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Simultaneous Detection of Multiple STR Loci on Sex Chromosomes for Forensic Testing of Sex and Identity

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ABSTRACT: The forensic usefulness of X and Y chromosomal STR loci has recently been demonstrated. One quadruplex-PCR, using 2 X- and 2 Y-STRs (STRX1/HPRTB and DYS390/DYS393), and 2 duplex-PCRs, each using an X- and a Y-STR (ARA/DYS390 and ARA/DYS393), and detection of PCR products by using an automated DNA sequencer are reported herein. This approach allows us to determine not only the sex of the donor of a sample, but also the X- and/or Y-STR genotypes of the sample. A male biological specimen yields 4 amplified products in quadruplex-PCR and 2 amplified fragments in duplex-PCRs, whereas a female biological specimen yields only 2 amplified fragments of X-STR in quadruplex-PCR and one fragment, also of X-STR, in duplex-PCRs. Our study thus provides useful information for many activities in forensic practice, such as identity testing, paternity testing, especially of deficiency cases, compilation of population data, and sex determination of a biological sample from a single PCR.

KEYWORDS: forensic science, sex determination, DNA typing, polymerase chain reaction, multiplex, X chromosome, Y chromosome, short tandem repeats

Identity testing by analysis of polymorphic DNA loci by the polymerase chain reaction (PCR) has become a routine procedure in the field of forensic medicine because it is more sensitive and less labor intensive than traditional Southern blotting or AmpFLP analysis. Using PCR in variable numbers of tandem repeats (VNTR) systems for personal identification yields more precise allele determinations (1,2), but a relatively large product size makes it difficult to incorporate them into multiplexing or co-amplification, which was first introduced by Chamberlain et al. in 1988 (3). Co-amplification of genetic loci, when possible, is advantageous to forensic scientists, because it not only can be used on the small quantities of poor quality DNA encountered in forensic practice, but also yields genetic information on additional loci without consuming additional DNA. In addition, multiplexing is time and cost effective (4–6). Short tandem repeats (STR), which consist of simple tandemly repeated sequences of 2–7 bp with fragment sizes less than 350 bp (7) and are widely spread throughout the human genome, may even be amplified from a very small amount of highly degraded DNA samples. Moreover, shorter fragment sizes of STR enable us to amplify 2 or more loci simultaneously in one reaction from a single DNA sample. Thus, STR amplified in multiplex-PCRs are of special interest to forensic scientists because they can be used on poor quality DNA, and they also facilitate in compiling population databases of several loci at a time. Forensic testing of any genetic marker requires establishing a database of the relevant population for proper analysis of the results.

The use of fluorescently labeled primers and detection with highly sensitive automatic DNA sequencers have proven powerful tools for detection of multiplex-PCR products and analysis of genetic polymorphisms (8). We herein present a strategy for multiple detection of X and Y chromosomal STR loci for sex and personal identification using forensic biological materials. In this study, we focus on 3 X chromosomal STR (7) and 2 Y chromosomal STR loci (9).

Materials and Methods

DNA Extraction

Genomic DNA was extracted from the blood of healthy unrelated individuals and various forensic samples by the phenol-chloroform method (10); from saliva samples by chelex extraction (11,12); and from hair and fresh tissue samples using an Isotissue DNA extraction kit (Nippon Gene, Tokyo, Japan), according to the manufacture's instructions. Extracted DNA was quantified by measuring its optical density at 260 nm; a value of 1.0 corresponds to approximately 50 μg/mL DNA (Beckman-DU®70 Spectrophotometer, Fullerton, CA, USA).

PCR Amplification for STR Typing

There are many STR loci within the X and Y chromosomes. Appropriate sets of amplification were determined and designed

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TABLE 1—*STR loci, number of repeats, size range of known alleles and of allelic ladders used in the present study.*

Locus	No. of Repeats of Known Alleles	No. of Alleles	Size Range of Known Alleles (bp)	No. of Repeats & Size Range of Allelic Ladder	Reference†
HumSTRX1	ND	13	308–332*	11(308)–17(332)*	Edwards (7)
HumHPRTB	6–16	11	263–299	10(275)–17(303)*	Edwards (6)
DYS390	18–27	10	191–227	21(203)–27(227)	Kayser (9)
DYS393	9–15	6	108–132	12(120)–15(132)	Kayser (9)
HumARA	11–35	27	255–327	17(273)–32(318)	Kishida (13)

ND = not determined.
* From detected allele and sequence data of present study.
† For known alleles.

