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SWGDAM Developmental Validation of a 19-Locus Y-STR System for Forensic Casework

ABSTRACT: A Scientific Working Group on DNA Analysis Methods (SWGDAM) developmental validation study was carried out on two Y-STR multiplex systems (MPI and MPII) that collectively permit the co-amplification of 19 Y-STR markers, including DYS393, DYS392, DYS391, DYS389I, DYS389II, Y-GATA-A7.2 (DYS461), DYS438, DYS385a and DYS385b (MPI); DYS425, DYS388, DYS390, DYS439, DYS434, DYS437, Y-GATA-C.4, Y-GATA-A7.1 (DYS460), Y-GATA-H.4, and DYS19 (MPII). Performance checks subsequent to PCR parameter optimization indicated that MPI and MPII were suitably reproducible, precise and accurate for forensic use. The sensitivity of the systems was such that a full 19-locus Y-STR profile was obtainable with 150–200 pg of male DNA, and some loci were detectable even with as little as 20–30 pg of input DNA. Primate specificity was demonstrated by the lack of cross-reactivity with a variety of commonly encountered bacterial and animal species, with the single exception of a monomorphic canine product that was outside of the size range of human alleles from any of the 19 loci. Not surprisingly, cross-reactivity was observed with a number of male and female nonhuman primates. Environmentally compromised samples produced full or partial Y-STR profiles. For example, a semen stain exposed to the outdoor elements for six months still gave a 13-locus Y-STR profile. Although a limited number of female DNA artifacts were observed in mixed stains in which the male DNA comprised 1/300 of the total, the full 19-locus male profile was easily discernible. Even at a 1500-to-2000-fold dilution of male DNA with female DNA partial Y-STR profiles were obtained. Furthermore, the potential utility of MPI and MPII for forensic casework is exemplified by their ability to dissect out the male haplotype in a variety of case-type samples, including, *inter alia*, post-coital vaginal swabs, admixed male and female bloodstains, the nonsperm fraction from a differentially extracted semen stain, and determination of the number of male donors in mixed semen stains.

KEYWORDS: forensic science, SWGDAM validation, multiplex Y-STR analysis, post-coital cervicovaginal samples, DYS19, DYS385, DYS388, DYS389, DYS390, DYS391, DYS392, DYS393, DYS425, DYS434, DYS437, DYS438, DYS439, Y-GATA-C4, Y-GATA-A7.1 (DYS460), Y-GATA-A7.2 (DYS461), Y-GATA-H4

Although autosomal short tandem repeat (STR) markers—the current loci of choice for the forensic analysis of biological evidence—are normally able to fully discriminate between unrelated individuals, there are several circumstances in which Y-STR polymorphisms are a useful adjunct to this technology. First, Y-chromosome-specific systems can enable the determination of the genetic profile of the male component in mixed male/female specimens in those instances in which the female portion is present in overwhelming quantities relative to the male, and where a differential extraction to separate sperm from nonsperm cells is not possible. This circumstance could be due to the deposition of semen by an azoospermic or oligospermic male, to cases of oral sodomy where only trace amounts of male buccal epithelial cells may be present, or to the normal post-coital degradative and sample loss processes that occur with the passage of time. Second, the hemizygous nature of Y-chromosome markers could facilitate determination of the number of semen donors in cases of multiple perpetrator rape. A third reason for employing Y-chromosome polymorphisms would be in disaster victim identification. The Y-chromosome haplotype of a missing individual may be determined by typing a male relative such as a son, brother, father, nephew, or uncle. Fourth, the ability to specifically detect a male profile could obviate the need for the time-consuming and ofttimes inefficient differential extraction procedure for the separation of sperm and nonsperm fractions. Finally, male-specific systems may aid the investigation of cases involving mixtures or close biological relatives by providing additional statistical discriminatory power.

Sample quantity limitations with forensic specimens require that candidate Y-STR loci be analyzed together in a parallel fashion by incorporating them into a multiplex polymerase chain reaction (PCR) assay format, the design and optimization of which is complicated by primer interactions and cross-reactivity with the evolutionarily related X-chromosome. Although more than 100 STR loci have been described on the Y-chromosome, a much more limited number have been appropriately evaluated for potential forensic use in multiplex formats (1–6). Even fewer of these Y-STR multiplex systems have undergone the extensive developmental validation exercises required by national standards prior to their use in forensic casework (7,8). Validation is the process by which the scientific community acquires the necessary information to assess the ability of a procedure to obtain reliable results, determine the conditions under which such results can be obtained, and define the limitations of the procedure (9). The validation process identifies aspects of a procedure that are critical and must be carefully controlled and monitored. Developmental validation, in contradistinction to a less comprehensive internal validation, precedes the usage by the general forensic community of a novel DNA procedure for forensic casework analysis. In addition to determining the accuracy, precision, and reproducibility of the method, species specificity, sensitivity, stability, population, PCR parameters and simulated casework

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studies are performed. Internal validation refers to the much less extensive study whose purport is to demonstrate a particular laboratory's capability in performing a previously established and already developmentally validated procedure (9).

In the present work we have sought to improve the discriminatory potential, and hence probative value, of multiplex Y-STR testing by developing an extended set of Y-chromosome STR loci available for forensic casework use. In accord with the requirements of a multiplex system developed for forensic use, we have attempted to maximize the number of loci capable of being co-amplified, ensure appropriate assay sensitivity (0.5–1 ng of input genomic DNA), balance inter-locus signals, and minimize confounding female DNA artifacts. Two systems have been developed, multiplex I (MPI) and multiplex II (MPII) (10,11), which allow for the robust co-amplification of 19 Y-STRs and their subsequent separation and detection using a standard capillary electrophoresis analytical platform. The loci include DYS19, DYS385 (a) and (b), DYS388, DYS389I and II, DYS390, DYS391, DYS392, DYS393, DYS425, DYS434, DYS437, DYS438, DYS439, Y-GATA-C4, Y-GATA-A7.1 (DYS460), GATA-A7.2 (DYS461), Y-GATA-H4. We report here the results of a comprehensive developmental validation study of the MPI and MPII systems in accordance with industry-wide standards. The unique biology of the Y-chromosome required the incorporation of additional performance checks on analytical specificity that are unnecessary or irrelevant with autosomal genetic markers.

One of the chief goals in the development of Y-chromosome polymorphisms for forensic use is to permit the specific amplification of male DNA in a background of excessive quantities of female DNA, rendering unnecessary a separation of the two fractions prior to analysis. However, due to its evolutionary history, the Y-chromosome is not only home to a variety of intra-chromosomal segmental duplications, but it also retains a considerable degree of sequence homology with the X-chromosome (12). Accordingly, most primers designed to recognize specific Y-STR loci, such as those incorporated into MPI and MPII, possess homologous sequences on the X-chromosome. The degree of homology will determine to what extent confounding X-chromosome derived artifacts are produced by DNA isolated from male (XY) versus female (XX) individuals. The object in Y-STR assay design is to remove, or at least minimize, such artifacts and this is accomplished by judicious primer design (to maximize differences with X-chromosome sequences) and by stringent PCR cycling conditions (to reduce nonspecific hybridization to homologous sequences). The success of our MPI and MPII design strategy was verified during the developmental validation process by checking the performance of the systems with high concentrations of female DNA in the presence and absence of male DNA.

The results of these validation studies indicate that MPI and MPII demonstrate considerable promise for use in forensic casework. Specifically, both systems were able to dissect out the genetic profile of the male donor in the presence of a vast excess of female DNA and to determine the number of male donors in mixed stains.

Materials and Methods

DNA Isolation and Purification

Blood was collected from human subjects by venepuncture in accordance with the procedures established by the University's Institutional Review Board. Fifty μL drops of blood were aliquotted onto cotton cloth and dried overnight. Semen swabs were collected by soaking sterile swabs in liquid semen deposited in a plastic cup and allowing them to dry overnight. Buccal swabs were collected by a swabbing of the subject's inside cheek with sterile swabs and allowing them to dry overnight. DNA was extracted from the samples using a standard organic extraction protocol (13). Swab cuttings were placed in a Spin-Ease tube (Gibco-BRL, Grand Island, NY) and incubated overnight at 56°C in 400 μL DNA extraction buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml Proteinase K and, for semen containing samples, 39 mM DTT). The cuttings were removed from the tube, placed in a Spin-Ease basket and placed back in the original tube and centrifuged (Eppendorf Centrifuge 5415C) at 14,000 rpm for 5 min to ensure removal of the absorbed fluid.

The extract was purified using 25:24:1 phenol/chloroform/isoamyl alcohol (Fisher Scientific, Norcross, GA). DNA was recovered by precipitation in absolute ethanol at −20°C for at least 1 h. Precipitated DNA was washed twice with room temperature 70% ethanol, which was removed by evaporation in a Speed-Vac (Savant, Albertville, MN). Alternatively, extracts originating from bloodstains or compromised samples were purified using a Microcon concentrator (Millipore, Bedford, MA) according to the manufacturer's instructions. Samples were stored in TE^{-4} (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5) and kept at 4°C until analysis.

DNA Quantitation—Yield Gel

Extracted DNA was electrophoresed in a 1% agarose gel and stained using 1% ethidium bromide (Fisher Scientific). Samples were visualized on a shortwave ultraviolet (UV) transilluminator. Quantitation was accomplished by a comparison of the intensity of the unknown bands with that of a set of known standards run concurrently with the samples.

Standard PCR Conditions

Reaction Components—The standard multiplex PCR reactions were as follows: *MPI*: 25 μL total volume reaction containing 3 ng template DNA, 0.69–1.25 μM primers (see below), 250 μM dNTPs, 1X PCR Buffer II (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 3.25 mM $MgCl_2$, 10 μg nonacetylated BSA (Sigma-Aldrich, St. Louis, MO), and 2.5 units AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA). *MPII*: 25 μL total volume reaction containing 3 ng template DNA, 0.081–0.63 μM primers (see below), 250 μM dNTPs, 1X PCR Buffer II, 2.5 mM $MgCl_2$, 10 μg nonacetylated BSA, and 2.5 units AmpliTaq Gold DNA polymerase.

