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Comparative Analysis of the HV1 and HV2 Regions of Human Mitochondrial DNA by Denaturing High-Performance Liquid Chromatography

ABSTRACT: Denaturing high-performance liquid chromatography (DHPLC) was evaluated as a sequencing-independent means of detecting the presence of sequence differences in pair-wise mixtures of nonconcordant amplicons of human mitochondrial DNA (mtDNA). A total of 920 pair-wise combinations of HV1 and HV2 mtDNA amplicons from 95 individuals were assayed by DHPLC for sequence concordance/nonconcordance. For the 72 combinations of amplicons from different individuals who shared identical DNA sequences, DHPLC assays consistently indicated sequence concordance between the samples. This was in 100% agreement with sequencing data. For the 849 combinations of amplicons which differed in sequence, DHPLC detected the presence of sequence nonconcordance in all but 13 assays to yield 98.5% concordance with sequencing. Thus, DHPLC can be used to detect a diversity of sequence differences (transitions, transversions, insertions, and deletions) in the mtDNA D-loop. Accordingly, DHPLC may have utility as a presumptive indicator of mtDNA sequence concordance samples, as a screen for heteroplasmy/situational mixtures, and as a means for the physical fractionation of the individual contributors to an mtDNA mixture prior to sequencing.

KEYWORDS: forensic science, DHPLC, mitochondrial DNA, comparative analysis, polymorphisms, presumptive test, screening

Analysis of mitochondrial DNA (mtDNA) for forensic casework is relatively expensive, time-consuming, and requires the expertise of skilled analysts currently employed by a limited number of public and private laboratories. Many efforts to streamline the processing of forensic mtDNA casework have sought to develop rapid, cost-effective, and reliable methods of screening for mtDNA concordance/nonconcordance between samples or for the presence of mtDNA mixtures within a single sample prior to sequence characterization.

A variety of methods for mtDNA characterization have been investigated in recent years. These include hybridization to linear arrays of sequence-specific oligonucleotides (SSO) (1,2), denaturing gradient gel electrophoresis (DGGE) (3,4), single-strand conformational polymorphism (SSCP) analysis (5,6), time-of-flight mass spectrometry (7), and microarray-based analysis (8). These approaches suffer from a number of limitations which include: the interrogation of only a subset of variant sites, cross-hybridization to nontarget sequences, laborious manipulations of gels (2,9), and interference by primer impurities (7). Most critically, however, all of these approaches suffer from the fact that they consume often precious forensic evidence while not necessarily providing a reliably comprehensive assessment of all possible sequence differences across the entire amplicon.

Denaturing high-performance liquid chromatography (DHPLC) circumvents these limitations while offering the potential to characterize mtDNA amplicons in an accurate, rapid, and cost-effective manner that can be automated. Moreover, DHPLC makes it possible to recover the assayed DNA from the column eluent at the end of an assay. As currently employed in a variety of biomedical applications, the technique facilitates discrimination between amplicons that differ in sequence (i.e., single nucleotide substitutions, insertions, deletions) but which may be identical in length. When all DNA molecules in a sample are identical, denaturation and renaturation produce only homoduplices which elute simultaneously to yield a single chromatographic peak in a DHPLC assay. Conversely, a mixture of nonidentical DNA amplicons yields a combination of homo- and cross-hybridized heteroduplices. Heteroduplices elute more readily than homoduplices (10,11) from the DHPLC DNASep® column. Thus sequence nonconcordance between DNA molecules is readily indicated by the appearance of early eluting heteroduplex peak(s) on the resulting chromatogram (10–12).

In comparative studies of DHPLC-based detection of sequence polymorphisms versus alternate approaches such as SSCP and DGGE, DHPLC has often been found to provide superior sensitivity and accuracy (10,13,14). Using carefully designed assay conditions, the concordance between direct DNA sequencing and comparative sequence analysis by DHPLC typically ranges from 95 to 100%. In some cases, DHPLC has proven to have greater sensitivity than direct DNA sequencing for the detection of minor component sequence variants. The accuracy and sensitivity with which sequence variants can be detected by DHPLC can be significantly influenced by the length, GC content, and other thermodynamic characteristics of the amplicon being assayed. The current study, therefore, investigated the potential utility of DHPLC to specifically detect sequence variants throughout the forensically relevant portions of the HV1 and HV2 regions of human mtDNA. A high degree of concordance between DHPLC and direct sequencing for the comparative analysis of those amplicons commonly used by forensic practitioners could facilitate the development of less consumptive approaches to mtDNA screening.

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Methods

This research was conducted in compliance with U.S. Federal Policy for the Protection of Human Subjects (56 FR 28003). Blood samples were collected from 103 unrelated subjects who had provided informed consent; of these, eight samples were used to generate mtDNA subclones. All samples were stored at −20°C until DNA extraction.

Mitochondrial DNA Extraction from Dried Blood

Total DNA was extracted from dried reference blood stains using a QIAmp® 96-Well Blood Kit (Qiagen Inc., Valencia, CA) as per the manufacturer's recommended protocol. For consistency, the extraction was performed using a Biomek® 2000 automated liquid handler (Beckman Coulter Inc., Fullerton, CA). Purified DNA was eluted into 200 μL of nuclease-free ddH_2O and stored at −20°C until PCR amplification.

