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Multiplex Systems for the Amplification of Short Tandem Repeat Loci: Evaluation of Laser Fluorescence Detection

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Marie D. Ricciardone[1,2], Ann M. Lins[3], James W. Schumm[3] and Mitchell M. Holland[1]
[1]Armed Forces Institute of Pathology, Rockville, MD, USA; [2]Bilkent University, Bilkent-Ankara, Turkey; [3]Promega Corporation, Madison, WI, USA

ABSTRACT

Short tandem repeat (STR) loci are ideal markers for personal identification and for genomic mapping. Two fluorescent multiplex systems, each designed for simultaneous PCR amplification of four polymorphic STR loci (HUMCSF1PO, HUMTPOX, HUMTH01 and HUMVWFA31, and HUMF13A01, HUMFESFPS, HUMBFXIII and HUMLIPOL), were evaluated on three laser fluorescence detection instruments. Concordant DNA typing results were obtained with all three detection methods. These fluorescent multiplex STR systems offer an accurate, reproducible and versatile method of DNA profiling that is well-suited for forensic identity testing and other genetic analyses.

INTRODUCTION

Short tandem repeat (STR) loci consist of tandemly repeated sequence motifs, 3–7 bp in length, that are widely distributed throughout the human genome (2,4,5,16–18,24). Since the number of repeat motifs present at a particular locus within a population of individuals is highly polymorphic, STRs are ideal genetic markers for personal identification. Individual profiles are generated by polymerase chain reaction (PCR) amplification of the STR loci using specific primers that flank the repeat motif. The amplified fragments, each with a discrete length that reflects the number of repeat motifs, are separated by denaturing gel electrophoresis and subsequently detected by silver stain or fluorescence methods. STR profiles have been used for personal identification in forensic DNA typing (4,6–8,10,11), parentage determination (1) and genetic mapping (14).

STR analysis offers several advantages for personal identification. Whereas conventional DNA typing methods, such as restriction fragment length polymorphism (RFLP) analysis, require relatively large amounts of intact DNA, PCR typing methods are often successful when the quantity of DNA is limited (15) or when the DNA is highly degraded (3,9). Although individual STR loci are only moderately discriminating, multiple STR loci can be highly discriminating (10,12,23). The optimal STR profiling system should be accurate, sensitive, discriminating and robust. With these goals in mind, we have evaluated two multiplex STR systems, each designed for the simultaneous amplification of four polymorphic STR loci (12,21), on three laser fluorescence detection-instruments: the Model 373 DNA Sequencer, the FluorImager™ Scanner and the FMBIO® Fluorescent Scanner.

The Model 373 DNA Sequencer detects fluorescent PCR fragments as they migrate through the gel past a scanning laser. The DNA fragments are automatically sized by the integrated GENESCAN™ software using co-electrophoresed standards labeled with a second fluorescent dye (13,25). This method offers a precise, accurate, sensitive and reproducible method of DNA fragment detection that has been successfully used with several STR systems for personal identification (6,7,10,22,23). The FluorImager and FMBIO instruments detect fluorescent PCR fragments by scanning the gel after electrophoresis is completed. The DNA fragments are sized by visual or automated comparison with adjacent allelic ladders (19,21). Because the fluorescence detection is independent of electrophoresis, several gels can be analyzed rapidly with the same instrument. This study evaluated the consistency and accuracy of the STR profiles obtained using the three fluorescence detection methods.

Table 1. STR Precision Data

Multiplex (Dye)	Locus	No. of Alleles	CV[c] (%)	Range[d] (bp)
CTTV (FL)[a]	CSF1PO	9	0.07	1.10
	TPOX	5	0.11	1.30
	TH01	7	0.06	0.62
	VWF	8	0.13	0.85
CTTV (HEX)[b]	CSF1PO	9	0.07	0.97
	TPOX	8	0.10	1.20
	TH01	7	0.06	0.60
	VWF	8	0.14	0.92
FFFL (FL)[b]	F13A	12	0.08	1.20
	FESFPS	8	0.10	1.20
	F13B	6	0.07	0.45
	LPL	7	0.14	0.67

[a]CTTV (FL) data represent 90 replicate samples and 12 electrophoretic runs.

