TECHNICAL NOTE

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Melting Curve SNP (McSNP) Genotyping: a Useful Approach for Diallelic Genotyping in Forensic Science

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ABSTRACT: The increasing availability of Single Nucleotide Polymorphisms (SNPs) and Deletion/Insertion Polymorphisms (DIPs), as well as the outstanding progress in SNP genotyping technologies, will impact forensics profoundly. We have developed a new method for genotyping SNPs and DIPs, which is based on the determination of melting curve profiles of amplified DNA in solution. We have termed this method Melting curve SNP (McSNP) genotyping. Melting curve profiles are composites of the particular melting temperatures (T_m) of the individual fragments that comprise the DNA sample. Simple mixtures of DNA can be resolved in a very robust and efficient fashion, since the samples can be scored in the plates in which they were amplified with no or very few post-PCR manipulations. As such, McSNP is one of the least expensive genotyping methods available and can and should be useful in forensic science.

KEYWORDS: forensic science, DNA typing, SNPs, DIPs, Melting curve SNP (McSNP) genotyping

The incorporation of DNA analysis into criminal investigations and paternity testing has been tremendously important in forensics, and the current advances in molecular biology are expected to bring easier, faster, and cheaper DNA technologies, while increasing the already high discriminatory power currently available in many forensic labs.

Single Nucleotide Polymorphisms (SNPs) represent the most abundant source of sequence variation in the human genome (1,2). When comparing two genomes, it has been estimated that SNPs occur approximately every 1000–2000 bases (1). Over 1.4 million and 2.0 million SNPs have been mapped in the human genome as a result of the public and private sequencing efforts, respectively (1,2). The primary motivation for the SNP discovery and detection advances has been the potential applicability of these markers in many fields, including the study of complex disease traits, pharmacogenomics, and human evolution (3–8). Obviously, the field of forensics can and should take advantage of the vast amount of information that has been gathered in the past few years, not only in terms of the increasing number of markers available in the databases, but also the breakthroughs in the technologies for SNP genotyping. Given the diallelic nature of these polymorphisms, it will be necessary to genotype a larger number of SNPs than multiallelic markers, like VNTRs or STRs, in order to have the same discriminatory power. However, this disadvantage is minor if SNP typing technologies are used that allow easier, faster, cheaper, and more automated genotyping than is currently possible for STR typing. Not surprisingly, in parallel with the identification and mapping of diallelic polymorphisms, the past few years have witnessed an incredible explosion in technologies for SNP genotyping (for a recent review, see Shi (6)), which undoubtedly will have a profound impact in forensics in the near future.

In this paper a novel SNP genotyping method is described, with a special emphasis on its applicability to forensic science. We refer to this method as Melting Curve SNP (McSNP) Genotyping (9). McSNP combines a classic approach for discriminating alleles, restriction enzyme digestion, with a more recent method for detecting DNA fragments, Melting Curve Analysis (MCA). In the case of deletion/insertion polymorphisms (DIPs), the use of restriction enzymes is not necessary, and the only required step for genotyping after PCR is the analysis of the melting curve patterns of the PCR products. McSNP is a simple, inexpensive, and robust high-throughput method for genotyping diallelic markers.

Materials and Methods

McSNP: The Principle

The principle of McSNP is very simple, and is represented schematically in Fig. 1. The most important aspect of the method is the use of Melting Curve Analysis (MCA) to detect the genotype of the samples. Briefly, SYBR Green I (Molecular Probes, Eugene, OR), a double stranded DNA (dsDNA) intercalating dye, is used. When SYBR Green I is bound to dsDNA, it emits a fluorescence signal on excitation (10). As the PCR products are heated and reach their respective melting temperatures (T_ms), the two strands of the

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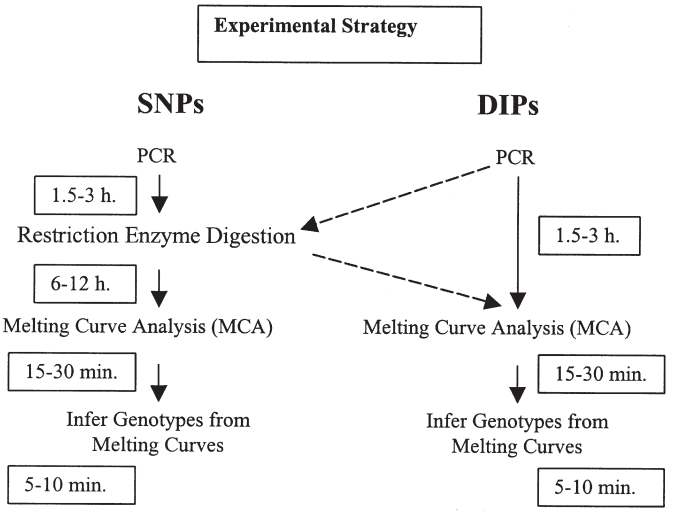

