J Forensic Sci, November 2007, Vol. 52, No. 6
doi: 10.1111/j.1556-4029.2007.00561.x
Available online at: www.blackwell-synergy.com

CASE REPORT

Jodi A. Irwin,[1] M.S.; Mark D. Leney,[2,†,‡] Ph.D.; Odile Loreille,[1] Ph.D.; Suzanne M. Barritt,[1] M.S.; Alexander F. Christensen,[2] Ph.D.; Thomas D. Holland,[2] Ph.D.; Brion C. Smith,[1] D.D.S.; and Thomas J. Parsons,[1,§] Ph.D.

Application of Low Copy Number STR Typing to the Identification of Aged, Degraded Skeletal Remains*

ABSTRACT: Low copy number (LCN) STR typing was successfully applied to four interesting cases during developmental validation of the approach for degraded skeletal remains. Specific questions were addressed in each case, with the acquisition of STR data largely serving as additional confirmatory or investigatory information in any specific situation, and not necessarily providing the definitive evidence to establish identity. The cases involve missing U.S. service members from World War I, World War II, and the Vietnam War. The variety of these cases, in terms of the questions addressed, the age of the remains, and the type of reference material available for comparison, demonstrates the broad utility of LCN STR typing in the identification of degraded skeletal remains from missing persons.

KEYWORDS: forensic sciences, DNA typing, low copy number, Y chromosome, mitochondrial DNA, short tandem repeat, degraded skeletal remains

Mitochondrial DNA (mtDNA) typing is regularly employed at the Armed Forces DNA Identification Laboratory (AFDIL) to assist in the identification of skeletal remains of service members missing from previous military conflicts (1–3). In general, the age and condition of these remains preclude the use of standard DNA typing methods that target autosomal short tandem repeats (STRs). Not only are the remains too degraded for STR typing, but also in many of these decades-old cases references from immediate family members are no longer available. As a result, sequence data from the mitochondrial control region is regularly targeted. In many cases, however, the limited discriminating power of mtDNA alone may not adequately reconcile the incomplete or nonspecific anthropological, odontological, or contextual findings. As a result, we have applied low copy number (LCN) STR methods to four such historic cases in order to verify its potential value in these situations (4–8). These cases span three of the four major military conflicts in which the United States was involved during the 20th century. They represent the extremes in quality of sample material that are regularly encountered in AFDIL casework and present very specific requirements in terms of the application of autosomal and Y-chromosomal data.

[1]Armed Forces DNA Identification Laboratory, Armed Forces Institute of Pathology, AFIP Annex, 1413 Research Blvd., Rockville, MD 20850.
[2]Joint POW/MIA Accounting Command—Central Identification Laboratory, 310 Worchester Avenue, Hickam AFB, HI 96853.
[†]Current address: Massachusetts Biologic Laboratories, University of Massachusetts Medical School, Jamaica Plain, MA 02310.
[‡]Current affiliation: Scientific Advisor, The National League of Families of American Prisoners and Missing in Southeast Asia, 1005 Glebe Road, Suite 170 Arlington, VA 22201.
[§]Current address: International Commission on Missing Persons, Alipašina 45 A, 71000, Sarajevo, Bosnia and Herzegovina.
*The opinions and assertions contained herein are solely those of the authors and are not to be construed as official or as views of the U.S. Department of Defense, the U.S. Department of the Army, or the U.S. Department of the Navy.

Received 24 Dec. 2006; and in revised form 29 April 2007; accepted 29 April 2007; published 21 Dec. 2007.

Materials and Methods

Skeletal material was extracted with either a standard phenol/chloroform protocol as described in Edson et al. (3), or a modified extraction protocol that includes a complete demineralization step (9). LCN amplifications were conducted using the PowerPlex 16 system (Promega Corporation, Madison, WI) or the Yfiler system (Applied Biosystems, Foster City, CA). Thermal cycling temperatures and times were performed according to the manufacturer's recommendations. However, for each multiplex, twice the recommended Taq concentration and six additional polymerase chain reaction (PCR) cycles were used (36 cycles total for each multiplex). Additional Taq was included to overcome inhibition from the large volumes of DNA extract added to the PCR; extract volumes were maximized in order to also maximize allele sampling and recovery. PCR products were separated on an Applied Biosystems 3100 and analyzed using Genescan software version 3.7 (Applied Biosystems, Foster City, CA). Genotyper version 3.7 (Applied Biosystems) was used to assign allele calls to electropherograms, using the allelic ladders provided in the respective kits as references. Y-chromosomal profiles were evaluated against the Applied Biosystems Yfiler database of 3561 individuals. Depending on the quantity of evidentiary material, results were replicated from independent extracts or specimen samplings.