[b]CTTV (HEX) and FFFL (FL) data represent 49 replicate samples and 8 electrophoretic runs.

[c]CV or coefficient of variation is the ratio of the standard deviation to the mean. Values represent the maximum CV observed for all the alleles tested at that locus.

[d]Range is the difference in length between the longest and shortest fragments observed for an individual allele. Table values represent the maximum range observed for all the alleles tested at the locus.

MATERIALS AND METHODS

STR Systems

The CTTV multiplex (Promega, Madison, WI, USA) contained the HUMCSF1PO, HUMTPOX, HUMTH01 and HUMVWFA31 primer pairs. Both fluorescein-labeled and HEX-labeled CTTV primers were evaluated. The FFFL mul-

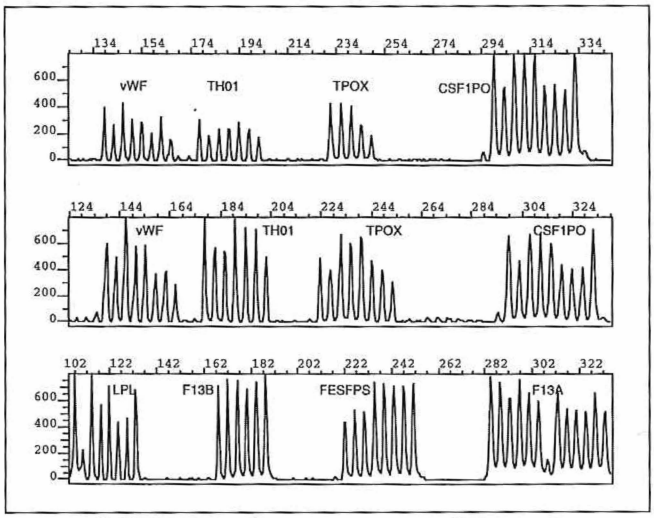

Figure 1. GENESCAN analysis of fluorescent multiplex STR allelic ladders. Top panel: electropherogram of fluorescein-labeled CTTV allelic ladders (TPOX ladder contains alleles 8–12). Center panel: electropherogram of HEX-labeled CTTV allelic ladders (TPOX ladder contains alleles 6–13). Bottom panel: electropherogram of fluorescein-labeled FFFL allelic ladders. Values along x-axis represent the size of DNA fragments; values along y-axis represent the intensity of fluorescence.

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Table 2. FFFL STR Fragments: GENESCAN Analysis and Allele Assignment

Sample	LPL		F13B		FESFPS		F13A01	
	Size[a]	Allele[b]	Size[a]	Allele[b]	Size[a]	Allele[b]	Size[a]	Allele[b]
6	117.32	10	177.67	8	239.27	11	287.10	5
	125.29	12	181.97	9	242.87	12	290.95	6
8	125.01	12	182.04	9	239.00	11	290.96	6
			186.00	10			294.83	7
10	117.30	10	169.50	6	235.77	10	291.23	6
	124.91	12	186.00	10	243.06	12	295.11	7

[a]Fragment size (bp) determined by GS analysis.
[b]Allele assigments according to band size measurements determined by precision studies (Table 1).

tiplex (Promega) contained the HUMF13A01, HUMFESFPS, HUMBFXIII and HUMLIPOL fluorescein-labeled primer pairs. Allelic ladders (19,21) containing a mixture of DNA fragments representing most or all of the known alleles at each locus were used to assist with allele size determination and measurement precision.

