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CASE REPORT

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The Use of Mitochondrial DNA Single Nucleotide Polymorphisms to Assist in the Resolution of Three Challenging Forensic Cases

ABSTRACT: Mitochondrial DNA (mtDNA) single nucleotide polymorphisms (SNPs) in an 11-plex assay were typed in three missing person cases involving highly degraded human remains. Unlike the traditional forensic approach to analyzing mtDNA which focuses on sequencing portions of the noncoding Control Region, this assay targets discriminatory SNPs that reside principally in the coding region. In two of the cases, the SNP typing successfully excluded one of two reference families that could not be excluded on the basis of mtDNA hypervariable region sequencing alone, and resulted in the final resolution of both decades-old cases. In a third case, SNP typing confirmed the sorting and reassociation of multiple commingled skeletal elements. The application of a specific mtDNA SNP assay in these cases demonstrates its utility in distinguishing samples when the most common Caucasian hypervariable region type is encountered in forensic casework.

KEYWORDS: forensic science, DNA typing, mitochondrial DNA, degraded DNA, coding region, single nucleotide polymorphisms

Forensic laboratories often encounter samples that, due to their high degree of degradation, prove difficult to analyze using standard DNA typing methods targeting nuclear DNA. This is especially true of laboratories that respond to mass disasters and missing persons cases. Efficient DNA extraction techniques, increased polymerase chain reaction (PCR) cycle number, and/or the use of reduced length amplicons have permitted short tandem repeat (STR) typing to be a highly effective tool in identifying large numbers of missing person cases involving degraded remains (1–6). However in more difficult cases, standard STR typing frequently results in partial profiles or, in cases of extreme degradation, no profile at all (7,8).

Therefore, the typical protocol at the Armed Forces DNA Identification Laboratory (AFDIL) and other laboratories is to perform mitochondrial DNA (mtDNA) sequencing in such cases. Although the mitochondrial genome (mtGenome) is not a unique identifier (due to maternal inheritance and a lack of recombination), the accumulation of mutations in noncoding portions of the genome over time has resulted in substantial variation among nonrelated individuals. The sequencing performed by forensic laboratories usually targets hypervariable regions 1 and 2 (HV1 and HV2) of the noncoding Control Region (CR), as these approximately 600 base pairs provide the single greatest opportunity within the mtGenome for inter-individual differentiation (9–12). When sufficient discrimination is not obtained by HV1 and HV2 sequencing, additional portions of the CR may be sequenced (13,14). However, the presence of common mtDNA haplotypes results in situations for which even entire CR sequencing does not provide enough variation for sample discrimination (15–18).

Over the past several years, numerous publications have proposed methods by which the coding region of the mtGenome may be interrogated to uncover additional variation to assist in the individuation of samples in such cases (15,17,19–25). This paper presents casework applications of a multiplex allele-specific primer extension (ASPE) assay; "Multiplex A" targets 11 single nucleotide polymorphisms (SNPs) chosen specifically to reveal additional variation in samples of the most common Caucasian HV type, H:1 (263G, 315.1C) (17,21). We describe three missing persons cases involving highly degraded human remains possessing the most common HV type. The resolution of each of these cases was assisted by typing the Multiplex A mtDNA SNPs.

Materials and Methods

We have previously described the selection of the 11 mtDNA SNPs typed by Multiplex A (477, 3010, 4580, 4793, 5004, 7028, 7202, 10211, 12858, 14470, and 16519), the possible variants at each position, primer design methodology, primer sequences used in the amplification and ASPE reactions, and primer quantitation (17,21). Genomic DNA was extracted from reference sample dried bloodstains on paper with Chelex 100 beads (BioRad Laboratories, Hercules, CA) and from skeletal material using a standard phenol/chloroform method, both as described in Edson et al. (14). The SNP assay was performed as described by Vallone et al. (21), with

