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Testing and Evaluation of 43 "Noncore" Y Chromosome Markers for Forensic Casework Applications

ABSTRACT: A developmental validation study was performed on three Y-STR multiplex systems, Multiplex III (MPIII), Multiplex IV (MPIV), and Multiplex V (MPV), to ascertain their potential applicability to forensic casework. MPIII contains eight Y-STRs, including DYS426, DYS435, DYS436, DYS441, DYS442, DYS446, DYS462, and Y-GATA-A10, and one InDel, YAP (DYS287). MPIV contains 21 Y-STR loci, including DYS443, DYS444, DYS445, DYS447, DYS448, DYS449, DYS452, DYS453, DYS454, DYS455, DYS456, DYS458, DYS463, DYS464, DYS468, DYS484, DYS522, DYS527, DYS531 DYS557, and DYS588. MPV contains 13 Y-STR loci, including DYS459, DYS476, DYS488, DYS513, DYS549, DYS561, DYS570, DYS575, DYS576, DYS590, DYS594, DYS598, and DYS607. Full genetic profiles were consistently obtained for all three multiplexes with 25–50 pg of male DNA. No significant amplification was observed with 1 μg of female DNA. Each multiplex permitted the determination of the number of male donors in male:male DNA admixtures. Species specificity studies demonstrated some cross-reactivity with some primate samples. Environmentally compromised blood samples produced full or partial profiles after exposure to various conditions for up to 1 year. Full profiles were recovered from simulated casework specimens including cigarette butts and postcoital cervicovaginal swabs. Population data were collected to determine individual loci gene diversity and multiplex discriminatory capacity.

KEYWORDS: forensic science, Y chromosome STR, multiplex, SWGDAM validation, MPIII, MPIV, MPV

The unique biology of the Y chromosome has led to the widespread use of genetic markers thereon in determining patrilineal relationships within and between population groups (1,2). A subset of these markers, Y-STRs, are now used routinely in forensic casework, albeit under restricted circumstances (3–10). Y-STR analysis benefits include genetic profiling of a minor male contributor among an overwhelming quantity of female DNA in body fluid admixtures, determination of a missing person's male haplotype by typing male relatives, and the ability to provide additional discriminatory power when combined with (often partial) autosomal STR profile results.

A set of nine Y-STR loci, commonly referred to as the ''minimal haplotype loci'' set (MHL), were recommended for use in the forensic community (2). Subsequently, several additional loci were reported that, in conjunction with the MHL loci, proffered increased discriminatory capacity (5,11–16). A number of commercial Y-STR kits are available and most incorporate 12–17 markers into single multiplex systems, which have been validated for casework (10,17,18). These commercial kits incorporate all twelve of the Y-STR ''core'' markers that were recommended for forensic use by the U.S. Scientific Working Group on DNA Analysis Methods (SWGDAM) (19). Despite the robustness of these commercial multiplex Y-STR systems and the ability to discriminate two male individuals in most cases, the coincidence match probabilities are modest compared with a set of standard autosomal STR markers. Hence, there is still a need to develop new multiplex systems to supplement these for those cases where additional discriminatory power is desired or where there is a coincidental Y-STR match between potential male participants. Although more than 300 STR loci have been described on the Y chromosome (19), most of these loci still have not been well characterized for their forensic utility. The few novel ''noncore'' markers that have been incorporated into multiplex PCR systems have yet to undergo the extensive ''developmental validation'' studies (4,17) required by U.S. national standards (19), despite the availability of limited population data on some of them (11–13,20–23). The aim of the development validation study is to assess the ability of the ''noncore'' multiplex STR system to obtain reliable results, to determine the conditions under which such results can be obtained, and to define the limitations of the procedure (19). Such developmental validation studies must be performed on a novel DNA procedure before use in forensic casework analysis (19).

In the present work, we describe the results of a full SWGDAM developmental validation on three ''noncore'' multiplex Y-STR systems, Multiplex III (MPIII), Multiplex IV (MPIV), and Multiplex V (MPV), according to the SWGDAM Revised Validation Guidelines (19). Previously, we described the development and preliminary performance testing of the MPIV system (24). This is the first report describing and characterizing the MPIII and MPV systems. MPIII, MPIV, and MPV allow for the coamplification of eight, 21, and 13 Y-STR markers, respectively. MPIII uniquely contains the YAP (DYS287) *indel* locus. The Y-STR loci include DYS426, DYS435, DYS436, DYS441, DYS442, DYS443, DYS444, DYS445, DYS446, DYS447, DYS448, DYS449, DYS452, DYS453, DYS454, DYS455, DYS456, DYS458, DYS459, DYS462, DYS463, DYS464, DYS468, DYS476, DYS484, DYS488, DYS513, DYS522, DYS527, DYS531, DYS549, DYS557, DYS561, DYS570, DYS575, DYS576, DYS588, DYS590, DYS594, DYS598, DYS607, and Y-GATA-

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A10. The results indicate that all three multiplex systems (and the loci involved) are suitable for use in forensic casework applications.

Methods

Preparation of Body Fluid Stains

Body fluids were collected from volunteers using procedures approved by the University of Central Florida's Institutional Review Board. Buccal samples were collected from donors using sterile swabs by swabbing the inside of the donor's mouth. Neat semen samples were provided in sealed plastic tubes and stored frozen until they were dried onto sterile cotton swabs. Postcoital cervicovaginal swabs were taken from female participants at various specified time periods subsequent to sexual intercourse. Blood samples were collected by venipuncture into sterile preservative-free vacutainers (Becton Dickinson, Franklin Laks, NJ) and 50 μL aliquots were placed onto cotton cloth and dried at room temperature. Population samples for gene diversity studies were obtained from the Virginia Division of Forensic Science (bloodstains), Richmond, VA. All samples were stored at −47°C until needed.

DNA Isolation and Purification

DNA was extracted from the buccal swabs, the vaginal swabs, and the semen swabs using a standard phenol:chloroform method (25).

Differential Cell Lysis for the Recovery of Sperm DNA

Sperm and nonsperm cells were separated using a standard differential lysis protocol, with minor modifications (26). Postcoital cervicovaginal swabs were incubated overnight at 37°C in 400 μL of DNA extraction buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS) and 0.1 mg/mL proteinase K. Swab remnants were removed to a spin-ease basket, the basket inserted back into the original tube, and centrifuged at 14,000 *g* for 5 min. The resulting supernatant, containing the nonsperm DNA fraction, was removed into a separate tube for further analysis. The sperm pellet was resuspended in 400 μL of DNA extraction buffer, 0.1 mg/mL proteinase K, and 40 μL of 0.39 M DTT and incubated for 1 h at 56°C. DNA from both the sperm and nonsperm fractions was isolated and purified using the phenol:chloroform method described above.

DNA Isolation and Purification of Dried Blood Samples

The dried bloodstains were incubated overnight at 56°C in 400 μL of DNA extraction buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS) and 0.1 mg/mL proteinase K. The swab pieces were placed into a spin-ease basket and centrifuged at 14,000 *g* for 5 min. An equal volume of phenol/chloroform/isoamyl alcohol was added to the crude extract. The aqueous phase extracts containing the DNA were purified using Centricon 100™ concentrators (Millipore, Bedford, MA) according to the manufacturer's instructions.

DNA Quantitation

The amount of DNA was estimated using ethidium bromide-induced fluorescence on a 1% agarose yield gel, using a reference set of DNA standards of known concentration (27). DNA quantitation estimates based on this method are known to have a 10–20% margin of error. This should be taken into account when interpreting the results of the studies presented in this paper.

Characterization of Genetic Markers

Locus Nomenclature—All locus characteristics, including repeat unit structure and size and general chromosome location, were obtained from published sources.

Mapping—All individual Y-STR loci have been previously mapped to the Y chromosome using standard BLAT searches (28). BLAT searches also allowed for an assessment of a locus' degree of homology with other chromosomes, specifically the X chromosome (29). Five of the loci are located within the ampliconic palindrome regions (30). These include DYS 448 (MPIV), DYS 464 (MPIV), DYS 527 (MPIV), and DYS 459 (MP5), which are located within the P1–P2–P3 amplicon, and DYS 607 (MPV), which is in the P6 amplicon.

Inheritance Study—Males within the same paternal lineage should possess the same Y-STR haplotype as the NRY region of the Y chromosome does not undergo genetic recombination with the X chromosome. To demonstrate this male lineage concordance, five males from the same paternal lineage were analyzed with the multiplex system. The males included in this study involved a father/son pair and two grandfather/grandson pairs.

Standard Conditions

Reaction Components—Optimization of the multiplex systems resulted in a set of standard conditions. (MPIII)—The 25 μL reaction mix contained 3 ng of template DNA, 0.08–0.80 μM primers, 1 mM dNTPs, 1 × PCR buffer II (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 1.6 mM $MgCl_2$, 10 μg of nonacetylated bovine serum albumin (Sigma-Aldrich, St. Louis, MO), and 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). (MPIV)—the 25 μL reaction mix contained 3 ng of template DNA, 0.12–1.2 μM primers, 1 mM dNTPs, 1 × PCR buffer II, 1.5 mM $MgCl_2$, 10 μg nonacetylated bovine serum albumin, and 3.75 U of AmpliTaq Gold DNA polymerase. (MPV)—the 25 μL reaction mix contained: 3 ng of template DNA, 0.064–0.80 μM primers, 1 mM dNTPs, 1 × PCR Buffer II, 2.0 mM $MgCl_2$, 10 μg nonacetylated bovine serum albumin, and 1.5 U of AmpliTaq Gold DNA polymerase.

Primers—Primer sequences were obtained from published sources, the Human Genome Database, or redesigned using Oligo 6 Primer Analysis Software (Lifescience Software Resource, Long Lake, MN). The primers were tested initially as singleplexes, and subsequently in the multiplex system. The primer concentrations for MPIII were as follows: DYS426–0.10 μM; DYS436–0.08 μM; DYS462 (G09411), 0.20 μM; DYS441, 0.24 μM; YAP, 0.80 μM; Y-GATA-10, 0.24 μM; DYS446, 0.24 μM; DYS435, 0.10 μM; and DYS442, 0.24 μM (Invitrogen, Grand Island, NY). The primer concentrations for the MPIV loci were as follows: DYS443, 0.32 μM; DYS444, 0.40 μM; DYS445, 0.32 μM; DYS447, 0.48 μM; DYS448, 0.40 μM; DYS449, 0.48 μM; DYS452, 0.64 μM; DYS453, 0.56 μM; DYS454, 0.36 μM; DYS455, 0.48 μM; DYS456, 0.16 μM; DYS458, 0.48 μM; DYS463, 0.56 μM; DYS464, 0.20 μM; DYS468, 0.80 μM; DYS484, 0.24 μM; DYS522, 0.51 μM; DYS527, 1.2 μM; DYS531, 0.12 μM; DYS557, 0.56 μM; and DYS588, 0.20 μM (Invitrogen and Applied Biosystems). The primer concentrations for the MPV loci were as follows: DYS459, 0.56 μM; DYS476, 0.096 μM; DYS488, 0.32 μM; DYS513, 0.064 μM; DYS549, 0.80 μM;

DYS561, 0.56 μM; DYS570, 0.096 μM; DYS575, 0.48 μM; DYS576, 0.128 μM; DYS590, 0.096 μM; DYS594, 0.64 μM; DYS598, 0.48 μM; DYS607, 0.32 μM (Invitrogen and Applied Biosystems).

Cycling Conditions—MPIII—(1) 95°C 11 min, (2) 32 cycles of 96°C 30 s, 59°C 1 min, 72°C 45 s, (3) and a final extension 72°C for 45 min. MPIV—(1) 95°C 11 min, (2) 34 cycles of 96°C 30 s, 52°C 45 s, 72°C 1 min, (3) and a final extension 72°C for 45 min. MPV—(1) 95°C 11 min, (2) 32 cycles of 96°C 30 s, 61°C 1 min, 72°C 1 min, (3) and a final extension 72°C for 55 min. All samples were amplified using an Applied Biosystems 9700 GeneAmp PCR System thermocycler.

PCR Product Detection

MPIII—An aliquot (1.5 μL) of the amplified product was added to 24 μL of deionized formamide (Applied Biosystems) and 1 μL GeneScan 500 TAMRA internal lane standard (Applied Biosystems). Tubes containing the above were heated at 95°C for 3 min and snap cooled on ice for 3 min. Samples were injected onto an ABI Prism 310 Genetic Analyzer using Module C (5-s injection, 15 kV, 60°C) and analyzed with GeneScan Analysis Software v3.7 using Filter Set C (Applied Biosystems) and the Local Southern Method smoothing option. A peak detection threshold of 50 RFUs was used for allele designation.