PCR Amplification of STR Loci

Model 373 DNA Sequencer. STR loci were co-amplified in a 25-µL reaction mixture containing approximately 2 ng of genomic DNA, four primer pairs, STR buffer (Promega), 1 U *Taq* DNA Polymerase (from either Perkin-Elmer, Norwalk, CT, USA or Boehringer Mannheim, Indianapolis, IN, USA) and overlaid with mineral oil. Amplifications were performed in 0.5-mL Thin-Walled GeneAmp® PCR Tubes in a GeneAmp 9600 System (Perkin-Elmer) as follows: denaturation at 96°C for 2 min; 10 cycles of 94°C for 1 min, 60°C for 1 min and 70°C for 1.5 min; 20 cycles of 90°C for 1 min, 60°C for 1 min and 70°C for 1.5 min; and final extension at 70°C for 10 min.

FMBIO and FluorImager. STR loci were co-amplified as described above except that the reactions contained approximately 10 ng template DNA; amplifications were performed in 0.5-mL GeneAmp PCR Reaction Tubes in a DNA

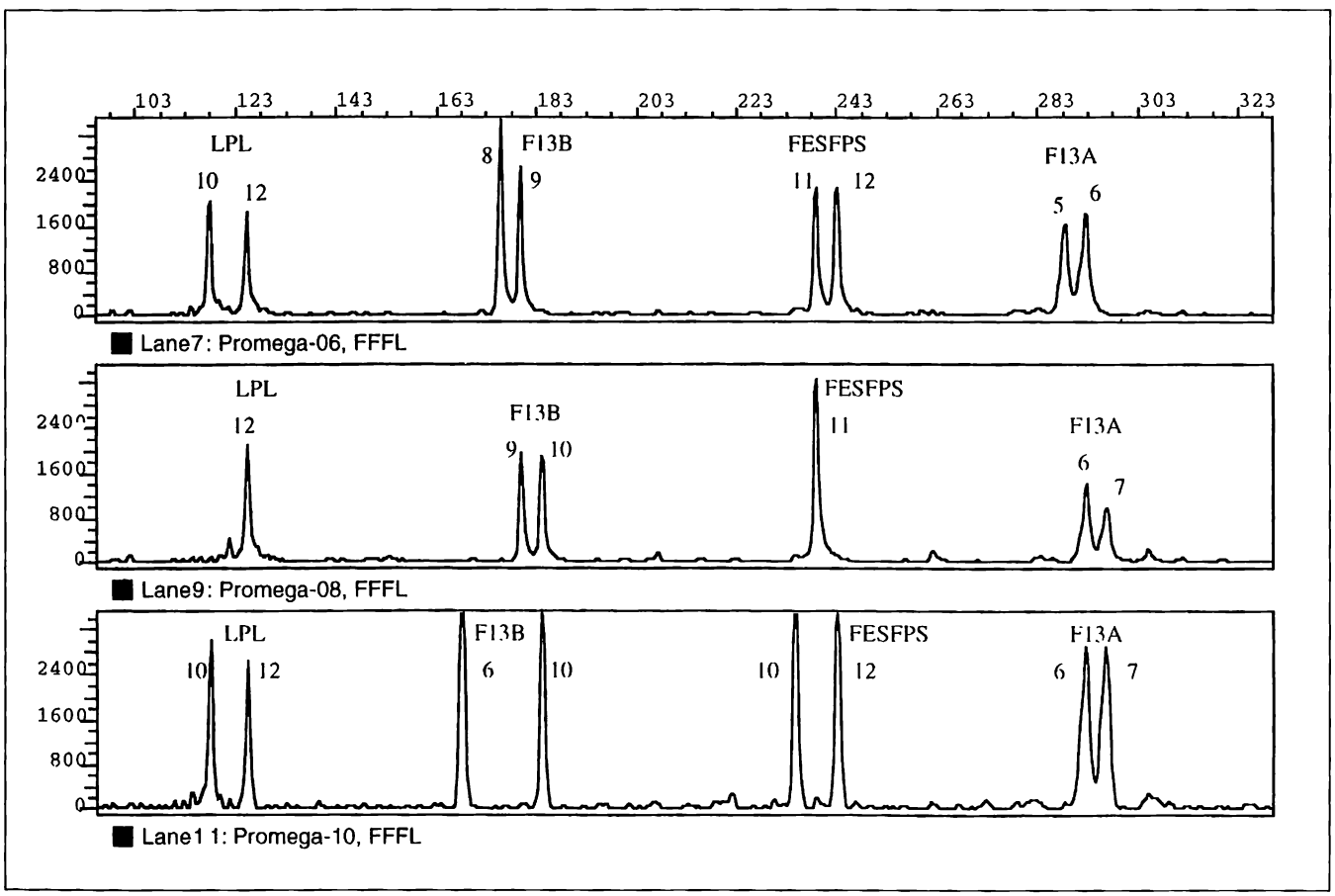

Figure 2. Fluorescence detection of FFFL-amplified DNA fragments using the Model 373 DNA Sequencer with GENESCAN software. Three representative electropherogram tracings for DNA samples 6 (top), 8 (center) and 10 (bottom).

Thermal Cycler 480 (Perkin-Elmer); the final extension at 70°C for 10 min was omitted.

Fluorescence Detection of Amplified Products