The results presented in this study are based on preliminary guidelines for reporting of alleles, based on sensitivity data and numerous test amplifications of degraded extracts. These guidelines are currently being refined, but the basic parameters are described here. Depending on the specific case, the available evidentiary material and the question at hand, between two to five amplifications of any particular extract were conducted. In general, three amplifications

were performed and only alleles observed in at least two of the three amplifications were reported. More than three amplifications of any single extract were sometimes conducted to clarify particular issues related to allelic dropout or homozygosity. In these situations, we used a conservative requirement that the "final" alleles had to be observed in the majority of amplification products. Preliminary sensitivity and developmental validation data generated with these LCN protocols (data not shown) indicate that the chance of observing a stochastic drop-in allele on a per amplification basis is ~0.3. If we assume 150 potential sites at which drop-in can occur, then the chance of duplicating a random drop-in allele in up to five amplifications is less than one one-hundredth of a percent (conservatively estimating 10 possible alleles at each of the 15 loci). Thus, alleles observed in two amplifications are almost certainly authentic and could be included in final profiles. In the cases presented here, however, we have applied the more conservative requirement of replication of alleles in the majority of amplification products. Therefore, when five amplifications were conducted, the reported alleles were observed in at least three of the five products.

Cases

Case 1—In July 1918, a young private, participating in a combined French-American attack on German positions near Soissons, France, was killed in action. His remains were never recovered.

In August 2003, a French national turned over to the U.S. Army mortuary in Germany human remains and artifacts that had been found during a construction project near Soissons. The remains of two individuals, as well as associated evidence were transported to the Joint POW/MIA Accounting Command's Central Identification Laboratory (CIL) for analysis. Among the artifacts recovered were a leather wallet bearing the soldier's name and a military boot fragment consistent with sizes 5 or 5.5. Based on the material evidence associated with the recovery site (the wallet and the boot fragment), dental analysis of the remains, and the unusually small stature of the skeleton that was consistent with the personnel records for the soldier, a presumptive identification of one of the skeletons was made.

In order to acquire additional supporting evidence, a dental sample was submitted to the AFDIL in August 2004. Mitochondrial DNA testing was successfully conducted on an extract from 170 mg of dentin. A sample of femoral bone (mass) was also taken from the second skeleton yielding a different mtDNA profile. Unfortunately, a known maternal relative for the presumed missing serviceman could not be located and the mtDNA investigation stalled for lack of reference material. Without supporting genetic evidence, the biological basis for identification hinged almost exclusively on the anthropometric data. However, the skeleton exhibited such extreme gracility that it appeared *a priori* more consistent with a female than a male. Therefore, the anthropologist conducting the initial analysis of the skeletal remains raised some doubt regarding the sex determination. This analyst was working blind, knowing only that the remains dated to the World War I era. He had not been informed of the name association to a specific casualty, nor was he aware of the dental evidence supporting the association of the remains to this man. A *post hoc* comparison of the biological profile of the remains to the service records of the individual, in accordance with CIL Standard Operating Procedures, clarified the issue. The size of the skeleton was consistent with the casualty presumptively identified; he was simply a small, gracile male. However, in the interests of conducting a thorough investigation and given the fact that a DNA sample had already been taken from the remains, the CIL requested that AFDIL attempt DNA confirmation of the sex. The resulting STR profiles were unambiguous for amelogenin in the PowerPlex 16 system and nearly complete for the Yfiler multiplex, establishing that the skeletal remains were indeed male. Data were recovered from all but one Y-STR locus, and 29 alleles were confirmed with PowerPlex 16. The DNA data produced from this set of remains were particularly notable both because the extract derived from such a small quantity of dentin and because the remains were significantly older than other cases that had produced far less data (Fig. 1). It is likely that data recovery with this sample was facilitated by two factors: one, the moderate climate in which the remains lay for over 80 years and two, the protective anatomical environment that dental DNA enjoys (2,10), a factor that is highlighted by the more fragmentary profile recovered from the much larger osseous sample obtained from the second skeleton.