MPIV—An aliquot (2.0 μL) of the amplified product was added to 24 μL of deionized formamide and 1.5 μL GeneScan 500 LIZ internal lane standard. Tubes containing the above were heated at 95°C for 3 min and snap cooled on ice for 3 min. Samples were injected onto an ABI Prism 310 Genetic Analyzer using Module G5 (5-s injection, 15 kV, 60°C) and analyzed with GeneScan Analysis Software v3.7 using Filter Set G5 and the Local Southern Method smoothing option. A peak detection threshold of 50 RFUs was used for allele designation.

MPV

An aliquot (1.75 μL) of the amplified product was added to 24 μL of deionized formamide and 1.5 μL GeneScan 500 LIZ internal lane standard. Tubes containing the above were heated at 95°C for 3 min and snap cooled on ice for 3 min. Samples were injected onto an ABI Prism 310 Genetic Analyzer using Module G5 (5-s injection, 15 kV, 60°C) and analyzed with GeneScan Analysis Software v3.7 using Filter Set G5 and the Local Southern Method Smoothing option. A peak detection threshold of 50 RFUs was used for allele designation.

Optimization of PCR Parameters

Magnesium—The effect of magnesium concentration on the multiplex system was assessed using 3 ng of male DNA and 300 ng of female DNA. The magnesium concentrations tested ranged from 1.0 to 4.0 mM (in 0.25 mM increments).

DNA Polymerase—Various amounts of DNA polymerase were tested in order to achieve optimal amplification efficiency. Three nanograms of male DNA and 300 ng of female DNA were amplified with a range of DNA polymerase amounts: MPIII—1.0–.5 U, in 0.25 U increments; MPIV—1.0–4.0 U, in 0.25 U increments; and 1.0–2.0 U, in 0.25 increments.

Cycling Conditions—Annealing temperatures and times, as well as number of amplification cycles were varied in order to determine the most efficient cycling conditions for coamplification of all loci.

The annealing temperatures tested for MPIII included 55°C, 57°C, 59°C, 61°C, and 63°C, for MPV 50°C, 52°C, 54°C, and 56°C for MPIV, and 59°C, 61°C, and 63°C. These temperatures were each tested using 3 ng of male DNA and 300 ng of female DNA. The ranges were selected based on preliminary multiplex testing at 59°C, 52°C, and 61°C for MPIII, MPIV, and MPV, respectively. Annealing times of 30, 45, 60, and 90 s were tested in separate reactions using 3 ng of male DNA and 300 ng of female DNA for both multiplex systems.

Various numbers of amplification cycles for both multiplexes were also tested with 3 ng of male DNA and 300 ng of female DNA, including 28, 30, 32, 34, and 36 cycles. Initial multiplex testing was performed using 32 cycles, and the range of cycle numbers was selected to include several cycles less and several cycles more than the initial amount.

Stutter—The average percent stutter for each locus was evaluated by calculating a ratio of stutter peak heights to true allele peak heights. An alternative method for determination of stutter percentages is to use ratios of peak height areas (31). No significant differences in stutter calculations have been observed between stutter calculations using peak heights versus peak areas (31). Peak height values were used in stutter calculations in the present study as this is the method of choice in previous stutter studies (32). Twenty male samples (using 3 ng input DNA) were included in these calculations.

Multiplex System Performance

Sensitivity—Different input quantities of male template DNA were tested using the standard multiplex reaction conditions. The amounts tested included 100, 10, 5, 3, 1 ng, 500, 250, 150, 125, 100, 50, and 25 pg.

Specificity—To evaluate possible female DNA cross-reactivity, the following amounts of female template DNA were tested: 3 ng, 30 ng, 300 ng, and 1 μg.

Species Specificity—To determine any cross-reactivity among nonhuman species, 3 ng DNA from the following animal species were analyzed: dog, cat, sheep, horse, bull, male chimpanzee, male gorilla, male orangutan, male crab-eating macaque, male spider monkey, female lowland gorilla, and female orangutan.

In addition, several bacterial samples were examined for potential cross-reactivity with the multiplexes. These samples included *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterobacter aerogenes*, and *Pseudomonas aeruginosa*. The surface of the bacterial culture was swabbed using a sterile cotton swab, and the DNA was extracted using the standard phenol:chloroform method described previously. Three nanograms of DNA was amplified.

Stability Studies

Recovery of Y-STR Profiles from Different Body Fluids from the Same Individual—To ensure that reproducible Y-STR haplotypes would be produced from various body fluids from the same individual, DNA was extracted from blood, semen, and saliva from the same male individual and typed with the Y-STR multiplex systems.

Environmentally Compromised Blood Samples—Fifty microliter aliquots of human blood were previously dried onto cotton cloth. These samples were exposed to different environmental conditions including various temperatures, light sources, and environmental influences including humidity and rain. The environmental conditions were as follows: RTED (room temperature,

envelope dried), HLHR (placed outside for exposure to heat, light, humidity, and rain), and HLH (placed outside and covered exposure to heat, light, and humidity). Samples from all sets of conditions were collected at varying lengths of time, including 3 days, 1 week, 1, 3, 6 months, and 1 year. Samples were also exposed to short-wave UV at room temperature for 3 days, 1 week, 1, 3, 6, and 18 months.

Precision

To demonstrate the ability of the analytical system to measure reproducibly the size of the detected fragments, the positive control male sample (GM9948) was injected 20 times using capillary electrophoresis and the average standard error was calculated for each individual locus (33).

Reproducibility

To assess the reproducibility of the multiplex systems, five DNA extracts from bloodstains were given to two experienced analysts for analysis, and five DNA extracts from buccal swabs were also given to two experienced analysts for analysis, to ensure that the same male haplotype was obtained.

Mixture Studies

Male/Female—Three nanograms of male DNA was coamplified with increasing amounts of female DNA in the following ratios: 1/2 (3 ng male DNA/3 ng female DNA); 1/10 (3 ng male DNA/27 ng female DNA); 1/100 (3 ng male DNA/297 ng female DNA); 1/500 (3 ng male DNA/497 ng female DNA); and 1/1000 (3 ng male DNA/997 ng female DNA).

Male/Male—Three nanograms total DNA from two individual males was coamplified in the following ratios: 1/2 (1.5 ng male-1 DNA/1.5 ng male-2 DNA), 1/3 (1.0 ng male-1 DNA/2.0 ng male-2 DNA), 1/6 (0.5 ng male-1 DNA/1.5 ng male-2 DNA), 1/12 (0.25 ng male-1 DNA/2.75 ng male-2 DNA), and 1/30 (0.10 ng male-1 DNA/2.9 ng male-2 DNA).

Nonprobative Casework Samples

Cigarette Butts—In order to determine whether the multiplex could accommodate low copy number forensic samples, cigarette butts were collected from an ashtray after being deposited by a male volunteer. The outer paper wrapping was removed from the cigarette and DNA was extracted using the standard phenol:chloroform method described previously. Quantitation was performed as described previously, but the yield of DNA was below the detection limits of the system (<1 ng/μL in a total extract volume of 100 μL). Five microliters of the extract was used for amplification.

Postcoital Cervicovaginal Samples—Postcoital cervicovaginal swabs were collected by participants (one male/female couple) at various time intervals, including 0 h (immediately), 12, 24, 48, and 72 h, and stored at -47°C until needed. Participants were instructed to collect two swabs after each individual sexual encounter, and were asked to collect samples no less than 3 days apart. Only one set of swabs representing a particular time interval was taken after each sexual encounter to ensure that the amount of semen present at the time of collection was not affected by prior semen removal. One swab of the set was extracted using a differential extraction protocol, separating the male sperm component from the nonsperm "female" component. The second swab for the 72-h samples was extracted using a nondifferential extraction, co-extracting the male sperm and the nonsperm "female" component.

Population Studies

Descriptive Statistics—One hundred and ninety-eight male individuals were used for all population studies (98 Caucasian, 100 African Americans). All data were checked for duplicate samples. A small number of duplicate samples ($n = 4$) were removed from the data set after confirmation by the submitting laboratory of their duplicate status by autosomal STR analysis. The following formulae were used (1) discriminatory capacity = no. of individuals/no. of different haplotypes; (2) gene diversity (h), equivalent to the expected frequency of heterozygotes with autosomal diploid loci, was calculated as $[n/(n-1)) \times (1 - \Sigma p_i^2]$, where p_i = allele frequency at the ith locus (34).

Results and Discussion

Characterization of Genetic Markers

Forty-three "noncore" Y-chromosome markers (42 Y-STRs and one InDel, YAP) were selected for inclusion into three novel multiplex systems, MPIII (Fig. 1*A*, left panel), MPIV (Fig. 1*B*, left panel), and MPV (Fig. 1*C*, left panel). MPIII contains eight Y-STR loci and, uniquely, one InDel, including DYS426 (12,35,36), DYS435 (11), DYS436 (11), DYS441 (21), DYS442 (21), DYS446 (14), DYS462 (16,37,38), Y-GATA-A10 (16,38), and DYS287 or YAP (39). MPIV (MPIV) permits the coamplification of 25 segregating sites from 21 Y-STR loci, including a bi-local locus (DYS527) and a tetra-local locus (DYS464). The MPIV loci include DYS443, DYS444, DYS445, DYS446, DYS448, DYS449, DYS452, DYS453, DYS454, DYS455, DYS456, DYS458, DYS463, DYS464, DYS468, DYS484, DYS522, DYS527, DYS531, DYS557, and DYS588 (24). MPV (MPV) permits the coamplification of 14 segregating sites from 13 Y-STR loci, including one bi-local locus (DYS459). The MPV loci include DYS459 (14), DYS476 (40), DYS488 (40), DYS513 (20), DYS549 (22), DYS561 (20), DYS570 (22), DYS575 (22), DYS576 (40), DYS590 (40), DYS594 (22), DYS598 (23), and DYS607 (23). GDB (Genome data base) accession numbers, number of alleles, allelic size range, average percent stutter, and primer sequences with associated dye labels are provided for MPIII (Table 1), MPIV (Table 2), and MPV (Table 3).

Minor modifications have been made to MPIV since its original development (24). The multiplex was initially developed on an NT-based ABI Prism 310 Genetic Analyzer, and was subsequently transitioned to a MAC-based format. As a result of this transition, minor modifications were necessary to increase efficiency including an increase in cycle number from 32 to 34, movement of DYS468 to the green channel (VIC) from the red channel (PET) with redesigned primer sequences, and also movement of DYS588 from the blue channel (FAM) to the yellow channel (NED) due to overlapping allele ranges. These minor modifications increased both the amplification efficiency of DYS468 and the analytical sensitivity of the multiplex system.

Inheritance—The 43 Y-chromosome markers from MPIII, MPIV, and MPV are found in the nonrecombining region of the Y chromosome. As a result, these markers are inherited unchanged, barring rare spontaneous mutations, as a physical block of linked multilocus haplotypes from father to son. A family study was used to assess this possible mode of inheritance in both multiplex systems. A father/son and two grandfather/grandson pairs were included in this study. The 43-locus haplotypes resulting