Primers—Either the forward or reverse primer was labeled with a fluorescent phosphoroamidite dye (Invitrogen, Grand Island, NY) (see Tables 1 and 2). The forward and reverse primer concentrations were as follows: *MPI*: DYS393–0.69 μM, DYS392–1.25 μM, DYS391–0.0815 μM, DYS389–0.1875 μM, DYS438–0.1875 μM, Y-GATA-A7.2–0.125 μM, and DYS385–1.25 μM. *MPII*: DYS388–0.1875 μM, DYS390–0.125 μM, DYS425–0.1125 μM, DYS434–0.1625 μM, DYS437–0.0875 μM, DYS439–0.0813 μM, Y-GATA-C4–0.01065 μM, Y-GATA-A7.1–0.0875 μM, Y-GATA-H4–0.1125 μM, and DYS19–0.63 μM. The primers can be added individually to the mix, or a master mix of all the constituent primers can be prepared, in which case it is denatured at 95°C for 3 min and snap-cooled on ice for 3 min immediately prior to use.

Cycling Conditions—The cycling conditions were: *MPI*: 95°C, 11 min hot start; 2 cycles: 96°C 30 s, 62°C 1 min, 72°C 1 min; 2 cycles: 96°C 30 s, 60°C 1 min, 72°C 1 min; 31 cycles: 96°C 30 s, 58°C 1 min, 72°C 1 min; final extension 72°C 45 min. *MPII*: 95°C,

TABLE 1—*MPI characteristics. Information provided includes the GDB accession number, the forward and reverse primer sequence and associated dyes, average percent stutter, repeat motif, and allele size ranges in bases. Original references for primer sequences are indicated in parentheses. An asterisk indicates that the primer was designed in house.*

Locus	Primer Sequences/Dyes	Average Stutter (%)	Repeat Structure	Range (Bases)
DYS393 GDB:455838	5′-FAMgtg gtc ttc tac ttg tgt caa tac (4) 5′-aac tca agt cca aaa aat gag g (4)	13	AGAT	108–132
DYS392 GDB:455698	5′-FAMtca tta atc tag ctt tta aaa aca a (4) 5′-aga ccc agt tga tgc aat gt (4)	12	TAT	236–263
DYS391 GDB:367966	5′-FAM cta ttc att caa tca tac acc ca (4) 5′-gat tct ttg tgg tgg gtc tg (4)	8	TCTA	275–295
DYS389I GDB:365241	5′-TET cca act ctc atc tgt att atc tat g (4) 5′-tct tat ctc cac cca cca ga (4)	9	(TCTG)(TCTA)	239–263
DYS389II GDB:365241	Same as 389I	23	Same as 389I	353–385
Y-GATA A7.2 GDB:9996572	5′-HEXagg cag agg ata gat gat atg gat (22) 5′-ttc agg taa atc tgt cca gta gtg a (22)	8	TAGA	174–190
DYS438 GDB:9899868	5′-tgg gga ata gtt gaa cgg taa (5) 5′-HEXgtg gca gac gcc tat aat cc (5)	3	TTTTC	203–233
DYS385 GDB:312502	5′-HEXgga agg aga aag aaa gta aaa* 5′-tag gta aag ctg gta agg g*	9	GAAA	252–300

TABLE 2—*MPII characteristics. Information provided includes the GDB accession number, the forward and reverse primer sequence and associated dyes, average percent stutter, repeat motif, and allele size ranges in bases. Original references for primer sequences are indicated in parentheses. An asterisk indicates that the primer was designed in house.*

Locus	Primer Sequences/Dyes	Avg. % Stutter	Repeat Structure	Range (Bases)
DYS19 GDB:196497	5′-atg act act gag ttt ctg tt* 5′-HEX-cac ctg gaa ata gtg g*	8	TAGA	242–254
DYS425 GDB:1386884	5′-FAMtgg aga gaa gaa gag aga aat (21) 5′-agt aat tct gga ggt aaa atg g (21)	1	TGT	104–110
DYS388 GDB:364862	5′-FAMgtg agt tag ccg ttt agc ga (4) 5′-cag atc gca acc act gcg (4)	18	ATT	119–131
DYS390 GDB:365248	5′-tat att tta cac att ttt ggg cc (4) 5′-FAMtga cag taa aat gaa cac att gc (4)	11	(TCTA)(TCTG)	200–251
DYS439 GDB:9899870	5′-FAMtcc tga atg gta ctt cct agg ttt (5) 5′-gcc tgg ctt gga att ctt tt (5)	7	AGAT	242–258
DYS434 GDB:9899860	5′-TETcac tcc ctg agt gct gga tt (5) 5′-gga gat gaa tga atg gat gga (5)	5	ATCT	106–116
DYS437 GDB:9899866	5′-TETgac tat ggg cgt gag tgc at (5) 5′-aga ccc tgt cat tca cag atg a (5)	2	TCTA	188–192
Y-GATA C.4 GDB:9996575	5′-TETagt gtc tca ctt caa gca cca agc ac (6) 5′-gca gca aaa ttc aca gtt gga aaa atg t (6)	8	TATC	250–269
Y-GATA A7.1 GDB:11498962	5′-gag gaa tct gac acc tct gac a (22) 5′- HEXtcc ata tca tct atc ctc tgc cta (22)	8	ATAG	104–112
Y-GATA H.4 GDB:9996580	5′-HEXatg ctg agg aga att tcc aa (22) 5′- cta ttc atc cat cta atc tat cca tt (22)	8	TAGA	130–143

11 min hot start; 2 cycles: 96°C 30 s, 62°C 45 s, 72°C 1 min; 2 cycles: 96°C 30 s, 60°C 45 s, 72°C 1 min; 30 cycles: 96°C 30 s, 58°C 45 s, 72°C 1 min; final extension 72°C 45 min.

Sample Electrophoresis and Data Analysis—The amplified product was detected using either a Windows NT or Macintosh-based ABI Prism 310 capillary electrophoresis system (Applied Biosystems). Macintosh system: 1.5 μL of PCR product was added to 24 μL Hi-Di formamide (Applied Biosystems) and 1 μL of the GeneScan 500 TAMRA internal lane standard (Applied Biosystems). NT system: 0.5 μL of PCR product was added to 12 μL Hi-Di formamide and 0.5 μL of the GeneScan 500 TAMRA internal lane standard. The samples were heated at 95°C for 3 min and snap-cooled for at least 3 min. Samples analyzed with both systems were injected using Module C (5 s injection, 15 kV, 60°C, Filter set C). Samples were subjected to capillary electrophoresis, detected by laser-induced fluorescence, and analyzed with GeneScan Analysis 3.1.2 software (Applied Biosystems).

Autosomal STR Analysis—Autosomal STR analysis was carried out with 2 ng of genomic DNA using a commercial kit (the AmpF*l*STR® Profiler™ PCR Amplification kit) to determine a nine-locus (plus amelogenin) genotype. The analysis was performed in accordance with the manufacturer's instructions.

PCR Parameters

Magnesium—The effect of magnesium concentration was evaluated by separately amplifying 3 ng male DNA and, to assess potential cross-reactivity, 300 ng female DNA with magnesium

concentrations ranging from 1.5 to 4.0 mM in 0.25 mM increments for MPI and 1.5 to 3.5 mM in 0.25 mM gradations for MPII.

DNA Polymerase—DNA polymerase titration experiments were carried out by separately amplifying 3 ng male DNA and, to assess potential cross-reactivity, 300 ng female DNA with enzyme concentrations ranging from 1.5 to 4.0 units in 0.5 unit increments.

Thermocycling Conditions—Annealing temperature was tested using a number of touchdown strategies, as well as constant-temperature cycling at 56°C, 58°C, 60°C, and 62°C, using 3 ng male DNA and 300 ng female DNA in separate reactions.

For Multiplex I, the various annealing times examined were 30 s, 45 s, 60 s, and 90 s. For Multiplex II, those examined were 15 s, 30 s, 60 s, and 90 s. All experiments were carried out using 3 ng male DNA and 300 ng female DNA in separate reactions.

The final cycling condition to be considered was the number of cycles. To maintain the PCR touchdown strategy, cycles were added to or subtracted from the final set of cycles using the 58°C annealing segment. For MPI the various cycle numbers tested were 32, 34, 36, and 38 and for MPII the different cycle numbers tested were 31, 33, 35, and 37, since the preliminarily determined standard cycling conditions were 35 (MPI) and 34 (MPII).

Primer Concentration—The effect of primer concentration on the performance of each multiplex was evaluated by either doubling or halving the standard reaction primer concentration at each locus in turn, while keeping the remaining primer concentrations constant.

Stutter—The percent stutter was determined by calculating the stutter peak height to parent peak height ratio at each locus. Twenty different male samples were used and the percentages were averaged.

Reproducibility

Five bloodstains were amplified in a standard reaction and analyzed by two experienced analysts. Analyst 1 used both Macintosh and Windows NT 310 Genetic Analyzer systems, and analyst 2 employed only the Macintosh system. The maximum variation in allele size estimates was calculated for each locus.

Precision and Accuracy

Precision, for our purposes, deals with the extent to which the analytical system's ability to measure the size of an amplified fragment is reproducible. To assess the precision afforded by the 310 analytical instrument, a male DNA sample was injected 20 times on the capillary electrophoresis system and estimates of the sizes of the alleles (in bases) were obtained. Measurement precision (expressed as a standard deviation) was calculated for each locus.

Accuracy describes the ability of the analytical system to obtain a correct result (i.e., genotype). The accuracy of analysis was confirmed by interlaboratory collaborative studies. Sixty male DNA samples from different individuals were typed by MPI and MPII as part of a SWGDAM collaborative exercise. Additionally, ten samples (5 "known" and 5 "unknown") were obtained from Dr. Lutz Roewer, Humboldt University, Berlin, Germany, as part of an exercise to demonstrate the ability to correctly genotype samples for inclusion into an international Y-STR database.

Species Specificity

Five bacterial strains, donated by The University of Central Florida Molecular Biology Department, were used for this study. The bacteria studied were *Enterobacter aerogenes, Escherichia coli, Bacillus cereus, Staphylococcus aureus,* and *Pseudomonas aeruginosa*. The bacteria were received in appropriate growth media. To extract bacterial DNA, a swab was taken from the media and extracted as described above. Each bacterial DNA sample was tested for MPI and MPII cross-reactivity using varying amounts of input DNA (3–450 ng) to represent a range that may be encountered in bona fide casework specimens.

Nonhuman blood was received from a number of sources: Tuscawilla Oaks Animal Hospital, Oviedo, FL (male cat, male dog); HemoStat Laboratories, Dixon, CA (male cow, male horse, male sheep); Charles R. Daniels, DeLand, FL (male deer, male coyote), and Coriell Cell Repository, Camden, NJ (all nonhuman primate samples, including male chimpanzee, male orangutan, male spider monkey, male macaque, female lemur, and female gorilla). The DNA was extracted using an organic procedure as described above. For amplification, 3 ng of male DNA or 300 ng of female DNA were used.