FIG. 1—*Schematic representation of the McSNP method, including information about the time involved in the different steps required for genotyping SNPs and DIPs, from PCR to genotype scoring. The dashed line represents an alternative approach to genotype DIPs.*

dsDNA dissociate, causing a rapid loss of fluorescence, which is monitored using the DASH instrument (ThermoHybaid, Ashford, Middlesex, United Kingdom). Since the T_m is a function of product length, sequence composition, and GC content, it is possible to distinguish DNA fragments that differ with respect to these parameters by MCA. The longer the PCR product and the higher the GC content, the higher the temperature at which the two strands melt (higher T_m). In the case of many DIPs, the difference in length between the allelic products is reflected as differences in T_m, which in many cases are large enough to be readily detected by MCA. In the case of SNPs, very often one of the alternative bases of the polymorphism alters the recognition sequence for a particular restriction enzyme, so that the enzyme recognizes and cuts only one allelic product but not the other, creating differences in size (and consequently in T_m) between the allelic products. Thus, it is possible to score the three genotypes (homozygous "cut," homozygous "uncut," and heterozygous "cut-uncut") by MCA since each demonstrates a distinctive T_m pattern. When there is no natural restriction site difference, one or both of the PCR primers can usually be modified so that a restriction site is engineered by converting the sequences directly flanking the SNP. It is notable that there is a high correlation between the observed T_m of a particular DNA fragment and the expected T_m as computed from theoretical formulations (9).

Experimental Protocol

Polymerase Chain Reaction—The sizes of the amplicons for McSNP conventionally range between 50 and 150 bp. Given this small fragment size, PCR works well under a number of conditions in terms of $MgCl_2$ concentrations and annealing temperature. Most of these reactions are conducted under a standard set of amplification parameters; PCR is carried out in 25 μL reactions (although it can be scaled down to 15 μL), consisting of 10–25 ng genomic DNA, 1.5–2.5 mM $MgCl_2$, 2.5 μL 10X PCR buffer (GibcoBRL, Rockville, MD), 0.1 mM dNTPs, 0.4 μM of each primer, and 2.5 units Taq polymerase (GibcoBRL, Rockville, MD). PCR is performed in either a ThermoHybaid MBS (ThermoHybaid, Ashford, Middlesex, United Kingdom) or a GeneAmp 9700 thermocycler (PE-Applied Biosystems, Foster City, CA). Primer sequences and PCR conditions for the markers presented in this report are shown in Table 1.

Restriction Enzyme Digestion—The entire PCR reaction is digested with the appropriate restriction enzyme (Table 1). It is not necessary to purify or quantitate the PCR products prior to restriction enzyme digestion. Restriction enzyme digestions are performed as recommended by the suppliers for a period of between 6 and 12 h.

Melting Curve Analysis—Typically, melting curve reactions contain 10 μL of restriction enzyme digested PCR product, 5 μL of 20X SYBR Green I, 10% formamide (Fisher Biotech, Fair Lawn, NJ) and ddH_2O to a final volume of 50 μL. All reactions are performed in polycarbonate 96-well plates (Omniplates, ThermoHybaid, Ashford, Middlesex, United Kingdom). Real time fluorescent monitoring is performed with specially designed software (McSNP software package, Thermohybaid, Ashford, Middlesex, United Kingdom), using a DASH machine. Melting curves are acquired by slowly ramping the temperature from 45 to 90°C at a rate of 0.04°C/sec. The total run time is approximately 20 min, but can be reduced by adjusting the thermal range, once the T_m is known. The fluorescence readings are captured during the slow ramp to 90°C. Final melting curves are reported graphically using the negative first derivative of fluorescence with respect to temperature on the Y-axis and temperature on the X-axis. The McSNP software package implements several methods to make classification of the genotypes more accurate, such as smoothing (which defines the width in number of data points of a sliding window for generating the derivative curve), sensitivity (which is the percentage of the height of the highest peak that will be considered for the classification of genotypes), and several baseline correction options (which use least squares fit algorithms to remove the downward trend of most MCA derivative curves). Finally, genotype scoring can be done manually or automatically, with or without baseline correction, using the McSNP software. The software classifies each well into one of the three possible genotypes, depending on the observed melting curve patterns. More information on this method can be found in Akey et al. (9).