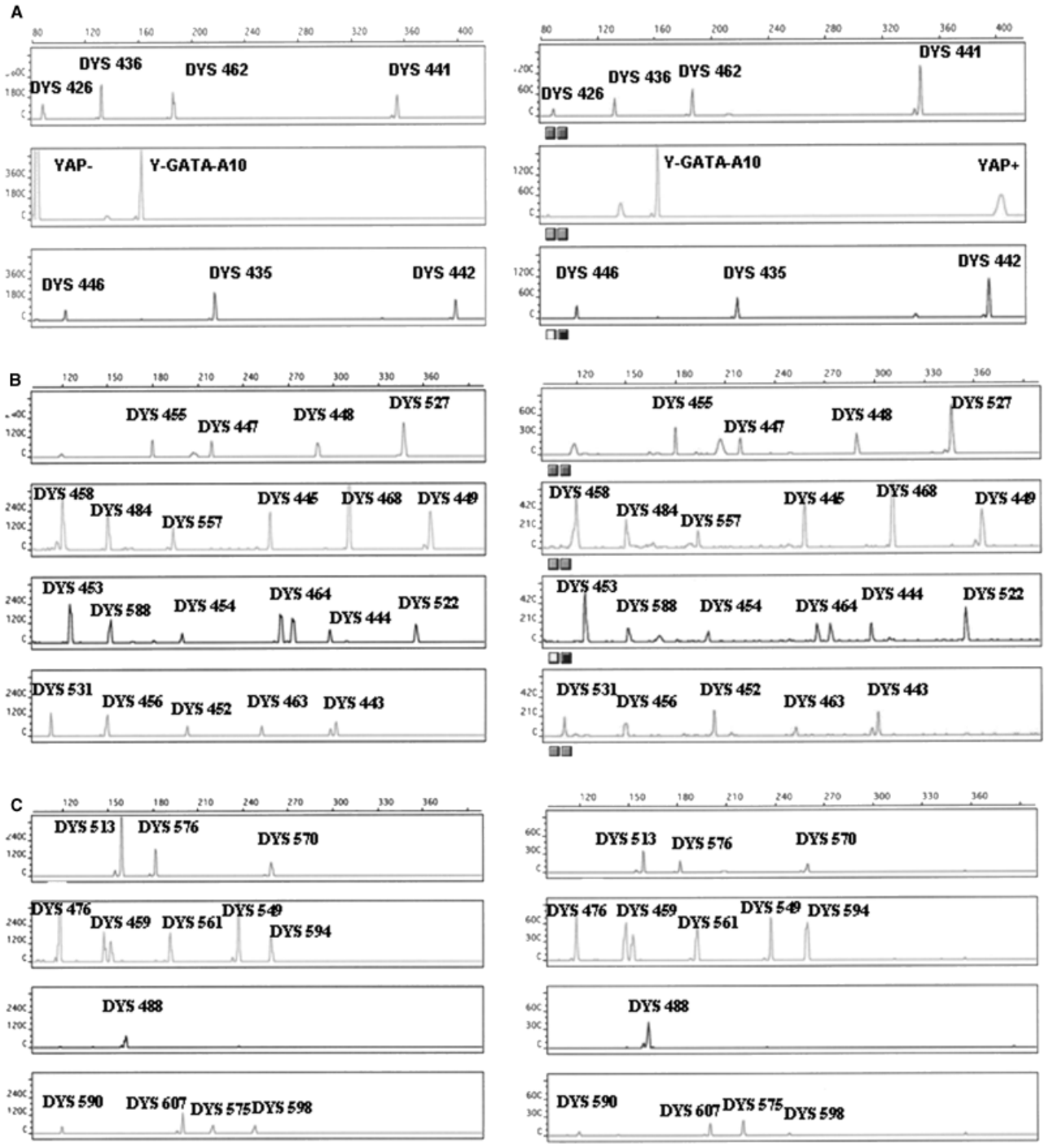

FIG. 1—*Multiplex appearance with optimal and minimum amount of input DNA. (A) Left panel: MPIII (YAP −)—A full nine-locus male haplotype with a YAP − sample obtained with 1 ng of template DNA, Right panel: a full nine-locus male haplotype with a YAP+ sample obtained with 25 pg of template DNA. (B) Left panel: MPIV – a full 21-locus male haplotype obtained with 1 ng of template DNA. Right panel: a full 21-locus male haplotype obtained with 25 pg of template DNA. (C) Left panel: MPV—a full 13-locus male haplotype obtained with 1 ng of input DNA, Right panel: a full 13-locus male haplotype obtained with 50 pg of template DNA. The x-axis of each electropherogram represents fragment size (bp) and the y-axis represents signal intensity (relative fluorescence units, RFU).*

from amplification with MPIII, MPIV, and MPV were identical for all the males tested for this study, supporting a patrilinear mode of inheritance. It is recognized that this limited study demonstrating patrilineal inheritance of the markers does not permit an estimate of the mutation rate.

Mapping—All individual loci have been previously mapped to the Y chromosome using a BLAT search (www.genome.ucsc.edu) (28). The BLAT search results also allowed for an assessment of the degree of homology with the X chromosome. No loci included in this study showed a 100% homology to the X chromosome.

Detection—Detection of the polymorphic Y-STR loci evaluated in this study was accomplished by laser-induced fluorescence of the labeled amplification products. One of the primers for each individual loci contained an attached fluorescent dye. MPIII was designed as a four-dye-based system, using FAM (blue), TET (green), HEX (yellow), and TAMRA (red, internal size standard).

TABLE 1—*Multiplex III loci characteristics.*

Locus	Repeat motif	# of alleles	PCR product size range (bp)	Average (%) stutter	Primer sequences
DYS426	GTT	5	82–95	6	FF:GGTGACAAGACGAGACTTTGTG (36)
GDB:1386838					R: TCAAAGTATGAAAGCATGACCA (36)
DYS436	GTT	5	125–138	3	FF: CCAGGAGAGCACACACAAAA (11)
GDB:9899864					R: GCAATCCAACTTCAGCCAAT (11)
DYS462	TATG	6	169–190	4	FF: TGTGCTGTACCAGTTGCCTA (37)
GDB:6449613					R: CCAGCCTGAGCAAGAGAGTA (37)
DYS441	CCTT	7	338–362	4	FF: TCAAATTCTCAGGCATTGCAG (21)
GDB:10013873					R: CTAGGCAACACAGGAAAACCC (21)
YAP	N/A	2	84–422	N/A	FT: AGGACTAGCAATAGCAGGGGAAGA (39)
GDB:196899					R: CAGGGCCAACTCCAACCAAG (39)
Y-GATA-A10	GATA	8	148–179	10	FT: ATAAATGGAGATAGTGGGTGGATT (38)
GDB:9996577					R: CCTGCCATCTCTATTTATCTTGCATATA (38)
DYS446	TCTCT	12	89–147	10	FH: TATTTTCAGTCTTGTCCTGTC (14)
GDB:10873760					R: GAGACTCTGTCTGAAGAGAG (14)
DYS435	TGGA	4	213–226	3	FH: AGCATGTCCACACAGCACAC (16)
GDB:9899862					R: TTCTCTCTCCCCCTCCTCTC (16)
DYS442	TATC	8	382–441	3	FH: GTGCACCCATCTCCTTAGCAG (21)
GDB:10030304					R: AATCACGGAACCAACCCAAAC (21)

Fluorescent dye label: F, FAM; T, TET; H, HEX.

Multiplexes IV and V were designed as five-dye-based systems, using FAM (blue), VIC (green), NED (yellow), PET (red), and LIZ (orange, internal size standard).

Polymorphism—The MPIII, MPIV, and MPV loci comprise tri-, tetra-, penta-, and hexa-nucleotide short tandem repeats. Nominal alleles are distinguished based on their size (in base

TABLE 2—*Multiplex IV loci characteristics.*

Locus	Repeat motif	No. alleles	PCR product size range (bp)	Average (%) stutter	Primer sequences
DYS455	AAAT	5	167–185	0	FF: GGGGTGGAAACGAGTGTT (24)
GDB:11498125					R: ATCTGAGCCGAGAGAATGATA (24)
DYS447	TAATA	9	199–239	0	F: GGTCACAGCATGGCTTGGTT (24)
GDB:10843977					RF: GGGCTTGCTTTGCGTTATCT (24)
DYS448	AGAGAT	8	266–310	0	FF: TGTCAAAGAGCTTCAATGGAGA (24)
GDB:10877524					R: TCTTCCTTAACGTGAATTTCCTC (24)
DYS527	TTCC	11	324–366	0	FF: TCGCAAACATAGCACTTCAG (24)
GDB:11503872					R: TTCTAGGAAGATTAGCCACAACA (24)
DYS458	GAAA	9	106–138	12	FV: AGCAACAGGAATGAAACTCCAAT (24)
GDB:11498131					R: CCACCACGCCCACCCTCC (24)
DYS484	AAT	4	144–155	0	FV: CCTATCATCCGCATGGACTT (24)
GDB:11503786					R: CCTGGTTGACAAAGCCAGAT (24)
DYS557	TTTC	8	179–206	2	F: TTTTCTGTGCCAAGCCTACA (24)
GDB:11503932					RV: TCTAATGCACCTTGAGGGATG (24)
DYS445	TTTA	6	250–270	0	FV: GAGCTGAGATTATGCCACCAAAA (24)
GDB:10807129					R: AGTTAAGAGCCCCACCTTCCTG (24)
DYS468	TCC	11	296–326	0	FV: CTGCTGCTGCTTCTTCTACT
GDB:11503754					R: GGGTAAGAGTCCAAAGTTGC
DYS449	TTTC	11	345–385	8	FV: TGGAGTCTCTCAAGCCTGTTCTA (24)
GDB:10879367					R: CCTGGAAGTGGAGTTTGCTGT (24)
DYS453	AAAT	3	122–130	0	FN: GGGTAACAGAACAAGACAGT (24)
GDB:11498119					R: CTAAAAGTATGGATATTCTTCG (24)
DYS588	GCATT	8	140–176	0	FN: GAATGCAGAACCCTCAAGGA (24)
GDB:11503994					R: AGCCTGGGTGACAGAAACAC (24)
DYS454	AAAT	3	196–204	0	FN: GACATGTAGCTCTTCACTTCAC (24)
GDB:11498123					R: GACTGACCTCACATTGTTGTTA (24)
DYS464	CCTT	9	250–282	0	FN: TTACGAGCTTTGGGCTATG (24)
GDB:11499416					R: CCTGGGTAACAGAGAGACTCTT (24)
DYS522	GATA	5	348–364	0	FN: CCTTTGAAATCATTCATAATGC (24)
GDB:11503862					R: TCATAAACAGAGGGTTCTGG (24)
DYS531	AAAT	3	108–116	0	FP: GACCCACTGGCATTCAAATC (24)
GDB:11503880					R: TGCTCCCTTTCTTTGTAGACG (24)
DYS456	AGAT	7	136–164	0	FP: CCCATCAACTCAGCCCAAAAC (24)
GDB:11498127					R: GGACCTTGTGATAATGTAAGATA (24)
DYS452	TATAC	9	182–220	0	FP: TTTATTATACTCAGCTAATTAATTGGTT (24)
GDB:11498121					R: GTGGTGTTCTGATGAGGATAAT (24)
DYS463	AARGG	10	222–266	0	FP: AATTCTAGGTTTGACCAAAGACA (24)
GDB:11499418					R: ATGAGGTTGTGTGACTTGACTG (24)
DYS443	TTCC	6	290–310	0	FP: TTTCATTGGCCACCTGACATTAC (24)
GDB:10807127					R: CGCTTCCATTTACACTTCCTGTG (24)

Fluorescent dye label: F, FAM; V, VIC; N, NED; P, PET.

TABLE 3—*Multiplex V loci characteristics.*

Locus	Repeat motif	No. alleles	PCR product size range (bp)	Average (%) stutter	Primer sequences
DYS513 GDB:11503844	TCTA	4	150–163	6	FF: ATTGATCCATCCGTCTGTCC (20) R: GTTGGATGAAGGGAGAGCAG (20)
DYS576 GDB:11503970	AAAG	10	166–200	5	FF: TTGGGCTGAGGAGTTCAATC R: GGCAGTCTCATTTCCTGGAG
DYS570 GDB:11503958	TTTC	10	242–279	4	FF: GAACTGTCTACAATGGCTCACG (22) R: TCAGCATAGTCAAGAAACCAGACA (22)
DYS476 GDB:11503770	GAT	4	114–124	5	FV: CGACTATGATTTGGGCTGTG (40) R: AGCTGGGAAGTACTCAATGCTC (40)
DYS459 GDB:11498133	TAAA	5	138–156	0	FV: CAGGTGAACTGGGGTAAATAAT (14) R: TTGAGCAACAGAGCAAGACTTA (14)
DYS561 GDB:11503940	GATA	5	183–199	3	FV: GCCTGATGCCATCTGAAAAT (20) R: TGATCCCAACAACTGCACTC (20)
DYS549 GDB:11503916	AGAT	6	225–245	4	FV: AACCAAATTCAGGGATGTACTGA (22) R: GTCCCCTTTTCCATTTGTGA (22)
DYS594 GDB:11503747	TAAAA	4	258–273	0	FV: GATGTGCCTAATGCCACAGA (22) R: CCCTGGTGTTAATCGTGTCC (22)
DYS488 GDB:11503794	ATA	5	154–167	1	FN: ATGGCACATGTATATCGATG R: TTGTGAGTACCCTGGTCCAC
DYS590 GDB:11503998	TTTTG	4	108–124	0	FP: GGGAACATAGTCGGGCTGTA (40) R: GGGTGACAGAGCAAGAATCC (40)
DYS607 GDB:11505463	AAGG	7	180–204	2	FP: AGCATACAGCGTAATCACAGC (23) R: TCAGACAAAGCCCAGTTGAG (23)
DYS575 GDB:11503968	AAAT	2	215–219	0	FP: GGTGGTGGACATCCGTAATC (22) R: AGTAATGGGATGCTGGGTCA (22)
DYS598 GDB:11505454	AGAAC	5	241–262	0	FP: CTTTATTAGGCAGGCAGTTTTG (23) R: CCAGACAATGTATGAGCAAGC (23)

Fluorescent dye label: F, FAM; V, VIC; N, NED; P, PET.

pairs), with fragments ranging from *c*. 80 to *c*. 425 bases (Tables 1–3).