Sensitivity Studies

DNA from three males and three females was tested. The quantities of male DNA used were 20 pg, 30 pg, 40 pg, 50 pg, 100 pg, 150 pg, 200 pg, 250 pg, 500 pg, 1 ng, 3 ng, 5 ng, 10 ng, 100 ng, and 500 ng. The quantities of female DNA tested were 3 ng, 30 ng, 300 ng, and 1 μg. The GeneScan Analysis threshold for these studies was set at 100 RFU (relative fluorescence units).

Stability Studies

Consistency of Y-STR Haplotypes in Different Tissues from the Same Individual—The consistency of Y-STR typing within different tissues of the same individual was confirmed by amplifying DNA extracted from blood, saliva, and semen collected from the same individual. Samples were obtained from two individual males.

Environmental Effects on Stability—Samples for the degradation studies were prepared by pipetting 50 μL of liquid blood or semen onto denim, cotton, or leather, and allowing them to dry overnight. The samples were then exposed to the environment on Jan. 13, 2002, in a small, fenced-in, wooded area in Deland, Florida. Samples were collected after 1 day, 3 days, 7 days, 16 days, 6 weeks, 10 weeks, and 6 months and stored at −20°C until analysis. Samples were extracted using a phenol/chloroform procedure and purified through a Microcon concentrator, as described. The amount of template DNA used for amplification varied depending on the condition of the sample.

Use of BSA—For inhibition studies, two samples of 50 μL blood extracts were amplified in the presence and in the absence of non-acetylated BSA (Sigma-Aldrich).

Mixture Studies

Male/Female—In previous work (14), we have shown that, when amplifying an admixed male/female sample arising from a post-coital swab, the best results, i.e., a complete male profile, were obtained from 300 ng of total input DNA. Therefore, in mixture experiments, the female DNA component was held constant at 300 ng, the male DNA added in varying quantities, and the entire sample amplified. Male/female ratios tested were 1:100 (3 ng male DNA/300 ng female DNA), 1:300 (1 ng male DNA/300 ng female DNA), 1:600 (0.5 ng male DNA/300 ng female DNA), 1:800 (0.375 ng male DNA/300 ng female DNA), 1:1000 (0.3 ng male DNA/

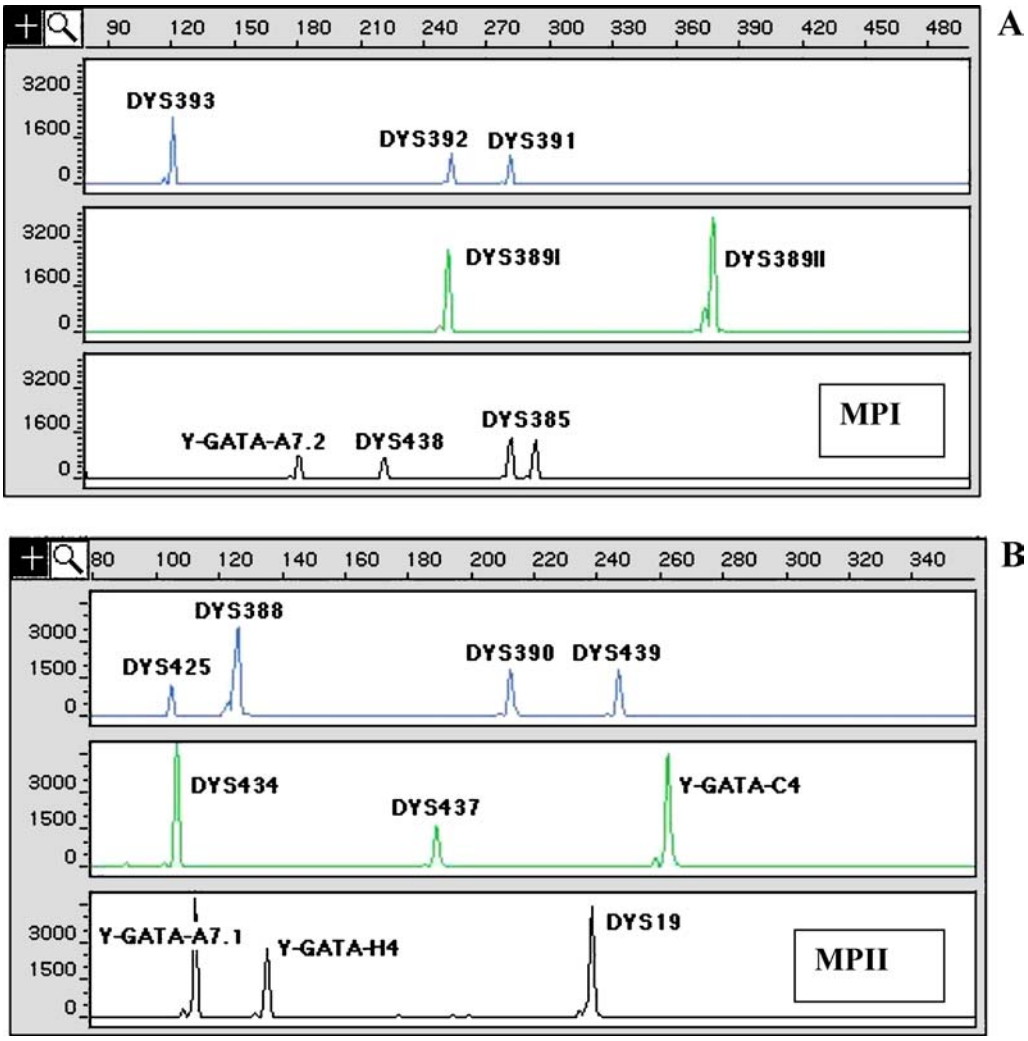

FIG. 1—*Typical electropherograms of Multiplex I* (A)*, and Multiplex II* (B)*. Three ng of male DNA was used as input DNA template. The* x*-axis represents allele size (bases) and the* y*-axis corresponds to peak height in relative fluorescence units (RFU).*

300 ng female DNA), 1:1500 (0.2 ng male DNA/300 ng female DNA), and 1:2000 (0.15 ng male DNA/300 ng female DNA).

Male/Male—For mixture studies, DNA from two males was combined for a total of 3 ng, and the entire volume amplified in standard MPI and MPII reactions. Ratios tested were: 1/2 (1.5 ng/1.5 ng), 1/3 (1 ng/2 ng), 1/6 (0.5 ng/2.5 ng), 1/12 (0.25 ng/2.75 ng), 1/15 (0.20 ng/2.80 ng), 1/20 (0.15 ng/2.85 ng), and 1/30 (0.10 ng/2.90 ng).

Case-type Samples

Five simulated case-type samples were prepared in-house and one set of samples from an adjudicated case (donated by the Florida Department of Law Enforcement, Orlando, FL) were analyzed. The amount of template DNA used varied depending on each individual case.

Results and Discussion

Characterization of Genetic Markers

The two highly discriminating multiplex systems, MPI (Fig. 1A) and MPII (Fig. 1B), collectively, include the loci DYS19, DYS385a, DYS385b, DYS388, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS425, DYS434, DYS437, DYS438, DYS439, Y-GATA-A7.1 (DYS460), Y-GATA-A7.2 (DYS461), Y-GATA-H4 and Y-GATA-C4. MPI comprises nine of these loci with an allelic size range of 115–387 bases (Table 1) whereas MPII contains ten Y-STR loci whose alleles range from 101 bases to 271 bases (Table 2).

Inheritance

The 19 Y-STR markers incorporated into MPI and MPII are located in the nonrecombining region of the Y-chromosome. Consequently, the allelic state at each of the 19 loci on a particular chromosome (a multilocus genotype) is transmitted unchanged from father to son as a physically and genetically linked "haplotype block." To confirm this mode of inheritance, we examined four father/son pairs, one sibling pair, and a lineage including a patriarch, his two sons, and his grandson (the child of one of his sons). The 19 locus profiles were identical within each family pair and within the three-generation family group studied. No intergenerational mutation events were observed, which is not surprising given the limited sample size and an empirically determined Y-STR mutation rate of $\sim 3 \times 10^{-3}$ mutations per locus per gamete per generation (15,16).

Mapping

The chromosomal location of each of the MPI and MPII Y-STR loci has been submitted to, or recorded by, the Nomenclature Committee of the Human Genome Organization (HUGO). Loci details can be accessed via the Human Genome Database (accession numbers are listed in Tables 1 and 2).

Detection

The 19 Y-chromosome loci evaluated in the present study comprise tandemly repeated microsatellite DNA and are, therefore, length polymorphisms. Detection is accomplished by the laser-induced fluorescence of dye-labeled PCR products corresponding to individual Y-STR alleles. Individual alleles are labeled by the incorporation of dye-labeled primers into the amplimers during the PCR process (primer sequences and dye labels for each locus are provided in Tables 1 and 2).

Polymorphism

The MPI and MPII short tandem repeat loci are tri-, tetra-, or pentanucleotide repeats (Tables 1 and 2). Alleles are differentiated according to their amplimer size, ranging from approximately 100 bases to approximately 400 bases (Tables 1 and 2).

PCR Parameters

Reaction Conditions—The robustness and specificity of the MPI and MPII PCR reaction conditions were investigated by altering the concentration of critical reagents or the thermocycling conditions (see below) and, in all instances, using both male (3 ng) and female (300 ng) DNA as input template. An attempt was made to "balance" the systems in the sense of reducing as much as possible the variation in peak heights between the individual loci. The use of a high concentration of female DNA (i.e., 300 ng) was included to test for nonspecificity. Since the MPI and MPII primers demonstrate varying degrees of homology with X-chromosome derived sequences (data not shown), it is important to test for the presence of potentially confounding female DNA artifacts caused by an excess of female DNA. Such female DNA excess is commonly found in casework, particularly in sexual assaults.

Magnesium—We tested MPI and MPII with magnesium concentrations ranging from 1.5 to 4.0 mM (MPI) or 1.5 to 3.5 mM (MPII) in 0.25 mM increments. With MPI, complete but imbalanced profiles were obtained up to 2.25 mM whereas balanced peaks were obtained between 2.5 to 3.75 mM. Higher magnesium concentrations, however, resulted in the amplification of X-chromosomal peaks, which could confound the analysis of male/female mixed specimens (data not shown). MPII performed similarly in that suboptimal concentrations of magnesium resulted in imbalanced profiles ($<$2 mM) or increased X-chromosome artifacts ($>$3.5 mM) (data not shown). Accordingly, 3.25 mM and 2.5 mM were chosen for the standard PCR reactions for MPI and MPII, respectively.