TABLE 2—*STR loci with repeated motif, chromosomal location, primer sequences, and fluorescent dye labels used in this study.*

Locus and Repeated Motif	Chromosomal Location	Primer Sequence (5′-3′)	Dye Label	Reference and Accession No.
HumSTRX1 (AGAT)n	X	F: 5′-CTCCTTGTGGCCTTCCTTAAATGG-3′	(Red)	Edwards et al. (7)
	(♂,♀)	R: 5′-CTTCTCCAGCACCCAAGGAAGTCA-3′	ROX	GenBank M38419
HumHPRTB (AGAT)n	X	F: 5′-ATGCCACAGATAATACACATCCCC-3′	(Blue)	Edwards et al. (7)
	(♂,♀)	R: 5′-CTCTCCAGAATAGTTAGATGTAGG-3′	FAM	GenBank M26434
DYS390 (CTG/AT)n	Y	F: 5′-TATATTTTACACATTTTTGGGCC-3′	(Red)	Kayser et al. (9)
	(♂)	R: 5′-TGACAGTAAAATGAACACATTGC-3′	ROX	GDB G00-366-115
DYS393 (GATA)n	Y	F: 5′-GTGGTCTTCTACTTGTGTCAATAC-3′	(Green)	Kayser et al. (9)
	(♂)	R: 5′-AACTCAAGTCCAAAAAATGAGG-3′	HEX	GDB G00-456-649
HumARA (AGC)n	X	F: 5′-TCCAGAATCTGTTCCAGAGCGTGC-3′	(Green)	Edwards et al. (7)
	(♂,♀)	R: 5′-GCTGTGAAGGTTGCTGTTCCTCAT-3′	HEX	GenBank M21748

to prevent overlap of fragment size and to minimize competitive amplification of one loci for another. One quadruplex-PCR using 2 X and 2 Y chromosomal STRS (STRX1/HPRTB and DYS390/DYS393, respectively) and 2 duplex-PCRs using one X and one Y chromosomal STR (ARA/DYS390-Duplex I and ARA/DYS393-Duplex II, respectively) were selected for use in the present study (Table 1).

Accurate and convenient allele determination requires comparison of allele fragments with an allelic ladder. The X-STR allelic ladders were constructed by mixing the selected PCR products of different alleles after confirming their simple repeated units and flanking region by direct sequencing, by the dye terminator method using a 377 DNA sequencer (Perkin Elmer, Applied Biosystems, Foster City, CA, USA). Y-STR allelic ladders were kindly provided by Peter de Knijff of Leiden University, the Netherlands. Allelic ladders for corresponding sets of multiplex-PCRs were loaded in lanes flanking the samples and electrophoresed with the samples.

PCRs were performed in a final 25 μL reaction mixtures containing 10–40 ng template DNA, 200 μM dNTPs, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 2U AmpliTaq Gold™ (PE, Applied Biosystems, Foster City, CA, USA) in a PE GeneAmp PCR System 2400 thermal cycler (Norwalk, CT, USA). All oligonucleotide primers used were synthesized commercially by Nippon Flourmills Co. Ltd., Japan and the reverse primers were labeled with different fluorescence dyes. In the quadruplex-PCR, 3–4, 2.5–3, 10–15, and 5–7.5 pmol each of STRX1, HPRTB, DYS390 and DYS393 primers, respectively, were used. For duplex-PCRs, 15 pmol each of ARA primers, 30 pmol each of DYS390 primers, and 5 pmole each of DYS393 primers were used. Recommended PCR cycling conditions were as follows:

For quadruplex-PCR (STRX1/HPRTB and DYS390/DYS393) − 94°C × 10 min, followed by 30 cycles of 94°C × 20 s, 55°C × 20 s, 72°C × 20 s and 72°C × 2 min. For duplex I (ARA/DYS390) − 94°C × 10 min, followed by 10 cycles of 94°C × 20 s, 55°C × 20 s, 72°C × 40 s and another 20 cycles of 94°C × 20 s, 60°C × 20 s, 72°C × 40 s, and 72°C × 2 min. For Duplex II (ARA/DYS393) − 94°C × 10 min, followed by 30 cycles of 94°C × 30 s, 60°C × 30 s, 72°C × 30 s and 72°C × 2 min.