Still, some question must remain over the authenticity of the profile obtained from the dentin, given that it was based on a single extract. In favor of authenticity are the clean negative controls and reagent blanks, the presence of a positive but different profile in the sample from the second skeleton and the absence of any mixtures in either sample. It remains possible, although we maintain unlikely, that a specific contaminant on the dentin sample resulted in the recovery of an exogenous profile with little or no evidence of endogenous DNA. Typically such a question mark would be addressed through replication of the initial result; a second dentin sample (or an osseous sample) from the same skeleton, independently sampled and processed could significantly narrow the room for doubt. However, at the point that the LCN analysis was completed, the circumstantial case for identification had been assessed and was found compelling. As a result, the identification was already in hand independent of any DNA data.

Case 2—A set of skeletal remains was unilaterally turned over by the Vietnamese government in 1989 as part of a large series of

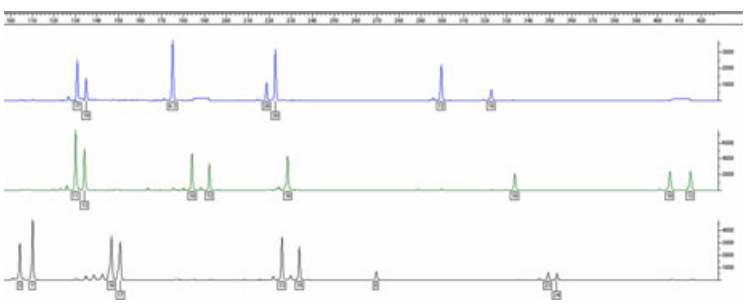


FIG. 1—*Sample Powerplex 16 profile generated from an extract of 180 mg of dentin. The tooth was recovered from a World War I battlefield in France. In this particular case, alleles larger than 400 bp were both recovered and reproducible.*

remains repatriated over several months that year. Some of these cases had very little associated information, while others were associated with erroneous information. The case in question had no data concerning the geographic location of the recovery but the remains returned were allegedly those of an American. With no circumstantial or material evidence to consider, the only meaningful data came from the anthropological examination which suggested that in the absence of definitive skeletal indicators of sex, the size and gracility of the remains most likely pointed to a female skeleton. Subsequently, mtDNA testing was successfully performed on a cranial sample. However, comparisons to the maternal references for all three female American casualties that remain unresolved as a result of the war Southeast Asia resulted in exclusions. The mtDNA sequence data were also inconsistent with the hypothesis that the casualty was indigenous to Southeast Asia. The mtDNA lineage was of western European origin (haplogroup H) and thus it was unlikely that the skeleton represented a Vietnamese female whose remains had been inadvertently turned over to the United States. There are however an unknown number of third country national females, deceased in Vietnam, both during the colonial period and the subsequent conflict whose remains might have been inadvertently included with the materials repatriated by the Vietnamese authorities.

In order to verify the suspected sex, typing of amelogenin and subsequently Y-chromosomal STRs was conducted on a femoral sample. LCN analysis established that the remains were, in fact, male. Duplicate extractions were performed in this case, but LCN typing was only conducted using the extract with the higher DNA concentration. Mitochondrial DNA testing was conducted on both extracts. The mtDNA data were concordant and the haplotype was unique in the SWGDAM database of 4839 individuals. This information redefined the list of potentially associated casualties by providing key data for this case, which had previously hit a dead end. The case remains unresolved, but has been reinvigorated by the genetic typing. Identification efforts will now proceed on the basis of the supposition that the remains could represent an unaccounted-for casualty who is a small male.

Case 3—On June 25, 1965, a single-seater, A-4C Skyhawk took off from the USS *Coral Sea* on an armed reconnaissance mission over North Vietnam. En route, the plane encountered both inclement weather and enemy anti-aircraft artillery fire. When the plane failed to return to the aircraft carrier, the pilot was listed as Missing in Action.