DNA Polymerase—The effect of DNA polymerase concentration was evaluated by the addition of 1.5 to 4.0 Units/reaction in increments of 0.5 U. With the goals again of obtaining balanced peaks around 1000 RFU and limiting artifact interference, the optimal enzyme concentration for both MPI and MPII was determined to be 2.0–3.25 U per PCR reaction volume. Below this range, male peak heights were imbalanced, and above it X-chromosome artifacts become prevalent (data not shown). The enzyme concentration selected for both the MPI and MPII standard PCR reactions was 2.5 U per in a 25 μL final PCR reaction volume (0.1 U/μL).

Thermocycling Conditions—A number of cycling parameters were evaluated, including annealing time (30 s, 45 s, 1 min, 1.5 min) and temperature (56°C–62°C), cycle number (MPI: 32–38: MPII: 31–37) and primer concentration (half and double the standard concentration) (data not shown).

Annealing Time—An annealing time of 30 s was insufficient for several loci to amplify including DYS425 (MPII), DYS434 (MPII), and Y-GATA-A7.2 (MPI). One minute (MPI) or 45 s (MPII) proved to be optimal for the production of balanced allelic signals of sufficient intensity at all loci whereas annealing for 1.5 min resulted in the amplification of a number of X-chromosome artifacts (data not shown).

Annealing Temperature—The optimal annealing temperature was found to be 58°C after an initial touchdown PCR process involving two cycles at 62°C followed by two cycles at 60°C. Lower or higher temperatures resulted in the preferential amplification of some loci (data not shown).

Cycle Number—MPI and MPII loci were amplified successfully with all cycle numbers tested with the optima at 35 and 34 cycles, respectively (data not shown).

Primer Concentration—In many instances, doubling or halving the concentration of each individual primer pair (while keeping the others constant) caused an imbalance, but no loss of signal, from the nine MPI and ten MPII loci. However, halving the 393, 425, A7.1, 390, 434, 437, and H4 primer concentrations resulted in allelic dropout (data not shown). Significantly, doubling the concentration resulted in the dropout of the affected loci. For example, doubling of the 439 primer concentration resulted in 390 and 434 dropout and, likewise, increases in A7.1 and 19 knocked-out H4 and A7.1, respectively (data not shown). Halving or doubling the primer concentration also resulted in the more prevalent appearance of X-chromosome-derived products particularly with the MPI system (data not shown). These experiments exemplify the critical need to ensure batch-to-batch consistency of primer mixes by checking their performance prior to use.

Potential for Differential, Preferential, and Stochastic Amplification

Since each Y-chromosome locus is hemizygous, the phenomenon of "pseudo-homozygotes'" being formed by allelic dropout is not a major concern. Stochastic effects (i.e., locus dropout due to low copy number templates) are not evident in good-quality samples unless the input DNA template is less than 150–200 pg (see Sensitivity section below). Stochastic effects caused by environmentally induced sample damage are addressed in the Stability section (see below).

Stutter

Stutter is a PCR artifact, attributed to DNA polymerase slippage, which is observed during the amplification of simple sequence repeats such as STRs (17). Stutter is characterized by the presence of an allelic-like signal that is typically one repeat shorter than the parent peak and is significantly less intense than the parent peak. It is important to be able to distinguish between stutter and a true allele in order to be able to resolve mixtures of DNA from at least two individuals. The average percent stutter was determined for all MPI and MPII loci (Tables 1 and 2). Average stutter was less than or equal to

13% for all loci with the exception of DYS389II and DYS388, which had an average stutter of 23% and 18%, respectively. The relatively high stutter observed with DYS389II and DYS388 would impair the ability of these loci to discern the presence of some mixtures, and consequently the authors do not recommend their use for mixture interpretation. However, for the other loci the stutter range observed is similar to that seen with autosomal STRs and consequently stutter signals should normally be distinguishable from allele signals.

Effects of Co-Amplification

A male DNA standard (see below) was amplified separately in 19 monoplex reactions corresponding to each of the MPI and MPII loci and the results were compared with those obtained from a standard MPI and MPII analysis. In all instances, identical results (in terms of allelic size estimates) were obtained at each locus using both the monoplex and multiplex formats.

Positive and Negative Controls—The positive amplification control consisted of a male DNA sample obtained from a voluntary donor whose Y-STR haplotype has been well characterized and confirmed on a number of occasions using external standards. Three negative controls were used: a 300 ng female DNA sample to control for specificity, a reagent blank, and an amplification blank. The external standards used in the determination of allelic state were obtained from an external source, and consisted of alleles of known size and defined repeat number, which had been confirmed by sequencing.

Detection of PCR Product—MPI and MPII PCR products were characterized directly by an assessment of their size in bases. This was accomplished for each sample individually by the incorporation of an appropriate internal lane standard (GeneScan 500 TAMRA).

Reproducibility

In order to check for consistency of results, DNA was extracted from bloodstains from five different individuals and subjected to MPI and MPII analysis by two different analysis scientists using both the Windows NT and Macintosh 310 analysis software packages. Equivalent results were obtained whether different individuals or different software packages were used (data not shown). To compare the results from these independent experiments, we calculated the maximum difference between peak sizes within a locus. These values ranged from 0.2 to 1.0 bases, with an average of 0.5 bases.

Precision and Accuracy

Precision—Allele sizes were determined with excellent precision by use of an internal lane standard (GeneScan TAMRA 500). Depending upon the locus, the standard deviation of the measurement was determined by replicate measurements of the same sample to be 0.05–0.12 bases (data not shown). Determination of the allelic state of a sample, including the ability to detect rare microvariant alleles that differ from the parental alleles by a nonintegral number of repeat units, was therefore straightforward. The allelic state of each of the 19 MPI and MPII loci in terms of the number of repeat units was determined by comparison with external standards consisting of alleles of known size and defined repeat number, which had been confirmed by sequencing.

Accuracy—The accuracy of analysis was confirmed by interlaboratory collaborative studies. Sixty male DNA samples from different individuals were typed by MPI and MPII as part of a SWGDAM collaborative exercise. Additionally, ten samples (5 "known" and 5 "unknown") were obtained from Dr. Lutz Roewer, Humboldt University, Berlin, Germany, as part of an exercise to demonstrate the ability to correctly genotype samples for inclusion into an international Y-STR database. In all instances, the determined MPI and MPII genotypes agreed with the concordance haplotype data (data not shown).

Species Specificity

Species specificity was checked by testing MPI and MPII using DNA isolated from various species of bacteria, several common mammals, and a number of nonhuman primates.

MPI and MPII failed to produce detectable products from the five bacterial species tested: *Enterobacter aerogenes, Escherichia coli, Baccillus cereus, Staphylococcus aureus,* and *Pseudomonas aeruginosa* (data not shown).

Several male animals were tested, including dog, cat, cow, horse, sheep, deer, and coyote. Subsequent to MPI amplification, no alleles were detected in any of the animal samples tested. MPII, however, produced a monomorphic canine-specific peak (seen in both dog and coyote) at 122 bases, which is outside the normal human allele ranges of the Y-STR loci employed (data not shown).

A high level of DNA sequence homology exists between members of the primate family. We tested DNA from four male nonhuman primates (chimpanzee, orangutan, spider monkey, and macaque) and two female nonhuman primates (lemur and gorilla) for the degree of cross-reactivity with the MPI and MPII systems (data not shown). In all nonhuman primate samples tested we observed MPI and MPII cross-reactivity of our multiplex primers with both male and female samples (Fig. 2). Of the simians tested, the chimpanzee is the human's closest evolutionary relative (18). At least 14 amplified products were obtained from male chimpanzee DNA using MPI (Fig. 2A) and MPII (Fig. 2B) with a number of them falling within the defined human allelic size ranges. Of the primates considered here, the gorilla is man's next closest evolutionary ancestor (18). The female gorilla DNA sample reacted with MPI (Fig. 2C) and MPII (Fig. 2D) and displayed at least 29 products. Again, several of these products were within the *Homo sapiens* allelic size ranges. Further removed on an evolutionary scale is the orangutan (18). Amplification of male orangutan DNA with both MPI (Fig. 2A) and MPII (Fig. 2B) produced distinguishable products, although only two of them fell within the human allelic size ranges. The final two simian species considered, the male macaque and the male spider monkey, diverged from man at an even earlier point in history (18). Combination MPI (Fig. 2A) and MPII (Fig. 2B) amplified only four products from each of the samples. One prosimian species was considered, a female lemur, which demonstrated at least 11 products after MPI amplification (Fig. 2C), some of which resided within the human allele range. Interestingly, no MPII amplification products were obtained (Fig. 2D).

Sensitivity

The sensitivity of the MPI and MPII systems was tested using three different male DNA samples and comparable results were obtained from each. While the optimal quantity of template DNA for MPI and MPII ranged from 1–3 ng, both proved effective over a wide range of input template DNA. The lower limit of template DNA necessary for a full nine-locus MPI profile was 200 pg, which is equivalent to approximately 33 epithelial cells (Fig. 3A). Even with as little as 50 pg (~8 diploid cells) and 20 pg (~3 diploid cells) of template it was possible to obtain a four- or three-locus partial