PCR Parameters

The MPIII, MPIV, and MPV reaction conditions were optimized by altering the concentration of critical reagents and the thermocycling conditions, using both male (3 ng) and female (300 ng) input DNA. This resulted in a set of standard reaction conditions, as described in the Methods section.

Magnesium

MPIII, MPIV, and MPV were tested with various magnesium concentrations, ranging from 1.0 to 4.0 mM, in 0.25 mM increments. MPIII performed well throughout with an input of 1.25–2.25 mM magnesium. With magnesium concentrations lower than 1.25 mM and greater than 2.25 mM, allelic drop-out was observed for several loci (data not shown). A final magnesium concentration of 1.6 mM was selected as the YAP insert appeared to be amplified most efficiently using this concentration. For MPIV, a drastic decrease in the amount of detectible products was observed for magnesium concentrations greater than 1.8 mM (data not shown). An optimal magnesium concentration of 1.5 mM was selected for MPIV, due to a good interlocus peak height balance and the elimination of X chromosome artifacts. For MPV, no amplification was observed with a magnesium concentration lower than 1.5 mM (data not shown). Using 1.5 and 1.75 mM, peak heights were generally lower compared with profiles obtained using higher concentrations of magnesium. Increased artifact and stutter product were observed when greater than 2.0 mM magnesium was used (data not shown). As a result, 2.0 mM magnesium was selected as the optimal concentration for MPV.

DNA Polymerase—Various concentrations of DNA polymerase were tested for each multiplex system. A range of 1.0–2.5 U was used for MPIII, 1.0–4.0 U for MPIV, and 1.0 to 2.0 U for MPV, all in 0.25 increments. For MPIII, 1.25 U was determined to be optimal due to the reduction in the amplification of an artifact at $\sim$ 343 bp on the yellow channel (data not shown). MPIV required a significant increase in the amount of polymerase added, with the optimal amount determined to be 3.75 U, in order to amplify efficiently all 21 loci. In previous work carried out by the authors, it was determined that 2.0 U of polymerase were sufficient for MPIV. However, when the system was changed from an NT-based detection platform to the MAC-based detection platform, a decrease in sensitivity was observed and modifications were made to overcome this lowered detection sensitivity. For MPV, the optimal amount of DNA polymerase was determined to be 1.5 U.

Thermocycling Conditions—Thermocycling conditions were varied in order to obtain conditions that resulted in the most efficient amplification of loci, determined by balanced peak heights and reduction in the appearance of X chromosome or stutter artifacts. These conditions included cycle number and annealing times and temperatures.

Annealing Time—For all three multiplex systems, annealing times of 30, 45 s, and 1 min were tested. For MPV, an annealing time of 1 min and 30 s was also tested. For MPIII, annealing times of 30 and 45 s resulted in allelic drop-out for DYS426, DYS436, and poor amplification of DYS446. An annealing time of 1 min was selected for MPIII, producing efficient amplification of all alleles with an absence of any X chromosome artifact production. For MPIV, full 21 locus haplotypes were obtained using 30 or 45 s annealing times. Annealing times of 1 min caused a significant increase in the number of artifacts observed in the male samples, and in the female samples as well. The 45 s annealing time produced a greater peak balance between loci, and as a result was selected for MPIV. For MPV, annealing times of 30 s and 45 s resulted in poor amplification efficiency of all loci in the multiplex. An annealing time of 1 min 30 s allowed for amplification of all loci; however, there was an increase in stutter product formation. Therefore, 1 min was selected as the optimal annealing time for MPV.

Annealing Temperature—Before multiplex development, Oligo6 Primer Design Software was used in order to determine an optimal annealing temperature for each individual locus. An average annealing temperature was calculated based on the optimal annealing temperatures for the loci included in each multiplex. The range of annealing temperatures used to validate the systems was constructed based on the initially selected temperature. For MPIII, an optimal annealing temperature of 59°C was calculated, and therefore a range of 55–63°C was tested, in 2°C increments. Increasing or decreasing the annealing temperature for MPIII from 59°C resulted in poor amplification of the YAP insert. As a result, 59°C was selected as the optimal annealing temperature for MPIII. For MPIV, an optimal annealing temperature of 50°C was calculated, and therefore a range of 50–56°C was tested, in 2°C increments. An annealing temperature of 52°C for MPIV resulted in a reduction in the appearance of artifacts in the male samples, and was thus selected for MPIV. For MPV, an optimal annealing temperature of 61°C was calculated, and therefore a range of 59–61°C was tested, in 2°C increments. Increasing or decreasing the annealing temperature for MPV from 61°C resulted in poor amplification of the yellow and red channels. An annealing temperature of 61°C was therefore selected for MPV.

Cycle Number—MPIII, MPIV, and MPV were amplified successfully with all cycle numbers tested. It was determined that 32 cycles were sufficient for MPIII and MPV; however, MPIV required 34 cycles selected to amplify all 21 loci efficiently.

Primer Concentration—Individual primer concentrations were adjusted in order to obtain sufficient peak balance across all loci contained within the multiplex. Once the optimal concentrations were determined, the use of a lower concentration of most primers resulted in a decrease in signal intensity, and in some cases a complete absence of signal (data not shown). The use of a higher concentration of most primers resulted in an increase in stutter and artifact production, particularly with the MPIV loci (data not shown).

Potential for Differential, Preferential, and Stochastic Amplification

Y-STR loci are hemizygous in nature, with only one allele observed for most individual loci. As a result, a false ''homozygous'' classification due to allelic drop-out is not an issue. However, for the bi-local (DYS527) and tetra-local (DYS464) loci contained in MPIV and the bi-local locus (DYS459) in MPV, where the potential for ''false homozygous'' classifications is possible, no instance of allelic drop-out was observed throughout this study.

Stutter

Stutter is a commonly observed PCR artifact when amplifying microsatellite repeats. Attributed to DNA polymerase slippage during the amplification process, stutter typically appears as a peak one repeat unit less in size than the true allele peak (32). The appearance of stutter may confound mixture interpretation due to the fact that a stutter peak is found in a true allele position. The ability to distinguish between stutter and true allele peaks is critical in order to resolve mixtures of DNA from two or more individuals. Loci with stutter $<15\%$ are suitable for forensic use. The average percent stutter was calculated for all loci in MPIII, MPIV, and MPV using 3 ng input DNA (Tables 1–3). Stutter was more prevalent with the MPIII Y-STR loci, with stutter averages ranging from 3% to 10%. For MPIV, only three of the 21 loci had any observed stutter peaks (DYS458, DYS557, DYS449). DYS458 had the highest calculated stutter (12%) for MPIV. DYS458 is labeled with VIC, and a dye blob artifact is consistently observed within the allele range for this locus and this may contribute to the enhanced stutter. For the MPV loci, the average stutter ranged from 0% to 6%.

Effects of Coamplification

To determine whether coamplification of the Y-STR loci would produce any differences in detected allele size or produce any nonspecific amplification, each locus was amplified in a monoplex reaction, followed by subsequent amplification in a multiplex reaction. All detected sizes for the 43 loci were identical between the monoplex and multiplex reactions. Any artifacts produced within the multiplex reaction were addressed during the developmental stages and largely eliminated from the optimized reactions. In MPIII, an artifact produced at *c*. 343 bp in the yellow channel, arising from an interaction of the primers for DYS442 and another primer from a different locus (unknown), could not be eliminated despite primer redesign, primer relabeling, and optimization of standard reaction components and PCR conditions. This artifact was not observed in any of the monoplex reactions and does not fall within any of the allele ranges for the loci.

Positive and Negative Controls

Two male samples were selected as positive controls throughout the validation of both multiplex systems. One of the male controls (GM9948) was obtained commercially (Promega, Madison, WI) (33), whereas the other male positive control was obtained from a male volunteer whose Y-STR haplotype had been well characterized in the initial developmental stages of the multiplex systems.

Various negative controls were used throughout the validation, including a female DNA control sample for specificity (300 ng used), a reagent blank from each set of extractions performed, and an amplification blank. No amplification was observed in any of the negative controls throughout the validation.

Reproducibility

Sets of samples were given to two experienced analysts to obtain genetic profiles independently for all three multiplexes used in the study. All samples were amplified and detected with the same instruments. For MPIII, DNA extracted from five buccal swabs was given to two experienced analysts within the laboratory and identical haplotypes were obtained for all samples. Maximum differences in allele sizes were calculated and ranged from 0.0 to 0.4 bases for individual loci. For MPIV, DNA extracted from five bloodstains was given to two experienced analysts and identical multilocus profiles were obtained. Maximum differences in allele size were calculated and ranged from 0.0 to 0.3 bases. For MPV, DNA extracted from five bloodstains was given to two experienced analysts and identical multilocus profiles were obtained. Maximum differences in allele size were calculated and ranged from 0.0 to 0.2 bases. There was no correlation between the magnitude of the above differences and amplicon size or locus structure for any of the multiplexes.

Precision

The precision of the detection system was evaluated by replicate measurement of the same sample (GM9948) 20 times by means of capillary electrophoresis. The average standard deviation was

TABLE 4—*Primate DNA cross-reactivity. For those amplification products falling with allele ranges, the loci that these products were observed in are listed below the number of products for each primate sample.*

	Male chimp	Male gorilla	Male orangutan	Male crab-eating macaque	Male spider monkey	Female orangutan	Female gorilla
MPIII							
Amplification products within allele ranges	4 DYS426 DYS436 DYS462 DYS442	3 DYS462 DYS441 DYS442	2 DYS462 DYS442	0	1 DYS442	0	0
Amplification products outside allele ranges	3 326.7 (B) 132.5 (G) 343.5 (Y)	1 196.8 (Y)	0	0	0	0	0
MPIV							
Amplification products within allele ranges	3 DYS458 DYS453 DYS464	1 DYS522	2 DYS449 DYS464	0	2 DYS458 DYS557	0	0
Amplification products outside allele ranges	3 151.2 (B) 112.5 (G) 230.8 (G)	2 287.2 (B) 288.5 (G)	0	0	0	0	0
MPV							
Amplification products within allele ranges	4 DYS576 DYS476 DYS549 DYS488	1 DYS607	1 DYS476	0	0	1 DYS513	1 DYS488
Amplification products outside allele ranges	5 133.4 (B) 142.8 (B) 129.1 (G) 162.7 (G) 144.2 (R)	3 127.1 (G) 131.1 (G) 207.2 (Y)	1 159.4 (G)	0	1 176.0 (R)	2 119.6 (B) 137.5 (Y)	2 128.9 (G) 308.2 (R)

(B), blue channel; (Y), yellow channel; (G), green channel; (R), red channel.

For those amplification products outside of allele ranges, the size of the amplification product (bp) is provided, as well as the channel it appears on in each multiplex.

calculated for each individual locus. For MPIII, the standard deviation values ranged from 0.05 to 0.11 bases. For MPIV, the standard deviation ranged from 0.03 to 0.24 bases, except DYS444 (0.38) and a DYS464 allele (0.30). For MPV, the standard deviation values ranged from 0.05 to 0.10 bases, with the exception of DYS575 (0.45). The reason for the significantly higher variation at some loci is unknown but may be reflective of allele sequence heterogeneity (32) or may be due to reduced precision of size estimates of larger alleles at larger-sized STR loci (41).

Species Specificity

The species specificity of each multiplex was checked by amplifying DNA from five bacterial species, several male animal species, and various male and female primate samples.

There were no observed MPIII, MPIV, and MPV amplification products for any of the five bacterial species tested: *Esherichia. coli*, *Staphylococcus aureus*, *Streptococcus Pyogenes*, *Enterbacter aerogenes*, and *Psedomonas. aeruginosa*.

Several animal species were also tested, including dog, cat, sheep, horse, and bull. MPIII, MPIV, and MPV all failed to produce any detectible amplification products from these samples.

Several primate samples, both male and female, were also tested as there is a high-degree Y chromosome sequence homology between humans and primates. The primates tested included: male—chimpanzee, gorilla, orangutan, crab-eating macaque, and spider monkey; female—lowland gorilla, orangutan. There was no detectible product for either of the female primates used in this study for either MPIII or MPIV. However, for MPV, amplification was observed for the female orangutan and the female gorilla (Table 4). These products were found both within and outside of designated allele ranges.

All of the male primates tested, with the exception of the crab-eating macaque, produced amplification products with MPIII, MPIV, and MPV (Table 4). The male chimpanzee sample, in particular, produced 22 amplified products using MPIII, MPIV, and MPV. Of these products, 11 fell within allele ranges determined for human DNA samples. Amplification of the male gorilla sample produced seven detectible products using MPIII, MPIV, and MPV as well, five of which fell within human allelic size ranges. The male orangutan and spider monkey samples produced fewer amplified products with all three multiplexes. These results are consistent with evolutionary studies that show that chimpanzees are the closest relatives, having diverged from humans 6 M years ago (42,43). Thus, the chimpanzee demonstrated more cross-reactivity products than the other nonhuman primates tested. Similarly, the gorilla has diverged from humans more recently than the orangutan and spider monkey. This is again reflected in the results obtained in that there were less cross-reactivity products observed with gorilla compared with chimpanzee, and then less observed for orangutan and spider monkey compared with gorilla and chimpanzee.