In October 1993, a team from the CIL traveled to Thanh Hoa Province, Vietnam, to investigate the incident. The CIL team interviewed two local Vietnamese nationals who recalled an aircraft crash and led the team to the site. A survey of the area revealed both aircraft wreckage and pilot-related artifacts. During a complete forensic archaeological recovery of the crash site, four small fragments of weathered human postcranial bone were recovered. The condition of these remains precluded any further meaningful anthropological characterization. At the time of recovery the largest fragment was considered too small for mtDNA analysis. With nothing but circumstantial data associated with the case, the evidence lay dormant for half a decade. In the light of significant improvements in the capabilities of the AFDIL during the late 1990s (11), the remains were reevaluated and in January 2000 the largest bone fragment was sampled, submitted for DNA testing and typed for mtDNA. The mtDNA haplotype reflected a western European lineage (haplogroup H), which supported the theory that the remains were from an American serviceman. Based on this information, as well as wreckage analysis and circumstantial evidence linking the crash site to this specific loss incident, a presumptive identification was established. However, because no maternal reference was available for mtDNA comparison, the investigators sought additional genetic lines of evidence. In this instance, the wife and children (son and daughter) of the presumed missing individual were available to provide reference material for autosomal and Y markers. Therefore, with lowered expectations given the poor condition of the bone fragments, both autosomal and Y STRs were typed on two extracts of the same skeletal sample. LCN data from the duplicate extracts were concordant. The PowerPlex 16 amplifications provided data at 15 of 16 loci, and a complete genetic profile was recovered with the Yfiler LCN amplification (Fig. 2). The Y haplotype was consistent between the remains and the putative son, and also unique in a database of 3561 individuals. The autosomal data further confirmed the identity of the missing serviceman, resulting in a likelihood ratio of 9.9 billion to one, in support of the stated genetic relationship.

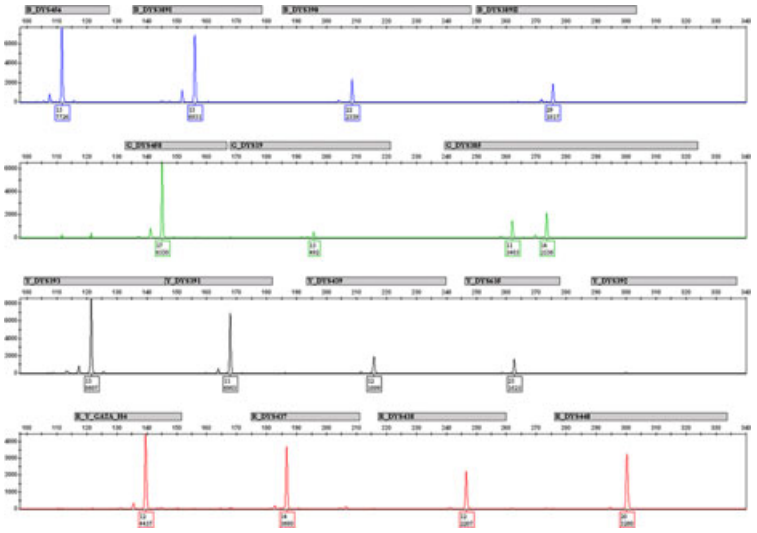
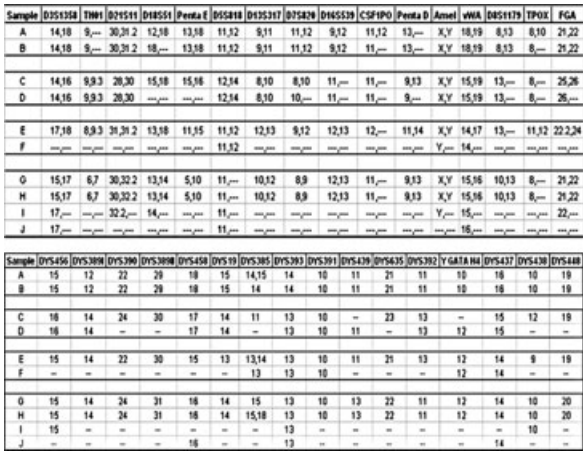


FIG. 2—*Low copy number Y-chromosome STR results from postcranial element recovered from Vietnam. This incomplete haplotype is consistent with the Y haplotype of the suspected casualty's son and unique in the Applied Biosystems Yfiler database of 3561 individuals.*



