations ($P < 0.005$) mainly due to significant differences in allele frequency for alleles HUMTH01*9 ($P < 0.005$) and HUMTH01*9.3 ($P < 0.005$). Similar pairwise analyses did not reveal significant differences between the Caucasian populations from Victoria and the United States (Puers et al. 1993) ($0.8 < P < 0.9$) or between the Asian populations from Victoria and the United States (Puers et al. 1993) ($0.25 < P < 0.30$).

Species specificity

The species origin of forensic samples cannot be assumed. Samples of animal or mixed origin do occur and it is therefore of importance to know if there is any cross species amplification by the HUMTH01 primers. We therefore attempted to amplify approximately 10 ng of genomic DNA from a series of non-human species, using our standard protocols. The HUMTH01 primers amplified fragments in 2 primate species that are relatively closely related to humans, gorilla and chimpanzee, but not in 3 other more distantly related primate species, orangutan, baboon and pig tail macaque. The primers also failed to amplify fragments in a range of other earlier diverged eutherian species (horse, cow, pig, goat, sheep, dog, fox, cat, rabbit, guinea pig, mouse), metatherian species (kangaroo, possum), avian species (ostrich, emu, chicken, goose) and microorganisms (Escherichia coli, Lactobacillus, 2 species of Saccharomyces). Of the 2 gorillas that were typed one exhibited 2 fragments of equal size to human alleles HUMTH01*6 and *7. The other exhibited only one fragment equivalent in size to HUMTH01*6. The single chimpanzee that was typed also exhibited only one fragment equivalent in size to HUMTH01*6.

Forensic case studies

HUMTH01 typing has been used in 19 cases in our Laboratory; 6 rapes, 8 homicides and 5 serious assaults. Three of the rapes formed part of a serial investigation in which a succession of suspects was eliminated followed by a successful inclusion and subsequent confession. In one assault case both suspect and victim were excluded as the source of the stain. In all other cases, typings of probative value were obtained on some or all of the items examined. Out of 86 items tested 81 gave positive HUMTH01 results. The majority of these samples had already been typed previously for the HLA-DQA1 and/or D1S80 systems decreasing the significance of this success rate. Interestingly one sample which gave a positive HLA-DQA1 result was negative for HUMTH01 but 3 out of 12 samples first screened for HUMTH01 were positive for HUMTH01 and negative for HLA-DQA1. An attempt to amplify from a DNA sample extracted from an old bone was unsuccessful. However amplification of the same sample for HLA-DQA1 and D1S80 was also unsuccessful.

Discussion

The amplification, electrophoretic separation and detection methods used in this study are reproducible, reliable and sensitive. We routinely use 1–10 ng in our amplification mixes. While the differing concentration of BSA did not have any obvious effects in our experiments using DNA extracted from bloodstains, Hagelberg et al. (1989) and Hochmeister et al. (1991 a, b) have shown that unknown contaminants inhibit amplification of DNA extracted from bone and cigarette butts, and this can be overcome by the inclusion of BSA in the amplification mix. It is now routine practice in our laboratory to include 8 µg BSA per 50 µl reaction.

The inability to amplify the DNA extracted from bone may have been due to the absence of BSA in the amplification mix. Alternatively the DNA was more of bacterial or fungal origin than human origin or the excess calcium that may inhibit amplification (Hagelberg et al. 1989; Hochmeister et al. 1991 b) was not sufficiently removed during the DNA extraction procedure.

The extra fragments in the higher molecular weight region of the gel ("ghost bands") are thought to be generated by the oligonucleotide primers binding to regions of the genome other than HUMTH01 and/or heteroduplex formation. Further studies are required to determine their true identity. It has been shown that the occurrence of these ghost bands can be reduced by limiting the amount of DNA template to under 5–10 ng.

As it cannot be totally relied upon that these ghost bands do not appear in a given set of forensic case samples, it is not advisable to multiplex HUMTH01 with a system that amplifies allelic fragments that are larger than HUMTH01 fragments. The HUMTH01 ghost bands may interfere with the phenotyping of the second system. A

system using different coloured fluorescent dyes attached to different primers within a multiplex reaction mixer (Sullivan et al. 1992) would circumvent this problem.

A further shortcoming of the methods described here is the limited separation among the alleles especially between alleles HUMTH01*9.3 and *10. Whilst these are distinguishable, especially when using appropriate mixes for confirmation, an increase in separation would be desirable. The use of different gel compositions, gel length, denaturing gels as used by Puers et al. (1993) or sequencing should be considered as alternatives.

From the broad range of species tested it seems that the HUMTH01 primers used in this study are specific for humans and at least 2 species of higher primates (gorilla and chimpanzee). It may be of interest from an evolutionary point of view to examine other primate species, to type more unrelated gorillas and chimpanzees, and to sequence some of their amplified products.

The minor differences between the Victorian Asian and the United States Asian population (Puers et al. 1993) databases may be due to differences in founder effect or sample collection. Those classified as Asian in the Victorian population are mainly of Vietnamese and Chinese origins with the former being the most prevalent. The mix and ratio of subpopulations may be different in the sample set of Asians from the United States used by Puers et al. (1993). This is relevant as significant differences among different Asian populations have been found for other genetic markers (Mourant et al. 1976).

For forensic casework, where the identity of the perpetrator is unknown, we routinely make use of a "Victorian General Database". This database currently consists of samples (in excess of 200) mainly obtained from persons involved in criminal proceedings in Victoria as well as a few samples obtained from the Victorian Red Cross and Laboratory volunteers. All available samples are used, without selection, except when they originate from persons already in the database or their relatives.

Australia is a multicultural country and persons from several ethnic origins are present within the Victorian population, including many from Great Britain, Italy, Greece, former Yugoslavia and Vietnam (Australian Bureau of Statistics, 1991 Census). Due to statements from within our legal system that there is statistically significant substructure within the Victorian population, we are currently in the process of acquiring databases from several of these groups. This will enable us to determine the degree of variation at the HUMTH01 locus among populations as well as allow us to use such databases in forensic casework on the rare occasions where the ethnicity of the perpetrator is known from other evidence.

. HUMTH01 is the third PCR marker to be used in our laboratory. Currently it is only used on those samples for which no exclusion has been possible using the marker systems HLA-DQA1 (AmpliType HLA DQ, Perkin Elmer-Cetus) and D1S80 (Budowle et al. 1991), thus increasing the power of discrimination in the General Victorian population from 0.9958 (HLA-DQA1 plus D1S80) to 0.9996 with the addition of HUMTH01.

As there will be many highly polymorphic STR systems to select from (Edwards et al. 1991) and because they are less likely to exhibit the problems associated with some of the commonly used AmpFLP systems, it is clear that a selection of STRs will provide forensic scientists with a useful tool to provide the discrimination power required by the courts.

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