Results

SNP Markers

We have used the McSNP method to genotype more than 30 SNPs in several population groups, with successful results. Figure 2 shows the smoothed melting curves for four autosomal SNP markers, after PCR amplification and digestion with the appropriate restriction enzyme. These markers have been selected to represent different strategies to type SNPs, and to show typical MCA patterns. Two of these markers (WI-16857 and WI-7423) have a natural restriction site in the DNA sequence (*Spe*I and *Sma*I, respectively), and in the remaining two SNPs (WI-11153 and WI-14981), the site has been introduced by means of converting primers (creating *BsaA*I and *Hpa*I sites, respectively, in the PCR products). For each marker, we show the three genotypes, homozygote "uncut," homozygote "cut," and heterozygote, in three different colors. It can be seen that the patterns of the three genotypes are clearly distinguishable, as a consequence of the difference in T_m between the resulting fragments. Homozygous "uncut" genotypes typically show a single peak, while homozygous "cut" genotypes typically show one or two peaks (depending on the difference in T_ms between the fragments resulting from the cleavage

TABLE 1—*SNPs and Deletion/Insertion Polymorphisms (DIPs) analyzed in the present study, including information on the nature of variation, primer sequences, PCR conditions, and enzymes used to characterize their genetic polymorphisms. Sizes are indicated for the resulting PCR products, both for SNPs typed using restriction enzymes (sizes given for uncut and cut fragments) and DIPs (sizes given for the alternative allelic products). For the SNPs that do not have a natural restriction site, the bases that have been altered in the primer sequence to introduce a restriction site polymorphism are indicated with bold letters.*

Markers	**Variation** base change or indel sequence	**5'-3' Forward/ Reverse Primers**	**PCR** (30 cycles)	**$MgCl_2$** (mM)	**Enzyme Frag sizes**
Autosomal SNPs					
WI-7423	C → T	F-CTCCTTGGCAGGGATTTGT R-CTGGTATCCACGGTGCAAG	94°30s 62°40s 72°40s	2.0	*Sma*I 80, (47+33)
WI-16857	A → C	F-CCATCCTCCAACACACACAC R-TTGAAGCACAGGTCTTGTGAA	94°30s 65°40s 72°40s	2.5	*Spe*I 79, (42+37)
WI-11153	C → G	F-CTTCAAATTGCTTTAAGTAC R-ATCCAACAGTCAAGGTCT**A**C	94°30s 55°45s 72°45s	2.5	*BsaA*I 45, (25+20)
WI-14981	G→T	F-TTGATTCAGTGGTGTTTATTG R-CTCTGACATAATACTTAGC**G**TT**A**	94°30s 48°30s 72°30s	2.5	*Hpa*I 53, (31+22)
DIPs					
MID187	CGTGAAGTCC	F-ATATTCACCTCCAGCCACCA R-CTGAGTCCAGAGGTCCCAAC	94°30s 60°30s 72°30s	2.0	52, 62 (*Dra*III)opt 52, (28+35)
MID 154	TCCCACGCGAGTGTGGTG GGACCTTG	F-TCTGCTTCTCTTGAGATACATAGTT R-AACAGGCAATCCTCCTAAGTCT	94°30s 60°30s 72°30s	2.0	62, 88
MID 944	TTAGATAAGAGAAACTA AATTCTTAAAAGC	F-TCAGTAAAAGGGTTTCCTTG R-AAAGCAAAGCCAAATATAAAAA	94°30s 55°30s 72°30s	2.5	55, 85
Y-SNPs					
M170	A → C	F-TGTTTTCATATTCTGTGCATT R-GACACAACCCACACTGAAAAA**G**A	94°30s 60°45s 72°45s	2.0	*Mnl*I 84, (63+21)
M9	C → G	F-CAAAGAAACGGCCTAAG R-GACATTGAACGTTTGAACA	94°30s 60°45s 72°45s	2.0	*Hinf*I 73, (24+49)

Note on PCR conditions: After an initial denaturation for 5 min. at 94°C, DNA samples were amplified for 30 cycles at the denaturation/annealing/extension temperatures specified for each marker, followed by a final extension for 5 min. at 72°C.

by the digestion enzyme), with lower T_ms that the peak corresponding to the homozygous "uncut" genotype. Heterozygotes always present a composite pattern, showing the peaks present in "uncut" and "cut" homozygotes.