Model 373 DNA Sequencer. Amplified fragments were separated by gel electrophoresis on a Model 373 DNA Sequencer equipped with GENESCAN 672 Fragment Analysis Software (both from PE Applied Biosystems, Foster City, CA, USA). The 0.4-mm denaturing gel contained 6% polyacrylamide (19 acrylamide:1 bisacrylamide; Bio-Rad, Hercules, CA, USA), 8 M urea (Mallinckrodt, Paris, KY, USA) and 1× Tris-borate EDTA buffer (AMRESCO, Solon, OH, USA). Two microliters of the amplification reaction were mixed with 3 fmol of GENESCAN-2500 [ROX] Lane Standard (PE Applied Biosystems) and an equal volume of formamide, denatured at 95°C for 2 min, chilled on ice and loaded on the gel. Electrophoresis was at 30 W constant power for approximately 4 h.

FluorImager and FMBIO. Amplified fragments were separated by electrophoresis in a 0.4-mm denaturing gel containing 4% polyacrylamide (19 acrylamide:1 bisacrylamide; Promega), 7 M urea (Promega) and 0.5× Tris-borate EDTA buffer. A 2.5-µL portion of the amplification reaction was mixed with 2.5 µL loading solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue), denatured at 95°C for 2 min and chilled on ice. A 3-µL portion was loaded on the gel. Electrophoresis was at 40 W constant power for 50–60 min. After electrophoresis, DNA fragments were detected using the FluorImager Scanner (Molecular Dynamics, Sunnyvale, CA, USA) or the FMBIO Fluorescent Scanner (Hitachi Software Engineering America, San Bruno, CA, USA).