Mitochondrial DNA Amplification and Sequencing

Mitochondrial DNA control region amplification reactions (50 μL) were prepared using 50 pmol each of forensically validated PCR primers (15,16); 2.25 U Ampli*Taq* GOLD® DNA polymerase (Applied Biosystems, Foster City, CA) supplemented with 0.25 U *Pfu* DNA polymerase (Stratagene, La Jolla, CA); Ampli*Taq* GOLD® Buffer (Applied Biosystems); 10 nmol of each dNTP (Stratagene) and 10 μL of an approximately 10 pg/μL DNA extract. Amplifications were performed on a GeneAmp® 9700 thermocycler (Applied Biosystems) with an initial denaturation at 95°C for 10 min, followed by 32 cycles of 95°C for 20 sec, 60°C for 30 sec, and 72°C for 45 sec. The final extension at 72°C was for 15 min. The resulting PCR yield for each fragment was then quantified by HPLC under a nondenaturing column temperature of 50°C. The near perfect correlation ($R^2 = 0.9982$) between chromatographic peak area and DNA quantity made it possible to reliably determine the DNA quantity from each amplification reaction. This approach, which requires ≤7 min per assay, has been internally validated for fragments ranging in size from 200 to 1000 bp and for quantities ranging from 1.25 to 140 ng (data not shown).

Approximately 0.8 ng of amplified DNA was labeled for sequencing using the Big-Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems). Labeling reactions were primed using the PCR primers employed above or additional internal sequencing primers (5′-GGAACCAGATGTCGGATACAGTTC-3′ and 5′-CATCTGGTTCCTACTTCAGGGTCA-3′) developed in-house. Labeled products were purified by ethanol precipitation or on Performa® DTR V3 96-Well Short Plates (Edge BioSystems, Gaithersburg, MD) and then resolved on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems). Appropriate positive and negative controls were included at all stages of the process. The resulting sequence data were analyzed using the Sequencher™ DNA analysis software (Gene Codes Corp, Ann Arbor, MI).

Mitochondrial DNA Subcloning

Primers L15997 (5′-CACCATTAGCACCCAAAGCT-3′) and H408 (5′-CTGTTAAAAGTGCATACCGCCA-3′) were used to amplify an mtDNA fragment of *c*. 1021 bp which encompassed both HV1 and HV2. The resulting amplification products representing eight mtDNA haplotypes were purified using Wizard® PCR Preps (Promega, Madison, WI), ligated into the pGEM®-T vector (Promega) and electroporated into DH5α electrocompetent cells. Cells were selected on LB agar plates containing 50 μg/mL ampicillin and screened by PCR to identify plasmids containing target inserts. Plasmids were isolated from overnight cultures of these colonies by alkaline lysis and captured on glass-filter binding plates (Millipore, Bedford, MA). DNA was sequenced as described above.

DHPLC-Based Temperature-Modulated Heteroduplex Analysis

DNA sequence data for each of four forensically relevant amplicons (HV1A, 278 bp: L15997–H16236; HV1B, 271 bp: L16159–H16395; HV2A, 278 bp: L48–H285; and HV2B, 277 bp: L172–H408) were compared to identify individuals with different mtDNA haplotypes (16). Fragments were then combined at a 1:1 molar ratio to generate a series of pair-wise mixtures which were cross-hybridized by denaturation at 95°C for 4 min and gradual cooling (1.5°C/min) to a final temperature of 25°C (15).

The cross-hybridized mixtures were analyzed by temperature-modulated heteroduplex analysis (17) on a WAVE® 3500HT DNA Fragment Analysis System (Transgenomic Inc., Omaha, NE) using a DNASep® analytical column (12). Triethylammonium acetate (TEAA) pH 7.0 at a final concentration of 0.1 M served as an ion-pairing reagent. Cross-hybridized mixtures were analyzed under partially denaturing conditions at the empirically determined optimal temperatures for each of the four mtDNA amplicons (i.e., HV1A, 58°C; HV1B, 59.2°C; HV2A, 56.5°C, and HV2B, 57°C). The optimal acetonitrile linear gradient generated from differential mixing of buffer A (0.1 M TEAA) and buffer B (0.1 M TEAA, 25% ACN) was a 56% to 65% buffer B increase in 3.5 min for HV1A, 4.5 min for HV2A and HV2B and a 55% to 64% buffer B increase in 3.5 min for HV1B. All samples were eluted at a 0.9 mL/min flow rate and detected by UV absorbance at 260 nm. Standard DHPLC controls included: zero-volume injections to screen for DNA carryover between assays; no-template PCR controls to check for reagent contamination; and manufacturer mutation control standards for buffer and column quality control. Where necessary for enhanced sensitivity, temperate titrations were conducted in ±0.5°C increments using an integrated oven accurate to ±0.1°C.

The resulting chromatograms were visually examined. The presence of a single homoduplex peak was scored as consistent with sequence concordance. The presence of more than one chromatographic peak or a shoulder on a homoduplex peak was scored as consistent with the presence of one or more sequence differences between the two amplicons in the sample being assayed, i.e., sequence nonconcordance (10,11).

Results and Discussion

Essential Modification of Forensically Validated Protocols

In evaluating the utility of DHPLC for comparative mtDNA sequence analysis, the current study employed forensically validated oligonucleotide primer pairs and standard operating procedures (SOPs) (18). Two minor modifications to these procedures were made, the exclusion of bovine serum albumin (BSA) from the amplification reaction and the supplementation of Ampli*Taq* GOLD® with higher fidelity *Pfu* DNA polymerase.

The presence of >10% w/v BSA in a sample reduces the functional lifespan of the DNASep® column. BSA, however, is often used to ameliorate the effects of endogenous and environmental PCR inhibitors in some DNA extracts. These include IgG and heme in blood (19), melanin in hair (20), collagen in bone (21),

and such environmental inhibitors as humic acid in soil (22) which are not efficiently removed by inorganic extraction methodologies (e.g., Chelex™). In our laboratory, both endogenous and environmental inhibitors have been effectively removed from hair, bone, and environmentally contaminated samples by the use of organic extraction, silica-gel membranes or by systems that employ paramagnetic particles such as the Qiagen® EZ1 DNA tissue kit.

