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Characterization of 26 MiniSTR Loci for Improved Analysis of Degraded DNA Samples

ABSTRACT: An additional 20 novel mini-short tandem repeat (miniSTR) loci have been developed and characterized beyond the six previously developed by our laboratory for a total of 26 non-CODIS miniSTR markers. These new markers produce short PCR products in the target range of 50–150 base pairs (bp) by moving the primer sequences as close as possible—often directly next to the identified repeat region. These candidate loci were initially screened based on their small amplicon sizes and locations on chromosomes currently unoccupied by the 13 CODIS STR loci or at least 50 Mb away from them on the same chromosome. They were sequenced and evaluated across more than 600 samples, and their population statistics were determined. The heterozygosities of the new loci were compared with those of the 13 CODIS loci and all were found to be comparable. Only five of the new loci had lower values than the CODIS loci; however, all of these were much smaller in size. This data suggests that these 26 miniSTR loci will serve as useful complements to the CODIS loci to aid in the forensic analysis of degraded DNA, as well as missing persons work and parentage testing with limited next-of-kin reference samples.

KEYWORDS: forensic science, DNA typing, DNA profiling, degraded DNA, short tandem repeats, STR, miniSTR, D1GATA113E02, D1S1627, D1S1677, D2S441, D2S1776, D3S3053, D3S4529, D4S2364, D4S2408, D5S2500, D6S474, D6S1017, D8S1115, D9S1122, D9S2157, D10S1248, D10S1435, D11S4463, D12ATA63A05, D14S1434, D17S974, D17S1301, D18S853, D20S482, D20S1082, D22S1045

The development of novel mini-short tandem repeat (miniSTR) loci has become important in forensic DNA casework when degraded DNA samples are present. Six new miniSTR markers have been previously described and characterized (1) to serve as complements to the 13 CODIS loci in the analysis of badly damaged DNA samples. It would be valuable to evaluate additional miniSTR loci for a multitude of reasons (2). All of the chromosomes in the human genome have a variety of unique repeat regions that can all potentially serve as forensic identifiers. The commercial DNA typing kits currently used have loci that occupy only 14 of the 22 autosomal chromosomes (3). This leaves eight unoccupied chromosomes with potential markers as well as regions on the other chromosomes with previously untapped STR markers that could prove useful. Also, it would be beneficial to find additional small markers with high heterozygosities (4). Ultimately, obtaining STR typing results with additional loci would allow for an elevated level of confidence for an association, especially in a case where the DNA is badly degraded.

Recently, a collaborative study with the European DNA Profiling Group evaluated several methods of analysis to assess how effective each was for genotyping degraded DNA (5). STR systems (miniSTR assays and standard STR kits) and single nucleotide polymorphisms were compared and in general, miniSTR systems were observed to be the most effective in the analysis of degraded DNA (5). Several of the miniSTR loci reported here (D10S1248, D2S441, and D22S1045) have been recommended for adoption by the European DNA community as new core loci (6,7). In addition, many of these miniSTR markers have the potential to provide additional discrimination in complex paternity cases or missing persons cases (8).

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For this study, 26 new loci (20 in addition to the six that were previously characterized) were screened and selected as candidate markers to be developed as miniSTR loci. A larger group of loci derived from the literature was initially narrowed down to 32 loci based on their small amplicon sizes, their location on autosomal chromosomes that are well spaced from currently used STR markers (3), and their potential to have high heterozygosities within various populations (1). The target amplicon sizes for the candidate loci were between 50 and 150 base pairs (bp). The primers for these markers were all designed to be as close as possible, and often directly next to, the STR repeat region to allow for the smallest possible amplicon (1). As in our previous work, the newly characterized miniSTR loci are all positioned on chromosomes that differ from the 13 CODIS loci or at least 50 Mb apart from an existing locus on the same chromosome and considered unlinked from that marker. Each of the potential loci were previously reported in the literature (e.g., Marshfield Clinic) to have high heterozygosities. Once these "miniSTR" markers were selected, primers were designed and they were multiplexed into sets of three, with each locus in a different dye color. These loci were then run across more than 600 population samples (9) and a few homozygotes were sequenced to determine their repeat structure. They were all genotyped to determine population statistics, including their heterozygosities. Once characterized in this fashion, they were then compared to the 13 CODIS loci by amplicon size, chromosomal location, and heterozygosities. The results of this comparison are reported here.

Six of the original 32 markers were dropped from this study because they were problematic in various ways that will be further discussed; leaving 20 characterized novel miniSTR loci in addition to the six previously described (1). It was determined from the results that almost all of the 20 remaining miniSTR loci were comparable to the 13 CODIS loci in their population statistics, with only five of the new loci having lower heterozygosity values. These 20 newly developed loci when included together with the six loci previously described (1), yield a total of 26 miniSTR markers with

amplicon sizes <140 bp in all instances except for one. In most cases these markers are smaller in size (base pairs) than the 13 CODIS loci, therefore allowing them to serve as valuable supplements in the analysis of degraded DNA where there is too little intact DNA present to get full profiles with larger loci.

In addition, there have recently been some nomenclature changes to five of the six previously described miniSTR loci (1). Repeat number differences were discovered after re-sequencing the 12 DNA components of the National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 2391b (10) with the D1S1677, D2S441, D4S2364, D10S1248, D14S1434, and D22S1045 markers. D2S441 is the only locus that did not warrant a nomenclature alteration; however the remaining five loci all had differences. These repeat adjustments will be further addressed and discussed in this paper.

Materials and Methods

Selection of Loci and Primer Design

The criteria for selection of these 20 new candidate loci (i.e., high heterozygosity and minimal allele spread) and their primer design was described previously (1) and will be briefly stated here. The Marshfield Clinic Center for Medical Genetics (http://research.marshfieldclinic.org/genetics/) originally published a variety of STR marker sets and these were used to screen several potential miniSTR markers. For the loci selected in this study, the original amplification primer sequences were obtained from the Genome Database (GDB) (http://www.gdb.org/) utilizing information from the Cooperative Human Linkage Center (CHLC—http://lpgws.nci.nih.gov/CHLC/index.html). Several hundred base pairs of sequence information including the target repeat were saved as a text file from a BLAST-nucleotide (BLASTn) search (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). The internet program BLAT (http://genome/ucsc.edu/cgi-bin/hgBlat) and the May 2004 assembly of the human genome were used to determine the chromosomal location and bp position of each marker. The GenBank® accession numbers for each STR locus examined here are listed in Table 1.