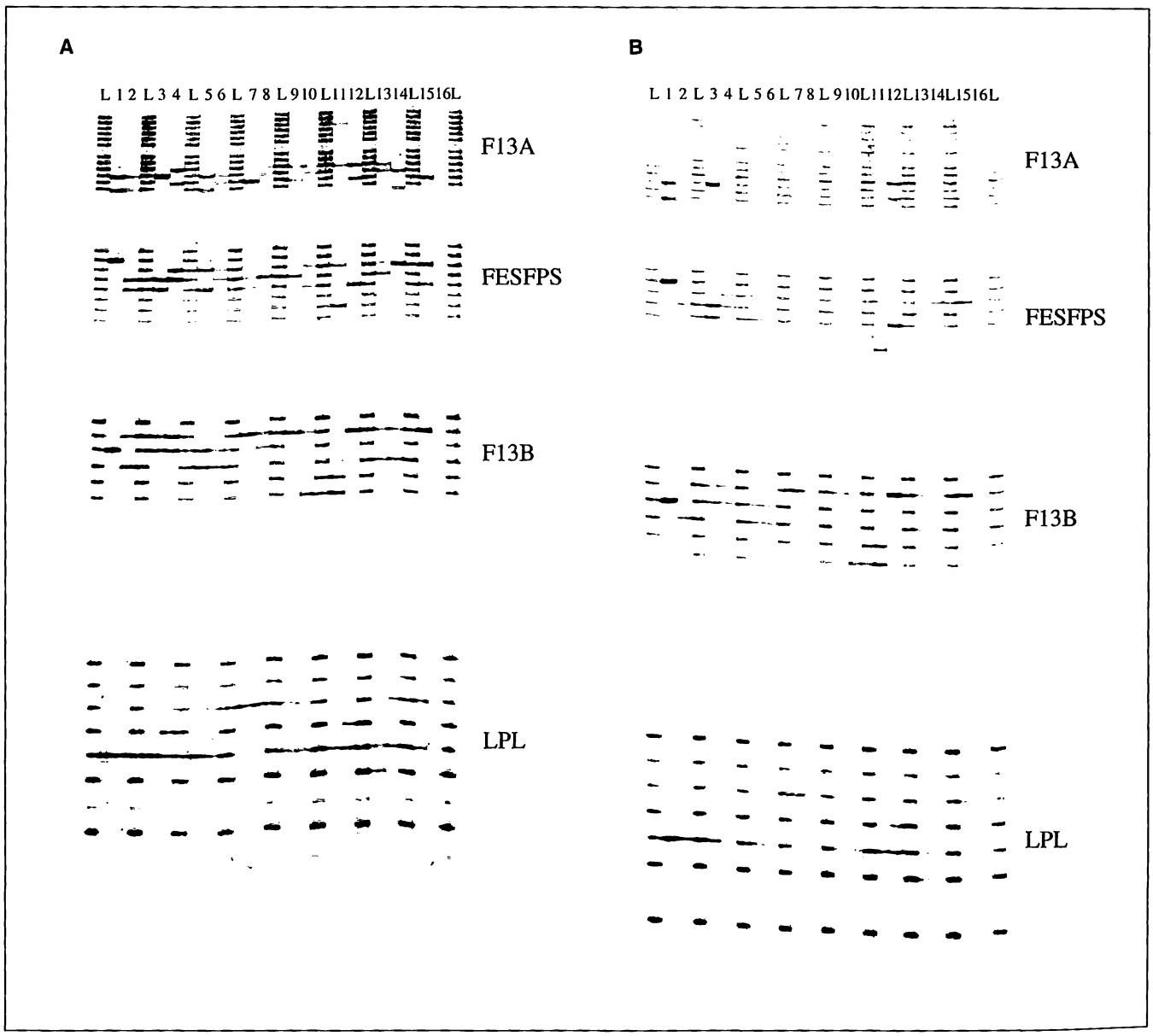

Figure 3. Fluorescence detection of FFFL-amplified DNA fragments. (a) Fluorescence detection using the FluorImager. Lanes labeled L contain locus-specific allelic ladders. Lanes numbered 1–14 contain individual-amplified DNA samples. Lane 15 contains amplified DNA from the K562 cell line. Lane 16 contains negative-control amplification reaction (no template DNA). (b) Fluorescence detection using the FMBIO scanner. Lanes labeled L contain locus-specific allelic ladders. Lanes numbered lanes 1–14 contain individual-amplified DNA samples. Lane 15 contains amplified DNA from the K562 cell line. Lane 16 contains negative-control amplification reaction (no template DNA).

RESULTS AND DISCUSSION

Model 373 DNA Sequencer Precision

The precision of DNA fragment-length sizing by the GENESCAN software on the Model 373 DNA Sequencer was evaluated by performing replicate analysis of allelic ladders. Figure 1 shows representative electropherogram tracings for the CTTV ladder alleles (labeled with both fluorescein [FL] and HEX) and the FFFL ladder alleles. Each allele is resolved as a discrete peak and, as expected, the size ranges of the alleles of different loci do not overlap. Fragment-length sizing is very reproducible, as indicated by the small coefficients of variation (CV) (Table 1). The maximum CV observed was 0.14%. This value indicates a measurement precision of 99.86%, which is comparable to that observed by other investigators with this instrument (10). Another measure of the precision of the method is the tight range of fragment lengths observed for individual alleles. For most of the alleles tested, this value was less than one base (Table 1). The maximum range observed for any allele was 1.3 base, which is sufficient to ensure unambiguous allele assignment based on the length of the DNA fragment.