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the modifications listed here. Reference sample amplifications were performed in a 15-μL total volume reaction, using 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) and 2-μL sample extract. Skeletal extract amplifications were performed in a 25-μL total volume reaction, using 6.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and 1-μL sample extract. PCR reaction cleanup was performed using exonuclease I (EXO) and shrimp alkaline phosphatase (SAP; USB Corporation, Cleveland, OH) in a ratio of 3 units EXO and 0.17 units SAP for each 1-μL PCR product. Unincorporated dideoxynucleotide removal was performed using 2 units of SAP per reaction. Extension product separation and detection was performed on either an ABI PRISM 3100 or 3130 Genetic Analyzer using POP6 performance optimized polymer (Applied Biosystems). The volume of the LIZ-120 internal sizing standard (Applied Biosystems) used per sample for the capillary electrophoresis (CE) run varied from 0.1 to 1.0 uL, depending on manufacturer lot. Data were analyzed using either GENESCAN v3.7 or GENEMAPPER v3.2 (Applied Biosystems) and alleles were assigned automatically using either a customized GENOTYPER v3.7 (Applied Biosystems) macro or a customized panel and bin set in GENEMAPPER.

Cases

Case 1

In the fall of 1966, a U.S. Air Force pilot led a flight of F-105D Thunderchief aircraft on an armed reconnaissance mission over North Vietnam. As the flight neared the target area, the pilots of the other "Thuds" observed an explosion on the fuselage of the aircraft, and shortly thereafter the plane crashed in Lang Son Province. Although the pilots of the remaining planes did not see a parachute or detect any emergency beeper signal, the ultimate fate of the Thunderchief pilot was still a mystery. He was therefore initially listed as Missing in Action (MIA), but later declared Killed in Action. In late 1991, a joint U.S./Socialist Republic of Vietnam (S.R.V.) team traveled to Lang Son Province and spoke with local villagers about possible U.S. aircraft crash sites. Joint U.S./S.R.V. investigation teams returned in 1994 and 1995, and in February of 1995 were led by two villagers to a rocky hillside where human remains were recovered. The remains were eventually accessioned at the Central Identification Laboratory (CIL; Hickam AFB, Hawaii) in April of 1995.

In 2000, a single long bone sample was submitted to the AFDIL, and the mtDNA sequence compared to seven maternal references representing four casualties that could not be excluded as the source of the remains on the basis of historical and circumstantial evidence. Comparison of the HV1 and HV2 sequences excluded only two of the four families. The bone specimen and the references from the two remaining families shared the most common Caucasian HV type and therefore could not be further resolved. Additional control region sequencing revealed that the case sample and references from both families shared a 16519C polymorphism. The additional sequencing also identified a single difference between the reference families at position 477. However, based on domestic and international mtDNA sequence interpretation guidelines, AFDIL policy dictates that two differences must be observed in order to report an exclusion (14,26,27). Multiplex A typing of the references was performed and confirmed the difference between the families at position 477, and also identified an additional difference between the families at position 3010 (Table 1).

Case 2

On the morning of 10 May 1968, a fierce ground battle between a multinational strike force and North Vietnamese soldiers took place at Ngok Tavak, an abandoned French fort that an Australian captain had been commanding as a base of operations. After 10 h of intense fighting, the captain led a group of survivors to safety, leaving behind 12 dead or injured Americans and a 13th U.S.

TABLE 1—*Differences from the revised Cambridge reference sequence (rCRS) as identified by HV region sequencing, additional CR sequencing, and typing of Multiplex A SNPs.*

		Differences from rCRS*		
	Sample	HV1/HV2	Additional CR	Mutliplex A SNPs
Case 1	Reference Family 1	263G, 315.1C	16519C	16519C
	Reference Family 2	263G, 315.1C	16519C, 477C	16519C, 477C, 3010A
	Case Sample A	263G, 315.1C	16519C, 477C	16519C, 477C, 3010A
Case 2	Reference Family 1	263G, 315.1C, 334C	16519C	16519C, 14470A
	Reference Family 2	263G, 315.1C	16465T†, 16519C	16519C
	Case Sample A	263G, 315.1C	16519C	16519C
	Case Sample B	263G, 315.1C	16519C	16519C
	Case Sample C	263G, 315.1C	16519C	16519C
Case 3	Case Sample A‡	263G, 315.1C		5004C
	Case Sample B	263G, 315.1C		5004C
	Case Sample C	263G, 315.1C	16519C	16519C
	Case Sample D	263G, 315.1C	16519C	16519C
	Case Sample E	16093C, 16221C, 263G, 315.1C	16519C	16519C, 14470A
	Case Sample F	16093C, 16221C, 263G, 315.1C	16519C	16519C, 14470A
	Case Sample G	16093C, 16221C, 263G, 315.1C	16519C	16519C, 14470A
	Case Sample H	16093C, 16221C, 263G, 315.1C	16519C	16519C, 14470A
	Case Sample I	16093C, 16221C, 263G, 315.1C	16519C	16519C, 14470A
	Case Sample J	16093C, 16221C, 263G, 315.1C	16519C	16519C, 14470A

HV, hypervariable region; CR, control region; SNP, single nucleotide polymorphism.