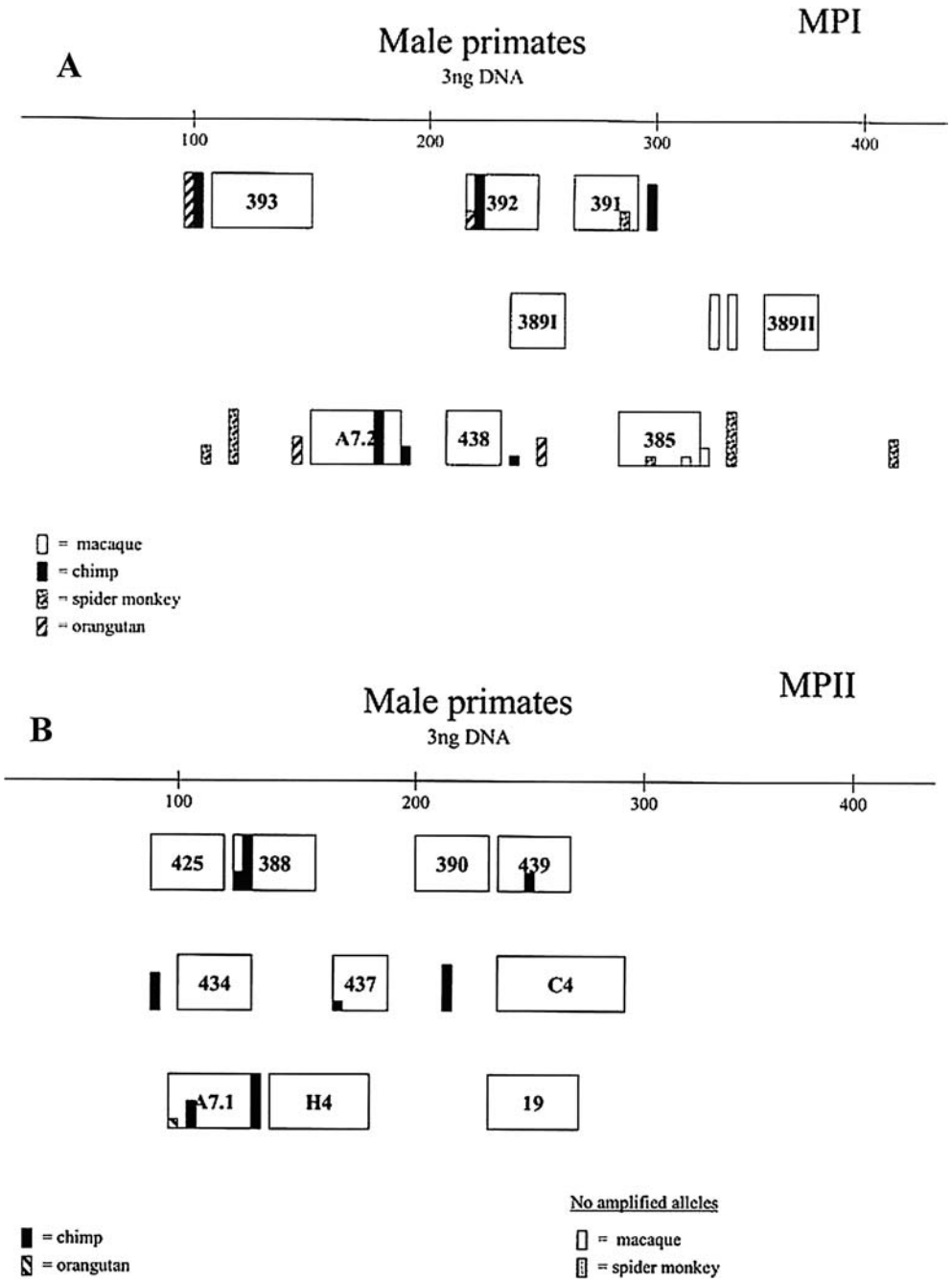

FIG. 2—*Primate DNA cross-reactivity. The products obtained from 3 ng of male primate DNA with MPI* (A) *and MPII* (B) *and from 300 ng of female primate DNA with MPI* (C) *and MPII* (D) *are diagrammed. The large open boxes show the ranges of human alleles for each locus, while the small boxes depict the location of the nonhuman primate alleles. The heights of the large open boxes represent the signal intensities (in RFU) of typical human alleles. Nonhuman primate alleles are depicted with a box height proportional to the signal intensities observed in relation to the human alleles. The number line at the top indicates fragment size (bases).*

profile, respectively. A full ten-locus MPII profile was obtained with 150 pg template DNA, the equivalent of approximately 25 epithelial cells (Fig. 3B). Similarly, partial MPII profiles were obtainable with considerably less input DNA. For example, an eight-locus or two-locus profile was discernible with 50 pg (~8 diploid cells) or 30 pg (~5 diploid cells) of DNA, respectively.

Stability

Consistency of Y-STR Haplotypes in Different Tissues from the Same Individual—The consistency of MPI and MPII Y-STR typing within different tissues of the same individual was confirmed by typing blood, semen, and saliva stains from the same individual. This was repeated with another unrelated male. In both instances, the 19 locus Y-STR haplotypes obtained were identical within the tissues of the same individual (data not shown).

Environmental Effects on Stability—The ability to obtain an autosomal DNA profile from biological samples recovered from a variety of substrates and subject to various environmental insults has been well documented. Therefore, a limited study was carried

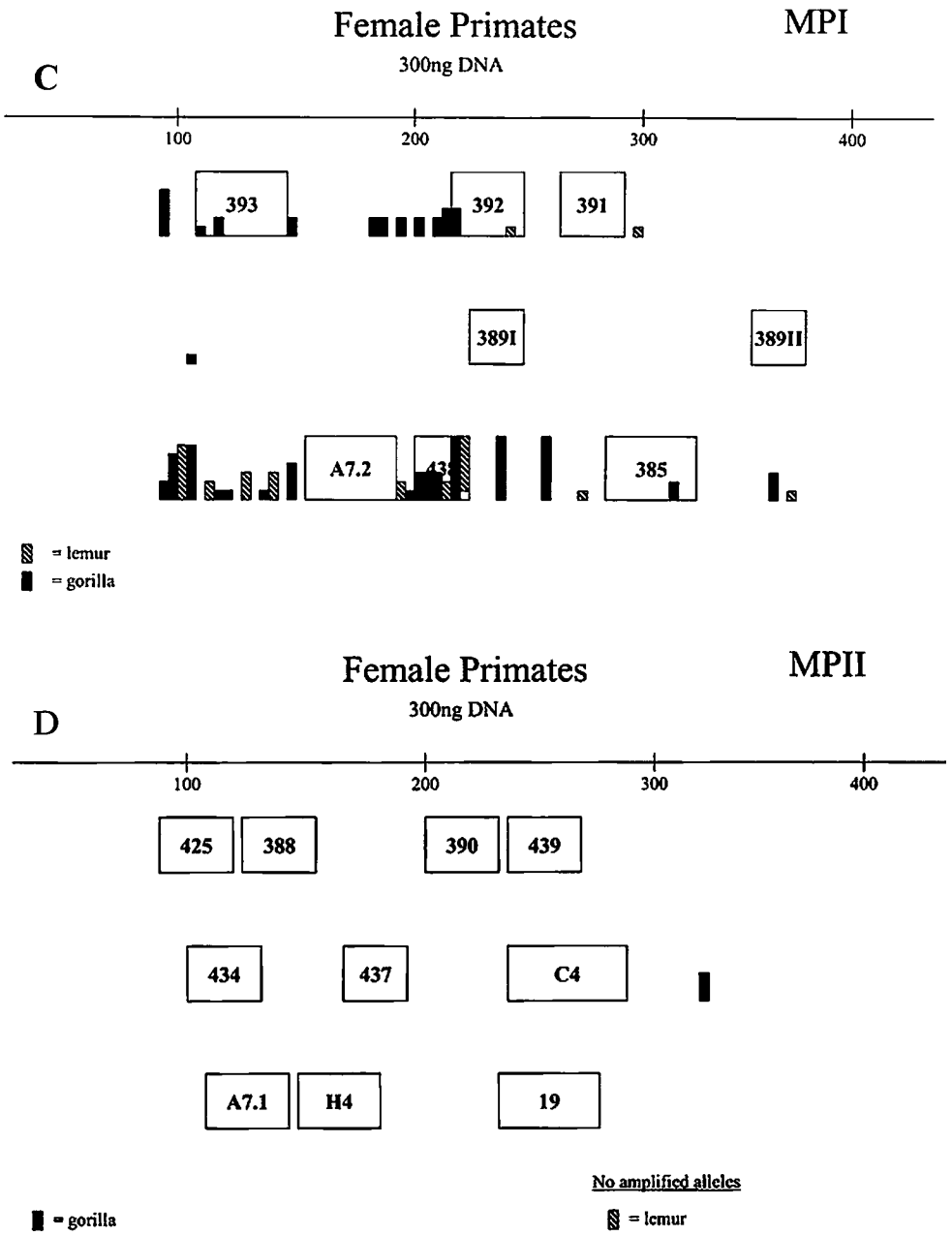

FIG. 2—*Continued.*

out to assess the effects of these factors on the ability to obtain MPI and MPII Y-STR profiles using particularly harsh environmental insults. In the study, 50 μL drops of liquid blood and semen from an individual whose MPI/II haplotype was known were deposited separately on leather, cotton and denim clothing items and allowed to dry at room temperature overnight. The samples were placed in an outdoor, wooded area in Central Florida and completely exposed to the elements, which comprised a combination of high levels of heat, light, and humidity (including precipitation in the form of drenching rain). Exposure of the samples continued from January to June, with temperatures ranging from 53°F to 84°F (78°F average high; 62°F average low). Humidity and precipitation for this period averaged 77% and 22 in. (8.6 cm), respectively. Samples were removed after 1 day, 3 days, 7 days, 16 days, 6 weeks, 10 weeks, and 6 months and subjected to Y-STR analysis. The results are summarized in Table 3, which indicates the number of Y-STR loci that were successfully typed from each substrate at each exposure time.

TABLE 3—*MPI and MPII typing of environmentally compromised blood and semen stains. The number of loci successfully typed (out of a possible 19) is indicated. "No data" signifies that no sample was taken for testing.*

	1 Day	3 Days	7 Days	16 Days	6 Weeks	10 Weeks	6 Months
Jeans—semen	19	19	19	19	19	19	13
—blood	19	19	14	0	0	0	No data
Cotton—semen	19	19	19	19	19	3	No data
—blood	19	19	15	0	0	0	No data
Leather—semen	19	15	15	14	0	6	No data
—blood	19	16	9	7	0	0	No data

With semen it was possible to obtain a full 19-locus Y-STR profile from stains on jeans, or on a cotton T-shirt, after 10 weeks or 6 weeks, respectively. This is in contradistinction to leather, where the full 19-locus profile was obtainable only up to 1 day's exposure