In the current study, the silica-gel membrane-based QIAamp® DNA blood purification kit was used to prepare all DNA extracts from whole blood. The purity of the resulting extracts was evaluated by PCR amplification of mtDNA in the presence and absence of BSA at a final concentration of 0.16 μg/μL. Yields for individual amplification reactions were generally in the range of 20–25 ng/μL as determined by optical absorbance at 260 nm. The amplification of mtDNA from QIAamp® prepared extracts was equally efficient regardless of whether or not BSA was added (data not shown). If the use of BSA is unavoidable, purification of the PCR amplification products by organic extraction or other protein removal method may be needed prior to DHPLC analysis.

The fidelity of the DNA polymerase used for amplification may also impact the accurate interpretation of DHPLC chromatograms. The relatively high nucleotide misincorporation rate of *Taq* DNA polymerase (8.0×10^{-6} mutations/bp/duplication) (23) typically produce DHPLC chromatograms from single sequence samples which erroneously appear to indicate a DNA mixture. A mixture of *Taq* and *Pfu* DNA polymerases at a 9:1 ratio effectively eliminates these anomalies. The $3' \rightarrow 5'$ exonuclease activity and lower nucleotide misincorporation rate (1.3×10^{-6} mutations/bp/duplication) of *Pfu* yield greater uniformity in amplification products (23).

DHPLC Assay Development

Recognizing that mtDNA heteroplasmy is detected by DHPLC as a mixture of nonconcordant sequences, the initial optimization

TABLE 1A—*Sequence polymorphisms in HV1 subclones based on the revised Cambridge Reference Sequence.*

HV1	Nucleotide Position (rCRS)																			
	HV1A							HV1B												
Sample	16086	16104	16224	16126	16128	16129	16146	16182	16183	16189	16193.1	16223	16249	16294	16296	16304	16311	16323	16342	16359	16362
rCRS	**T**	**C**	**T**	**T**	**C**	**G**	**A**	**A**	**A**	**T**	**:**	**C**	**T**	**C**	**C**	**T**	**T**	**T**	**T**	**T**	**T**
Subclone 1	*	*	*	*	*	A	*	*	C	C	C	T	C	*	*	*	C	*	*	C	*
Subclone 2	*	*	*	C	*	*	*	*	*	*	*	*	*	T	T	C	*	*	*	*	*
Subclone 3	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Subclone 4	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Subclone 5	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C
Subclone 6	*	T	*	*	*	*	G	*	*	*	*	*	*	*	*	*	*	*	C	*	*
Subclone 7	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*
Subclone 8	*	*	*	*	*	*	*	C	C	C	*	*	C	*	*	*	*	*	*	*	*

TABLE 1B—*Sequence polymorphisms in HV2 subclones based on the revised Cambridge Reference Sequence.*

HV2	Nucleotide Position (rCRS)												
	HV2A									HV2B			
Sample	57.1	64	73	146	152	195	263	282	285	309.1	310	315.1	385
rCRS	**:**	**C**	**A**	**T**	**T**	**T**	**A**	**T**	**C**	**:**	**T**	**:**	**A**
Subclone 1	*	*	G	*	*	C	G	*	*	C	*	C	*
Subclone 2	*	*	G	*	*	*	G	*	*	C	*	C	*
Subclone 3	*	*	*	*	*	*	G	*	*	*	*	C	*
Subclone 4	*	*	*	*	C	*	G	*	*	*	*	C	*
Subclone 5	C	T	*	*	*	*	G	*	*	*	*	C	*
Subclone 6	*	*	G	*	*	*	G	C	*	C	*	C	*
Subclone 7	*	*	G	C	C	*	G	*	*	C	C	C	*
Subclone 8	*	*	G	*	*	*	G	*	T	*	*	C	G

Due to a 285C>T mutation in the native sequence from which Subclone 8 was derived, a 1nt truncated D2 primer was used for all amplification reactions.

of DHPLC assay parameters was conducted using a group of eight mtDNA subclones as templates for PCR amplification of the HV1 and HV2 amplicons (Table 1). Collectively these subcloned sequences differed from each other at an average of 9.4 positions (0–7 positions in HV1A; 0–10 positions in HV1B; 0–5 positions in HV2A; 0–4 positions in HV2B). The use of subcloned sequences ensured that each sample represented a single distinct haplotype. This eliminated heteroplasmy as a potentially confounding variable for the purpose of assay optimization.

Optimization of DNASep® Column Temperatures

Comparative DHPLC-based DNA sequence analysis relies on temperature-dependent chromatographic separation of the nucleic acids under partially denaturing conditions. In accordance with manufacturer recommendations and empirical observations by the authors and other researchers, optimal resolution is generally obtained using amplicons of less than 400 bp in length and a column temperature where the average of nucleic acid helicity across an amplicon is approximately 75–85% (24,25). Under these conditions, heteroduplices with a single base mismatch elute from the DNASep® column before identical-length homoduplices.

The mtDNA primer pairs used in the current study yield amplicons within a size range that are well suited for DHPLC analysis (i.e., HV1A = 278 bp, HV1B = 271 bp, HV2A = 278 bp, HV2B = 277 bp). Although the appropriate partially denaturing temperature for a given amplicon is approximated by the system software based on nearest neighbor interactions (26), the optimal temperature must be empirically confirmed. This was done by monitoring the separation of homo- and heteroduplices in multiple mixed samples at temperatures flanking the predicted value. The optimal temperature is that at which the greatest chromatographic resolution is achieved. This is illustrated in Fig. 1, where the greatest resolution for the mixture of HV1B amplicons being assayed is at 59.2°C. This is not to suggest that all mixtures of HV1B amplicons will be optimally resolved at this temperature. Rather, this represents the optimal initial assay temperature at which mixtures of HV1B amplicons of unknown haplotype can be screened. Similarly, the optimal initial assay temperatures for the HV1A, HV2A, and HV2B amplicons were determined to be 58.0°C, 56.5°C, and 57.0°C, respectively.