*Excludes HV2 poly-C length variation.

†Subsequent to SNP typing, additional portions of the control region were sequenced for the case samples, and a 16465T polymorphism was also identified in case samples. A, B, and C, thus confirming their match to this reference.

‡Samples for Case 3 are shown here sorted based on all markers typed, including low copy number STR and Y-chromosome typing (6).

soldier last seen alive tending to the wounded. One additional U.S. serviceman, a Marine Corps pilot, was also killed in the vicinity of the battle zone. From 1993 to mid-1998, joint U.S./S.R.V. teams interviewed local Vietnamese and performed site investigations. In August and September of 1998, a team excavated the Ngok Tavak base camp and recovered human remains, which were accessioned at the CIL in November of 1998. Excavations at Ngok Tavak continued in 1999, and additional human remains were recovered and returned to the CIL later that year.

In 2000 and 2001, 14 specimens were submitted to the AFDIL, and the resulting mtDNA sequence data were compared with 16 maternal references representing the families of the 14 U.S. servicemen lost in the battle at Ngok Tavak. For three of the submitted skeletal elements, HV1/HV2 sequencing revealed the most common Caucasian HV type. These three specimens were consistent with the sequence data for one reference family (263G, 315.1C; ignoring HV2 poly-C length variation) and differed from a second reference family at only a single position (−334C). Because two differences are required to report an exclusion, the comparison between the three skeletal elements and the second family reference was reported as inconclusive. Multiplex A typing of the two references and the three case samples was performed to acquire additional information. The SNP results revealed a second position, 14470, at which the case samples differed from the second reference family (Table 1). Subsequent variable region sequencing identified a third position (16465) that also provided discrimination between the two mtDNA lineages.

Case 3

On 4 November 1943, a U.S. B-24D Liberator with nine men on board departed Dobodura, New Guinea on an armed reconnaissance mission over the Bismark Sea. The aircraft was last heard from the following day, when they reported having engaged the enemy. When the aircraft failed to return, the nine-man crew was placed in MIA status. The MIA status of the crew was subsequently amended to Presumed Dead, and in 1948 the remains were declared nonrecoverable. In March 2002, the Papua New Guinea (P.N.G.) government notified the U.S. Embassy that human remains had been found amid the wreckage of a WWII aircraft in the Morobe province. These remains and other material evidence were accessioned at the CIL in May 2002. From August to September 2003, a joint U.S./P.N.G. team excavated the crash site in the Morobe province, uncovering aircraft wreckage of a B-24 bearing a tail number. Additional human remains and identification media were recovered, and the items accessioned at the CIL in September 2003.

In late 2003, 25 specimens were submitted to the AFDIL from the CIL, along with 16 maternal references representing the families of the nine servicemen killed in the B-24 crash. These were the only U.S. service members believed to have perished in the vicinity of the crash. Fourteen of the 25 samples could be confidently associated with five of the nine reference families on the basis of HV1/HV2 sequencing. Ten of the 25 samples, possibly representing the four unaccounted for servicemen, could not be distinguished with the HV1/HV2 data. Four of these 10 case samples and two reference families matched the most common Caucasian HV type. Additional CR sequencing identified a single position which distinguished two of the four samples (16519C), but an additional sequence difference was required to report an exclusion. Multiplex A typing of these four case samples confirmed the difference at position 16519, and also identified an additional position at which the samples varied (5004), thus verifying that these four samples originated from two individuals (Table 1). Multiplex A typing did not provide any additional discrimination for the remaining six samples, which differed from the most common Caucasian HV type by two polymorphisms (16093C and 16221T) and matched the two remaining reference families. These samples, however, were distinguished by low copy number (LCN) PP16 (Promega Corporation, Madison, WI) and Y-filer (Applied Biosystems) typing performed at the AFDIL. LCN typing was also performed on four the samples distinguished by Multiplex A, and the results confirmed the sample Multiplex A sorting (6).