Sample	D3S1358	TH01	D21S11	D18S51	Penta E	D5S818	D13S317	D7S820	D16S539	CSF1PO	Penta D	Amel	vWA	D8S1179	TPOX	FGA
A	14,18	9,---	30,31.2	12,18	13,18	11,12	9,11	11,12	9,12	11,12	13,---	X,Y	18,19	8,13	8,10	21,22
B	14,18	9,---	30,31.2	18,---	13,18	11,12	9,11	11,12	9,12	11,---	13,---	X,Y	18,19	8,13	8,---	21,22
C	14,16	9,9.3	28,30	15,18	15,16	12,14	8,10	8,10	11,---	11,---	9,13	X,Y	15,19	13,---	8,---	25,26
D	14,16	9,9.3	28,30	---,---	---,---	12,14	8,10	10,---	11,---	11,---	9,---	X,Y	15,19	13,---	8,---	26,---
E	17,18	8,9.3	31,31.2	13,18	11,15	11,12	12,13	9,12	12,13	12,---	11,14	X,Y	14,17	13,---	11,12	22.2,24
F	---,---	---,---	---,---	---,---	---,---	11,12	---,---	---,---	---,---	---,---	---,---	Y---	14,---	---,---	---,---	---,---
G	15,17	6,7	30,32.2	13,14	5,10	11,---	10,12	8,9	12,13	11,---	9,13	X,Y	15,16	10,13	8,---	21,22
H	15,17	6,7	30,32.2	13,14	5,10	11,---	10,12	8,9	12,13	11,---	9,13	X,Y	15,16	10,13	8,---	21,22
I	17,---	---,---	32.2,---	14,---	---,---	11,---	---,---	---,---	---,---	---,---	---,---	Y---	15,---	---,---	---,---	22,---
J	17,---	---,---	---,---	---,---	---,---	11,---	---,---	---,---	---,---	---,---	---,---	---,---	16,---	---,---	---,---	---,---

Sample	DYS456	DYS389II	DYS390	DYS389I	DYS458	DYS19	DYS385	DYS393	DYS391	DYS439	DYS635	DYS392	Y GATA H4	DYS437	DYS438	DYS448
A	15	12	22	29	18	15	14,15	14	10	11	21	11	10	16	10	19
B	15	12	22	29	18	15	14	14	10	11	21	11	10	16	10	19
C	16	14	24	30	17	14	11	13	10	–	23	13	–	15	12	19
D	16	14	–	–	17	14	–	13	10	11	–	13	12	15	–	–
E	15	14	22	30	15	13	13,14	13	10	11	21	13	12	14	9	19
F	–	–	–	–	–	–	13	13	10	–	–	–	12	14	–	–
G	15	14	24	31	16	14	15	13	10	13	22	11	12	14	10	20
H	15	14	24	31	16	14	15,16	13	10	13	22	11	12	14	10	20
I	15	–	–	–	–	–	–	13	–	–	–	–	–	–	10	–
J	–	–	–	–	16	–	–	13	–	–	–	–	–	14	–	–

FIG. 3—*Low copy number STR results for Case 5. Skeletal elements are labeled A–J and sorted according to their autosomal and Y STR profiles. PowerPlex 16 profiles are shown in the upper frame. Yfiler profiles are shown in the lower frame. A sorts with B; C sorts with D; E sorts with F; and G, H, I and J sort together. These represent "consensus" profiles. Each profile was generated from multiple amplifications of duplicate extracts of a particular sample.*

Case 4— On November 4, 1943, an American B-24D Liberator departed Dobodura, New Guinea on an armed reconnaissance mission over the Bismarck Sea. The last transmission from the aircraft, on November 5, 1943, indicated that the crew had engaged the enemy. The aircraft failed to return and the nine-man crew was presumed dead. In 1948, the remains were declared nonrecoverable. In March of 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 World War II aircraft in Morobe Province. These remains and other material evidence were brought to the CIL in May of 2002. The CIL subsequently deployed a forensic archaeology team and performed an in-depth excavation of the B-24 crash site in August and September 2003. Additional human remains and identification media were recovered. They were accessioned at the CIL in September of 2003.

The CIL submitted 25 skeletal samples to AFDIL for mtDNA testing, along with maternal references for the nine missing individuals. On the basis of HVI/HVII sequence data, 15 of the 25 samples were confidently associated with five of the nine family references. The remaining 10 samples, possibly representing the remaining four unaccounted for servicemen, could not be completely distinguished with HVI/HVII sequence data alone. Four of these 10 evidentiary samples and two reference families matched the most common western European haplotype, and while two of these four samples could be distinguished by an HVII C-stretch length polymorphism, this difference alone provided weak evidence for sorting the four samples. Portions of the crash site critical to the origin of the samples in question had remained relatively undisturbed since the incident. Furthermore, the aircraft crash had occurred at relatively low speed. Thus, the application of forensic archaeological recovery methods allowed the association of individual bones into partial skeletons and further associated some of the questioned samples to dental and nonbiological evidence. The combination of mtDNA, archaeological, and nonbiological material evidence analysis permitted the association of some specific sampled remains to individual casualties. However, in other cases the combination of supporting lines of evidence was less comprehensive. Given that remains had already been sampled for mtDNA HVI/HVII testing, additional genetic data was sought to permit the segregation of commingled remains sharing common mtDNA HV types. Additional testing outside of HVI/HVII, using both sequence data and coding region single nucleotide polymorphisms (SNPs) (12,13), confirmed that four samples originated from two individuals. However, the remaining six case samples could not be resolved with available mtDNA data. As a result, LCN typing of Y and autosomal markers was conducted on all ten samples for further resolution. Amplification success among skeletal elements varied significantly, with some elements producing no more than three or four confirmed alleles. However, the combination of LCN data from both the autosomal and Y markers provided enough information to confidently sort elements and establish that the 10 elements originated from four individuals, not three (Fig. 3). For this particular case, a combination of mtDNA data and LCN autosomal and Y data contributed to the successful segregation of all nine missing servicemen.