The PCR primers for these candidate loci were designed against the downloaded reference sequences from BLASTn using Primer3 (11). The PCR product sizes were chosen to be as close to the target STR repeat region as possible (Table 2). The final miniSTR primers were then separated into groups of three and tested for potential binding issues with each other using AutoDimer (12). All of the forward primers were labeled with either 6FAM™, VIC™, or NED™ fluorescent dyes (Applied Biosystems, Foster City, CA). The reverse primers (Qiagen Operon, Alameda, CA) were unlabeled, with an additional guanine base added to the 5′ end to promote full adenylation (2,13) (Table 2, noted in bold and underlined). The final target concentration of the forward and reverse primers was 0.2 μM; however, the amounts of several forward primers were empirically decreased to generate balanced PCR products as measured with the ABI 3100.

Source of DNA Samples for Population Testing

A set of 665 U.S. population blood samples with self-identified ethnicities, including 265 Caucasian, 260 African-American, and 140 Hispanic, were purchased from Interstate Blood Bank (Memphis, TN) and Millennium Biotech, Inc. (Ft. Lauderdale, FL) and used in this study. They were extracted for DNA, quantified, diluted to *c.* 1 ng/μL, and separated into seven 96-well plates as previously described (9). An additional 190 (95 Caucasian and 95 African-American) samples were tested with the original six miniSTR loci previously described (1). Population data, including all allele frequencies, can be found on the NIST STRBase website (14).

TABLE 1—*Information on 20 new miniSTRs examined in this study in addition to the six miniSTR loci (boxed) reported previously (1).*

Locus	Allele range	Size range* (bp)	GenBank accession	GenBank allele	Repeat adjustments
D1GATA113	7–13	81–105	Z97987	11	
D1S1627	10–16	81–100	AC093119	13	
D1S1677	**9–19**	77–117	AL513307	15	+1
D2S441	9–17	78–110	AC079112	12	None
D2S1776	7–15	127–161	AC009475	11	
D3S3053	7–13	84–108	AC069259	9	
D3S4529	11–18	111–139	AC117452	13	
D4S2364	**7–11**	67–83	AC022317	9	−1
D4S2408	7–13	85–109	AC110763	9	
D5S2500	14–24	85–125	AC008791	17	
D6S474	13–20	107–135	AL357514	17	
D6S1017	7–14	81–109	AL035588	10	
D8S1115	9–20	63–96	AC090739	9	
D9S1122	9–17	93–125	AL161789	12	
D9S2157	7–19	71–107	AL162417	10	
D10S1248	**8–19**	79–123	AL391869	13	−1
D10S1435	5–19	82–139	AL354747	11	
D11S4463	10–17	88–116	AP002806	14	
D12ATA63	10–20	76–106	AC009771	13	
D14S1434	**9–17**	70–102	AL121612	13	−4
D17S974	5–12	95–123	AC034303	11	
D17S1301	9–15	114–138	AC016888	12	
D18S853	9–16	82–103	AP005130	14	
D20S482	9–19	86–126	AL121781	14	
D20S1082	8–17	73–100	AL158015	14	
D22S1045	**8–19**	76–109	AL022314	**17**	+3

Chromosomal location and base pair (bp) position of each marker was determined by using BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat) and the May 2004 assembly of the human genome. The observed allele ranges and size range (bp) values are based on the miniSTR amplicon sizes that were observed in this study relative to the GS500 LIZ size standard. The GenBank Accession numbers and reference alleles were obtained from nucleotide searches through NCBI. The GenBank allele correction for D22S1045 and the allele ranges for D1S1677, D4S2364, D10S1248, D14S1434, and D22S1045 are in bold. The repeat adjustments are listed for the loci that had nomenclature changes.

*Apparent size observed relative to GS500 LIZ size standard.

PCR Amplification

PCR amplification was carried out on a GeneAmp® 9700 (Applied Biosystems) using 1 ng of population sample DNA, 1× GeneAmp® PCR Gold buffer (Applied Biosystems), 2 μM $MgCl_2$ (Applied Biosystems), 250 μM dNTPs (USB Corporation, Cleveland, OH), *c.* 0.2 μM forward (Applied Biosystems) and reverse (Qiagen Operon) primers (see Table 2 for primer sequences), 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems), and 0.16 mg/mL bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) in a 10 μL total reaction volume. The PCR amplification conditions using the 9700 were as follows: denaturation for 10 min at 95°C, amplification for 28 cycles of 1 min at 94°C, 1 min at 59°C, and 1 min at 72°C, extension for 45 min at 60°C, and a final soak at 25°C.

Analysis on the ABI 3100 and 3130xl Genetic Analyzer

Amplification products were diluted in Hi-Di™ formamide (Applied Biosystems) by adding 1 μL PCR product and 0.35 μL