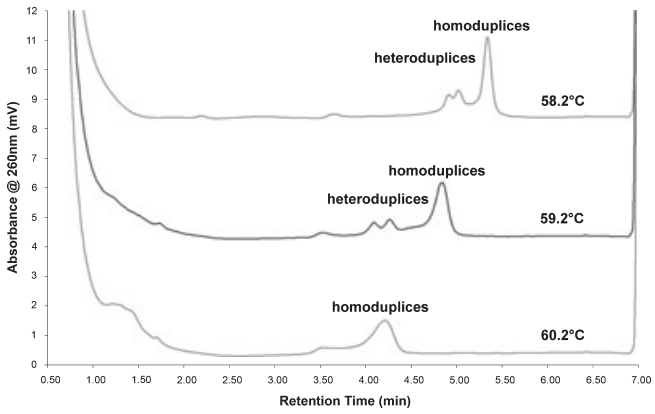

FIG. 1—*Empirical determination of the optimal column temperature for comparative sequence analysis of HV1B. While the hetero- and homoduplices can be resolved at 58.2°C, optimal resolution of clearly discernible chromatographic peaks is obtained at 59.2°C. At 60.2°C, the heteroduplices elute too readily to form distinct peaks.*

Reproducibility of DHPLC Chromatograms

To assess the reproducibility of peak height retention time and peak area across multiple injections, 10 replicate injections of a two-component HV2A mixture were sequentially assayed by DHPLC under the optimized conditions described above. The resulting data which encompass two early eluting heteroduplex peaks and two late eluting homoduplex peaks indicate that DHPLC analyses are highly reproducible in terms of both the maximum height and retention time of each individual peak (Table 2). Assay-to-assay variability was negligible between independent injections as evidenced by the small standard deviations in the parameters measured for each peak across the replicates tested. It should be noted that the excellent reproducibility of DHPLC assays does not require the use of added retention time standards but rather reflects the intrinsic reproducibility of the assay system. Commercial calibration standards are used, however, to regularly confirm and, if needed, to calibrate the DNASep® column oven.

Comparative Sequence Analysis of Subcloned mtDNA

As a preliminary assessment of the accuracy with which DHPLC is able to detect sequence differences, a matrix of all possible pair-wise combinations (i.e., 144 mixtures) of mtDNA fragments (i.e., HV1A, HV1B, HV2A, and HV2B) was prepared using the subcloned haplotypes as amplification templates. Following cross-hybridization and analysis by DHPLC, the resulting chromatograms were visually examined.

Figure 2 presents examples of chromatograms obtained from assays of 27 pair-wise comparisons between nonidentical and nine comparisons between identical HV2A amplicons. Sequence differences included a single-base insertion situated near the terminus of the amplicon (e.g., all comparisons with sample 5) and a variety of single and multiple base substitutions throughout the amplicon. Single base substitutions in both GC-rich and AT-rich regions were also assayed.

Combinations of each amplicon with itself, and the identical amplicons from subclones 2 and 8, yielded chromatograms with a single symmetrical peak (i.e., the homoduplex species). For two of the pair-wise combinations, nonconcordance was indicated by the presence of a shoulder on the homoduplex peak (e.g., subclones 2 and 4). For 24 of 27 pair-wise combinations of nonidentical amplicons, sequence nonconcordance was indicated by the presence of early-eluting and easily discernible heteroduplex peaks. In a few comparisons, the heteroduplex peak was much smaller than the homoduplex peak (e.g., subclones 1 and 5) making detection somewhat more challenging. In these cases, the presence of a heteroduplex peak was more readily discerned by comparing the chromatograms obtained for injections of each amplicon by itself with the chromatogram obtained when a mixture of the two amplicons was injected. This was done by superimposing these three chromatograms on top of each other.

Only the comparison between subclones 2 and 3 failed to show sequence nonconcordance by DHPLC at 56.5°C. This was expected, as the sequence difference between these amplicons (73G vs. 73A) was immediately adjacent to a short GC-rich region of predicted 100% helicity. The thermodynamic stability of this region is an intrinsic feature of the HV2A amplicon which interferes with the detection of base changes at position 73.

Pair-wise combinations of HV1A, HV1B, and HV2B fragments amplified from the eight mtDNA haplotype subclones were also assayed for sequence concordance/nonconcordance by DHPLC. As seen for the HV2A fragment, sequences that differed by one or more bases were successfully identified by DHPLC either by the

TABLE 2—*Ten sequential injections of a two-contributor HV2A mixture showing the reproducibility of DHPLC assays on a DNASep® column.*

		Injection											
HV2A		1	2	3	4	5	6	7	8	9	10	Average	SD
Peak 1	Peak height	2.82	2.82	2.78	2.75	2.76	2.79	2.70	2.78	2.74	2.76	2.82	0.004
	Ret. time	3.49	3.47	3.46	3.48	3.47	3.47	3.48	3.48	3.47	3.48	3.48	0.01
Peak 2	Peak height	8.34	8.53	8.37	8.31	8.44	8.28	8.41	8.37	8.34	8.47	8.39	0.08
	Ret. time	3.83	3.81	3.81	3.83	3.82	3.81	3.82	3.83	3.83	3.81	3.82	0.01
Peak 3	Peak height	4.50	4.51	4.47	4.51	4.48	4.47	4.49	4.45	4.45	4.51	4.48	0.02
	Ret. time	4.04	4.02	4.02	4.04	4.03	4.03	4.03	3.97	4.04	4.02	4.02	0.02
Peak 4	Peak height	11.13	11.13	10.91	11.02	11.22	11.25	11.33	11.11	11.31	11.35	11.17	0.14
	Ret. time	4.38	4.37	4.36	4.38	4.37	4.37	4.37	4.39	4.38	4.36	4.37	0.01