Discussion

The three cases described here represent applications of an mtDNA SNP assay to confirm, or add weight to, identifications of degraded human remains in cases that could not be resolved by mtDNA hypervariable region sequencing alone. In all three cases, the typing of 11 (mostly coding region) SNPs provided additional information that assisted in the identification of some skeletal elements. Cases 1 and 2, which were fully resolved through the application of the mtDNA SNPs, are typical cases for which an mtDNA SNP assay will prove highly useful: cases for which partial or entire CR sequencing cannot exclude multiple reference families as a match to the case sample(s). Case 3, however, represents an additional use for these assays: the sorting of samples and reassociation of skeletal elements. In this case, SNP typing added an additional variable position and thus confirmed that four samples had originated from two individuals. The six remaining samples were resolved as the final two missing individuals through the application of LCN STR typing (6).

The SNP assay utilized in the resolution of these cases was specifically designed to provide additional discrimination when the most common Caucasian HV type is encountered (17,21). In all three cases described here, there were some samples that matched the most common type. In Case 2, however, the HV1/HV2 sequence of one family was the most common type plus one additional polymorphism: 334C. A search of nearly 15,000 U.S. and Global CR samples in AFDIL's internal population database identified only six additional samples (or 0.04%) with the 334C mutation, none of which matched the case sample across the entire CR. The rarity of the HV type indicates that the 334C mutation in this instance is likely variation on the tip of the (HV type) H:1 branch of the haplogroup H tree (see [28] for a general overview of mtDNA phylogeography and haplogroups; see [29] for discussion of mtDNA haplogroup H specifically).

In Case 3, six of 10 samples had the most common type plus *two* additional polymorphisms: 16093C and 16221T. In contrast to the 334C mutation in Case 2, 16221T was observed in 0.57% of AFDIL's internal population database, and in 0.93% of the 2053 U.S. Caucasians in that database. 16221T has been previously associated with a particular haplogroup H subcluster (30,31). Additionally, the exact HV haplotype (16093C, 16221T, 263G, 315.1C) for these six samples in Case 3 was observed in 0.34% of U.S. Caucasians, making it a relative common haplotype.

It is not surprising, then, that Multiplex A typing identified an additional variable position in the H:1 plus 334C reference in Case 2, but did not provide any additional discrimination among the H:1 plus 16093C and 16221T samples in Case 3. The multiplex was designed to work well within a particular clade of haplogroup H sequences, within which the mutations it assays arose. However, it will seldom have utility for resolving constellations of polymorphisms that are outside that clade, nor will it be particularly useful for discriminating among random individuals (as was recently examined; [32]).

Finally, it is worth noting that in all three of the cases presented here, the SNPs included in Multiplex A would have been sufficient to distinguish the samples matching the most common HV type without the need for CR sequencing beyond HV1 and HV2. The acquisition of sequence data from degraded samples is both expensive and labor-intensive. Furthermore, extract volume is often a limiting factor in these cases. Given that more than one method is available for identification of degraded remains, the approach to destructive testing of precious evidentiary material should be considered carefully. Where references are available in advance and the pool of potential identities can be defined with some certainty, the risk to the limited evidentiary material can be minimized by processing the references first and thereby defining the most efficient set of loci capable of achieving the desired discrimination. Multiplex mtDNA SNP assays require very little extract and are inexpensive and quick to perform, so in many instances carefully selected SNP panels may be a better choice for added discrimination compared to additional CR sequence data.

The cases described here demonstrate the utility of Multiplex A when the most common Caucasian HV type is encountered. They also represent the exact situations for which this and similar multiplex mtDNA SNP assays were designed: the resolution of cases involving samples that match, or are closely related to, common HV types using carefully selected SNPs. Given that the combined frequency of HV type H:1 and closely related sequences (sequences that differ from H:1 by a single polymorphism) in U.S. Caucasians is >22%, this multiplex will likely be useful in many cases involving commingled remains.

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