The design of the primers is a key factor to the success of McSNP genotyping. It is important to assure that the differences in T_m of the PCR fragments are going to result in distinct peaks after digestion with the appropriate enzyme. Various T_m prediction formulae can provide good estimates on whether or not the difference in T_m between two DNA fragments will be resolvable by MCA (9). One such formula is implemented in the McSNP software package. Our experiments show that as a rough approximation, two DNA fragments can be consistently resolved if their predicted T_m difference is at least 3°C. This applies for SNPs with a natural site in the DNA sequence, SNPs in which the site is introduced by means of mismatched primers, and DIPs. Also, in order to ensure that the T_m of a fragment is reached under the experimental temperature range used in the MCA (35–90°C), it is necessary to add a destabilizing agent to the final PCR product. Both DMSO and formamide have very similar effects on melting curve characteristics and can be successfully used to lower the T_m. Finally, the sizes of the PCR fragments are critically important in McSNP genotyping. The best results are observed with PCR products in the range of 50–150 bp. Although in many cases larger fragments can be resolved by McSNP, in this size range it is easier to obtain large enough differences between the T_ms of the "cut" and "uncut" alleles, both for PCR products with a

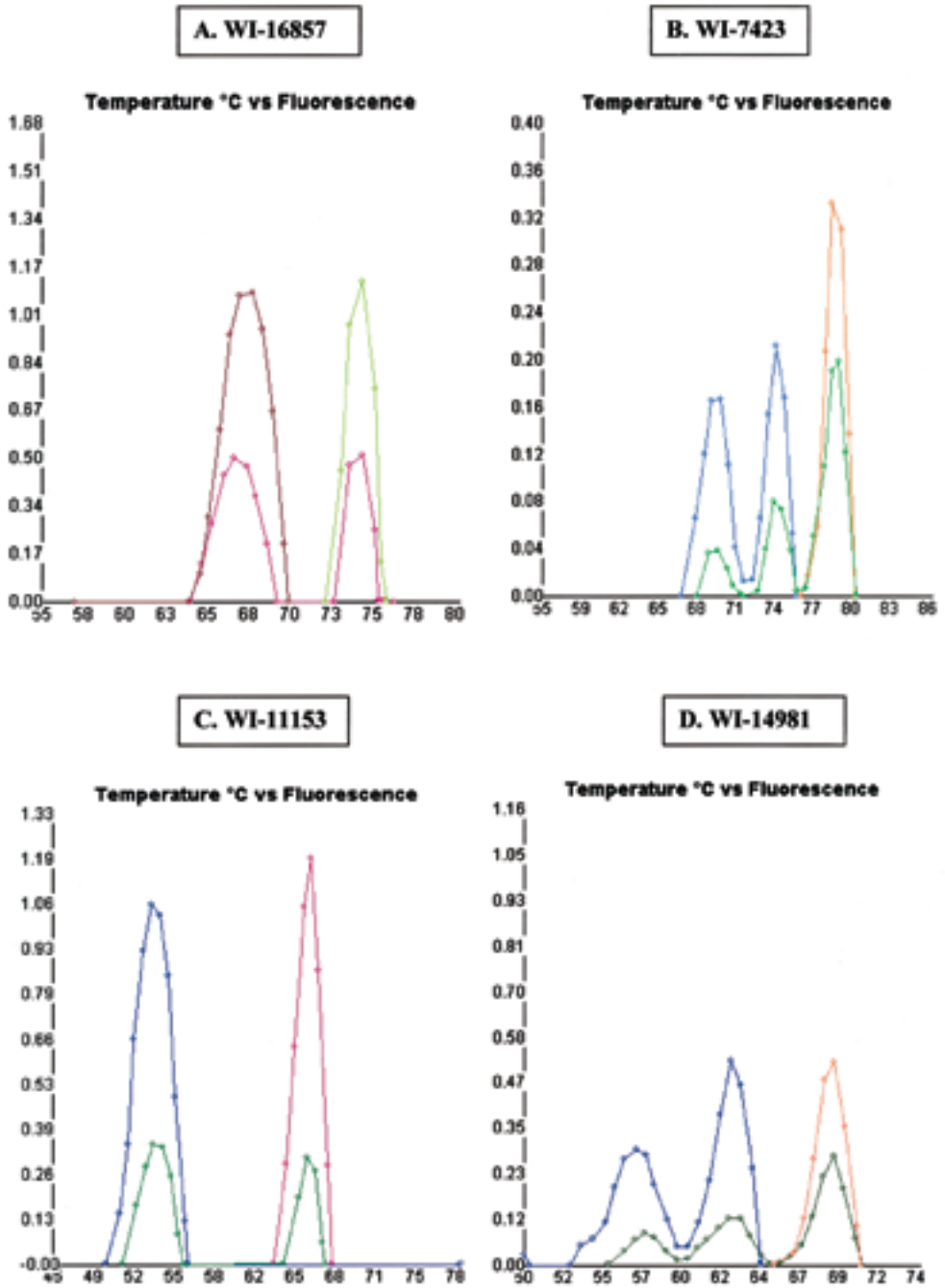

FIG. 2—*McSNP patterns of four autosomal SNPs, after PCR amplification and restriction enzyme digestion. A. WI-16857 (digested with SpeI), B. WI-7423 (digested with SmaI), C. WI-11153 (digested with BsaAI), D. WI-14981 (digested with HpaI). The three possible genotypes for each marker are depicted in different colors. The figures have been extracted directly from genotype files obtained using McSNP software, and resized for a better view.*

natural restriction site and PCR products in which the polymorphic site has been created by use of modified primers.

Under the aforementioned conditions, we have observed that McSNP is a fast, reliable, sensitive, and inexpensive method to genotype SNPs. Although partial digestion can be a potential problem when using methods based on restriction enzymes, the patterns resulting from partial digestion are typically different from the conventional patterns of the heterozygotes. The relative height of the peaks is different in heterozygotes than in partially digested "cut" homozygotes, in which there is only a residual peak corresponding to the undigested allele. Partial digestion can be minimized by controlling the digestion conditions and the number of PCR cycles and, in our experience, it is not a major problem in McSNP genotyping. In particular, it is important not to exceed 30 cycles of PCR so as to prevent the formation of heteroduplex DNA, one of the most common causes of incomplete digestion.