Allele Assignments

For Model 373 DNA Sequencer analysis, band sizes were defined for each ladder allele by the mean fragment length of the precision studies ±4 standard deviations. Statistically, approximately 99.99% of all observations should fall between these boundary values. Allele assignments for fluorescent PCR fragments were then made by comparing the fragment lengths calculated by the GENESCAN software with the band sizes defined for the individual ladder alleles. When using the FluorImager or FMBIO fluorescent scanners, alleles were assigned to the fluorescent PCR fragments by visual or software comparison of unknown samples to allelic ladders in adjacent lanes using Fragment Analysis Software and FMBIO Analysis Software supplied with the FluorImager and FMBIO instruments, respectively.

Inter-laboratory Validation

In an inter-laboratory validation study, 14 coded DNA samples (representing both pristine specimens and casework specimens) were amplified in two laboratories using the fluorescein-labeled CTTV, HEX-labeled CTTV and fluorescein-labeled FFFL. The amplified DNA fragments were then analyzed using the three laser fluorescence detection instruments. The Model 373 DNA Sequencer data can be viewed either as electropherograms (Figure 2), which display the amplified STR fragments as discrete peaks, or as tabulated data, which display the STR fragment length sizes in base pairs (Table 2). Data obtained with the FluorImager Scanner (Figure 3a) and the FMBIO Fluorescent Scanner (Figure 3b) are usually represented as gel images. A comparison of the FFFL data obtained using the three fluorescence detection instruments (Table 3; Figure 3, a and b; and data not shown) reveals iden-

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tical FFFL allele assignments. Fluorescence detection of CTTV fragments (both fluorescein-labeled and HEX-labeled) also resulted in identical CTTV allele assignments for the three analytical methods (data not shown). Thus, for all fourteen samples at all eight STR loci, there was 100% concordance in allele assignment among the three fluorescence detection methods.

These fluorescent CTTV and FFFL systems are well-suited for forensic identity testing. They meet the criteria of an optimal STR profiling system: accuracy, sensitivity, discrimination and robustness. In this study, we were able to distinguish consistently and unambiguously the TH01-9.3 and TH01-10 alleles and the F13A-3.2 and F13A-4 alleles (Table 3; Figure 3, a and b), whose PCR products differ by one or two base pairs, respectively. Reliable STR profiles were obtained with as little as 2 ng of template DNA. An eight-locus STR match using only moderately polymorphic markers offers significant power of discrimination. The combined matching probability of the CTTV and FFFL systems exceeds 1 in 17 000 000 in all groups tested to date (20).

Finally, this study demonstrates that the fluorescent CTTV and FFFL systems are compatible with a variety of instrumentation. The allelic ladders provide precise and reproducible results both within and across formats. Thus, electrophoretic gel conditions and fragment detection methods can vary considerably without compromising the accuracy of the genetic profile. The inter-laboratory comparison exhibited the consistency, reliability and robustness of the GenePrint™ STR systems (Promega) when detected using the Model 373 DNA Sequencer, FluorImager and FMBIO instruments.

Note added in proof. During the preparation, review and modification of this work, software available with the Hitachi FMBIO instrument has been expanded to allow line trace displays of individual bands as peaks, peak measurement, three-color detection and automated sizing in addition to the gel display format shown in this work. Also, updated versions of allelic ladders for the CSF1PO and VWF loci now contain 10 alleles each rather than the 9 and 8, respectively, included in Table 1.

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Address correspondence to Marie D. Ricciardone, Department of Molecular Biology and Genetics, Faculty of Science, Bilkent University, 06533 Bilkent-Ankara, Turkey. Internet: ricciard@fen.bilkent.edu.tr