TABLE 2—*MiniSTR primers used in this study.*

Marker name	Primer sequence (5′–3′)	Oligo distance from repeat (bp)	Amplicon size* (bp) with GenBank allele
D1GATA113	F-**[VIC]**-TCTTAGCCTAGATAGATACTTGCTTCC	5	98
	R-**G**TCAACCTTTGAGGCTATAGGAA	0	
D1S1627	F-**[VIC]**-CATGAGGTTTGCAAATACTATCTTAAC	4	92
	R-**G**TTTTAATTTTCTCCAAATCTCC**A**	−1	
D1S1677	F-**[NED]**-TTCTGTTGGTATAGAGCAGTGT**TT**	−2	103
	R-**G**TGACAGGAAGGACGGAATG	2	
D2S441	F-**[VIC]**-CTGTGGCTCATCTATGAAAACTT	−1	92
	R-**G**AAGTGGCTGTGGTGTTATGAT	1	
D2S1776	F-**[FAM]**-TGAACACAGATGTTAAGTGTGTATATG	45	146
	R-**G**TCTGAGGTGGACAGTTATGAAA	8	
D3S3053	F-**[VIC]**-TCTTTGCTCTCATGAATAGATCAGT	11	94
	R-**G**TTTGTGATAATGAACCCACTCA**G**	−1	
D3S4529	F-**[VIC]**-CCCAAAATTACTTGAGCCAAT	26	120
	R-**G**AGACAAAATGAAGAAACAGAC**AG**	−2	
D4S2364	F-**[FAM]**-CTAGGAGATCATGTGGGTATGATT	2	78
	R-**G**CAGTGAATAAATGAAC**GAATGGA**	−7	
D4S2408	F-**[NED]**-AAGGTACATAACAGTTCAATAGAAAGC	5	95
	R-**G**TGAAATGACTGAAAAATAGTAACCA	2	
D5S2500	F-**[NED]**-CTGTTGGTACATAATAGGTAGGTAGGT	−11	98
	R-**G**TCGTGGGCCCCATA**AATC**	−4	
D6S474	F-**[NED]**-GGTTTTCCAAGAGATAGACCAATT**A**	−1	125
	R-**G**TCCTCTCATAAATCCCTACTCATATC	7	
D6S1017	F-**[VIC]**-CCACCCGTCCATTTAGGC	13	96
	R-**G**TGAAAAAGTAGATATAATGGTTGGTG	−1	
D8S1115	F-**[FAM]**-TCCACATCCTCACCAACAC	0	66
	R-**G**CCTAGGAAGGCTACTGTCAA	0	
D9S1122	F-**[VIC]**-GGGTATTTCAAGATAACTGTAGATAGG	0	106
	R-**G**CTTCTGAAAGCTTCTAGTTTACC	8	
D9S2157	F-**[FAM]**-CAAAGCGAGACTCTGTCTCAA	1	84
	R-**G**AAAATGCTATCCTCTTTGGTATAAAT	6	
D10S1248	F-**[FAM]**-TTAATGAATTGAACAAATGAGTGAG	1	102
	R-**G**CAACTCTGGTTGTATTGTCTTCAT	0	
D10S1435	F-**[FAM]**-TGTTATAATGCATTGAGTTTTATTCTG	2	108
	R-**G**CCTGTCTCAAAAATAAAGAGATAGACA	8	
D11S4463	F-**[FAM]**-TCTGGATTGATCTGTCTGTCC	3	105
	R-**G**AATTAAATACCATCTGAGCACTGAA	0	
D12ATA63	F-**[FAM]**-GAGCGAGACCCTGTCTCAAG	0	89
	R-**G**GAAAAGACATAGGATAGCAATTT	7	
D14S1434	F-**[VIC]**-TGTAATAACTCTACG**ACTGTCTGTCTG**	−11	88
	R-**G**AATAGGAGGTGGATGGATGG	0	
D17S974	F-**[VIC]**-GCACCCAAAACTGAATGTCATA	1	121
	R-**G**GTGAGAGTGAGACCCTGTC	35	
D17S1301	F-**[FAM]**-AAGATGAAATTGCCATGTAAAAATA	30	129
	R-**G**TGTGTATAACAAAATTCCTATGATGG	0	
D18S853	F-**[NED]**-GCACATGTACCCTAAAACTTAAAAT	0	100
	R-**G**TCAACCAAAACTCAACAAGTAGTAA	8	
D20S482	F-**[FAM]**-CAGAGACACCGAACCAATAAG**A**	−1	108
	R-**G**CCACATGAATCAATTCCTATAATAAA	5	
D20S1082	F-**[VIC]**-ACATGTATCCCAGAACTTAAAGTAAAC	4	94
	R-**G**CAGAAGGGAAAATTGAAGCTG	0	
D22S1045	F-**[NED]**-ATTTTCCCCGATGATAGTAGTCT	1	105
	R-**G**CGAATGTATGATTGGCAATATTTTT	5	

The ‘‘Oligo distance from repeat’’ column refers to the distance of the 3′ end of the primer to the STR repeat region (in base pairs). A negative number indicates that the end of the primer is in the repeat region (with the nucleotide bases underlined at the 3′ end of the primer). The 5′ guanine residue in each reverse primer was added to promote adenylation (2,13). The GenBank reference allele in terms of repeat number is listed in Table 1.

*Amplicon size does not include the 5′ guanine residue added to the reverse primer.

GS500-LIZ internal size standard (Applied Biosystems) to 14 μL of Hi-Di. The samples were analyzed on the 16-capillary ABI Prism® 3100 or 3130xl Genetic Analyzer without prior denaturation of samples. Prior to testing, a five dye matrix was established under the ‘‘G5 filter’’ with the dyes 6FAM, VIC, NED, PET, and LIZ. POP™-6 (Applied Biosystems) rather than POP™-4 was utilized for higher resolution separations on a 36 cm array (Applied Biosystems) with 1× ACE buffer (Amresco, Solon, OH). Samples were injected electrokinetically for 10 sec at 3 kV. The STR alleles were then separated at 15 kV at a run temperature of 60°C. Data from the ABI 3100 and 3130xl was analyzed using GeneMapper®*ID* v3.2 (Applied Biosystems).