Sensitivity and Specificity

Sensitivity (Male)—Novel multiplex PCR systems developed for potential forensic use need to be able to produce a profile with subnanogram quantities of template DNA. To test the sensitivity of MPIII, MPIV, and MPV, various amounts of template male DNA were amplified, ranging from 25 pg (~ 4 diploid cells) to 100 ng. The optimal quantity of input DNA was determined to be 1–3 ng for MPIII (Fig. 1*A*, left panel), 1–4 ng for MPIV (Fig. 1*B*, left panel), and 1–3 ng for MPV (Fig. 1*C*, left panel). Representative electropherograms of each multiplex, using 1 ng of input DNA, have been provided for comparison with those profiles resulting from amplification of small amounts of input DNA. The sensitivity limit of MPIII and MPIV was determined to be at least 25 pg (Fig. 1*A* and 1*B*, right panels), with full multilocus haplotypes being detected using a 75 RFU peak detection threshold. Amplification of 25 pg for MPIII still resulted in relatively balanced peak heights (Fig. 1*A*, right panel). DYS426 demonstrated a reduced peak height (152 RFUs) in comparison with the rest of the loci (~ 300–2000 RFUs). Amplification of 25 pg for MPIV also produced relatively balanced peak heights (Fig. 1*B*, right panel). DYS557 and DYS463 were the least sensitive loci in the multiplex (183 and 125 RFUs, respectively). Owing to the reduced peak heights with the low-input DNA used for MPIV, the vertical scale was lowered in order to allow for better observation of the alleles. A "noisy" baseline can thus be observed, which would be reduced or eliminated if the vertical scale were adjusted. This adjustment of scale was not made in order to allow for observation of all loci within the multiplex. The sensitivity limit of MPV was determined to be 50 pg (Fig. 1*C*, right panel), with full haplotypes being detected using a 100 RFU peak detection threshold. When 25 pg was amplified with MPV, DYS590 and DYS598 were not detectible (data not shown). It can be seen from Fig. 1*C* (right panel) that these two loci demonstrated reduced peak heights compared with the other loci in the multiplex when 50 pg of input DNA was amplified. Additionally, for MPIII sensitivity, a YAP − and a YAP+ sample were tested in order to determine whether amplification of the ~ 400 bp insertion would be detectible with small quantities of template DNA. Both the YAP − (not shown) and YAP+ (Fig. 1*A*, right panel) samples produced full profiles with 25 pg. Detector saturation and associated artifacts were obtained with all three multiplexes when > 10 ng of input DNA was used.

Specificity (Female)—A major benefit of using Y-STR multiplex systems is the specific amplification of male DNA present in a male/female DNA admixture, such as in samples recovered from sexual assault cases. One of the main objectives in designing a Y-STR multiplex is to include loci that preferentially amplify Y-chromosome sequences, minimizing or eliminating interference from the X-chromosome. Loci were chosen as candidates for inclusion in both multiplex systems only after BLAT searches and singleplex testing were performed to determine the extent of their homology with the X chromosome (29).

To assess the ability of each multiplex system to target male DNA specifically, various amounts of female input DNA were amplified using MPIII, MPIV, and MPV. No significant female DNA amplification products were observed up to 300 ng of female DNA with either MPIII or MPIV (data not shown). However, a few artifacts of low signal intensity (< 200 RFUs) were observed with increased quantities of female DNA (> 300 ng), although most were nonspecific amplification products not falling within the allelic size ranges for any of the loci (data not shown). For MPV, no significant female DNA amplification products were observed up to and including 1 μg (data not shown).

Stability Studies

Recovery of Identical Multilocus Haplotypes from Various Body fluids—The ability of MPIII, MPIV, and MPV to produce identical multilocus haplotypes for DNA extracted from different

TABLE 5—*Recovery of DNA profiles from environmentally impacted blood stains.*

	3 Days	1 Week	1 Month	3 Months	6 Months	1 Year	> 1 Year
HLH							
Amount amplified (μL)	7 (7%)	7 (7%)	7 (7%)	7 (7%)	7 (7%)	7 (7%)	7 (7%)
MPIII	+	+	+	−	−	−	NA
MPIV	+	+	+	−	−	−	NA
MPV	+	+	+	−	−	−	NA
HLHR							
Amount amplified (μL)	7 (7%)	7 (7%)	7 (7%)	7 (7%)	7 (7%)	7 (7%)	7 (7%)
MPIII	+	+	+	−	−	−	NA
MPIV	+	+	+	−	−	−	NA
MPV	+	+	+	−	−	−	NA
UV							
Amount amplified (ng)	3	3	3	3	3	3	3
MPIII	+	+	+	+	+	NA	+
MPIV	+	+	+	+	+	NA	+
MPV	+	+	+	+	+	NA	+
RTED							
Amount amplified (ng)	3	3	3	3	3	3	3
MPIII	+	+	+	+	+	+	NA
MPIV	+	+	+	+	+	+	NA
MPV	+	+	+	+	+	+	NA

Fifty microliter bloodstains were extracted using a standard phenol:chloroform method and purified using Centricon 100™ concentrators. DNA was quantitated was estimated using ethidium bromide induced fluorescence on 1% agarose yield gels. The yield of DNA for the HLH (exposed to heat, light, and humidity) and the HLHR samples (exposed to heat, light, humidity, and rain) was below the detection limits of the system (< 1 ng/μL in a total extract volume of 100 μL). Seven microliters of the extract was used for amplification. The yield of DNA for the UV (exposed to UV-light) and RTED samples (stored at room temperature) was above the detection limits of the quantitation system and therefore the amount of input DNA (ng) is provided.

+ full profile obtained.

− no profile obtained.

NA, not available for testing; MP, multiplex.

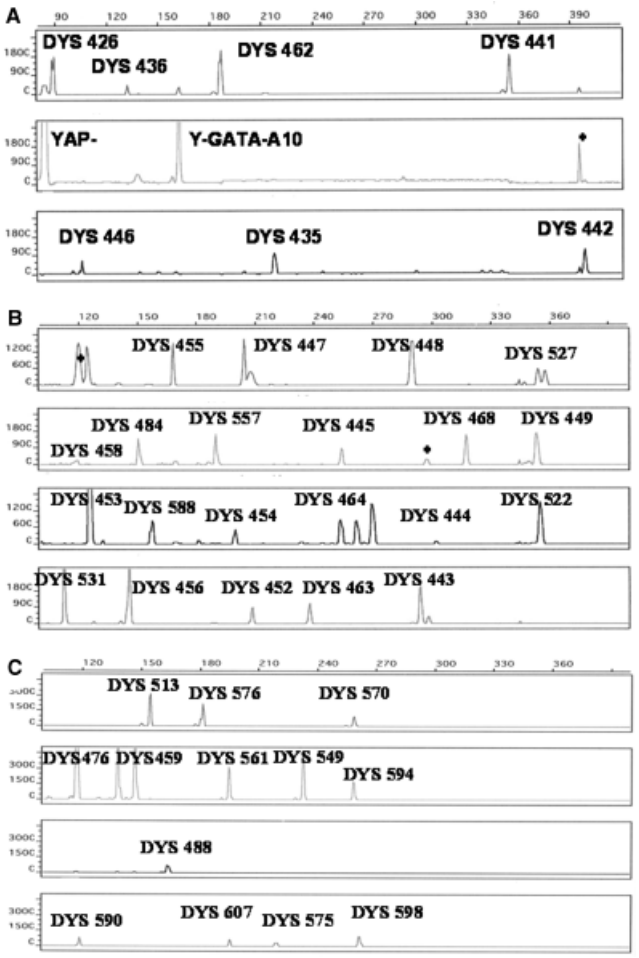

FIG. 2—*Stability of environmentally impacted blood samples. Full nine-locus, 21-locus, and 13-locus male haplotypes for MPIII (A), MPIV (B), and MPV (C) obtained from blood samples that had been exposed to heat, light, humidity, and rain for 1 month.* *Represent nonspecific amplification products.

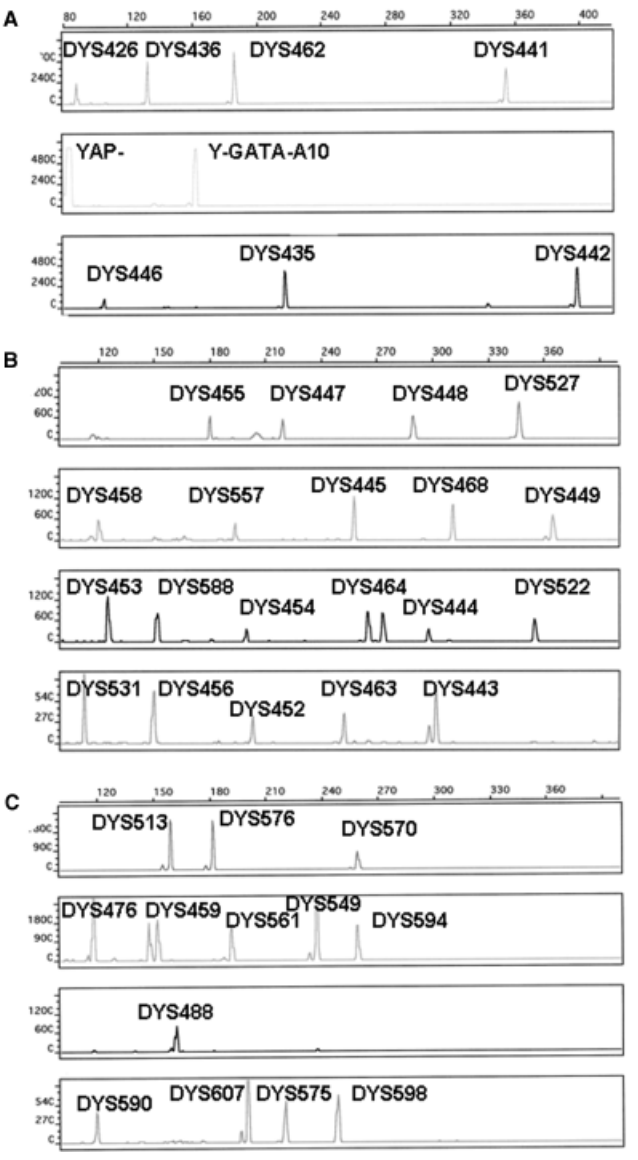

FIG. 3—*Male/female admixed DNA samples. Full nine-locus, 21-locus, and 13-locus male haplotypes for MPIII (A), MPIV (B), and MPV (C) obtained at a male/female DNA ratio of 1/1000.*

body fluids from the same individual was evaluated by typing blood, semen, and saliva from the same male. Within the tissues from the same male, identical haplotypes were obtained for all three multiplexes (data not shown).

Environmentally Impacted Blood Samples—For a multiplex system to be useful in forensic casework, it must permit the recovery of DNA profiles from samples that have been exposed to various environmental conditions. To assess the ability of MPIII, MPIV, and MPV to recover Y-STR haplotypes from environmentally compromised samples, bloodstains originating from the same male individual were exposed to different environmental conditions including various temperatures, light sources, and environmental influences including humidity and rain. For this study, samples were grouped into four categories: (1) dried blood stains RTED; (2) dried blood stains left outside and uncovered (HLHR), exposed to heat, light, humidity, and rain; (3) dried blood stains exposed to UV light (UV); and (4) dried blood stains left outside and covered (HLH), exposed to heat, light, and humidity. Samples were left exposed to these conditions and collected at various time intervals including 3 days, 1 week, 1 month, 3 months, 6 months, 1 year, and 18 months (available only for the UV exposure set). The results are summarized in Table 5.

For the dried blood stains RTED, full profiles for MPIII, MPIV, and MPV were recovered for all samples up to and including the 1-year samples, with no observed reduction in allelic signal intensities as the time interval increased. Full profiles were also obtained using MPIII, MPIV, and MPV for the samples exposed to UV light, up to and including the 18-month samples (data not shown).

The HLHR (exposed to heat, light, humidity, and rain) and HLH (exposed to heat, light. and humidity) samples were placed in an outdoor, unwooded area in Central Florida. During the course of this study (1 year), these samples were exposed to temperatures ranging from 32°F to 94°F (average high of 88°F and average low of 37°C) and exposed to rain fall on 137 days out of the year (3-day samples received 1 day of rain; 1-week samples received 1 day of rain; 6-month samples received 73 days of rain; and the 1-year sample received 137 days of rain). For MPIII, MPIV, and MPV, full profiles were obtained from the HLHR (Figs 2*A–C*) and HLH (data not shown) samples for up to 1 month of exposure. These results were somewhat expected as heat, light, humidity, and rain are extremely detrimental to DNA recovery and subsequent analysis.