Discussion

These cases cover a wide range of material for which LCN STR typing was successful and represent a broad spectrum of circumstances in which LCN STR typing might prove a useful application. The majority of these cases involved remains that were ~40 to 60 years old. Case 1, however, involved remains that were more than 80 years old and was further distinguished by the fact that nearly complete STR profiles were obtained from only 170 mg of dentin.

Replication of the LCN results from multiple extracts and/or specimen samplings was limited in two of the four reported cases (Cases 1 and 2), despite our interest in reproducing the data. Replication in Case 1 was limited by the quantity of evidentiary material. The particular circumstances of this case precluded multiple samplings or extractions, and while other data generated during the testing supported the authenticity of the LCN results, the unreplicated data remain less than ideal. This case was important in encouraging us to move forward with more controlled LCN testing. Yet, the DNA evidence is ultimately weaker than we would have liked because the case contingencies did not allow for replication. Replication remains a central pillar of degraded human DNA studies and it certainly would have been desirable here. In general practice, we seek to reproduce LCN results whenever possible. However, in some instances, we will be required to make the best use of limited evidentiary material that does not allow for multiple extractions or samplings.

In terms of the specific challenges each case posed, Cases 1 and 2 presented the simple question of gender determination. However, in each of these cases, the question was posed for very different

reasons. In Case 1, the STR data confirmed the circumstantial finding of "small male" and further supported the identification. In Case 2, the STR results conflicted with the anthropological analysis that had indicated a probable female, but opened new avenues for investigation in a case that was previously at a standstill.

Case 3 represents the familiar application of STR typing in identification efforts: the DNA typing and comparison of evidentiary and reference materials, in order to establish genetic relationships and thereby identity, using markers with high statistical discrimination. This case was actually the first identification from the Vietnam War (and indeed any past war) for which nuclear DNA evidence was used as part of the official identification process. While promising, application of the new technology will by no means be unproblematic in the identification of POW/MIA cases. First of all, the overall strength of the DNA evidence is limited by the number of loci recovered. With degraded skeletal remains, the profiles obtained will sometimes be incomplete and will, for some and perhaps many cases, be too poor to generate useful statistical inference. Even if the recovered profiles are complete, the investigator may be faced with a lack of suitable references with which to make identifications with autosomal data. The U.S. military accounting mission has traditionally focused solely on acquiring reference specimens from maternal relatives for mtDNA comparison. Thus, the references currently available for any particular individual may be less than ideal for STR kinship analysis. That said, many of these references are derived from the siblings of casualties and are therefore suited to both autosomal STR and mtDNA analyses. Because the population of siblings from past war casualties is ageing, it is now increasingly important that sibling references are collected in a timely fashion given their broad utility in genetic testing. Increased emphasis should also be placed on suitable paternal line reference samples, as well as extended reference sample collection in particular cases. While the application of any DNA-based methods for identification is limited by both the quality of the evidence profile generated and the availability of appropriate references, in those cases for which the evidentiary data are good and adequate references are available, our studies demonstrate the potential to use STRs to make identifications. In fact, another recent case has demonstrated that data from a suite of markers and multiple, distant relatives can in particular instances definitively establish identity even when partial profiles are recovered (14).