Generation of Allelic Ladders and GeneMapper ID Bins and Panels

Allelic ladders were created for each miniSTR marker using a combination of individual samples that represent each allele observed in the population data sets. The samples were amplified separately for each locus according to the previously described

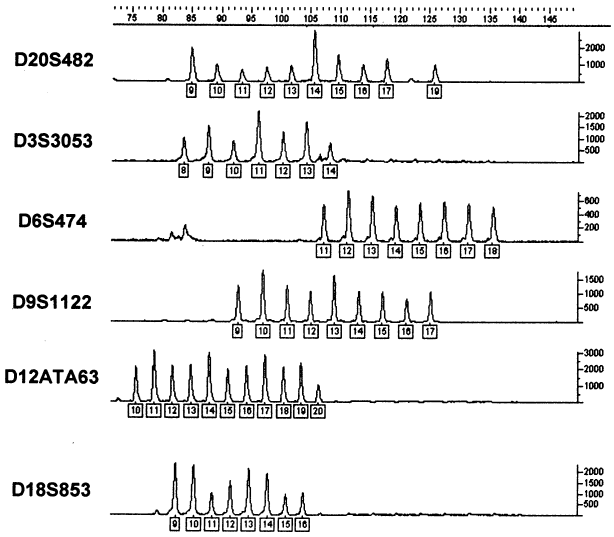

FIG. 1—*Examples of six miniSTR allelic ladders generated for this study.*

protocol. Each of the amplicons were diluted and mixed together by adding 1 μL of each PCR product to 1 mL of H_2O and re-amplifying under the same conditions with the following exceptions: 15 cycles instead of 28 cycles and a 60°C hold for 4 h. A few examples of allelic ladders created with this method are illustrated in Fig. 1.

Bins and panels were created in GeneMapper *ID* from population information using fixed bin allele sizes to determine allele calls for the population samples. The number of repeats was calibrated to allele size by sequencing. The bins and panels for each of the 26 miniSTR loci are available for use on http://www.cstl.nist.gov/biotech/strbase/miniSTR.htm#Protocols.

DNA Sequencing

At least two separate homozygote samples were sequenced at each STR locus to provide appropriate allele repeat numbers. Sequencing primers were designed to be outside of the miniSTR amplicon and used to characterize the number of repeat units and the consistency in the flanking region of each evaluated allele. The sequencing primers used for each marker are listed in Table 3.

The template DNA was amplified with the unlabeled primers listed in Table 3 as previously described in the PCR Amplification section using the GeneAmp 9700 with 20 μL PCR volumes containing 5 μL template DNA and 15 μL master mix. A total of 35 cycles of PCR were used to create sufficient amplicon quantities for sequencing. The amplification product was then further purified by using 2 μL of ExoSap-IT™ (USB Corporation) per 5 μL sample and the following conditions on the GeneAmp 9700: 90 min at 37°C, 20 min at 80°C, and a final soak at 25°C. Sequencing was then performed with the ABI PRISM® BigDye® Terminator, v3.1 cycle sequencing kit (Applied Biosystems). The sequencing mixture consisted of a 20 μL total reaction volume: 5 μL purified PCR product, 6 μL of 2.5× Sequencing Dilution Buffer (Edge Biosystems, Gaithersburg, MD), 3.8 μL dH_2O, 2.0 μL BigDye Terminator kit mix (Applied Biosystems), and 3.2 μL of the forward or reverse primer at a concentration of 1.0 μM. The GeneAmp 9700 was used for thermal cycling with the following conditions: denaturation for 5 min at 94°C, amplification for 25 cycles of 10 sec at 94°C, 5 sec at 50°C, and 2 min at 60°C, extension for 5 min at 60°C, and a final soak at 4°C. Finally, the DNA product was filtered through a Performa® DTR (dye terminator removal) gel filtration cartridge (Edge Biosystems).

TABLE 3—*Unlabeled primers used in this study for sequencing purposes. These primers bind outside the mini STR typing primers and have larger PCR product sizes.*

Marker name	Primer sequence (5′–3′)	PCR product size with reference allele (bp)
D1GATA113	F-TCATTTCTCCATACATTGATGAGC R-TGAACTCATTGGCAAAAGGA	247
D1S1627	F-CAATACAAAAACACTGTAACTGTGGA R-ACGTGCACAATACCCTGGA	277
D1S1677	F-GTAGTGCTGGTGCAGCGTAA R-TGCAATAGCAAATATCAGAATGTGT	261
D2S441	F-CTGTTCCTGAGCCCTAATGC R-CACCACACCCAGCCATAAAT	290
D2S1776	F-GGAGGAAGAGAAAACCGAATG R-ACCAAGACCTGGGGTGCTA	295
D3S3053	F-TTCCTGCTATGGTTCGAAGG R-AGCAAGAGAGGGCTGAACTC	245
D3S4529	F-AAGAAATTCTCCCTGACACTGC R-GGGAGCAGGAAGTTCATAGTG	390
D4S2364	F-TGTTGTCTGTAGGAGCTGAGAA R-GGTGTTTGGAGATGGCTGTT	258
D4S2408	F-GCACTATGATTCATTTCCATAGG R-ATCTTAGAAGAGTGCTTGGCATA	300
D5S2500	F-ATCATCCCTGCAAAGTAACG R-GTTGAATTGTTTGTAACTTAAAGGGTAA	375
D6S474	F-CCTGTTGCCACAGCAACTAA R-CTCCAGCCTTTGGACTCTTG	626
D6S1017	F-ATTGAACCAGATGGGAACGA R-GTGGATGGACATAATGGATGC	307
D8S1115	F-CCACAGAACTGTGAGCCAAA R-GCCTAGGAAGGCTACTGTCAA	321
D9S1122	F-GGTACAGTAGCAATATAAAAAGGAAGA R-TGGCAGGTGATATGGCATTA	237
D9S2157	F-GCCAACATGATGAAATGCTG R-GCAGCATAACCCTTTCTCCA	497
D10S1248	F-AGCAAACCTGAGCATTAGCC R-AGTGCTTGGCAAAGAGCAGA	257
D10S1435	F-AGTGAGCCCTCGAAGAGGTT R-GTGGTGGTGTGCACCTGTAGT	326
D11S4463	F-GCAATGCTGCTATTGGCACT R-TGATGATCTGCAAAGGAGGA	246
D12ATA63	F-TCACTTCAGCCCAGAAGGTT R-ATTTCCACCAAGCTCAGCAC	242
D14S1434	F-TTCCCAGCCTCCATAATCAG R-TGCAAATGCACACAGATTTC	262
D17S974	F-CTGAAGGATGGACTGGGGTA R-GCTCAGAGAGGCGCTGTTAT	487
D17S1301	F-CTGGGTGACAGAGCAAGGTT R-TTCAAAATTTCAGAATAAACCATGA	251
D18S853	F-GGGTGCAACACACCAACAT R-GAAGGCAAATCTGGCTTTACA	247
D20S482	F-GGCTGCAAAGGAACCTAAAA R-CCTGGTTCTCAGGTTTTGGA	303
D20S1082	F-GGTTGACATGTGCAGCAAAC R-GCAGAAGGGAAAATTGAAGCTG	160
D22S1045	F-CCCACTATGGGCAAACCTTA R-TGTGCTTCAGTCTCCTCAGC	342