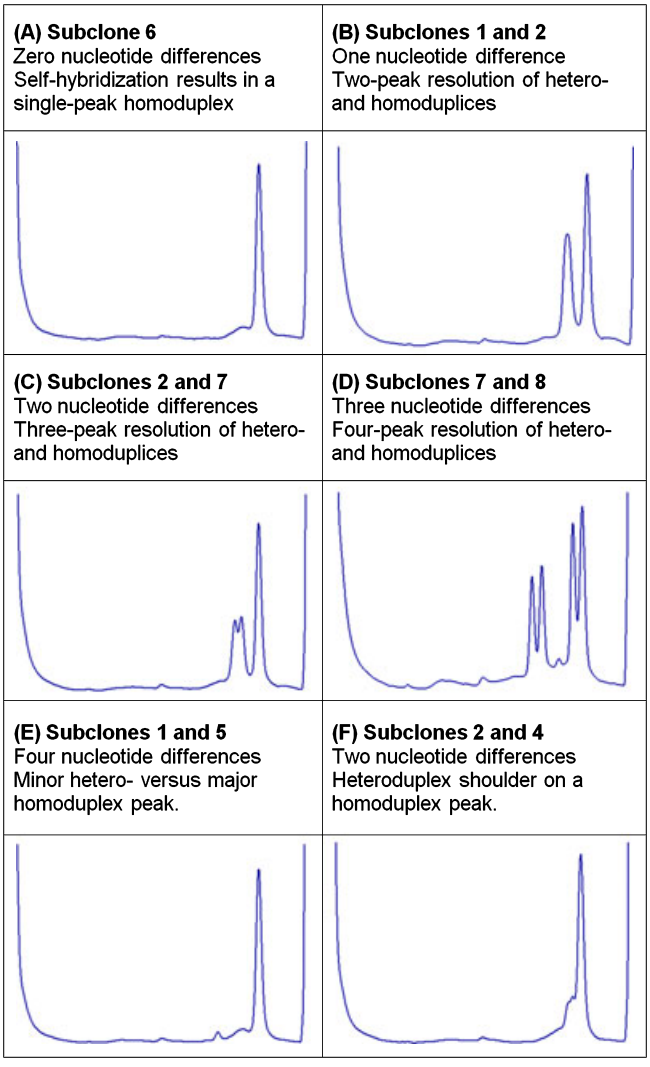

FIG. 2—*Examples of DHPLC chromatograms from pair-wise hybridization of the HV2A fragment amplified from eight mtDNA subclones. (A) Sequence concordance is indicated by the presence of a single homoduplex peak. (B–E) Sequence nonconcordance is indicated by the presence of more than a single peak. (F) Sequence nonconcordance can also be indicated by a shoulder on a homoduplex peak.*

presence of early eluting heteroduplex peaks or by an early eluting heteroduplex shoulder on a homoduplex peak. One sequence difference between subclones 3 and 5 (16362T vs. 16362C) was not readily detected without overlaying the chromatograms for the individual versus the mixed amplicons. This was not unexpected given that HV1B has a biphasic melt profile and position 16362 is within a region of higher GC-content that remains relatively stable at the standard assay temperature of 59.2°C.

Comparative Sequence Analysis of mtDNA from Blood Samples

The primary objective of the current study was to evaluate the potential utility of DHPLC as a means of screening amplified human mtDNA samples for sequence concordance/nonconcordance in the context of a forensic laboratory. Initial experiments using subcloned mtDNA haplotypes demonstrated that DHPLC accurately detects even single base differences between two mtDNA amplicons. Subclones, however, are artificially pure templates which do not reflect the molecular complexity of a human tissue-derived DNA extract. Furthermore, the eight haplotypes used encompass only a small fraction of known variant positions. To more thoroughly assess the reliability of DHPLC as a tool for comparative sequence analysis, the approach was tested on blood samples from 95 research volunteers.

Exclusive of length polymorphisms associated with the homopolymeric cytosine-stretch in HV2, the 95 subjects in the current study represented 83 distinct haplotypes. Seventy-seven of these were unique within the study population. Of the six haplotypes which occurred more than once, the most common (263G, 315.1C) was observed eight times while the remaining five haplotypes each occurred twice. Relative to the revised Cambridge Reference Sequence (27,28), the haplotypes represented in the current study encompass 84 polymorphisms in HV1 and 46 polymorphisms in HV2, including cytosine-stretch length polymorphisms.

Based on pair-wise comparisons, individual haplotypes differed from each other at 0–22 positions (0–11 in HV1A; 0–13 in HV1B; 0–13 in HV2A; and 0–12 in HV2B). On average, there were 8.71 positional differences between haplotypes. *In toto*, the study population encompasses a broad diversity of haplotypes and thus is well suited for evaluating the utility of DHPLC for the accurate detection of sequence polymorphisms throughout the mtDNA control region. This is essential for the validation of DHPLC as a tool for comparative sequence analysis.