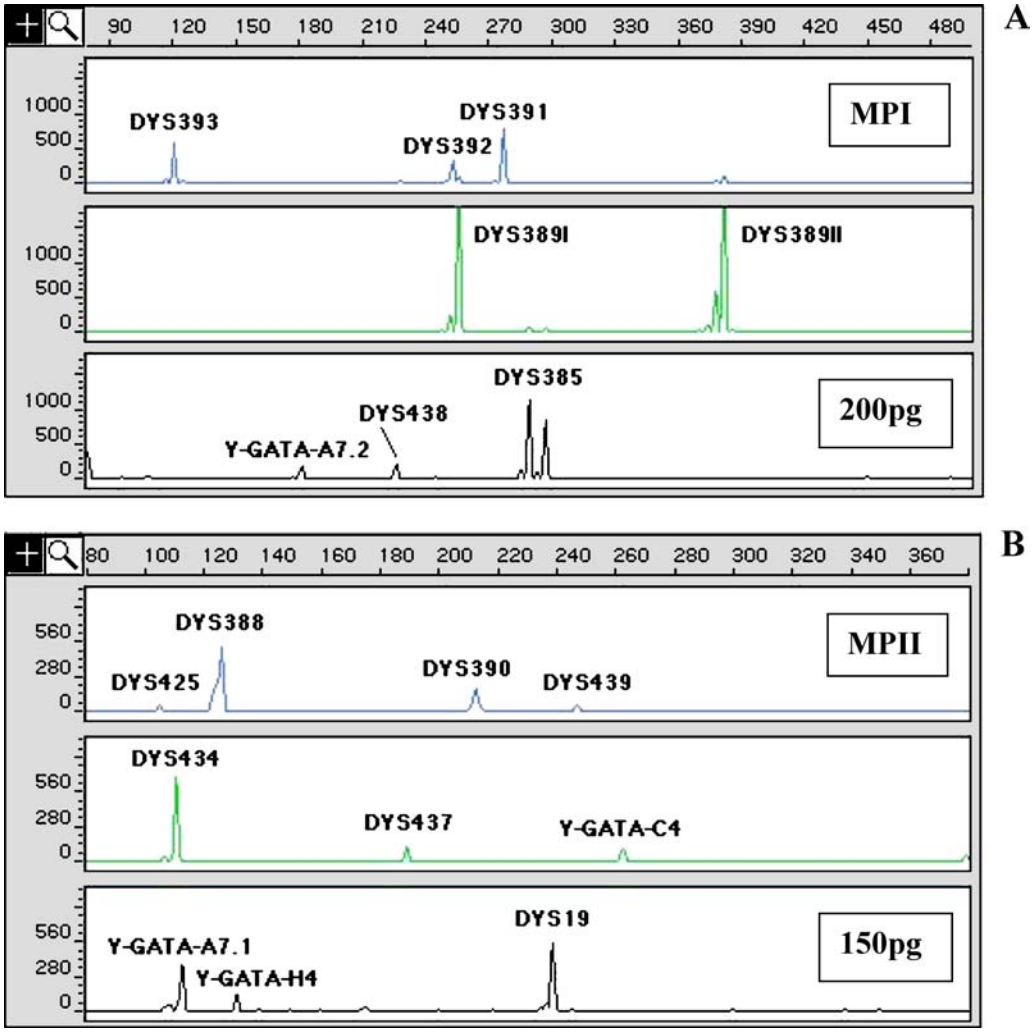

FIG. 3—*MPI and MPII sensitivity. Complete MPI* (A) *and MPII* (B) *Y-STR profiles are obtained with 200 pg and 150 pg of male DNA, respectively.*

although it was still possible to obtain a partial profile (6/19 loci) after 10 weeks. This reduction in success rate with the leather was probably due to the weather-induced washing out of the sample observed as a result of the nonporous nature of the substrate. Interestingly, 13 of the 19 MPI/MPII loci were typeable from a semen stain recovered 6 months after exposure to the elements (Figs. 4A, 4B), despite the inability to obtain an autosomal STR profile from the same sample (Fig. 4C). The improved success rate with the Y-STR systems was probably due to the increased PCR cycle number used for MPI/MPII analysis (35/34 cycles) compared to the autosomal system (28 cycles).

Less success was obtainable with bloodstains exposed to the elements. It was possible to obtain a full 19-locus Y-STR profile from bloodstains on jeans, or on a cotton T-shirt, after 3 days although a significant number of partial profiles were obtainable after 7 days (14 or 15 of the 19 loci). However, exposure of 16 days or longer resulted in the inability to obtain a Y-STR profile. With leather, a full profile could be obtained only up to 1 day's exposure although a partial profile was obtained up to 16 days (7/19 loci). The reduced success rate from bloodstains compared to semen stains may be due to the differential tenacity of binding of these physiological stains to their substrates under the environmental conditions encountered. Sperm, for example, occasionally are known to be able to persist in semen stains after washing despite the disappearance of the seminal fluid component (J. Ballantyne, personal communication). Alternatively, or additionally, the reduced success rate with bloodstains may reflect a differential stability of leukocyte and spermatozoa cellular structures when exposed to harsh environmental insults.

Use of BSA—Bovine serum albumin (BSA) is a commonly used PCR additive, believed to act by effectively removing inhibitors and stabilizing the DNA polymerase, thus lending greater specificity to the reaction (19). To determine if its addition would affect MPI or MPII amplification, 10 μg of nonacetylated BSA per PCR reaction (0.4 μg/μL) was added to samples originating from single-source DNA isolated from blood and semen stains that had been exposed to the environmental insults described above. The sensitivity and specificity of the MPI and MPII assay in the presence of BSA either remained unchanged or, particularly for environmentally compromised bloodstains, improved (data not shown). Consequently, BSA was included in the standard PCR reaction.

Female DNA Cross-Reactivity

One of the principal uses of Y-STR systems is to determine the genetic profile of the male donor in the presence of a vast excess of female DNA. However, due to its evolutionary history, the Y-chromosome is not only home to a variety of intrachromosomal segmental duplications, but also retains a considerable degree of

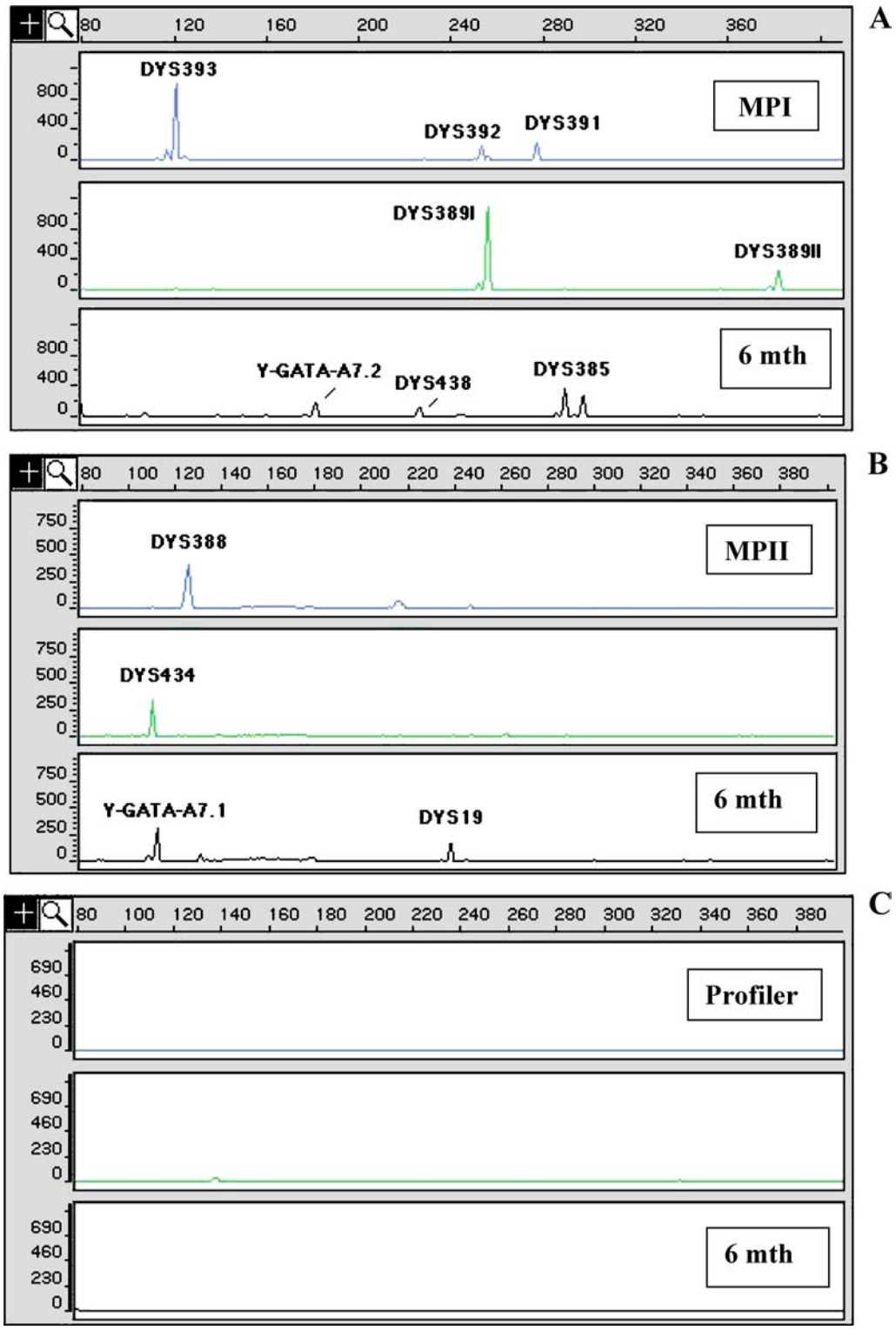

FIG. 4—*Environmental effects on MPI and MPII stability. MPI* (A)*, MPII* (B)*, and autosomal STR* (C) *electropherograms obtained from a semen stain on jeans subjected to the outdoor environment for six months. MPI* (A) *gave a complete 9-locus haplotype, and four of the ten MPII* (B) *loci were amplified. An autosomal multiplex* (C) *gave no result.*

sequence homology with the X-chromosome (12). Accordingly, most primers designed to recognize specific Y-STR loci, such as those incorporated into MPI and MPII, possess homologous sequences on the X-chromosome. The degree of homology will determine to what extent confounding X-chromosome derived artifacts are produced by DNA isolated from male (XY) versus female (XX) individuals.