Electrophoresis and DNA sequencing were performed on the ABI 3100 Genetic Analyzer using POP-6 on a 36 cm capillary array with the appropriate dye set. Sequences were initially analyzed using Sequence Analysis v3.7 (Applied Biosystems). Sequences were then aligned with the GenBank reference allele and edited using Sequencher Plus 4.0.5b11 (GeneCodes, Ann Arbor, MI) or SeqMan (Lasergene, DNA Star, Madison, WI).

In addition, confirmation sequencing to verify the correct allele designations was performed with the 20 new miniSTR loci, as well

as the six previously described loci (1) for a total of 26 markers. This confirmation sequencing was accomplished by using the NIST SRM 2391b DNA Profiling Standard (10) instead of population samples (9). First, the components of SRM 2391b, including eight genomic DNA samples, 9947A, and 9948 (10), along with the K562 (Promega Corporation, Madison, WI) and ABI 007 (Applied Biosystems) positive controls were genotyped as described for the population samples using all 26 of the miniSTR markers. Next, these SRM 2391b samples and positive controls were sequenced to verify the allele repeat designations initially determined by population sample sequencing. All samples with homozygous alleles (82 total) were sequenced as previously described; however, the alleles for the heterozygous samples (71 total) were separated on a polyacrylamide gel and further sequenced. These samples were initially amplified in the same manner as the homozygous samples and these products were run on a 32 cm × 0.4 mm polyacrylamide gel that was *c.* 9% total acrylamide with 3% cross link of bis-acrylamide gel in 50 mM Tris/Formate gel buffer with 1.7% glycerol added as a mobility modifier. The percentages were adjusted depending on the size differences of the alleles being separated. The samples were then run with a bromophenol blue/50% glycerol loading dye along with a DNA ladder in Sodium Borate running buffer (15) at 25 mA until the dye front reached the bottom of the gel. When finished, the gel was silver stained and the separated allele bands were cut from the gel. The gel bands were soaked in TE (10mM Tris, 0.1mM EDTA, pH8) buffer overnight and re-amplified and sequenced as separate samples as previously described. The sequencing results were then compared with the genotypes for these samples.

Data Analysis

A total of 665 unique miniSTR profiles were evaluated for each new marker: 663 males and two females. All allele calls for each of the population samples were made using GeneMapper *ID*. Once the genotypes were reviewed and edited, data tables were exported into Excel (Microsoft, Redmond, WA). The resultant data were evaluated using the PowerMarker statistics program (16). The observed heterozygosities are listed in Table 4 and Hardy–Weinberg exact test *p*-values in the three U.S. populations are listed in Table 5. The complete data set, including all allele frequencies, is available on the NIST STRBase website (14).

Results and Discussion

"*Problematic*" *Loci*

A total of 26 new miniSTR loci were evaluated in this study, in addition to the six that were discussed in by Coble and Butler (1), for a total of 32 potential miniSTR markers. All of these loci were analyzed across the complete set of 665 U.S. population samples; however, only 26 of the 32 new markers are recommended for further use. Problematic loci (D6S1027, D9S324, D10S1430, D10S2327, D14S297, D15S817) were eliminated from consideration due to various reasons including obvious allelic dropout and null alleles, excessively complex repeat motifs, tri- or quad-allelic patterns, and heterozygosity values <0.5. For example, allelic dropout was observed to occur in numerous population samples for the D9S324 locus. In some cases, there were no alleles showing at all, and in others a sample would appear to be a homozygote, when it was actually a heterozygote (null allele). These null alleles were discovered by re-amplifying certain samples using the sequencing reverse primers (see Table 3) with the dye-labeled miniSTR forward primers (see Table 2) and re-genotyping these samples. After sequencing several different alleles, the D9S324 locus was found to have complex repeats and a common 8 bp deletion near the 5′ end of the reverse primer shown in Table 2. While allele dropout due to this deletion could be corrected by using a reverse primer sequence further away from the STR repeat region, it would create an amplicon size over 150 bp and thus be too large for consideration as a miniSTR marker for the purposes of this study.

The D10S1430 locus was also eliminated as a potential marker due to very complex repeat patterns. In addition, there were two loci, D10S2327 and D15S817, which resulted in tri- and quad-allelic profiles for several of the samples (see Fig. 2). This could be as a result of extra chromosomal occurrences (duplication) such as has been observed with various Y-STR and autosomal loci (17,18). Because this duplication phenomenon was consistently observed in all populations examined, these loci were rejected from further consideration. Two additional loci, D6S1027 and D14S297, were eliminated from consideration due to poor heterozygosity values. They were both examined across the entire set of population samples, however, their heterozygosity values were too low (<0.5) to be considered as candidate markers.

There were also a few loci that were initially problematic but were eventually characterized and successfully used in this study after careful troubleshooting. An example of one such locus was D17S974. Initially, there was allelic dropout occurring with some select population samples that had no observed alleles in their profile for this locus. After sequencing, it was found that there was a 12 bp deletion in the reverse primer binding region. A revised reverse primer was used to generate PCR products that were only 22 bp larger than the original amplicons, but still <150 bp. Additionally, the D5S2500 locus was potentially problematic because it has a very complex repeat: $[GGTA]_n[GACA]_n[GATA]_n[GATT]_n$. This actually did not turn out to be a serious problem worthy of rejection because these repeat patterns appeared consistent and no variant alleles were observed. In fact, D5S2500 has a heterozygosity value of 0.747 and ranks well compared to other loci characterized in additional populations (19).