However, these types of cases introduce additional levels of complexity to the overall identification effort. When only a single DNA technology was available (mtDNA analysis), limited evidentiary material could be carefully managed in relation to that single line of analysis. In Case 3, the mtDNA testing of evidence recovered in 1994 was delayed until 2000 to provide a reasonable balance between the need to complete identifications in a timely manner and the risk of consuming all the usable evidence in a test that might fail to yield a result. Now, given the potential of LCN typing on degraded specimens, balance is required between multiple consumptive tests with differing likelihoods of success and differential payoffs in terms of the likely statistical weight, if successful. A more complex calculus of effort, risk and return is now required. Given that the total biological evidence in this type of situation might support only a single sample, the outcome might be drastically different if all evidentiary material is consumed by mtDNA testing. This would be particularly unfortunate if, as in Case 3, no mtDNA reference sample ever became available. While the single (mtDNA) locus approach allowed for a single-minded pursuit of mtDNA family reference samples, the availability of alternate technologies raises the question of how to determine the optimal distribution of effort and resource in such a large humanitarian identification process. Without being dogmatic, we suggest that a thorough review of the availability of different reference types for candidate casualties should be undertaken prior to the initiation of evidence testing, particularly in cases where the volume of evidence available for testing is limited.

Case 4 represents what will probably be one of the most immediately implementable applications of LCN testing to degraded remains identification at the AFDIL: the sorting and re-association of skeletal elements. On a case-by-case basis, but particularly in closed situations like Case 4, LCN typing may greatly assist in sorting elements when mtDNA data alone do not resolve individuals. In the particular instance of Case 4, and in the general re-association of skeletal elements where the candidate population is known to be limited, complete STR profiles are not necessary. Even limited data, such as those obtained from degraded skeletal elements, can prove useful. This model is, in fact, applied very successfully in large scale skeletal remains re-association by the International Commission on Missing Persons, using short amplicon STR multiplexes with only five to seven loci (15).

Low copy number STR methods will be integral to one particular set of cases at the CIL. These cases are colloquially referred to as the "K208," for Korea 208. These 208 boxes of skeletal remains were unilaterally transferred to the United States by North Korea between 1990 and 1994. Anthropological examination suggested that skeletons supposedly repatriated as single individuals had been assembled from portions of multiple individuals. In addition, early mtDNA results suggested that parts of single individuals were spread among several boxes and that any given box likely contained the remains of multiple individuals. In fact, it is likely that far more than 208 individuals are represented. An additional complication is that remains subsequently recovered by U.S. military teams operating in conjunction with North Korean personnel inside North Korea have, in some cases, clearly involved faked recovery sites (16). Some of these sites have been shown to contain remains believed to be the recycled residue of skeletal remains already repatriated by the North Koreans in the "K208" series. Because mtDNA is not a unique identifier, the particular problems that challenge some of the remains complexes originating from North Korea will require autosomal STRs for sorting and re-association of skeletal elements. Overall, a combination of mtDNA, autosomal STRs and Y STRs will likely be required to support identifications.

DNA typing of aged, degraded skeletal remains presents specific challenges over and above those commonly encountered in LCN casework. Not only are DNA templates from degraded skeletal elements short and in LCN, but in many cases the DNA is also severely affected by cross-linkage, deamination, dimers, and other insults resulting from extensive environmental exposure and various mortuary treatments (17–19). These types of damage further limit the already diminished rate of success with STR typing of LCN specimens. Therefore, the demonstration of success on damaged templates is extremely encouraging. It is particularly promising that LCN techniques have managed to leverage data from loci that were characterized for their utility in nondegraded samples (e.g., FGA, which is comprised of a large repeat structure) and using primer sets that were similarly never optimized for use on degraded DNA. While smaller amplicon approaches to the same loci or characterization of smaller loci (15,20,21) may certainly improve the chances that an individual degraded DNA sample will yield results, the cost and effort of implementing such specialized methods has to be offset against the utility of using widely available STR kits under modified protocols. Because such kits are currently commercially manufactured in high volumes and to stringent

quality control standards, they offer an excellent opportunity to conduct testing efficiently and effectively. The aforementioned cases represent the very casework issues that will continue to confront the AFDIL and others who work to identify skeletonized remains recovered from harsh environmental conditions. These issues were often irresolvable prior to LCN implementation. In any particular case, the relevant question may be something as simple as gender determination or the problem may be much more complex, involving extended family trees and the difficult acquisition of new reference specimens. The demonstrated success of LCN typing on degraded skeletal remains suggests that a hopeful new avenue may exist in a realm that was previously the sole purview of mtDNA sequencing.

Acknowledgments

Thanks to Dr. Charles Brenner for assistance with likelihood ratio calculations and numerous individuals who contributed to the acquisition of mtDNA sequence data. We also thank the American Registry of Pathology, James Canik, Kevin Carroll, Brion Smith, Louis Finelli, and James Ross (AFDIL) for logistical, administrative, and computer support.