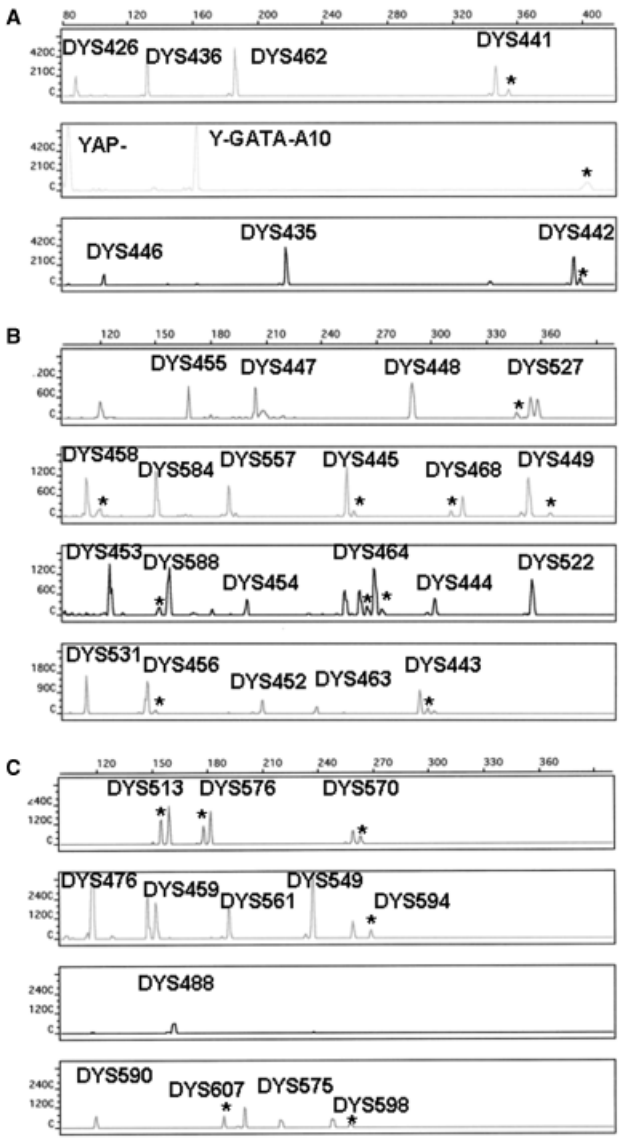

FIG. 4—*Male/Male admixed DNA samples. Two full 9-locus, 21-locus, and 13-locus male haplotypes for MPIII (A), MPIV (B), and MPV (C) obtained at a male/male DNA ratio of 1/6. When multiple alleles are present at an individual locus, the alleles originating from the minor male contributor are indicated with a *. Loci with no (*) indicate loci where a common allele was shared between the two males.*

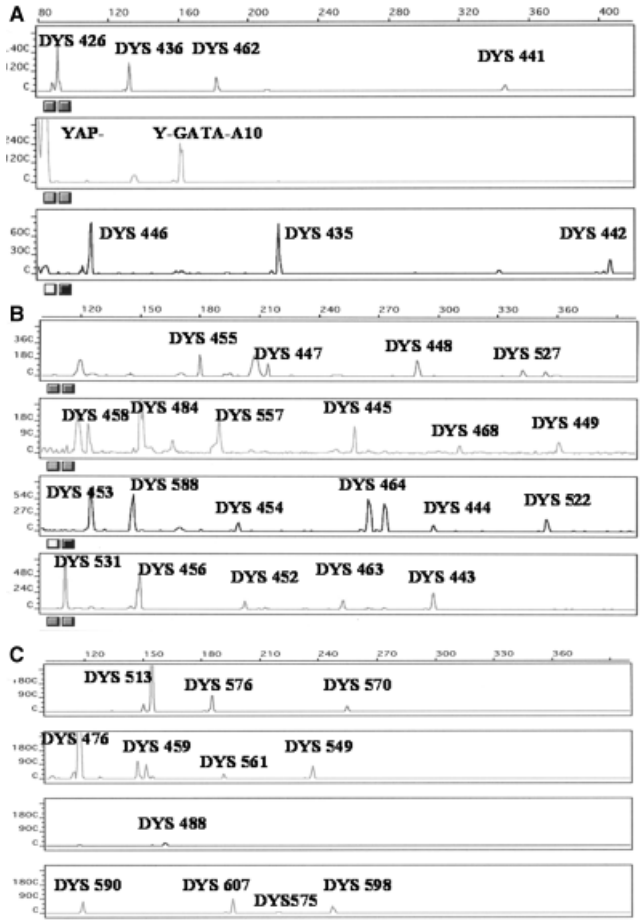

FIG. 5—*Nonprobative casework samples. Full MPIII (A), MPIV (B), and MPV (C) profiles obtained from male DNA recovered from a smoked cigarette butt.*

Mixture Studies

Male/Female—To test the ability of MPIII, MPIV, and MPV to determine the genetic profile of the male donor in the presence of large quantities of female DNA, a situation akin to that found in *bona fide* forensic casework, a series of samples were prepared in which 3 ng of male DNA was admixed with increasing amounts of female DNA and amplified in a single reaction. For all three multiplexes, full male haplotypes were obtained from the admixed samples when the male component comprised 1/2, 1/10th, 1/100th, 1/500th, and 1/1000th (Fig. 3*A*, 3*B*, and 3*C*) of the total. These experiments demonstrate that even in the presence of a significant amount of female DNA, it is possible, using MPIII, MPIV, and MPV, to recover a full Y-STR profile of the male contributor.

Male/Male—Often in forensic casework, more than one male contributor can be found in a sample. Using autosomal STR analysis, mixture interpretation becomes increasingly more difficult when the number of contributors exceeds two. As Y-STRs are inherited in a hemizygous manner (i.e., with only one allele found at most loci), determining the number of male donors in a sample is made relatively facile.

In order to assess the feasibility of detecting multiple male contributors in the same sample, and also to determine the limits of the detection of the minor component, male/male DNA admixtures from two individuals were prepared at various ratios (1/2, 1/3, 1/6, 1/12, and 1/30). For MPIII, two male profiles were detectable in the 1/2, 1/3, 1/6 (Fig. 4*A*), and 1/12 samples. No minor component profile was detected in the 1/30 sample (data not shown). The two males, using MPIII, exhibited different alleles for DYS441, YAP, and DYS442. They shared common alleles at all other loci. For MPIV, two male profiles were detectable in the 1/2, 1/3, and 1/6 (Fig. 4*B*) samples. No minor component profile was detected in the 1/12 or 1/30 samples (data not shown). MPIV contains one bi-local and one tetra-local locus, DYS527 and DYS464, respectively. Owing to multiple, overlapping alleles, it is more difficult to determine the number of donors using these loci, and their use for this purpose is not recommended. For MPV, two male profiles were detectable in the 1/2, 1/3, 1/6 (Fig. 4*C*), and 1/12 samples. No minor component profile was detected in the 1/30 sample (data not shown).

Three male donors could be discerned by the presence of three alleles at a single locus (excluding the multicopy loci). While not

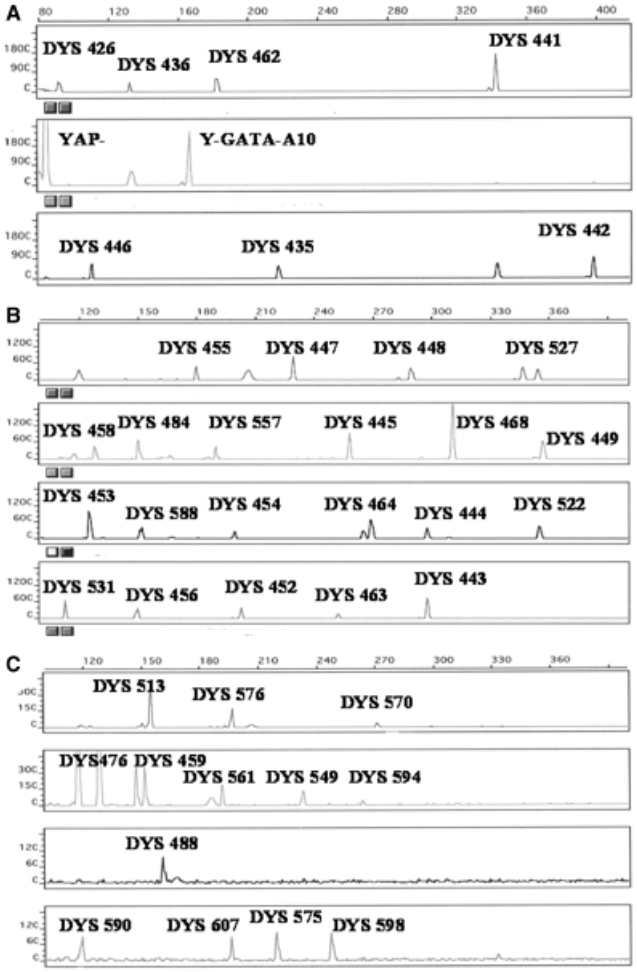

FIG. 6—*Postcoital cervicovaginal samples. Full MPIII (A) and MPIV (B) profiles recovered 48 h after intercourse, and a full MPV (C) profile recovered 72 h after intercourse.*

specifically tested in this study, previous studies with additional Y-STR multiplexes have demonstrated the ability to discern three male contributors (4,5).

Case-Type Samples

Recovery of a Male Y-STR Profile from a Cigarette Butt—Often in forensic casework, limited amounts of DNA are recovered from evidentiary materials. Thus, it is important to demonstrate that a novel multiplex system is capable of obtaining genetic profiles from minute quantities of DNA. A male participant provided a smoked cigarette butt from which DNA was isolated (<1 ng/μL in a total extract volume of 100 μL). Five microliters of the extract was used for amplification. A full nine-locus, 21 locus, and thirteen locus haplotype was recovered from the cigarette butt using MPIII, MPIV, and MPV, respectively (Fig. 5*A*–5*C*). Owing to the reduced peak heights with the low-input DNA used, the vertical scale was lowered in order to allow for better observation of the alleles. A ''noisy'' baseline can thus be observed particularly for MPIV, which would be reduced or eliminated if the vertical scale were adjusted. This was not performed in order to allow for observation of all loci within the multiplex.

Postcoital Cervicovaginal Samples—A series of postcoital cervicovaginal samples were collected from a female donor 0 to 72 h (in 12 h intervals) after intercourse. The participant was instructed to carry out normal daily functions to allow for regular drainage and degradation to simulate most accurately the type of samples encountered in forensic casework. Owing to the much greater discriminatory power compared with Y-STRs, most forensic laboratories in the first instance would perform autosomal STR analysis with postcoital cervicovaginal swabs. This necessitates an attempt at separating the sperm and nonsperm cells by a differential lysis method (26). Subsequent Y-STR testing would often use these separated cell fractions. Using a differential extraction, the sperm and nonsperm DNA fractions were separated. For the sperm fractions, *c*. 3–5 ng of male template DNA were as amplified, and for the non-sperm fractions *c*. 100 ng of template DNA was amplified. For both MPIII and MPIV, full profiles were recovered from the sperm fractions of the 0-, 12-, 24-, and 48-h samples (Figs. 6*A* and *B*). For MPIII, a six-locus partial profile was recovered from the 60-h sperm fraction and no profile was recovered from the 72-h sperm fraction (data not shown). For MPIV, no profiles were recovered for either the 60 or 72-h sperm fractions. For MPV, full profiles were obtained for the 0-, 12-, 24-, 48-, and 72-h samples (Fig. 6*C*). (No 60-h sample was available for MPV analysis.) Even though the limit of detection was not reached for MPV (full profile at 72 h), no additional samples beyond 72 h were available for testing. Typically in postcoital samples, the recovery of male profiles at extended intervals (60–72 h postcoitus) is difficult because very few sperm may be remaining in the vaginal canal and those that are present may be in an extremely fragile state. As a differential lysis procedure may result in premature lysis of the sperm fraction into the nonsperm fraction, the nonsperm fraction from each of the samples was amplified to determine whether the sperm component could be detected. No detectible products were recovered from the nonsperm fractions using MPIII or MPIV (data not shown). For MPV, a full male profile was detected in the female fraction for the 24-h sample.