Negative control reactions were also added to each PCR amplification set. The STR loci and corresponding primer sequences are listed in Table 2.

Electrophoresis

Amplification products were verified using post-amplification electrophoresis in 1.5% agarose gels (SeaKem®, FMC BioProducts, Rockland, ME, USA) stained with ethidium bromide (Figures). Then 0.5–1.5 μL of amplified PCR product was mixed with 0.5 μL of GS 500 TAMRA, a yellow labeled 500-base-pair internal size standard (PE, Applied Biosystems, Foster City, CA, USA), 2.5 μL formamide and 1 μL loading buffer. After denaturation at 90°C for 3 min, the mixtures were applied to a 6% polyacrylamide/bisacrylamide (19:1), 8 M urea, sequencing gel (24 cm well-to-read) and run in 1 × TBE buffer (0.09M Tris, 0.09M boric acid, and 0.001M EDTA) for 3.5 h at 2500 V, 45 mA, 30 W, by using a 373A automated DNA sequencer (PE, Applied Biosystems, Foster City, CA, USA). Electrophoresis data were collected and automatically analyzed by 672 Genescan software (Applied Biosystems) which employs Southern local method for calculation of fragment sizes utilizing known internal size standards.

Results and Discussion

After optimization of various PCR parameters, 2 X-STR loci (STRX1, HPRTB) and 2 Y-STR loci (DYS390, DYS393) as quadruplex-PCR, and 2 sets of one X- (ARA) and one Y-STR (DYS390

or DYS393) as duplex-PCRs were successfully amplified, and the sex of the donor as well as X- and Y-STR genotypes were determined accordingly.

A DNA sample derived from a male, who carries both X and Y chromosomes, yielded 4 fragments in quadruplex-PCR and 2 fragments in duplex-PCRs, whereas the DNA derived from a female, who carries 2 X chromosomes, yielded only 2 fragments of X-STR in quadruplex-PCR and one fragment in duplex-PCRs (Fig. 1). Female fragments may either be homozygous or heterozygous, but all male fragments are hemizygous. Electropherograms of a male and a female sample and allelic ladders used in quadruplex-PCR are presented in Fig. 2. One should suspect contamination or a male false positive result if there is detection of any extra peaks in the electropherogram results of either sample, i.e., a total of 5 peaks or more in quadruplex-PCRs or 3 peaks or more in duplex-PCRs.

Although the STR loci in this study were amplified faithfully in simplex PCR, the ARA locus, which is more polymorphic than others, was difficult to amplify in multiplexing. Moreover, to achieve successful multiplexing, optimization of primer concentration, annealing temperature and usage of AmpliTaq Gold™ (Perkin Elmer) were crucial in this study. In the present authors' experience, however, it is more important to adjust the primer concentration in multiplexing to achieve a PCR product without preferential amplification. If there is a strong preferential amplification in any locus, stutter bands or artifacts may lead to erroneous interpretation in typing and result. One of the reasons that we chose only tri- and tetranucleotide repeat STR is that they amplify more faithfully than (AC)n repeats and display less stutter following amplification (7). And also, appearance of PCR products in a multiplex-PCR system depends mainly on 2 factors; the primer concentration and the type of the dye used. For example, the HumHPRTB locus in quadruplex-PCR is very easy to amplify, having a wide range of annealing temperature and requires least amount of primers. However, if its peak height is significantly higher than the others, one can lower its primer concentration without interfering any other amplification, and vice versa. The 4 fluorescent dyes used in the present study have different intensity of fluorophore emission. For example, the HEX, is the most sensitive to be detected in the DNA sequencer, whereas ROX is the least sensitive to be detected. Therefore, it is usual to obtain a somewhat unbalanced appearance of PCR products in electropherogram even after adjusting the matrix file (Fig. 2A). Detection of fluorescent labeled PCR products in a DNA sequencer is very accurate and sensitive that it does not disturb the DNA typing nor affect the quality of PCR, as long as a clear colored peak between the size range of a corresponding locus is detected. Some variations (ranges) in primer concentration of quadruplex system are therefore given in materials and methods section. No discrepancy was found between the results after single and multiplex-PCR (data not shown). Furthermore, no PCR products were detected in the negative control samples.