To assess female DNA cross-reactivity, MPI and MPII were tested with increasing quantities of female DNA (3 ng, 30 ng, 300 ng, 1 μg). MPII proved to be highly specific for the Y-chromosome in that no significant female DNA products were observed (data not shown). However, female DNA artifacts were produced by MPI, especially at higher levels of input female DNA (≥300 ng) (data not shown). Specifically, two monomorphic products of 246 and

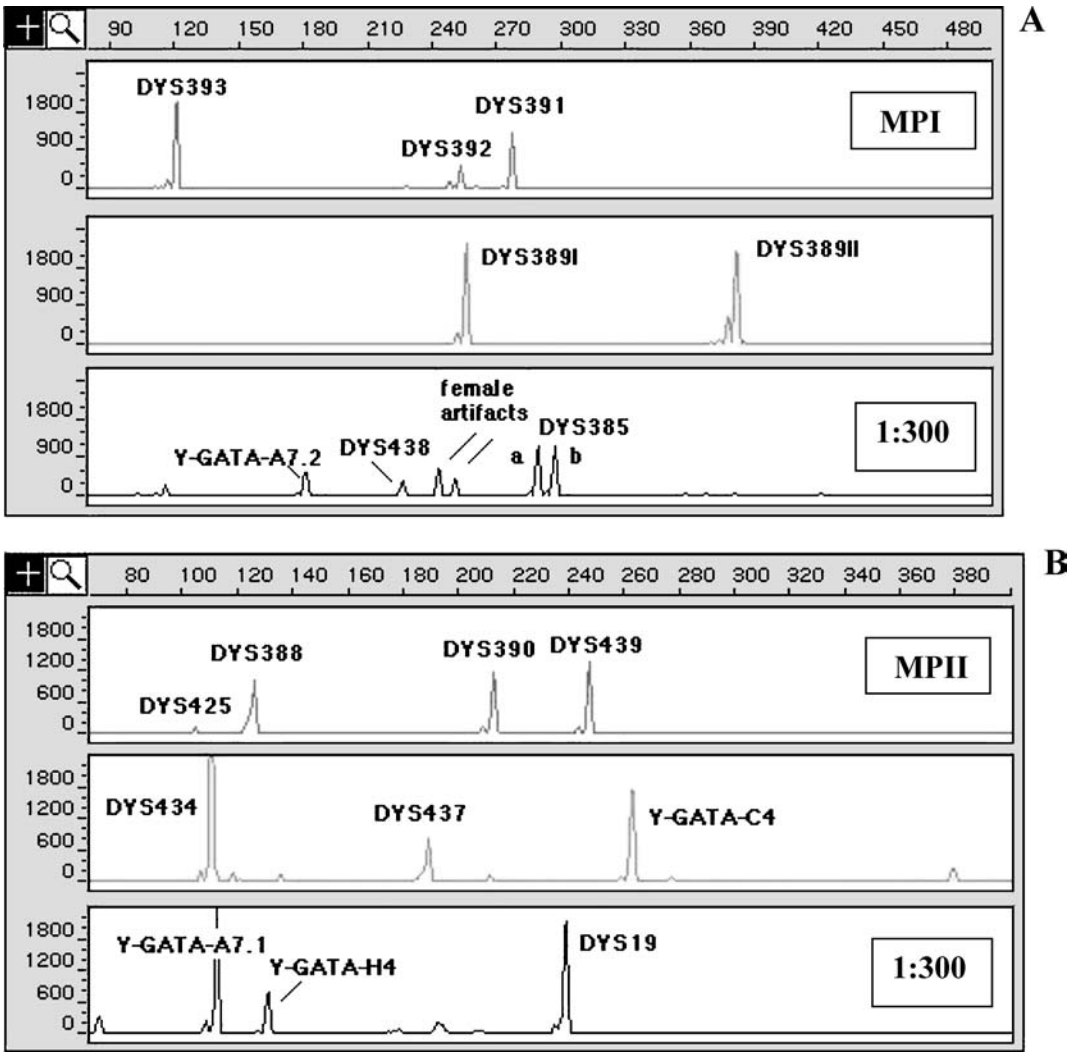

FIG. 5—*Male/female DNA admixtures. MPI* (A) *and MPII* (B) *profiles of the male donor were obtained from a 1:300 male/female DNA admixture. The profiles were generated using 1 ng male DNA in the presence of 300 ng female DNA. Female DNA artifacts observed with the MPI system are indicated.*

261 bases were detected in yellow. Two additional peaks were detected in blue with sizes of 248 and 256 bases. At least one of the artifacts detected in yellow overlaps the allelic size range of the DYS385 locus (252–300 bases) whereas both artifacts detected in blue overlap the allelic size range of the DYS392 locus (236–263 bases). Primer subtraction experiments revealed that the yellow channel artifacts result from an interaction between the labeled 438 primer and another unlabeled MPI primer from a different locus in the presence of female DNA. The blue artifacts were due to the binding of the DYS391 primers to a homologous region of the X-chromosome in the absence of male DNA (20). Female DNA artifacts can be explained by the binding of certain Y-STR primers to homologous, but not identical, sequences on the X-chromosome. Such binding is found only with female DNA since the presence of male DNA would permit the efficient binding of the primers to their perfectly complementary sequences on the Y-chromosome only.

We have demonstrated previously that female nonhuman primate DNA (gorilla and lemur) produces MPI and/or MPII allelic signals (Figs. 2*C*, 2*D*). However, these were clearly distinguishable from the human female products described here.

In summary, only three of the 19 MPI and MPII loci, DYS392 and DYS385 (a, b), were found to be affected by the presence of large quantities of female DNA in the absence of competing male DNA.

Mixture Studies

Male/Female DNA Mixtures—To test the ability of the Y-STR multiplex systems to determine the genetic profile of the male donor in the presence of large quantities of female DNA—a situation which is more akin to bona fide forensic casework—a series of samples was prepared in which 300 ng of female DNA was admixed with varying quantities of male DNA. One of the chief advantages of Y-STR multiplexes is their ability to dissect out a male profile from an overwhelming amount of admixed male/female DNA, especially that derived from post-coital vaginal swabs. The design of MPI and MPII allows for the inclusion of a much greater quantity of total DNA than can be accommodated by autosomal systems, thereby supplying enough Y-chromosomal DNA for amplification. Our experiments have shown that the input of 300 ng total DNA is the optimal in situations such as these, so we have used this as a basis for the mixture experiments. A full 19-locus Y-STR profile was obtained when the male DNA component comprised 1/300th of the total (1 ng male DNA/300 ng female DNA) (Fig. 5). Significantly, partial Y-STR profiles were obtained upon further dilution of the

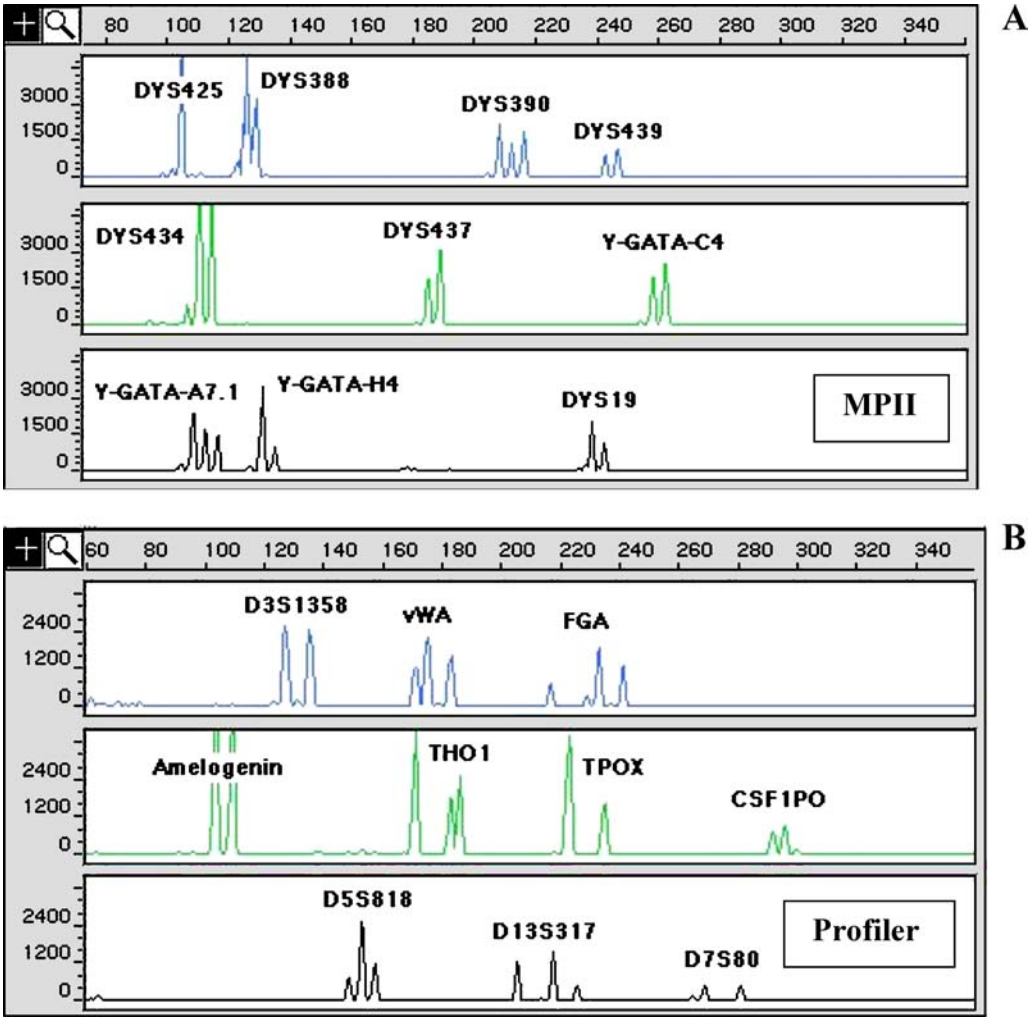

FIG. 6—*Male/male DNA admixtures. A mixed DNA sample (3 ng total) derived from three males (in a 1:1:1 ratio) was analyzed by the MPII Y-STR* (A) *and autosomal STR* (B) *systems.*

male DNA. The number of loci from which a male profile could be obtained decreased as the proportion of female DNA present in the admixture increased. For example, eight, six and four of the nine MPI loci were successfully typed at a 600- (0.5 ng male DNA/300 ng female DNA), 1000- (0.3 ng male DNA/300 ng female DNA) and 2000-fold (0.15 ng male DNA/300 ng female DNA) female DNA dilution, respectively (data not shown). With MPII, nine, eight and five of the ten loci were successfully typed at a 600-, 1000- and 1500-fold (0.2 ng male DNA/300 ng female DNA) female DNA dilution, respectively (data not shown).

Importantly, these experiments demonstrate that even in the presence of a vast excess of female DNA it is possible, with MPI and MPII, to obtain a full or partial Y-STR profile of the male donor. Significantly, these data show that female-derived artifacts that could confound allelic assignments do not normally arise in the presence of male DNA. However, due to their unique characteristics they would be recognized as such if they were produced and their presence should not result in an incorrect Y-STR profile determination in a female DNA containing casework sample, whether or not that sample contained multiple, or a single, male contributor(s).

Male/Male DNA Mixtures—Since the Y-chromosome is, by definition, hemizygous, the presence of a single allele from each male should facilitate the determination of the correct number of male donors. To determine the level at which two male samples in a mixed sample could be detected and typed, DNA from two males was mixed in various ratios (1/2, 1/3, 1/6, 1/12, 1/15, 1/20, 1/30) and a total of 3 ng amplified and typed using MPI and MPII. The presence of two individuals, as determined by the presence of two allelic signals at a single locus (except DYS385), was clearly discernible when the minor donor was present at 1/2, 1/3, 1/4 and 1/6 the concentration of the major donor. However, when the minor contributor comprised 1/12 or less of the total DNA, generally only the major contributor's Y-STR profile was discernible (data not shown).