A total of 920 pair-wise combinations of amplicons from the 95 individuals in this study were prepared, denatured, and allowed to gradually reanneal. Of these, 72 (22 in HV1A, eight in HV1B, 17 in HV2A, and 25 in HV2B) represented combinations of amplicons that were from different individuals but which had identical DNA sequences. DHPLC analyses of these samples all produced clear chromatograms consisting of a single symmetrical homoduplex peak. This pattern is consistent with sequence concordance and is in 100% agreement with direct sequencing data for these amplicons.

To assess the reliability of DHPLC to detect sequence nonconcordance, 849 combinations of amplicons (209 in HV1A, 222 in HV1B, 213 in HV2A, and 205 in HV2B) which differed in

TABLE 3—*Sequence polymorphisms assayed by DHPLC in the HV1 and HV2 regions.*

	HV1										HV2					
Transitions	16041G	16051G	16069T	16093C	16104T	16111T	16114T	16124C	16126C	16129A	55C	64T	72C	73G	89C	92A
	16136C	16144C	16145A	16147T	16148T	16153A	16154C	16163G	16172C	16173T	93G	114T	131C	143A	146C	150T
	16176T	16179T	16186T	16187T	16189C	16192T	16193T	16209C	16215G	16217C	152C	153G	185A	185A	188G	189G
	16221T	16222T	16223T	16224C	16239T	16241G	16243C	16244A	16249C	16256T	192C	194T	195C	196C	198T	199C
	16258G	16261T	16266T	16270T	16274A	16278T	16286T	16287T	16290T	16291T	200G	204C	207A	217C	222T	225A
	16293G	16294T	16295T	16296T	16298C	16299G	16301T	16304C	16309G	16311C	226C	228A	234G	239C	242T	250C
	16316G	16320T	16325C	16327T	16343G	16352C	16353T	16354T	16355T	16357C	285T	293C	295T	319C	340T	
	16359C	16362C	16390A													
Transversions	16111A	16129C	16176A	16182C	16183C	16184A	16265C	16290G			57G	207C	280G			
Insertions	16192.1T	16193.1C									309.1C	309.2C				
Deletions											249:	299:	309:			

sequence were assayed. These differences encompassed a broad diversity of polymorphisms distributed throughout the HV1 and HV2 regions including transitions, transversions, insertions, and deletions (Table 3). Positional differences were located internally as well as near the termini of amplicons and encompassed regions of varying GC richness. In all, the mixtures assayed in this study included sequence variants at 39, 62, 38, and 30 different positions in HV1A, HV1B, HV2A, and HV2B, respectively. Figure 3 illustrates the distribution of the variant positions and the frequency with which they were assayed.

Using the aforementioned initial assay temperature for each mtDNA amplicon, DHPLC analyses correctly indicated the presence of a mixture of nonconcordant amplicons in 836 (209 in HV1A, 222 in HV1B, 203 in HV2A, and 202 in HV2B) of the combinations tested. The remaining 13 mixtures (10 in HV2A and three in HV2B) of nonidentical amplicons yielded chromatographic traces with a single eluent peak, a result erroneously suggesting sequence concordance. Careful examination of the amplicon mixtures which were not detected by DHPLC reveals that these aberrant results are limited to a very small number of challenging positions. Mirroring results obtained with subcloned mtDNA, it was not possible to detect as nonconcordant, combinations of HV2A amplicons that differ only at positions 72 or 73. Taken together, these two positions account for all of the undetected nonconcordant mixtures in HV2A.

In HV2B, DHPLC was not able to be detected as nonconcordant combinations of amplicons that differed only at position 295. This position lies in a narrow stretch of sequence immediately adjacent to a large GC-rich region. Given the thermodynamic stability of this region, a single base mismatch may not sufficiently destabilize the surrounding helix such that an early eluting peak can be discerned. This is a postulate supported by the observation that mixtures of amplicons that possess an additional mismatch in this same region are readily detected.

An inverse relationship was generally observed between the number of positional differences associated with a given pair of nonidentical amplicons and the relative heights of the hetero- versus homoduplex peak(s). The height and retention time of a heteroduplex peak is a function of the stability and base sequence of the helix. The more stable a heteroduplex, the more readily it should form relative to the competing homoduplices and thus the greater its peak height (indicative of quantity) on the DHPLC chromatograms. The corollary of this is that the formation of less stable helices is less favored and should be associated with smaller and earlier-eluting peaks on the DHPLC chromatograms. In theory, this could compromise the ability of DHPLC to detect as nonconcordant some combinations of amplicons that differ at a large numbers of positions. In the current study, however, no examples of such "heteroduplex dropout" were observed.

Heteroplasmy

As a naturally occurring combination of two haplotypes, heteroplasmy represents an important but potentially confounding factor for DHPLC-based analyses. Chromatograms obtained from assays of known heteroplasmic samples typically show the presence of early eluting heteroduplices. In these cases, DHPLC can often provide an indication of the nature of the heteroplasmy. Using either nondenaturing (column temperature = 50°C) or partially denaturing conditions, length heteroplasmy results in a chromatogram characterized by a markedly broadened peak often characterized by multiple shoulders (Fig. 4). This represents the combination of the various length amplification products and the characteristic shape of such peaks makes it possible to readily screen for heteroplasmy associated with the cytosine-stretches of HV1 and HV2B. Using partially denaturing conditions, point (i.e., base substitution) heteroplasmy can also be readily detected. It is typically indicated by the presence of a defined heteroduplex peak or a significant shoulder in association with the main homoduplex peak.

When analyzing questioned samples, however, it is not possible to reliably distinguish between heteroplasmy and a situational mixture where mtDNA has been contributed by two or more individuals. Even so, the presence of heteroplasmy does not necessarily preclude the use of a heteroplasmic reference sample for comparative sequence analysis by DHPLC. Sequence nonconcordance in such cases would be indicated by the presence of additional early eluting peaks and/or shoulders that were not evident in the chromatogram generated by cross-hybridization of the heteroplasmic sample with itself. Conversely, the absence of any additional peaks would be consistent with sequence concordance.