Until now, we have detected 7, 8, 6, 5, and 16 alleles in STRX1, HPRTB, DYS390, DYS393, and ARA loci, respectively, in a Japanese population. Quadruplex-PCR does not display any overlapping alleles as yet, but since the distance between the longest allele detected in the HPRTB locus and the shortest allele detected in the STRX1 locus is only 5 bp, we labeled the primers with different colors to avoid identification problems taking into consideration that additional alleles might later be detected. Designation and nomenclature of the alleles was carried out according to the recommendations of the International Society of Forensic Haemogenetics (14) and as described by Edwards et al. (6,7) and Kayser et al. (9).

The forensic usefulness of X and Y chromosomal STRs has recently been evaluated (7,9), and using at least one X and one Y

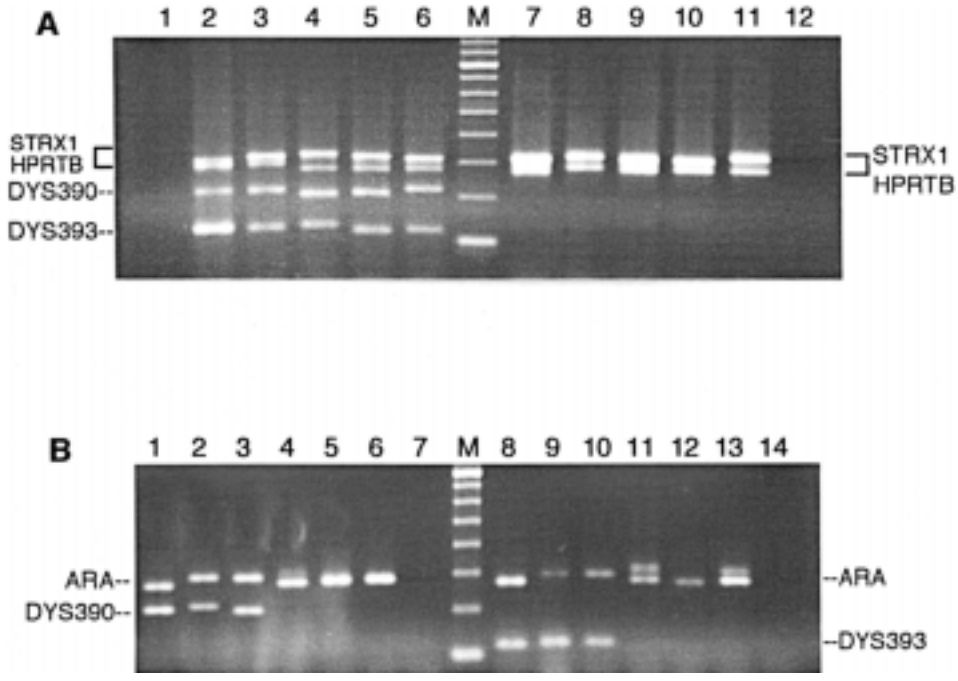

FIG. 1—*Multiplex-PCR products of blood samples of X and Y chromosomal STR loci run in 1.5% agarose gel. A. Quadruplex (2 X and 2 Y) PCR products. Lanes 1 and 12: negative amplification controls; lanes 2–6: male samples; lanes 7–11: female samples. B. Duplex (one X and one Y) PCR products. Lanes 1–3 and lanes 8–10: male samples; lanes 4–6 and lanes 11–13: female samples; lanes 7 and 14: negative amplification controls. M: 100 base-pair ladder. Note the stronger PCR products in female samples, although an equal amount of aliquot (8 μL) for each sample was applied for electrophoresis. It may be due to the fact that the electrophoretic conditions used here have not separated the 2 X products well, whereas the Y products are separated into distinct bands.*