Deletion/Insertion Polymorphisms (DIPs)

We have selected a number of DIPs from the Marshfield Short Insertion/Deletion Polymorphism database (11), to test the applicability of McSNP for genotyping these markers. PCR primers

were designed to flank the polymorphism, and the size of the PCR products was minimized to yield the maximum melting point difference between the two alleles. The size of the DNA that was inserted or deleted ranged from 4 to 26bp, and in GC content from 36 to 80%. As with restriction fragments, the key aspect for accurately scoring DIP genotypes using McSNP is the difference in T_m between the amplicons with and without the insertion. Markers showing T_m differences higher than 3°C are generally amenable to genotyping by McSNP. In some cases, amplicons with lower T_m differences (between 2 and 3°C) can be genotyped as well, adjusting the heating rate so that the number of fluorescence data points that are collected is increased (e.g., using a ramp rate of 0.02 rather than 0.04°C/sec). For several DIPs, the differences in T_m were too small and the resulting peaks too close to be distinguished by MCA. All assays were completed in less than 2 h, including PCR amplification time, and required only MCA buffer (including water, formamide, and SYBR Green I) in addition to the components of the initial PCR reaction.

It is important to note that in some cases the difference in sequence between the alleles with and without the insertion creates a polymorphism in a restriction enzyme site, and those DIPs can alternatively be genotyped by digestion with the appropriate enzyme prior to MCA. Figure 3 shows the MCA patterns of several DIPs that we have characterized in our laboratory. In the case of MID 187, we show the pattern of the three genotypes using these two alternative genotyping methods. In the first (Fig. 3, panel C: MID187A), MCA was carried out immediately after amplification. In the second (Fig. 3, panel D: MID187B), the PCR product was digested with the enzyme *Dra*II overnight before the final MCA step. Although in both cases it is possible to clearly distinguish the patterns of the three genotypes, the use of *Dra*III increases the T_m differences between the MID 187 alleles.