Heteroplasmy, however, can reduce the discriminatory power of DHPLC in some cases. In the current study, this was most often observed for samples with significant length heteroplasmy. The broad chromatographic peak associated with these samples can obscure the presence of additional heteroduplices formed as a result of cross-hybridization between the amplicons present in the known sample and additional amplicons in the questioned sample.

While DHPLC circumvents many of the limitations of alternate approaches to mtDNA screening, it is important to consider very carefully the types of samples for which such an approach might be indicated. Within an mtDNA sequencing laboratory, screening by DHPLC makes it possible to detect samples that contain mixtures of

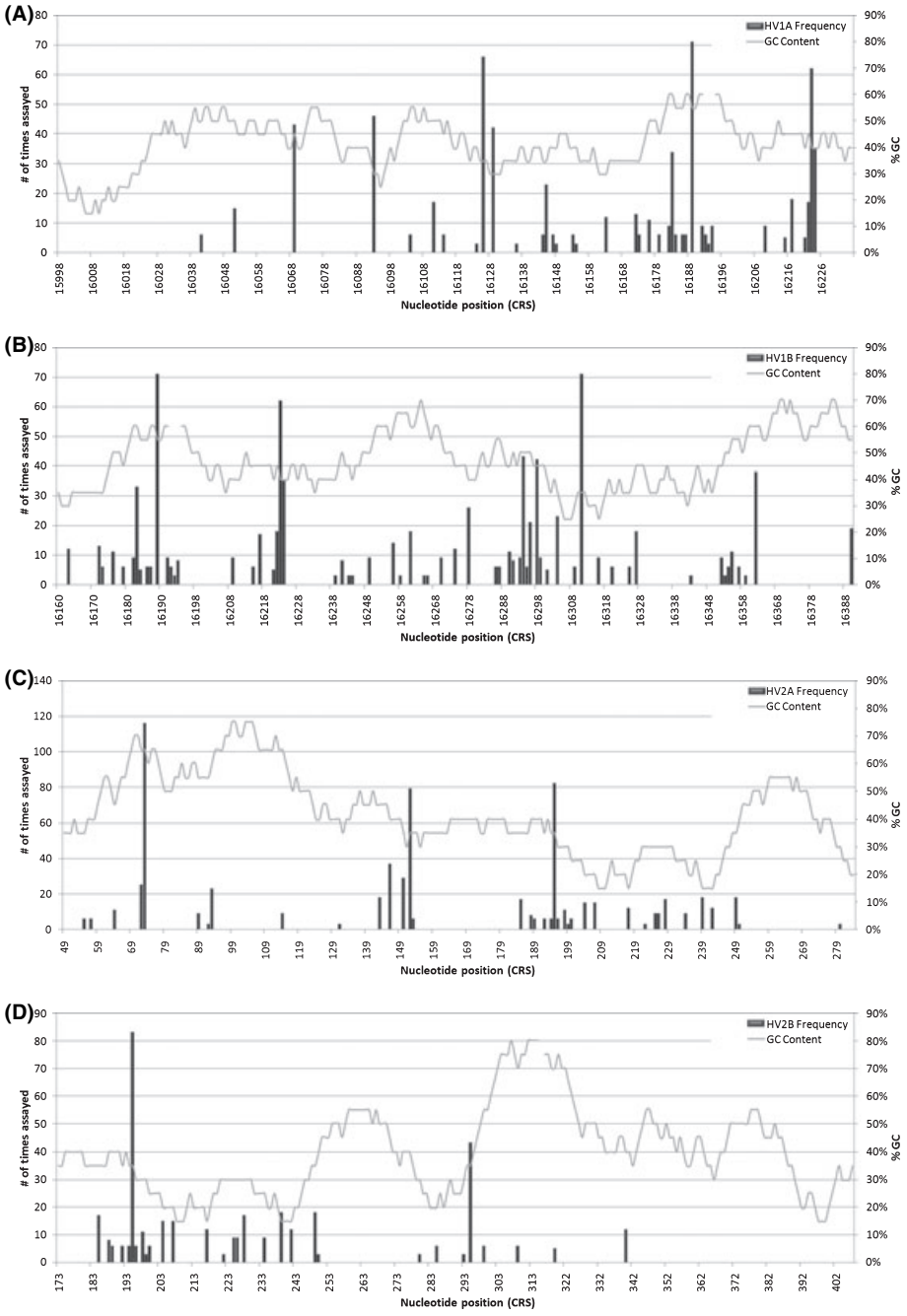

FIG. 3—*A histogram showing the distribution of nucleotide positions within the (A) HV1A, (B) HV1B, (C) HV2A, and (D) HV2B amplicons and the number of times that sequence polymorphisms at each position were assayed by DHPLC. The polymorphisms assayed are broadly distributed throughout both GC- and AT-rich regions as illustrated by the light gray line which indicates %GC content across each fragment.*

nonconcordant amplicons immediately after PCR amplification and without having to sequence them. For both heteroplasmic and situational mixtures, this approach would enable the analyst to identify potentially challenging samples and mark them for "special handling"—whether that be use of alternate sequencing primers to avoid C-stretch polymorphisms or the application of emerging technologies for resolving mixed samples (29–31).