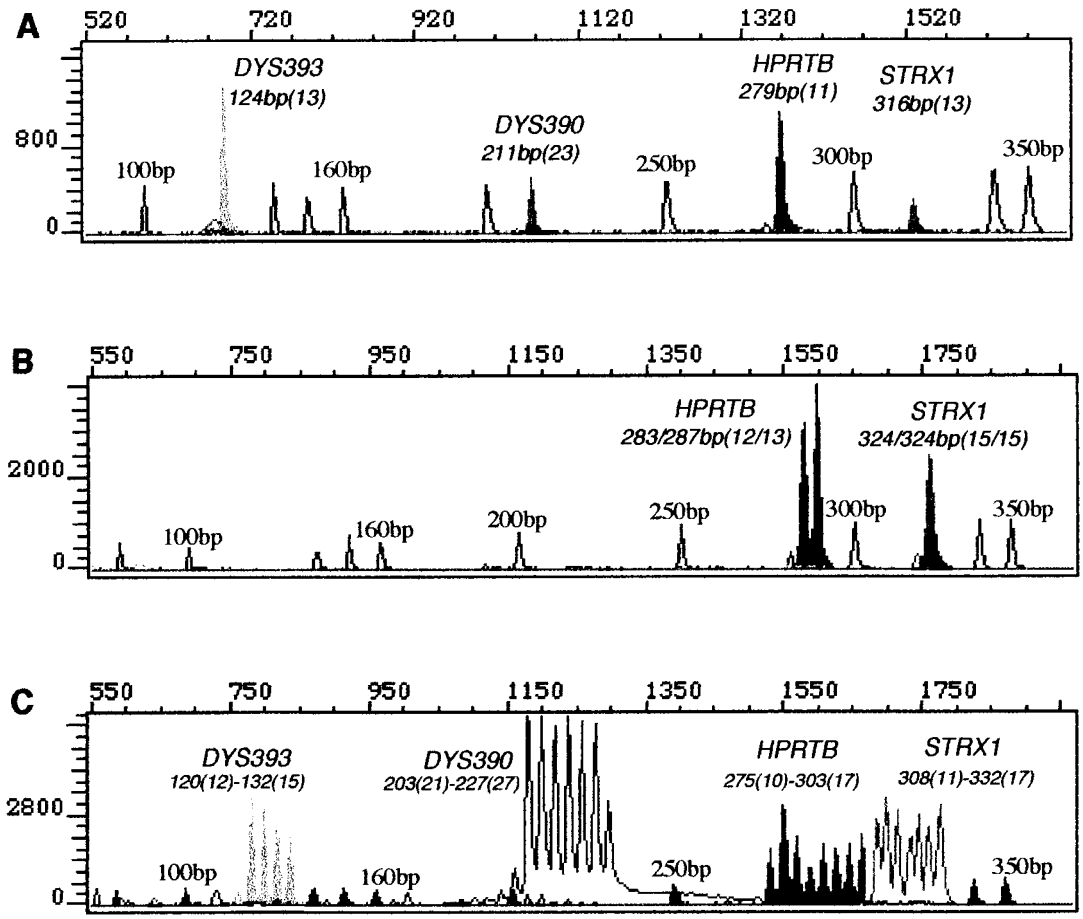

FIG. 2—*Electropherograms of quadruplex-PCR. A. Electropherogram of a male sample showing one peak at each locus. B. Electropherogram of a female sample, heterozygous for HPRTB, and homozygous for STRX1. No peak is detected in Y-STR loci. C. Allelic ladders used in quadruplex-PCR. Repeated units are shown in parenthesis.*