Y-specific SNPs

Determining the haplotypes of the non-pseudoautosomal region of the Y chromosome can be very important in some forensic situations, especially when the evidence consists of a mixture of male and female contributors. We have genotyped with McSNP a number of SNPs recently described (12–14). Two examples of the patterns typically observed for Y-specific SNPs are shown in Fig. 4 (M170 and M9, respectively).

Discussion

The use of SNPs and DIPs is expected to bring a new era in multiple scientific fields. Identifying and localizing most SNPs in the human genome is only the initial step to their application in biomedicine, evolutionary biology, and forensics. It is also fundamental to study the details of SNP variation in human populations if we want to understand the genetic basis of variation in human physiology and disease risk, and to use them to decipher the evolutionary history of our species. It is easy to see how the increased knowledge in SNP variation will have a profound impact in forensics. As mentioned before, SNPs can provide complementary information to that available using STRs. For example, in the cosmopolitan U.S. population, selecting a panel of 30 unlinked SNPs from an SNP database comprising information on European Americans, African Americans, Hispanics, Native Americans, and Asian Americans would be extremely useful in forensic and paternity testing. If the markers are selected so that allele frequencies range between 30–70% in all of these populations, the frequency of the most common genotype will never be higher than 50% for any of the markers and populations, assuming Hardy-Weinberg equilibrium. Consequently, genotyping 30 markers with the aforementioned characteristics and applying the product rule (15), the random-match probability for the most common multilocus genotype in any population group would be $(0.5)^{30}$, that is, less than 1 in 11 billion. SNPs will also be useful in many other ways. Selecting SNPs showing large frequency differences between major population groups will provide information about the biogeographical ancestry of individuals. Although it has been known for decades that the genetic variation between human populations is only a small percentage of the genetic variation of our species (around 10–15%, (16,17)), it will be possible to select, from the millions of SNPs dispersed in the human genome, enough informative SNPs to infer accurately biogeographical ancestry. That is, given a set of source populations and an individual from one of them, it will be possible to assign the individual to his/her population with high probability (18,19). Indeed, many of these markers have already been identified and verified as useful for estimating biogeographical ancestry (20–22). Finally, it is notable that SNPs likely underlie much of the physical phenotypic variability among persons. In most cases very complex gene-gene and gene-environment interactions, which we are only now beginning to understand, are involved in defining the final phenotype. In the not-so distant future, genotyping informative SNPs will give information on a variety of physical characteristics (23).

Determining the haplotypes of the non-pseudoautosomal region of the Y chromosome can be very important in forensic applications. The first SNP identified on the Y chromosome was described as recently as 1994 (23). Since then, better methods of SNP discovery, such as Denaturing High-Performance Liquid Chromatography (DHPLC), have provided the scientific community with an increasing number of Y-specific variants (12–14, 24). These male-specific genetic polymorphisms are already providing important insights in human evolution (14) and also will be very useful in forensic studies, in a way that is analogous to how mtDNA sequence data are used today. For forensic uses, panels of Y-specific binary markers can distinguish between mixtures of male and female contributors, be used as an initial profiling filter to rapidly exclude male individuals in paternity and assault cases, distinguish siblings from half siblings, and identify the presence of multiple unrelated male perpetrators via the appearance of apparent heterozygous signatures. It is also important to note that many Y-specific markers, and the haplotypes they define, display profound genetic variation between populations, and they can provide clues to possible biogeographical ancestry. Finally, such polymorphisms are potentially useful in reconstructing family genealogies.

As previously mentioned, a number of methods are currently available for SNP genotyping. The strategies for allelic discrimination, reaction format, and detection methods differ widely between the alternatives. Many of the available methods offer higher throughput and faster genotyping than the currently used STR typing technologies. However, in terms of cost, they offer no such improvement, since most SNP genotyping methods still require expensive reagents, equipment, and expertise, and therefore, do not significantly decrease the cost of the DNA analysis.

McSNP offers several advantages over competing methods when considering factors such as cost, time, and flexibility. The possibility of applying this method to genotyping deletion/insertion polymorphisms (DIPs) is of particular interest, because restriction enzymes are not often required and, therefore, the genotyping process can be carried out in just one or two steps. As mentioned in the Results section, the difference in length and se-

quence of the two alleles causes a shift in T_m that, in many instances, can be detected by MCA. This strategy offers tremendous advantages in terms of time and cost per reaction over alternative methods. After PCR, only 15–20 min are required to run the MCA and automatically (or manually) score the genotypes for 96 samples using the specially designed McSNP software.