References

1. Holland MM, Fisher DL, Mitchell LG, Rodriquez WC, Canik JJ, Merril CR, et al. Mitochondrial DNA sequence analysis of human skeletal remains: identification of remains from the Vietnam War. J Forensic Sci 1993;38:542–53.
2. Holland MM, Parsons TJ. Mitochondrial DNA sequence analysis: validation and use for forensic casework. For Sci Rev 1999;11:21–50.
3. Edson SM, Ross JR, Coble MD, Parsons TJ, Barritt SM. Naming the dead—confronting the realities of rapid identification of degraded skeletal remains. For Sci Rev 2004;16(1):64–89.
4. Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, et al. Reliable genotyping of samples with very low DNA quantities using PCR. Nucleic Acids Res 1996;24:3189–94.
5. Findlay I, Taylor A, Quirke P, Frazier R, Urquhart A. DNA fingerprinting from single cells. Nature 1997;387:767.
6. Gill P, Whitaker J, Flaxman C, Brown N, Buckleton J. An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. Forensic Sci Int 2000;112:17–40.
7. Gill P. Application of low copy number DNA profiling. Croat Med J 2001;42:229–32.
8. Schiffner LA, Bajda EJ, Prinz M, Sebestyen J, Shaler R, Caragine TA. Optimization of a simple, automatable extraction method to recover sufficient DNA from low copy number DNA samples for generation of short tandem repeat profiles. Croat Med J 2005;46(4):578–86.
9. Loreille OM, Diegoli TM, Irwin JA, Coble MD, Parsons TJ. High efficiency DNA extraction from bone by total demineralization. Forensic Sci Int: Genet 2007;1:191–5.
10. Leney MD. Sampling skeletal remains for Ancient DNA (aDNA): a measure of success. Hist Arch 2006;40(3):31–49.
11. Gabriel M, Huffine E, Ryan J, Holland M, Parsons T. Improved mtDNA sequence analysis of forensic remains using a ''mini-primer set'' amplification strategy. J Forensic Sci 2001;46:247–53.
12. Coble MD, Just RS, O'Callaghan JE, Letmanyi IH, Peterson CT, Irwin JA, et al. Single nucleotide polymorphisms over the entire mtDNA genome that increase the power of forensic testing in Caucasians. Int J Legal Medicine 2004;118:137–46.
13. Vallone PM, Just RM, Coble MD, Butler JM, Parsons TJ. A multiplex allele-specific primer extension assay for forensically informative SNPs distributed throughout the mitochondrial genome. Int J Legal Medicine 2004;118:147–57.
14. Irwin J, Edson S, Loreille O, Just R, Barritt S, Lee D, et al. The intersection of genetic identity: the application of multiple marker systems and new technologies to establish identity 50 years after death. J Forensic Sci 2007;52(5):1115–8.
15. Parsons TJ, Huel R, Davoren J, Katzmarzyk C, Pozder A, Smajlovič L, et al. Application of novel ''mini-amplicon'' STR multiplexes to high volume casework on degraded skeletal remains. Forensic Sci Int Genet 2007;1:175–9.
16. Pokines JT, Berg GE, Adams BJ, Bunch AW, Byrd JE, Holland TD, et al. How not to stage a burial: lessons from North Korea. Proceedings of the 54th Annual Meeting of the American Academy of Forensic Sciences; 2002 Feb 13–16. Atlanta, GA and Colorado Springs, CO: American Academy of Forensic Sciences, 2002.
17. Lindahl T. Instability and decay of the primary structure of DNA. Nature 1993;362:709–15.
18. Hofreiter M, Serre D, Poinar H, Kuch M, Paabo S. Ancient DNA. Nat Rev 2001;2:353–9.
19. Hansen A, Mitchell D, Wiuf C, Paniker L, Brand T, Binladen J, et al. Crosslinks rather than strand breaks determine access to ancient DNA sequences from frozen sediments. Genetics 2006;173:1175–9.
20. Butler JM, Shen Y, McCord BR. The development of reduced size STR amplicons as tools for analysis of degraded DNA. J Forensic Sci 2003;48(5):1054–64.
21. Coble MD, Butler JM. Characterization of new miniSTR loci to aid analysis of degraded DNA. J Forensic Sci 2005;1:43–53.

Additional information and reprint requests:
Jodi A. Irwin, M.S.
Armed Forces DNA Identification Laboratory
1413 Research Blvd, Building 101
Rockville, MD 20850
E-mail: jodi.irwin@afip.osd.mil