In a differential lysis procedure, there is a potential to lose the sperm component completely due to the physical manipulations during the extraction process itself. As no profile was recovered using either MPIII or MPIV for the 72-h sample, a nondifferential extraction was performed in order to reduce the amount of physical manipulation of the samples to determine whether the sperm component could be recovered. However, no profiles were recovered from the 72-h sample after performing the nondifferential extraction.

Population Studies

For a Y-STR multiplex system to be useful in forensic casework, it needs to be able to discriminate effectively between unrelated males, and the frequency of occurrence of any multilocus haplotype should be as small as possible. In order to evaluate the efficacy of the 43 ''noncore'' Y-STR loci contained in MPIII, MPIV, and MPV for individual discrimination, a limited population study was conducted. For this, 98 Caucasian and 100 African American samples were tested.

Individual Loci Gene Diversity—The gene diversity (h) for each locus is shown in Fig. 7. The striped bars represent values for the Caucasian population and the solid black bars represent values for the African American population. Twenty-seven of the loci possessed an h of >0.5 for at least one of the populations. Six of the loci were exceptionally discriminating in that they possessed an $h>0.8$ in at least one of the populations. DYS464, the tetra-local locus in MPIV, has the highest h value known for a Y-STR (>0.9). Interpretation of DYS464 profiles from samples containing

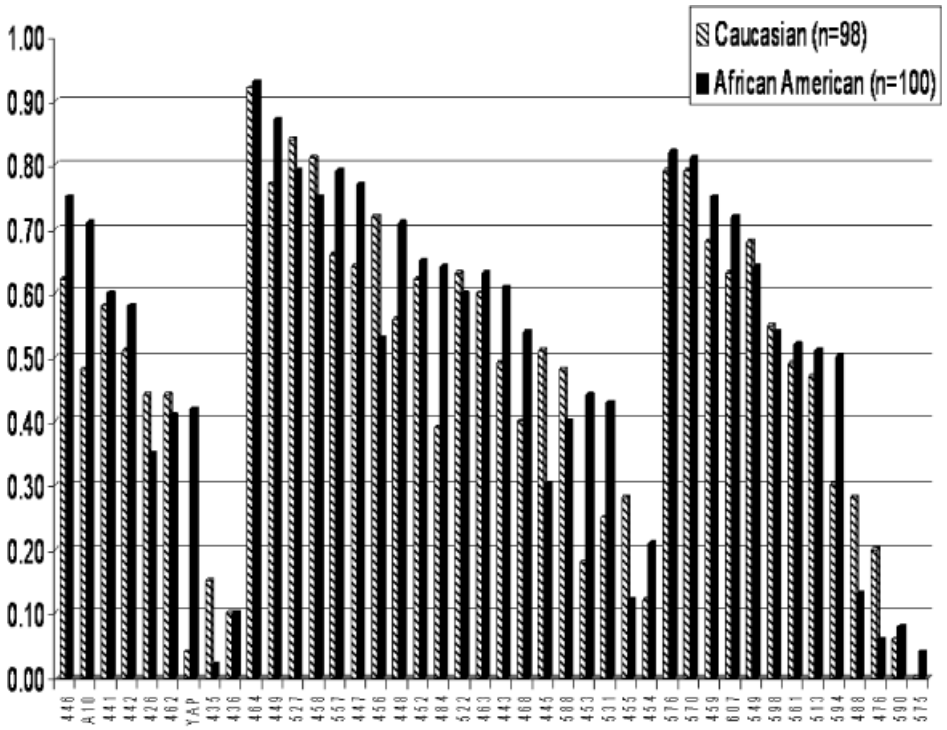

FIG. 7—*Gene diversity: graphic representation of the gene diversity values for the 43 loci contained in MPIII, MPIV, and MPV. The x-axis lists the loci and the y-axis represents the gene diversity values. Diversity values are provided for the Caucasian population (striped bars) and for the African American population (black bars).*

male–male admixtures or degraded DNA is difficult and its use for such samples is not recommended (14).

Allele Frequency Distribution—The allele frequency distributions for the Y-STR loci that possessed $h > 0.8$ values are graphically illustrated in Fig. 8*A*, for both the Caucasian (striped bars) and African American (solid bars) populations. DYS459, DYS464, and DYS527 are graphed according to multiallelic haplotypes rather than individual alleles as these are multicopy loci (Fig. 8*B*). The bi-allelic marker, YAP or DYS287, was also included for comparison purposes as it displays a high degree of genetic differentiation between the two populations and is uniquely coamplified in a Y-STR system (Fig. 8*A*). A majority of individuals in the Caucasian population do not possess the insertion. In contrast, a majority of individuals in the African American population do possess the insertion, with significantly less individuals not possessing the insertion. This observation is supported by numerous evolutionary studies that have examined the origins of the YAP insertion (39,44–48). The YAP is a unique event polymorphism that characterizes Groups D and E lineages, common among individuals of African and Asian descent (49).

The multiplex systems were designed such that the alleles of identically dye-labeled loci do not overlap. However, it is recognized that some of the allele ranges are in close proximity (e.g., DYS453 and DYS588 in MPIV differ by 10 bp) and it is possible that, with more population data, some rare or population-specific alleles will be discovered that do overlap other loci. In such cases, overlap is readily detected by the presence of a higher than normal number of alleles at a contiguous locus with the concomitant absence of an allele at the affected locus. In such cases, the sample would have to be reanalyzed using appropriate monoplex reactions.

Multilocus Haplotype Diversity—The discriminatory capacity of MPIII, MPIV, and MPV was 0.62, 1.0, and 0.94 for the African American and 0.61, 1.0, and 0.97 for the Caucasian populations, respectively. The number of unique haplotypes observed among the 198 samples was 114, 198, and 174 for MPIII, MPIV, and MPV, respectively.

MPIV was the most efficacious multiplex in discriminating unrelated Caucasian and African American males, followed by MPV and, lastly, MPIII. The 100% discrimination afforded by MPIV indicates its superb potential for casework analysis as an adjunct to the core Y-STR loci. The other two multiplexes could be used to provide additional discrimination power when needed.

Concluding Remarks

Three Y chromosome STR multiplex systems allowing for the co-amplification of eight (+YAP) (MPIII), 21 (MPIV), and thirteen (MPV) "noncore" Y-STRs, respectively, have undergone a full developmental validation according to revised guidelines proposed by the Scientific Working Group on DNA Analysis Methods. Rigorous performance checks on the optimized systems and an evaluation of the systems' abilities to discriminate between unrelated males have demonstrated their potential forensic casework utility.

There are two reasons why it may be necessary to use additional Y chromosome markers beyond the core set. The first is to provide additional discriminatory power to attempt to distinguish between two male suspects who share the same core loci haplotype. This sharing could be due either to pure coincidence or to the males (whether they know it or not) being close biological relatives of one another such as an uncle–nephew or brothers. Additional Y chromosome loci testing would help confirm the former (by discriminating the two individuals) or the latter (by failing to discriminate the individuals). The second reason for more markers is, akin to the philosophy underpinning autosomal STR typing, to provide stronger statistical support for the evidence sample and the known sample (or a biological male relative) having a common origin, due to the increased rarity of a coincidental match between them when additional markers are used. It is

hoped that studies such as these described herein will provide suitable candidate loci for possible inclusion into second-generation noncore loci commercial kits, so that the additional discriminatory power be made readily available to the forensic community.

Uniquely, a binary indel marker (YAP) is incorporated into one of the multiplex systems (MPIII). Such unique event polymorphisms (UEPs), including Y-SNPs, offer a number of advantages including the potential to determine the putative ethnogeographic ancestry of an unknown donor and also provide, through

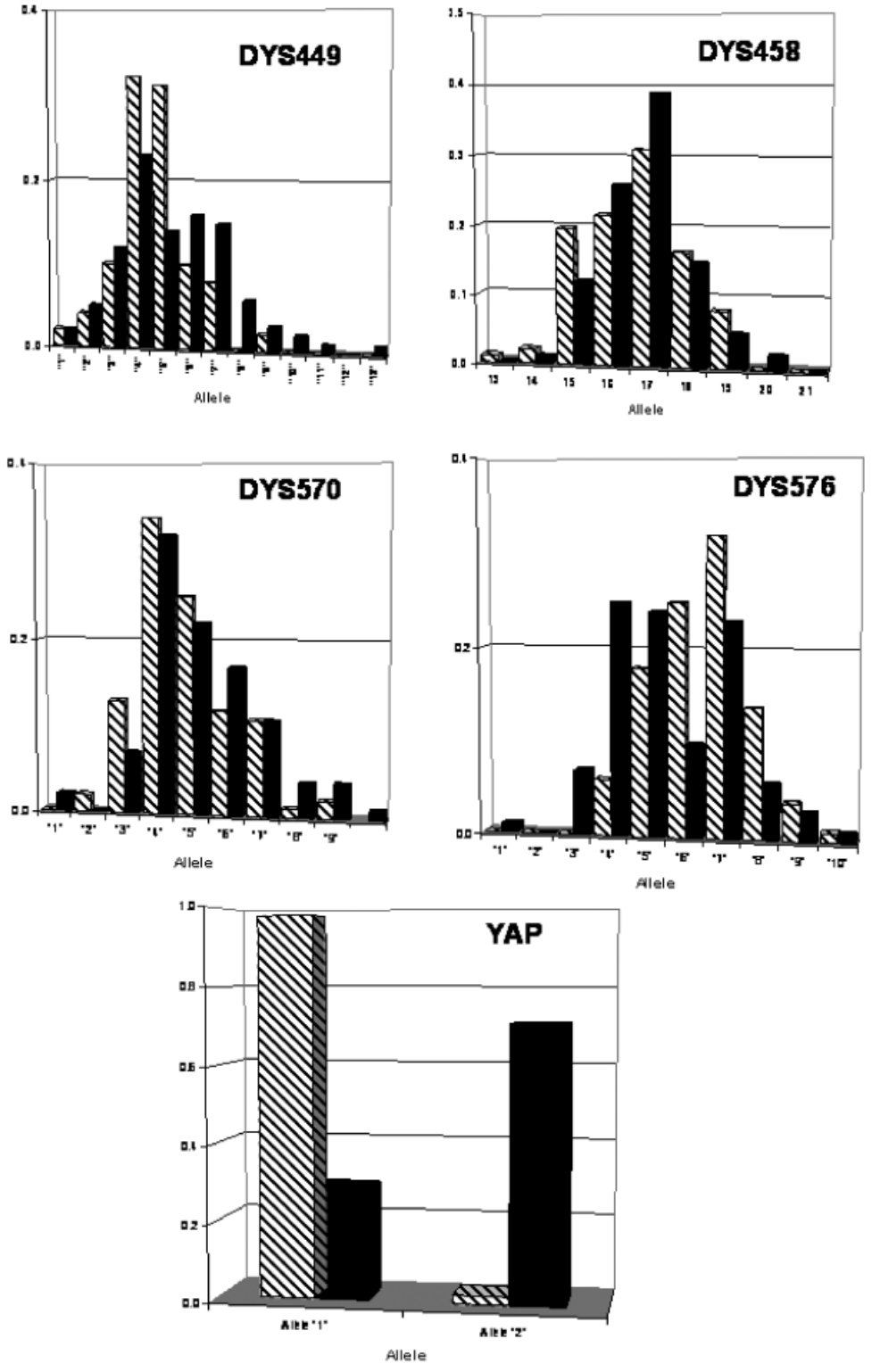

FIG. 8—*Allele frequency distributions: allele frequency distributions of single-copy loci with diversity values greater than 0.70 in at least one population (A) and multicopy loci (B) in the Caucasian population (n = 98, striped bars) and in the African American population (n = 100, black bars). For each distribution graph, the allele designations for the single-copy and nominal genotype designations for the multicopy loci are listed along the x-axis, and the frequency of occurrence is listed along the y-axis.*