Characterization of "*Final*" *Loci*

The primers for the 26 miniSTR markers that were characterized in this study are shown in Table 2. All of these loci have forward and reverse primers that are close to the repeat unit. There are six loci that contain a trinucleotide repeat unit: D1S1627, D8S1115, D9S2157, D12ATA63, D18S853, and D20S1082, while the remaining 14 consist of tetranucleotide repeats (Table 4). It is important to note that the stutter products for the loci with trinucleotide repeats were not observed to be significantly higher than the tetranucleotide markers in this study (data not shown). Nine of these new markers are found on chromosomes that have CODIS loci but are sufficiently spaced apart (*c.* 50 Mb) to be considered unlinked from these loci. The remaining 11 loci reside on chromosomes that were previously unoccupied. These markers were mapped onto each respective chromosome using the BLAT program (http://genome.ucsc.edu/cgi-bin/hgBlat) and the May 2004 assembly of the human genome as previously described (3). More information on these miniSTR loci and protocols can be found on the following website: http://www.cstl.nist.gov/biotech/strbase/newSTRs.htm.

The GenBank accession numbers previously reported for miniSTR markers (1) were taken from the GDB website and are based upon information from the CHLC. We have determined that these accession numbers are archaic, and do not necessarily reflect the true

TABLE 4—*Information on 20 new miniSTRs examined in this study in addition to the six miniSTR loci reported previously (1).*

Locus	Repeat type	Repeat motif	Chromosomal location	Chromosome position	Observed size range (bp)	Observed heterozygosity				
						n	African-American	Caucasian	Hispanic	Overall
FGA	*Tetra*	*CTTT*	*4q31.3*	*Chr 5 155.866 Mb*	*196–352*	*659*	*0.883*	*0.889*	*0.886*	*0.886*
D2S1338	*Complex Tetra*	*[TGCC][TTCC]*	*2q35*	*Chr 2 218.705 Mb*	*288–340*	*659*	*0.903*	*0.882*	*0.843*	*0.882*
D18S51	*Tetra*	*AGAA*	*18q21.33*	*Chr 18 59.100 Mb*	*264–344*	*659*	*0.860*	*0.870*	*0.914*	*0.876*
D9S2157	Tri	ATA	9q34.2	Chr 9 133.065 Mb	71–101	661	0.884	0.840	0.779	0.844
D21S11	*Complex Tetra*	*[TCTA][TCTG]*	*21q21.1*	*Chr 21 19.476 Mb*	*186–244*	*659*	*0.829*	*0.844*	*0.871*	*0.844*
D12ATA63	Tri	[TAA][CAA]	12q23.3	Chr 12 106.825 Mb	76–106	659	0.788	0.842	0.879	0.829
VWA	*Complex Tetra*	*[TCTG][TCTA]*	*12p13.31*	*Chr 12 5.963 Mb*	*152–212*	*659*	*0.802*	*0.836*	*0.850*	*0.826*
D7S820	*Tetra*	*GATA*	*7q21.11*	*Chr 7 83.433 Mb*	*253–293*	*659*	*0.763*	*0.817*	*0.864*	*0.806*
D19S433	*Tetra*	*AAGG*	*19q12*	*Chr 19, 35.109 Mb*	*106–140*	*659*	*0.876*	*0.752*	*0.764*	*0.803*
D10S1248 (NC01)	Tetra	GGAA	10q26.3	Chr 10 130.567 Mb	83–123	663	0.825	0.785	0.743	**0.792**
D22S1045 (NC01)	Tri	ATT	22q12.3	Chr 22 35.779 Mb	76–109	663	0.817	0.785	0.721	**0.784**
D2S441 (NC02)	Tetra	TCTA	2p14	Chr 2 68.214 Mb	78–110	660	0.798	0.780	0.721	**0.774**
D8S1179	*Complex Tetra*	*[TCTA][TCTG]*	*8q24.13*	*Chr 8 125.976 Mb*	*123–171*	*659*	*0.763*	*0.779*	*0.786*	*0.774*
D16S539	*Tetra*	*GATA*	*16q24.1*	*Chr 16 84.944 Mb*	*233–273*	*659*	*0.786*	*0.733*	*0.793*	*0.766*
D10S1435	Tetra	TATC	10p15.3	Chr 10 2.233 Mb	82–139	663	0.798	0.770	0.700	0.766
D3S1358	*Complex Tetra*	*[TCTG][TCTA]*	*3p21.31*	*Chr 3 45.557 Mb*	*97–145*	*659*	*0.767*	*0.763*	*0.757*	*0.763*
D2S1776	Tetra	AGAT	2q24.3	Chr 2 169.471 Mb	127–161	654	0.740	0.801	0.734	0.763
D3S4529	Tetra	ATCT	3p12.1	Chr 3 85.935 Mb	111–139	660	0.752	0.723	0.829	0.761
D6S474	Complex Tetra	[AGAT][GATA] [GGTA][GACA]	6q21	Chr 6 112.986 Mb	107–135	648	0.765	0.802	0.679	0.761
D5S2500	Complex Tetra	[GATA][GATT]	5q11.2	Chr 5 58.735 Mb	85–125	664	0.757	0.747	0.729	0.747
D13S317	*Tetra*	*TATC*	*13q31.1*	*Chr 13 81.620 Mb*	*193–237*	*659*	*0.693*	*0.748*	*0.843*	*0.747*
D1S1627	Tri	ATT	1p21.1	Chr 1 106.676 Mb	81–100	660	0.783	0.737	0.693	0.746
D1S1677 (NC02)	Tetra	**TTCC**	1q23.3	Chr 1 160.747 Mb	81–117	660	0.743	0.749	0.743	0.746
CSF1PO	*Tetra*	*TAGA*	*5q33.1*	*Chr 5 149.436 Mb*	*280–320*	*659*	*0.759*	*0.733*	*0.743*	*0.745*
TH01	*Tetra*	*TCAT*	*11p15.5*	*Chr 11 2.149 Mb*	*160–204*	*659*	*0.759*	*0.721*	*0.764*	*0.745*
D6S1017	Tetra	ATCC	6p21.1	Chr 6 41.785 Mb	81–109	664	0.807	0.698	0.693	0.740
D3S3053	Tetra	TATC	3q26.31	Chr 3 173.234 Mb	84–108	648	0.713	0.724	0.814	0.739
D9S1122	Tetra	TAGA	9q21.2	Chr 9 76.918 Mb	93–125	659	0.753	0.742	0.686	0.734
D17S974	Tetra	CTAT	17p13.1	Chr 17 10.459 Mb	95–123	664	0.757	0.702	0.743	0.732
D11S4463	Tetra	TATC	11q25	Chr 11 130.338 Mb	88–116	664	0.780	0.676	0.743	0.730
D4S2408	Tetra	ATCT	4p15.1	Chr 4 30.981 Mb	85–109	654	0.752	0.709	0.691	0.722
D5S818	*Tetra*	*AGAT*	*5q23.2*	*Chr 5 123.139 Mb*	*134–170*	*659*	*0.735*	*0.702*	*0.729*	*0.721*
D18S853	Tri	ATA	18p11.31	Chr 18 3.981 Mb	82–103	664	0.772	0.645	0.721	0.711
TPOX	*Tetra*	*GAAT*	*2p25.3*	*Chr 2 1.472 Mb*	*213–249*	*659*	*0.763*	*0.668*	*0.679*	*0.707*
D20S1082	Tri	ATA	20q13.2	Chr 20 53.299 Mb	73–100	664	0.792	0.653	0.600	0.696
D14S1434 (NC01)	Complex Tetra	**[CTGT][CTAT]**	14q32.13	Chr 14 93.298 Mb	70–98	663	0.685	0.721	0.650	0.696
D20S482	Tetra	AGAT	20p13	Chr 20 4.454 Mb	86–126	648	0.673	0.689	0.729	0.691
D1GATA113	Tetra	GATA	1p36.23	Chr 1 7.377 Mb	81–105	654	0.673	0.632	0.727	0.668
D8S1115	Tri	ATT	8p11.21	Chr 8 42.656 Mb	63–96	664	0.629	0.660	0.729	0.663
D17S1301	Tetra	AGAT	17q25.1	Chr 17 70.193 Mb	114–138	664	0.626	0.717	0.564	0.649
D4S2364 (NC02)	Tetra	**ATTC**	4q22.3	Chr 4 93.976 Mb	67–83	660	0.385	0.551	0.664	0.511