The identification of the number of male donors by Y-STR analysis is exemplified in the case of a three-male admixture. The electropherograms in Fig. 6 depict the results obtained after amplification of DNA from a three male admixture (1:1:1 ratio) with the MPII system (Fig. 6A), and an autosomal STR multiplex system (Fig. 6B). The MPII data clearly indicate the presence of three males whereas the autosomal STR data, although indicative of a mixture, is less clear as to the precise number of donors.

Case-type Samples

To assess the performance of MPI and MPII with specimens commonly encountered in forensic casework a variety of sample types were tested including a neat semen stain from a vasectomized

individual, a series of admixed male/female bloodstains, a 24-h post-coital cervicovaginal swab, the nonsperm (or "female") fraction of a 48-h post-coital cervicovaginal swab from a differential extraction, and a semen stain on a pair of panties from a rape victim. These studies complement the environmentally compromised case-type samples previously tested in connection with MPI and II stability assessment (see above) and exemplify the efficacy of Y-STR analysis.

Semen Stain from Vasectomized Male—Although the semen of a vasectomized male would be expected to contain no spermatozoa, it may be possible to obtain a Y-STR profile of the individual from contaminating epithelial cells originating from various tissues of the male reproductive tract. A neat semen stain from a vasectomized male who was cytologically negative for the presence of sperm was subjected to a nondifferential DNA isolation and purification procedure as described in the Materials and Methods section. Equal volumes of the DNA extract were subjected to autosomal and Y-STR analysis. Nine-locus MPI (Fig. 7A) and eight-locus MPII (Fig. 7B) profiles were obtained from this sample despite the inability to obtain a standard autosomal STR profile using the same quantity of input DNA (Fig. 7C). The Y-STR profile matched that of the donor (data not shown). The increased sensitivity observed with the Y-STR systems probably reflects the use of increased cycle number (34–35 cycles) with MPI and MPII compared with the standard 28 cycles used for the autosomal STR system as recommended by the manufacturer.

Admixed Male/Female Bloodstains—With the exception of semen-containing stains it is not possible to physically separate the male and female DNA from body fluids contributing to an admixed stain. Consequently, autosomal STR analysis yields a mixed profile that may confound interpretation of the data. One of the advantages of Y-STRs is the ability to dissect out the male profile in such admixed stains. To test this, we created a series of admixed bloodstains in which liquid blood from three different males was mixed separately (at a 1:1 ratio) with a female donor's blood to give a total volume of 50 μL and allowed to dry. DNA was isolated from the mixed stain and 3 ng subjected to MPI and MPII analysis. In all three cases a full 19-locus Y-STR profile was obtained that each matched its respective male donor (data not shown).

Post-Coital Cervicovaginal Sample—A post-coital cervicovaginal swab was obtained from a female volunteer who recovered the sample 24 h after intercourse. DNA was isolated using a standard nondifferential organic procedure, resulting in an extract containing both male and female DNA in an unknown ratio. MPI and MPII analysis was conducted using 300 ng of the nondifferentially extracted DNA as input template. A complete 19-locus Y-STR profile was obtained that matched the profile of the semen donor (data not shown).

Female Fraction Analysis from 48 h Post-Coital Cervicoaginal Sample—-Post-coital samples recovered $\geq$ 48 h are likely to contain few sperm and many of these could be in a structurally fragile state. Consequently, the differential extraction process normally used may result in premature lysis of the sperm DNA into the nonsperm or "female" fraction. The addition of 300 ng of DNA from the female fraction of a differentially extracted 48 h post-coital cervicovaginal sample gave a complete 19-locus MPI/MPII profile that was consistent with the male donor's profile (data not shown). The autosomal STR profile obtained from the same female fraction using 2 ng input DNA was consistent with the donor female and no allelic signals from the male donor were evident (data not shown). Significantly, the sperm or "male" fraction yielded no profile, which is consistent with the hypothesis of sperm loss due to premature lysis during the differential extraction process (data not shown). Laboratories may possess stored nonsperm DNA extracts from previously analyzed samples for which an autosomal STR profile was not obtained from the male donor due to an extended post-coital time interval. Such extracts may be subjected to Y-STR analysis using the strategies described herein, including, particularly, the incorporation of up to 300 ng of input template DNA.

Semen Stain from Underpants of Victim of Sexual Assault—A nonprobative bona fide casework sample consisting of a semen stain from the underpants of a victim of sexual assault was obtained from the Florida Department of Law Enforcement. Autosomal STR analysis had previously indicated the presence of a male/female admixture that could not be easily resolved into the constituent genotypes. MPI and MPII analysis was carried out using 300 ng of nondifferentially extracted DNA per reaction, and an 18-locus Y-STR profile was obtained (data not shown).

Casework Limitations of Y-STRs—Despite the demonstrated potential efficacy of MPI and MPII Y-STR analysis for casework as exemplified by the aforementioned cases, it should be stressed that Y-chromosome markers are not intended to supplant standard autosomal STR analysis for routine forensic casework. They should be used only when, due to the nature of the sample, autosomal STR analysis fails or is likely to fail. The limitations of the Y-STR systems stem from the basic biology of the Y-chromosome. Since, barring mutations, Y-STRs are inherited unchanged from father to son as a haplotype of physically linked markers, there is a lack of independence between the loci that results in a reduced degree of inter-individual genetic variation compared to autosomal STR systems. Thus, it is not possible to use the product rule to determine the frequency of occurrence of a particular multi-locus Y-STR haplotype in a population. Instead, a counting method is used which simply counts how many times a particular haplotype has been observed in a database of size N, thus limiting the minimal frequency estimate to $1/N$. In addition, some of the Y-STR loci are not very discriminating, which, together with the other limitations described, means that it would be premature to use Y-STR profiles to populate large databases of convicted offenders due to the large number of fortuitous matches that would be obtained.

Population Studies

The allelic distribution, genetic diversity, and 19-locus haplotype distribution for the MPI and MPII loci have been determined in various Caucasian, Hispanic, African, American, and Native American populations. The results of this analysis will be the subject of a separate report.

Concluding Remarks

A SWGDAM developmental validation was carried out on two Y-STR multiplexes that collectively permit the analysis of 19 loci. Subsequent to optimization of the PCR parameters, a number of performance checks proved that they possess the required degree of robustness, sensitivity, stability, and specificity for potential forensic casework use.

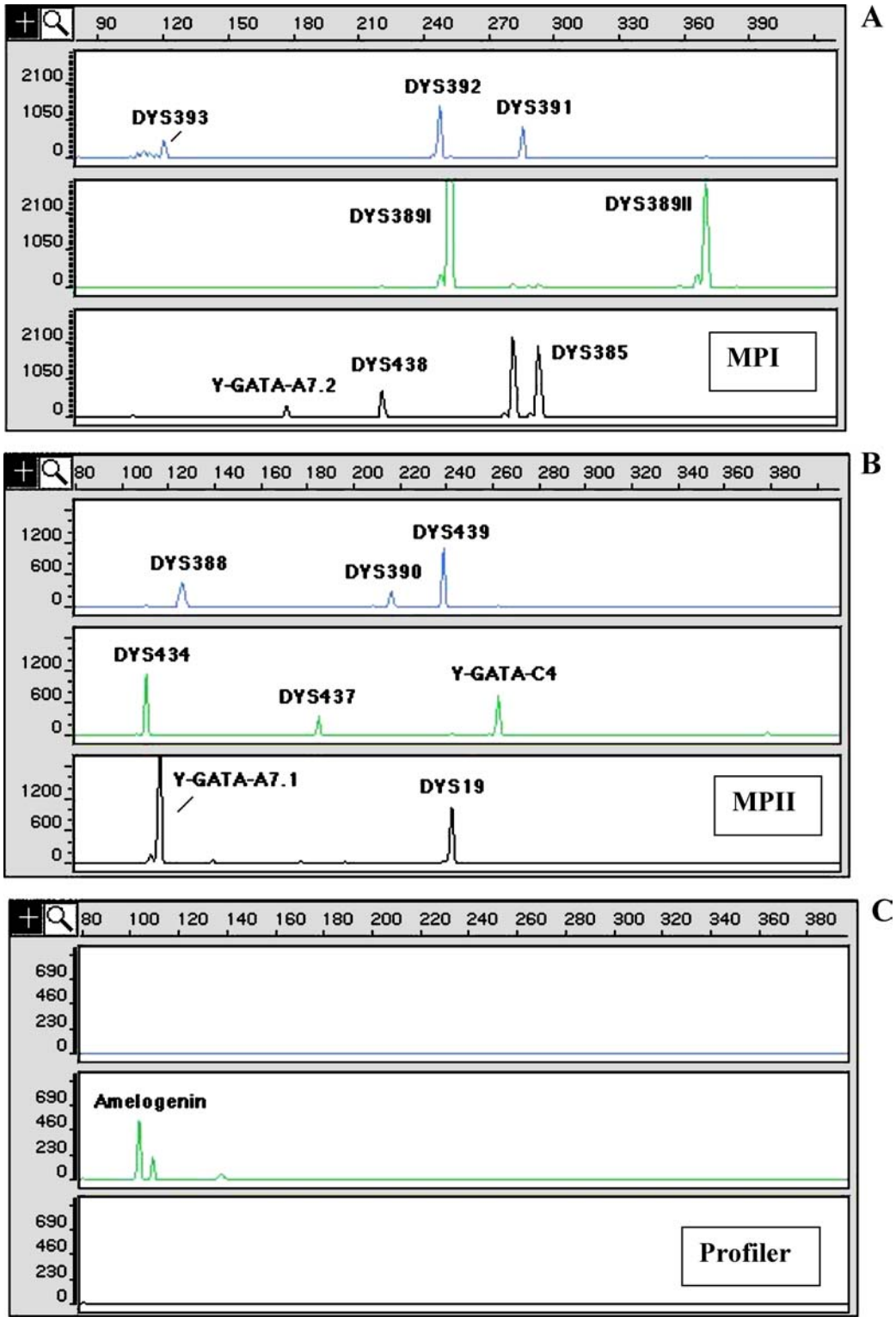

FIG. 7—*Y-STR profile from a vasectomized male. DNA extracted from a semen stain from a vasectomized male was analyzed using both Y-STR and autosomal STR systems. MPI yielded a full 9-locus profile* (A) *while eight of the ten MPII loci were amplified* (B). *The autosomal STR system did not produce any results with the exception of the non-STR amelogenin locus* (C).

Acknowledgments

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