Most SNPs are amenable to genotyping by means of McSNP—a combination of PCR amplification, restriction enzyme digestion, and MCA. This method has been used in our laboratory to type more than 20,000 genotypes for over 30 SNPs, with excellent results. Although we conventionally set the PCR reactions with 10–25 ng of template DNA for our anthropological studies, in which DNA availability is not a problem, McSNP works well with less than 1 ng template DNA (data not shown). Additionally, McSNP can also be carried out successfully after whole genome amplification, which is particularly important when the amount of DNA available for genetic analysis is limited, as in many forensic applications. Currently, in our laboratory only one marker is ana-

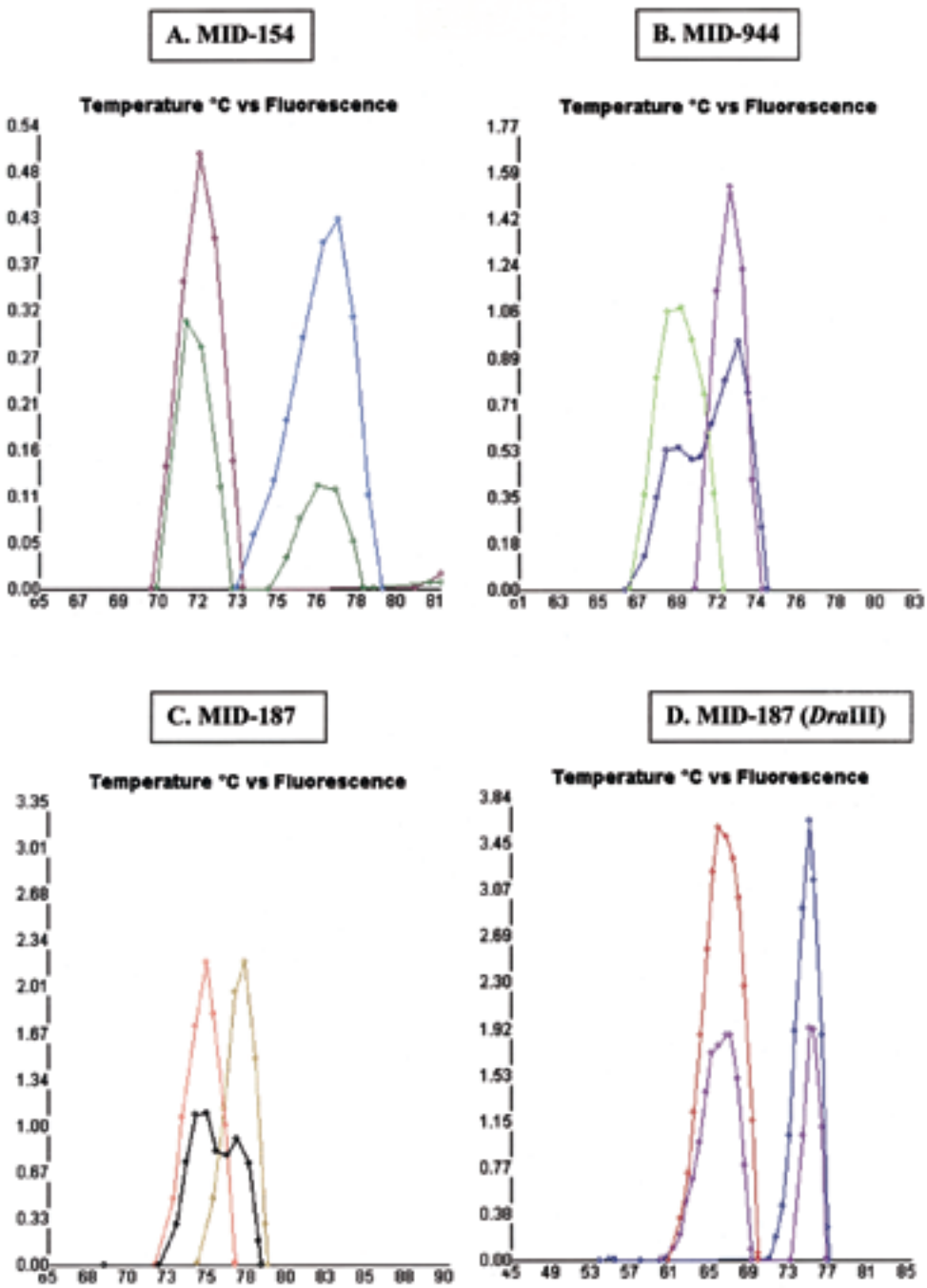

FIG. 3—*McSNP patterns of three Insertion/Deletion polymorphisms. A. MID 154, B. MID 944, C. MID 187, D. MID 187 (DraIII). The three possible genotypes for each marker are depicted in different colors. In A, B and C, melting curve analysis was carried out directly after PCR amplification. In D, melting curve analysis was carried out after PCR amplification and digestion with the enzyme DraIII. Note that the presence of the MID 187 insertion creates a DraIII site, so after digestion the peak at 78°C in figure C becomes a new peak at 65°C in figure D. The figures have been extracted directly from genotype files obtained using McSNP software, and resized for a better view.*

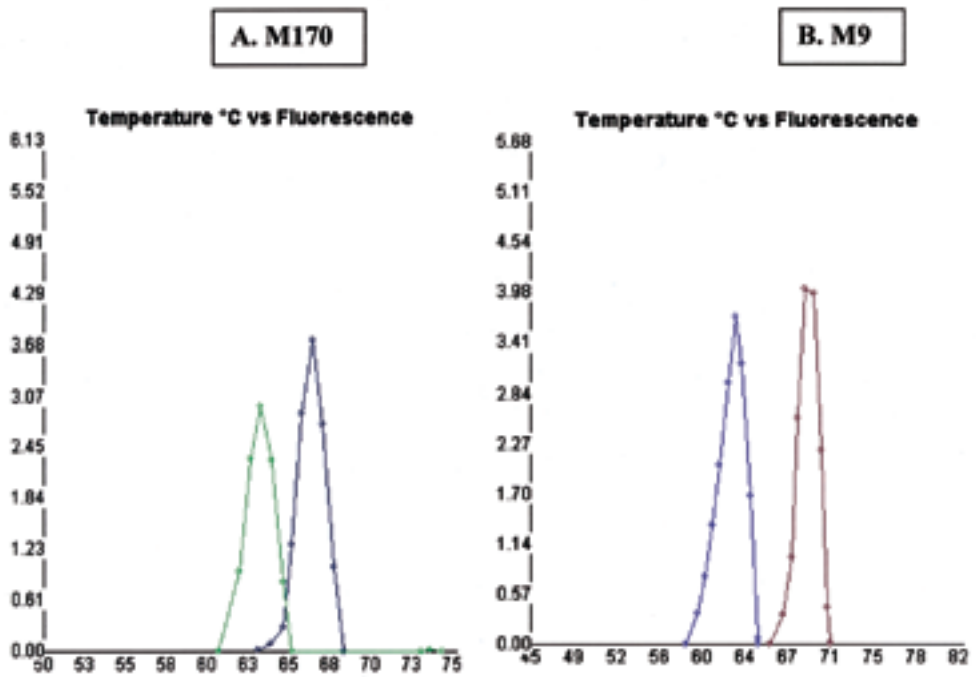

FIG. 4—*McSNP patterns of two Y-specific SNPs. A. M170, B. M9. The two possible genotypes for each marker are depicted in different colors. The figures have been extracted directly from genotype files obtained using McSNP software, and resized for a better view.*

lyzed per PCR reaction, but a careful selection of markers with different T_m profiles would potentially allow duplex analysis. However, the increased complexity of T_m patterns in reactions with more than two markers make this method unsuitable for genotyping more than two markers simultaneously. As with every other PCR-based method, extreme caution is necessary to avoid contamination, and in our laboratory the risk is minimized by devoting different rooms for setting up the PCR reactions and carrying out the genotyping of the PCR products, by using totally independent sets of pipettes for the pre- and post-PCR work, and by the use of filtered pipette tips, disposable latex gloves, and UV exposure of surfaces. It is notable that in the case of DIPs, where the formamide and SYBR green I can be added at the time of PCR setup, the risk of contamination will be dramatically reduced since there is no need to ever open the tubes of plates after the PCR reaction. An important advantage of McSNP is that a number of different markers can be typed with this method, including SNPs and DIPs. In summary, McSNP is a simple, inexpensive, and accurate method for genotyping SNP variation that can have important applications in the field of forensics.

Acknowledgments

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