Repeat types and motifs were determined by sequencing. Repeat motif corrections are in bold. Chromosomal location and base pair (bp) position of each marker was determined by using BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat) and the May 2004 assembly of the human genome. The observed size range (bp) values are based on the miniSTR amplicon sizes that were observed in this study relative to the GS500 LIZ size standard. The heterozygosity values are from this study. The Identifiler loci are in italics and all information regarding these markers is from Butler et al. (9). The loci are listed in order, highest to lowest, by the overall heterozygosity values. Allele frequencies are available on the NIST STRBase website (14). The heterozygosities of the three European recommended loci (6,7) are in bold.

repeat structure determined from updated versions of the human genome. The GenBank accession numbers in Table 1 represent the corrected information from previous and current markers studied.

Nomenclature Corrections for the NC01 and NC02 loci D1S1677, D4S2364, D10S1248, D14S1434, and D22S1045

During the course of work performed here in further characterizing the NC01 and NC02 loci, errors were discovered in the previously reported nomenclature for the D1S1677, D4S2364, D10S1248, D14S1434, and D22S1045 loci (1). In the case of D22S1045, after further review of the sequencing information, the original nomenclature has been changed to add an additional one ACT and two ATT repeats. Thus, the new allele range following the +3 repeats adjustment is 8–19 repeats and the GenBank reference allele is 17. In addition, there have been some recent allele designation changes for D1S1677, D4S2364, D10S1248, and D14S1434 markers based on confirmation sequence analysis of the SRM 2391b components with these loci. The following adjustments from what was previously reported (1) have been made: +1 repeat for D1S1677, −1 repeat for D4S2364, −1 repeat for D10S1248, and −4 repeats for D14S1434. The correct allele ranges for these

TABLE 5—*Summary of Hardy–Weinberg equilibrium (HWE) exact test p-values across all 26 loci and three populations reported in this study (p < 0.05 in bold).*

HWE	D1GATA113	D1S1627	D1S1677	D2S1776	D2S441	D3S3053	D3S4529	D4S2364	D4S2408	D5S2500	D6S1017	D6S474	D8S1115
p-Value Caucasian	0.5971	0.5662	0.7058	0.3671	0.7857	0.5148	0.2428	**0.0227**	0.1441	0.6897	0.2855	0.2105	0.1869
p-Value African-American	0.9676	0.8207	**0.0191**	0.3763	0.1520	0.8156	0.5752	0.7511	0.8213	0.6766	0.9903	0.4592	0.8742
p-Value Hispanic	0.7490	0.0986	0.5968	0.2598	**0.0399**	0.4772	0.7017	0.6312	0.1667	0.6364	0.7511	0.6923	0.5776

HWE	D9S1122	D9S2157	D10S1248	D10S1435	D11S4463	D12ATA63	D14S1434	D17S1301	D17S974	D18S853	D20S1082	D20S482	D22S1045
p-Value Caucasian	0.9674	0.8568	0.8021	0.6775	0.1991	0.0585	0.9052	0.5632	0.5242	0.6630	0.2827	0.1595	0.9191
p-Value African-American	0.4737	0.3648	**0.0456**	0.5520	0.6367	0.3194	0.1506	0.7422	0.6173	0.4394	0.5150	0.2887	0.2013
p-Value Hispanic	0.9547	0.5926	0.2857	0.7229	0.4182	0.7136	0.9944	**0.0338**	0.9528	0.9546	0.1146	0.2909	0.1133

All allele frequencies are listed on the NIST STRBase website (14).