DHPLC as a Presumptive Screen for Sequence Concordance

It has been reasonably argued by experienced practitioners in the field that it is best to avoid the use of mtDNA screening method on limited or irreplaceable evidentiary material (32,33). A presumptive DHPLC screen for mtDNA sequence concordance, however, could serve as a useful tool for investigators in special situations such as the investigation of property crimes. The limited budgets of many law enforcement agencies make it extremely difficult for investigators to justify the expense of mtDNA testing in the majority of criminal offenses that do not involve crimes against persons—particularly when there is no assurance *a priori* that the test results will necessarily advance an investigation. A presumptive screen for sequence concordance between a suspect and an item of evidence, however, could provide sufficient justification to submit the sample for confirmatory analysis by direct sequencing. Such

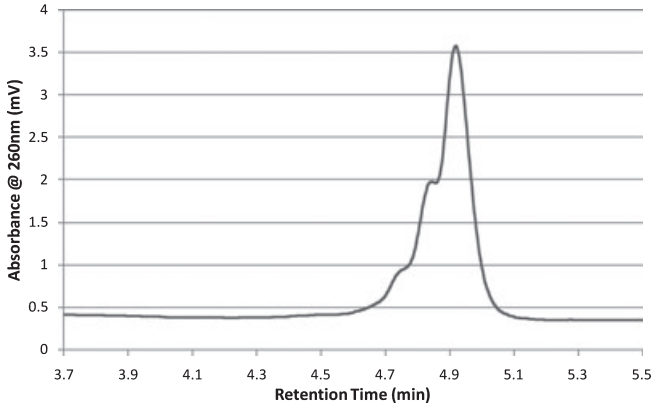

FIG. 4—*An example of length heteroplasmy as detected by DHPLC at a nondenaturing DNASep® column temperature of 50°C. Secondary and tertiary length variants are detected as shoulders prior to the primary length component.*

screening might also help to readily eliminate from consideration such nonprobative samples as hairs consistent with a victim that were recovered from the victim's home or vehicle in the case of a burglary or auto theft. In short, this could help investigators to focus their efforts on the most probative samples and thereby maximize the efficient use of investigative resources.

Employing a presumptive test of mtDNA sequence concordance in the manner described above shifts the process of DNA extraction from the dedicated mtDNA sequencing laboratory to the local law enforcement laboratory. This necessitates that additional consideration is given to the handling of these samples. The presence of evidentiary material with large quantities of mtDNA (e.g., blood, saliva, and seminal fluids, etc.) in local laboratories can pose a significant risk of cross-contamination. Accordingly, an mtDNA sequencing laboratory accepting a DNA extract for analysis would almost certainly require the submission of a co-extracted reagent blank control that could be tested to detect the presence of spurious mtDNA contamination. Similarly, the submission of amplified PCR products for direct sequencing would also need to be accompanied by the appropriate positive and negative PCR controls.

Conclusion

The results of the current study have demonstrated that DHPLC analysis of pair-wise combinations of identical mtDNA amplicons accurately and reliably produces a single chromatographic peak consistent with sequence concordance. These results were in 100% agreement with DNA sequence data. Conversely, in pair-wise combinations of nonidentical amplicons, DHPLC successfully detects a diversity of sequence differences throughout the HV1 and HV2 regions. These differences, which include a wide variety of base substitutions as well as insertions/deletions, are typically indicated by the presence of more than a single peak in the resulting chromatogram. As such, DHPLC may have significant forensic utility in several areas. These include: a presumptive test of mtDNA concordance between known and questioned samples, a screen for mixed samples prior to direct sequencing, and a preparative tool for the physical fractionation of the individual contributors to an mtDNA mixture prior to sequencing.

Although DHPLC is not a replacement for direct sequencing of mtDNA, it does offer some advantages as a potential screening tool. First, the assay is relatively simple and fast. It uses raw PCR products thereby avoiding the time and expense associated with amplicon cleanup. Following cross-hybridization, each assay takes only 7 min to run and interpretation of the results is straightforward. DHPLC provides a comprehensive assessment of sequence concordance across an entire amplicon without the often challenging task of trying to obtain quality base sequence information immediately adjacent to primer binding sites. Finally, the presence of heteroplasmy, while a potentially confounding variable, does not necessarily preclude comparative sequence analysis by DHPLC.

Compared to alternate mtDNA screening strategies based on oligonucleotide probes or linear arrays, DHPLC consumes less DNA and is not limited by the need to design probes for the detection of known mutations at predetermined polymorphic sites. This reduces the potential for false inclusions and eliminates the need to design custom probes for unique or rare sequence variants. Similarly, DHPLC assays are not subject to the "null" or "blank" results that arise when hybridization of the target sequence is impeded by other nearby polymorphisms. On the contrary, additional sequence variants typically make it easier to detect sequence nonconcordance between two amplicons.

Forensic laboratory implementation of a commercial DHPLC analysis system can be achieved with minimal training and initial equipment cost of just over $50K. The $0.50/run operating cost for DHPLC analyses is considerably less than that for alternative approaches. Depending on the nature of casework being analyzed and level of throughput, the acquisition of a DHPLC system may be a fiscally viable option for some forensic laboratories as is already the case in the molecular diagnostics arena.

Finally, the current study has also demonstrated that DHPLC can be used to resolve mixtures of nonidentical mtDNA amplicons into a series of homo- and heteroduplex peaks. As these can be differentially eluted in time, it should be possible to physically recover and concentrate the DNA contained in any given fraction of eluent. Subsequent sequence analysis of the recovered DNA may then provide a basis for determining the specific haplotype of each of the contributors to the mixture. The feasibility of this approach has already been demonstrated (29,30). Although, beyond the scope of the current study, ongoing research in the authors' laboratory is validating SOPs for DHPLC-based approaches to the resolution of mtDNA mixtures in accordance with guidelines of the Scientific Working Group on DNA Analysis Methods, the European DNA Profiling Group, and the International Society for Forensic Genetics.

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