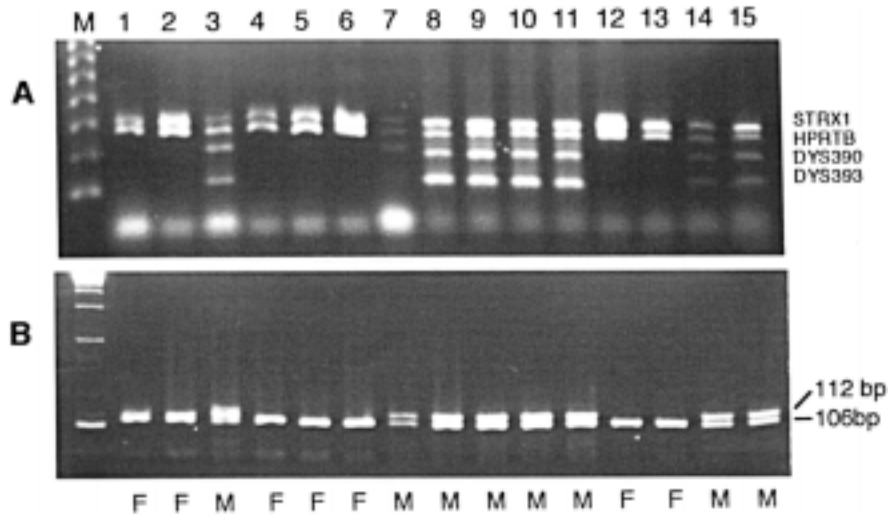

FIG. 3—*Validation study of the present method. A. Quadruplex-PCR applied to various forensic DNA samples run in 1.5% agarose gel. Lane 1: tooth; lane 2: hair; lane 3: bone marrow; lane 4: saliva; lane 5: vaginal swab; lane 6: spleen; lane 7: bone; lanes 8 to 15: sex undetermined blood samples. M: 100 base-pair ladder. B. XY homologous amelogenin PCR run in 4% metaphor gel. Each lane corresponds to the samples used in A. Left-most lane: 100 base-pair ladder; F = female, M = male.*

chromosomal STRs in single PCR not only yields sex identification but also additional DNA typing. Generally, routine sex determination tests are based on the X-Y homologous gene amelogenin which only gives information of sex (15). Some workers described multiplex systems in which amelogenin is incorporated to determine sex (4,16), and most multiplex STR systems are currently using somatic STR loci. The advantage of using X- and Y-STR in paternity testing over somatic STR loci is that male siblings directly express the father's Y type and one of the mother's X types, and all female siblings, including half siblings, carry the father's X type, which is common to all. Thus, this approach makes it easier to identify paternal and maternal lineages in deficiency cases. Moreover, in rape cases, Y markers are especially useful for they provide sex determination and specific information on the male assailant (9,17).

We applied the present method to DNA samples derived from various forensic and evidentiary materials, including hair, blood stains, tooth, bone, saliva and tissues, extracted by various procedures, and successfully determined the sex with corresponding X- and Y-STR DNA types. To confirm the reliability of present methods of sex identification, previously sex-determined forensic samples and 50 undetermined blood samples were typed by the present procedures. Then sex identification of these samples was confirmed by performing X-Y homologous gene Amelogenin PCR (15), and we found no discrepancy in sex determination between these two results (Fig. 3). Typing results of Fig. 3 are presented in Table 3. Moreover, we applied the present method to various DNA concentrations and found that only 1 ng of template DNA was enough for typing (Fig. 4).

In conclusion, STR polymorphisms are becoming the standard genetic markers used throughout the world for development of forensic databases. In forensic identification, it is sometimes a serious problem that material evidence obtained from the crime scene is restricted. Therefore, our method has a great advantage for foren-