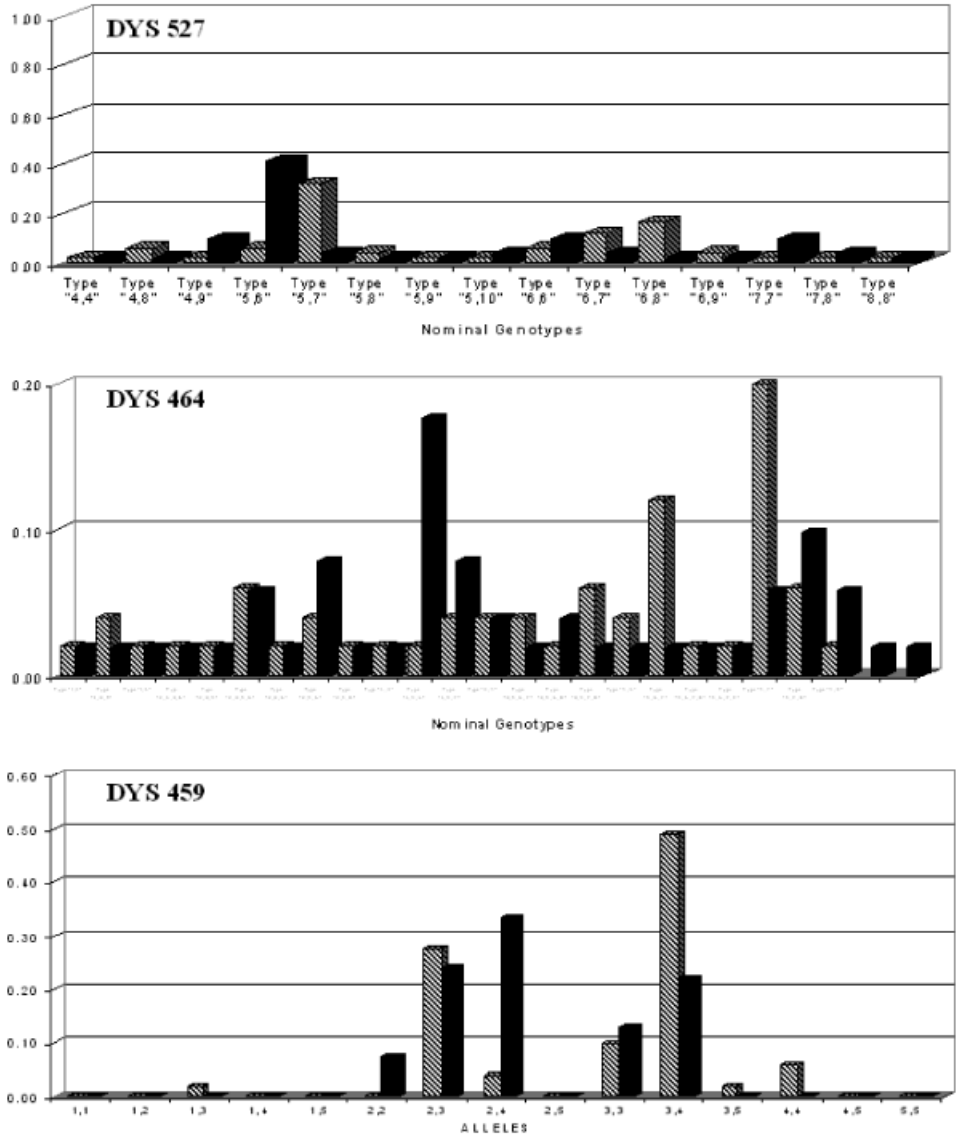

Fig. 8—Continued.

hierarchical haplogroup typing, the ability to discriminate subpopulations within these broader groups. Also, eliminating other haplogroups by UEP typing of necessity restricts the Y-STR haplotypes to that group and thus common Y-STR haplotypes that may be present in different, but closely related haplogroups, may be eliminated. This has the effect of increasing the discrimination afforded by Y-STR typing if combined with hierarchical Y-SNP typing. To this end, we are exploring the possibility of incorporating a number of Y-SNPs into other multiplex Y chromosome typing strategies.

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References

1. de Knijff P, Kayser M, Caglia A, Corach D, Fretwell N, Gehrig C, et al. Chromosome Y microsatellites: population genetic and evolutionary aspects. Int J Legal Med 1997;110(3):134–49.
2. Kayser M, Caglia A, Corach D, Fretwell N, Gehrig C, Graziosi G, et al. Evaluation of Y-chromosomal STRs: a multicenter study. Int J Legal Med 1997;110(3):125–9.
3. Corach DF, Filgueira Risso L, Marino M, Penacino G, Sala A. Routine Y-STR typing in forensic casework. Forensic Sci Int 2001;118(2–3):131–5.
4. Daniels DL, Hall AM, Ballantyne J. SWGDAM developmental validation of a 19-locus Y-STR system for forensic casework. J Forensic Sci 2004;49(4):668–83.
5. Hall A, Ballantyne J. The development of an 18-locus Y-STR system for forensic casework. Anal Bioanal Chem 2003;376(8):1234–46.
6. Kayser M, Brauer S, Willuweit S, Schadlich H, Batzer MA, Zawacki J, et al. Online Y-chromosomal short tandem repeat haplotype reference database (YHRD) for U.S. populations. J Forensic Sci 2002;47(3):513–9.
7. Prinz M, Ishii A, Coleman A, Baum HJ, Shaler RC. Validation and casework application of a Y chromosome specific STR multiplex. Forensic Sci Int 2001;120(3):177–88.
8. Prinz M, Sansone M. Y chromosome-specific short tandem repeats in forensic casework. Croat Med J 2001;42(3):288–91.
9. Roewer L, Krawczak M, Willuweit S, Nagy M, Alves C, Amorim A, et al. Online reference database of European Y-chromosomal short tandem repeat (STR) haplotypes. Forensic Sci Int 2001;118(2–3):106–13.

10. Sinha SK, Budowle B, Arcot SS, Richey SL, Chakrabor R, Jones MD, et al. Development and validation of a multiplexed Y-chromosome STR genotyping system, Y-PLEX 6, for forensic casework. J Forensic Sci 2003;48(1):93–103.
11. Ayub Q, Mohyuddin A, Qamar R, Mazhar K, Zerjal T, Mehdi SQ, et al. Identification and characterisation of novel human Y-chromosomal microsatellites from sequence database information. Nucleic Acids Res 2000;28(2):e8.
12. Butler JM, Schoske R, Vallone PM, Kline MC, Redd AJ, Hammer MF. A novel multiplex for simultaneous amplification of 20 Y chromosome STR markers. Forensic Sci Int 2002;129(1):10–24.
13. Iida R, Tsubota E, Sawazaki K, Masuyama M, Matsuki T, Yasuda T, et al. Characterization and haplotype analysis of the polymorphic Y-STRs DYS443, DYS444 and DYS445 in a Japanese population. Int J Legal Med 2002;116(3):191–4.
14. Redd AJ, Agellon AB, Kearney VA, Contreras VA, Karafet T, Park H, et al. Forensic value of 14 novel STRs on the human Y chromosome. Forensic Sci Int 2002;130(2–3):97–111.
15. Schoske R, Vallone PM, Kline MC, Redman JW, Butler JM. High-throughput Y-STR typing of U.S. populations with 27 regions of the Y chromosome using two multiplex PCR assays. Forensic Sci Int 2004;139(2–3):107–21.
16. White PS, Tatum OL, Deaven LL, Longmire JL. New, male-specific microsatellite markers from the human Y chromosome. Genomics 1999; 57(3):433–7.
17. Krenke BE, Viculis L, Richard ML, Prinz M, Milne SC, Ladd C, et al. Validation of a male-specific, 12-locus fluorescent short tandem repeat (STR) multiplex. Forensic Sci Int 2005;148(1):1–14.
18. Sinha SK, Nasir H, Gross AM, Budowle B, Shewale JG. Development and validation of the Y-PLEX 5, a Y-chromosome STR genotyping system, for forensic casework. J Forensic Sci 2003;48(5):985–1000.
19. SWGDAM. Revised validation guidelines. Forensic Sci Commun 2004;6(3):1–6.
20. Dai HL, Wang XD, Li YB, Wu J, Zhang J, Zhang HJ, et al. Characterization and haplotype analysis of 10 novel Y-STR loci in Chinese Han population. Forensic Sci Int 2004;145(1):47–55.
21. Iida R, Tsubota E, Matsuki T. Identification and characterization of two novel human polymorphic STRs on the Y chromosome. Int J Legal Med 2001;115(1):54–6.
22. Kayser M, Kittler R, Erler A, Hedman M, Lee AC, Mohyuddin A, et al. A comprehensive survey of human Y-chromosomal microsatellites. Am J Hum Genet 2004;74(6):1183–97.
23. Zhang J, Hou Y, Tang J, Li Y, Wu J. Haplotype frequencies for two new Y-STR loci in Chinese population. J Forensic Sci 2002;47(1):232–3.
24. Hanson EK, Ballantyne J. A highly discriminating 21 locus Y-STR ''megaplex'' system designed to augment the minimal haplotype loci for forensic casework. J Forensic Sci 2004;49(1):40–51.
25. Comey CK, Presely KW, Smerick JB, Sobieralski CA, Stanley DM, Baechtel FS. Extraction strategies for amplified fragment length polymorphism analysis. J Forensic Sci 1994;39:1254–69.
26. Gill P, Jeffreys AJ, Werrrett DJ. Forensic application of DNA 'fingerprints'. Nature 1985;318:577–9.
27. Kline MC, Duewer DL, Redman JW, Butler JM. NIST mixed stain study: DNA quantitation accuracy and its influence on short tandem repeat multiplex signal intensity. Anal Chem 2003;75(10):2463–9.
28. Hanson EK, Ballantyne J. Comprehensive annotated STR physical map of the human Y chromosome: forensic implications. Leg Med (Tokyo) 2006;8(2):110–20.
29. http://www.genome.ucsc.edu
30. Skaletsky H, Kuroda-Kawaguchi T, Minx PJ, Cordum HS, Hillier L, Brown LG, et al. The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. Nature 2003;423(6942):825–37.
31. Leclair B, Fregeau CJ, Bowen KL, Fourney RM. Systematic analysis of stutter percentages and allele peak height and peak area ratios at heterozygous STR loci for forensic casework and database samples. J Forensic Sci 2004;49(5):968–80.
32. Walsh PS, Fildes NJ, Reynolds R. Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA. Nucleic Acids Res 1996;24(14):2807–12.
33. Fregeau CJ, Aubin RA, Elliott JC, Gill SS, Fourney RM. Characterization of human lymphoid cell lines GM9947 and GM9948 as intra- and inter-laboratory reference standards for DNA typing. Genomics 1995;28(2): 184–97.
34. Nei M. Molecular evolutionary genetics. Columbia, NY: University Press, 1987.
35. Jobling MA, Samara V, Pandya A, Fretwell N, Bernasconi B, Mitchell RJ, et al. Recurrent duplication and deletion polymorphisms on the long arm of the Y chromosome in normal males. Hum Mol Genet 1996;5(11): 1767–75.
36. Thomas MG, Bradman N, Flinn HM. High throughput analysis of 10 microsatellite and 11 diallelic polymorphisms on the human Y-chromosome. Hum Genet 1999;105(6):577–81.
37. Bosch E, Lee AC, Calafell F, Arroyo E, Henneman P, de Knijff P, et al. High resolution Y chromosome typing: 19 STRs amplified in three multiplex reactions. Forensic Sci Int 2002;125(1):42–51.
38. Gusmao L, Alves C, Beleza S, Amorim A Forensic evaluation and population data on the new Y-STRs DYS434, DYS437, DYS438, DYS439 and GATA A10. Int J Legal Med 2002;116(3):139–47.
39. Hammer MF. A recent insertion of an alu element on the Y chromosome is a useful marker for human population studies. Mol Biol Evol 1994;11(5): 749–61.
40. http://www.gdb.org
41. Smith RN. Accurate size comparison of short tandem repeat alleles amplified by PCR. Biotechniques 1995;18(1):122–8.
42. Hughes JF, Skaletsky H, Pyntikova T, Minx PJ, Graves T, Rozen S, et al. Conservation of Y-linked genes during human evolution revealed by comparative sequencing in chimpanzee. Nature 2005;437(7055):100–3.
43. Li WH, Saunders MA News and views: the chimpanzee and us. Nature 2005;437(7055):50–51.
44. Bravi CM, Bailliet G, Martinez-Marignac VL, Bianchi NO. Origin of YAP+ lineages of the human Y-chromosome. Am J Phys Anthropol 2000;112(2):149–58.
45. Forster P, Rohl A, Lunnemann P, Brinkmann C, Zerjal T, Tyler-Smith C, et al. A short tandem repeat-based phylogeny for the human Y chromosome. Am J Hum Genet 2000;67(1):182–96.
46. Jobling MA, Pandya A, Tyler-Smith C. The Y chromosome in forensic analysis and paternity testing. Int J Legal Med 1997;110(3):118–24.
47. Weale ME, Shah T, Jones AL, Greenhalgh J, Wilson JF, Nymadawa P, et al. Rare deep-rooting Y chromosome lineages in humans: lessons for phylogeography. Genetics 2003;165(1):229–34.
48. Y-Chromosome-Consortium. A nomenclature system for the tree of human Y-chromosomal binary haplogroups. Genome Res 2002;12(2): 339–48.
49. Brion M, Sanchez JJ, Balogh K, Thacker C, Blanco-Verea A, Borsting C, et al. Introduction of an single nucleodite polymorphism-based ''Major Y-chromosome haplogroup typing kit'' suitable for predicting the geographical origin of male lineages. Electrophoresis 2005;26(23): 4411–20.

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