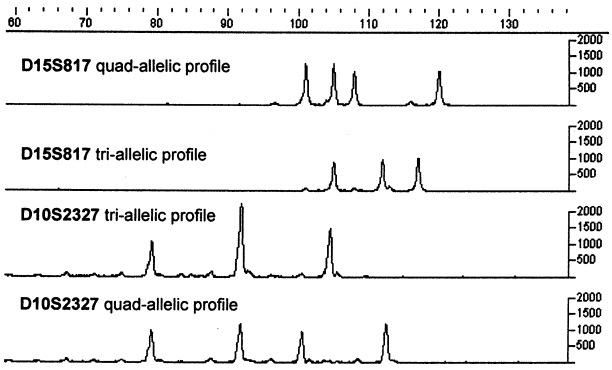

FIG. 2—*Examples of tri-allelic and quad-allelic profiles for problematic miniSTR loci (D10S2327 and D15S817) that were ultimately rejected for use in this study.*

loci are as follows: 9–19 for D1S1677, 7–11 for D4S2364, 8–19 for D10S1248, and 9–17 for D14S1434. The D2S441 remains the same as reported previously (1), with a 9–17 allele range. These adjustments are displayed in Table 1 and described on the NIST STRBase website: http://www.cstl.nist.gov/biotech/strbase/miniSTR.htm#Nomenclature_Errata as well as a recently published author's response to a letter to the editor regarding D10S1248 (20). All of the data reported in this paper reflects the updated nomenclature changes. Also, the bins and panels in GeneMapper *ID* were adjusted for these five loci according to their revised nomenclature.

In addition to sequencing all the components of SRM 2391b (including 9947A and 9948) (10) and the K562 and ABI 007 positive controls, these samples were genotyped with the 26 miniSTR loci as well. These standard types are all listed on http://www.cstl.nist.gov/biotech/strbase/miniSTR/miniSTR_NC_loci_types.htm and can be used for calibration of these miniSTR markers in laboratories wishing to examine these loci.

Heterozygosity Ranking and Hardy–Weinberg Equilibrium

Heterozygosity values were calculated for each locus (14) and are listed in Table 4. The D9S2157 locus showed the highest overall heterozygosity value (0.844) with the D12ATA63 marker a close second (0.829). Between these two loci, they showed the highest heterozygosity values among all of the population samples. It is interesting to note that the D12ATA63 African-American heterozygosity of 0.788 seems low compared to the other examined populations. Further studies with nonoverlapping primers may detect null alleles not observed with the current primers. The D4S2364 locus from NC02 has the lowest overall average heterozygosity value of 0.511. This is just above the lower limit for exclusion from this study, which was set at 0.5. In addition, the three European recommended loci (D2S441, D10S1248, and D22S1045) had high heterozygosities (0.774, 0.792, and 0.784, respectively) in comparison with the remaining miniSTR loci. Only the D9S2157 and D12ATA63 ranked higher among the 26 non-CODIS markers.

Exact tests to determine Hardy–Weinberg equilibrium (HWE) were performed on all of the new miniSTR loci for each population and the *p*-values are listed in Table 5. Across the 26 loci and three different populations (78 comparisons), there were five instances where *p*-values were below 0.05 suggesting possible deviation from HWE. However, if the Bonferroni correction is applied (21), then only *p*-values below 0.00064 (0.05/78) would be considered significant and all loci examined here would be deemed adequate in terms of this independence testing. Further characterization of the primer binding regions, using amplification and sequencing primers outside of the miniSTR amplicon (Table 3), will be necessary to identify potential mutations that create null alleles (22).

Impact of Adding Additional Samples to NC01 and NC02 Loci

In this study, an additional 190 population samples were evaluated with the NC01 and NC02 six loci: D1S1677, D2S441, D4S2364, D10S1248, D14S1434, and D22S1045. The extra population samples consisted of 95 African-Americans and 95 Caucasians. These supplementary samples did not significantly change the allele frequencies or heterozygosities from those that were reported previously (1), however they did yield more alleles in D1S1677, D10S1248, and D14S1434. The extra African-American samples resulted in the presence of both the nine and 18 alleles for D1S1677 and the eight allele for D10S1248, thus further spreading the initial range of alleles. The additional Caucasian samples resulted in the 17 allele for D14S1434.

Comparison to CODIS Loci

A comparison of heterozygosity values between the new miniSTR loci and those of the Identifiler kit (including the 13 CODIS loci) run on the same DNA samples (9) was performed, and the results are listed in Table 4. These markers are arranged from the highest heterozygosity values at the top to the lowest at the bottom. As shown, many of the new miniSTR loci have heterozygosities that are comparable and similar in range to the CODIS loci. There were seven newly characterized loci, including two that were previously described (1), that had values which fell below the

lowest Identifiler marker heterozygosity, but it is important to note that all seven markers have high enough values (>0.50) to be considered useful in future studies. These new miniSTR loci will likely be beneficial because of the small size amplicons that can be generated, thus increasing the likelihood of recovering information from degraded DNA samples.

Conclusions

This paper outlines the characterization of 20 new miniSTR loci for the analysis of highly degraded DNA, in addition to the six that were previously described (1). In this study, we have developed additional miniSTR markers with all but one locus under 140 bp. We have run these new loci across 665 U.S. population samples, including African-Americans, Caucasians, and Hispanics and determined population statistics for each of them. All 665 samples were resolved from one another with the 26 new loci. The heterozygosity values for all of these markers were comparable to those of the 13 core CODIS loci, with a few of the new marker values being lower. Allelic ladders were created for each of these new miniSTR loci using our population samples.

Now that each of these miniSTR loci have been characterized and optimized, the goal is to create larger multiplexes, with several loci present in different dye sets to maximize the effectiveness of these markers on reference samples. However, even used as single markers, their contribution to the forensic community to aid in the analysis of degraded DNA samples, as well as providing additional discrimination in complex paternity cases or missing persons cases (8) may prove useful.

Recently, several of these miniSTR loci (D10S1248, D2S441, and D22S1045) have been recommended for adoption by the European DNA community as new core loci (6,7). In addition, many of these miniSTR markers are currently being used in laboratories across the world as supplements to obtain full profiles from samples that generate partial profiles using commercial STR kits. These additional miniSTR loci should offer a potential tool for increasing the successful typing of degraded materials.

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