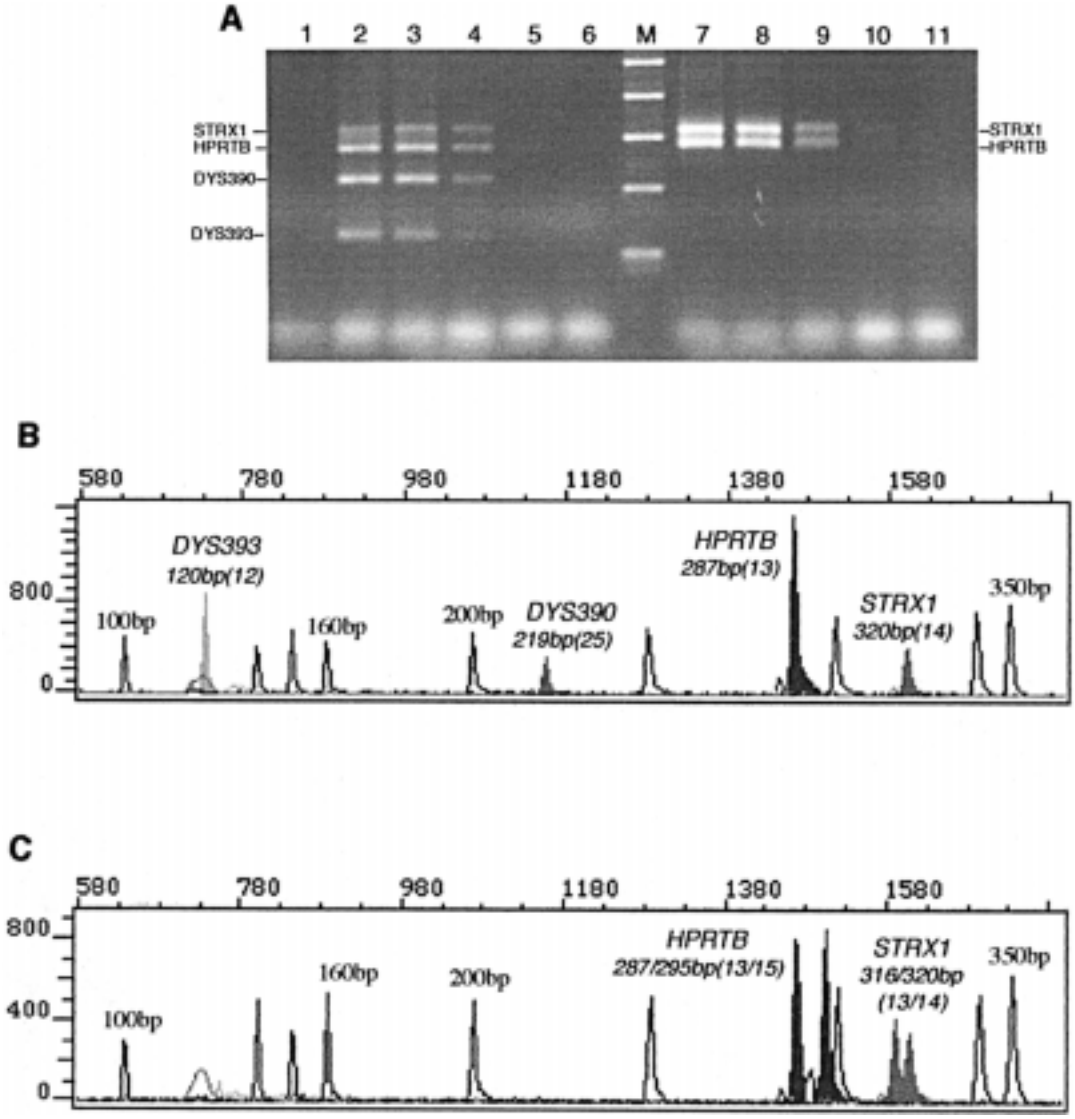

FIG. 4—*Sensitivity study of quadruplex-PCR. A. Electrophoresis run in 1.5% agarose gel. Lane 1: negative amplification control; lanes 2–6: male samples; lanes 7–11: female samples. DNA was serially diluted in the following concentrations: 20 ng, 10 ng, 1 ng, 0.1 ng, and 0.01 ng. Although there were only very faint bands detected in lanes 5 and 10, which correspond to 0.1 ng of template DNA in this photograph, they were detected in electropherograms. B. Electropherogram of PCR product of a male sample using 0.1 ng template DNA. C. Electropherogram of PCR product of a female sample using 0.1 ng template DNA.*

TABLE 3—*Typing results of DNA samples presented in Fig. 3.*

S/N	Sample	Sex before typing	STRX1	HPRTB	DYS390	DYS393	Sex after typing
1	Tooth	Female	14/15	14/14	—	—	Female
2	Hair	Female	14/15	12/13	—	—	Female
3	Bone Marrow*	Male	14	12	25	13	Male
4	Saliva	Female	15/16	13/13	—	—	Female
5	Vaginal Swab	Female	14/15	12/12	—	—	Female
6	Spleen	Female	12/13	11/13	—	—	Female
7	Bone*	Male	14	12	25	13	Male
8	Blood K1	Not known	13	13	23	12	Male
9	Blood K2	Not known	15	14	23	13	Male
10	Blood K3	Not known	13	13	24	12	Male
11	Blood K4	Not known	14	13	24	12	Male
12	Blood K5	Not known	12/16	13/15	—	—	Female
13	Blood K6	Not known	13/13	12/13	—	—	Female
14	Blood K7	Not known	15	12	23	13	Male
15	Blood K8	Not known	13	14	24	11	Male

* From same body.

sic practice because multi-loci information, including sex identification, can be detected by amplification in a single tube. Y markers alone can also determine the sex of a sample without female genetic information. Therefore, as long as some laboratory precautions are taken, especially to avoid contamination of samples, male/male or male/female, the procedures presented here can be applied for practical use.

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