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Mitochondrial DNA Analysis of Danforth Doe (CFS File #A-475-95) and Two Putative Sons

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Abstract

In the summer of 1995 human remains were recovered at a construction site on Danforth Avenue, East Toronto, Ontario. Evidence clearly showed that this female, who had died several decades earlier, had met a tragic end, as her skull had numerous unhealed impact fractures. Circumstantial evidence resulted in a presumptive ID which was the subject of a documentary aired on an episode of 'Exhibit A' entitled "The Danforth Lady". The episode not only concluded that the mystery of her identification had been solved, but that Danforth Doe had been positively identified by mtDNA. By stating that the mtDNA from the bones of Danforth Doe matched the DNA from her sons the documentary missed an important evidentiary item; the lab that conducted the DNA analysis does not have an mtDNA capability. This was confirmed by the lab director although he noted that some inconclusive nuclear DNA results had been obtained, but these were not reported in the documentary. In this study, mtDNA analysis of the "Danforth Doe" bones was conducted at two mtDNA laboratories in Thunder Bay. Comparison of the mtDNA hypervariable regions I and II in the bones and samples from the putative sons resulted in an exclusion (no match), which supported the preliminary nuclear DNA results that were revealed (in blind) following the mtDNA results. This case clearly shows the value of mtDNA in cold cases and also exposed the unreliability of documentary reporting. It warrants a pre-cautionary stance for forensic scientists involvement with the media despite the cold case nature of the investigation. Most important is the fact that the one surviving son in reality does not have closure on his mother's disappearance.

Keywords: Cold case; Toronto; Hypervariable regions; Exclusion; Exhibit "A"

Abbreviations: PCR: Polymerase Chain Reaction; HV: Hypervariable; STRs: Short Tandem Repeats; GTA: Greater Toronto Area; STR: Short Tandem Repeat; QIAGEN: QIAquick PCR Purification Kit; TBE: Tris Borate EDTA buffer.

Introduction

Few fields have evolved as rapidly and have had the public exposure as has forensic science. Its evolution over the past century has primarily been driven by advances in technology, both for recovering remains at crime scenes (e.g., total station, lumalite) and for analyzing evidentiary samples. Without doubt the most dramatic technological advancement has been the development of Polymerase Chain Reaction (PCR) by Mullis in the mid-1980s [1]. With PCR, biological evidence that was previously unsuitable for DNA testing because it was too small or degraded can now yield a DNA profile [2]. In Canada, this was best witnessed by the Guy Paul Morin case, where lawyers waited for the PCR to be validated prior to subjecting the little remaining sample of semen on Christine Jessops panties to DNA, since the amount was too small for restriction fragment length polymorphism analysis. Now even extreme low copy number DNA samples, such as sweat left on door knobs or saliva left on donut crumbs can be suitable for DNA amplification using PCR. Moreover, hair shafts that previously were submitted for microscopic hair analysis can now be analyzed using mitochondrial DNA (mtDNA) testing [3], which is the latest tool in DNA forensic. This paper presents the mtDNA results of a notorious cold case from Toronto, Ontario, called "The Danforth Lady". This case not only demonstrates the value of mtDNA testing, but reveals the tortuous path forensic cases often take when media exposure is involved. The media usually emphasizes the dramatic aspects of technology such as PCR, in criminal investigations, but often neglects the legal responsibilities of all authorized parties involved in criminal investigations. This paper illustrates the primary 'forensic' role in cold cases and emphasizes the responsibilities of forensic scientists to the process of human identification.

MtDNA in Forensic Science

MtDNA is primarily used for analyzing cold cases or recent cases in which the samples are highly degraded or represented only by hair shafts. Cold cases are ones that are removed by time from the crime and investigative scenes and for which identity of the victim is generally not known. They are normally represented only by skeletonized human remains. A number of recent cases have demonstrated the usefulness of mtDNA in solving forensic cold cases [4,5] and mtDNA analysis is now accepted in courts of law in Europe and North America, and recently in Canada [6,7]. The primary advantage of mtDNA over nuclear DNA in forensic cases is its high copy number/cell which provides a greater probability of obtaining DNA evidence. Other advantages include its clonal/maternal inheritance, lack of recombination and high mutation rate [8].

The mtDNA genome was fully sequenced in 1981 [9,10] and this sequence, now called the Cambridge reference sequence, is used in the comparison of evidentiary and comparison samples. Forensic scientists do not use the whole mtDNA genome, but focus their comparisons in the areas of the mtDNA genome known as the non-coding hypervariable regions 1 and 2 (HV-1 and HV-2) of the mtDNA control region. This region is selected because of its extremely high mutation rate compared to the rest of the mtDNA genome and paradoxically by its high stability over many generations. This and the lack of recombination facilitates linking individuals and families over many generations, such as the famous Czar Nicholas case [11]. Typically mtDNA mutations are single base substitutions such as transitions or transversions. Transitions are interchanges between pyrimidines (cytosine and thymine) or purines (adenine and guanine). Transversions are interchanges between purines and pyrimidines. Transitions occur approximately 40 times more frequently than transversions [12]. Deletions and insertions are also observed in mtDNA but are less common. However, with exception, small insertions are frequently found in 2 homopolymeric regions, HV-1 16183-16194 and HV-2 302-310 [12]. Usually, only one type of mtDNA sequence is observed in an individual but if an individual has a condition called heteroplasmy, they will have more than one mtDNA type [4]. It has been estimated that two maternally unrelated individuals will have at least 7 mutational differences between them, although it is higher in some populations.

Forensic scientists compare evidentiary and comparison samples against the base sequences in HV-1 and HV-2 in the Cambridge reference sequence. Two samples can be excluded from originating from the same maternal lineage if their mtDNA profiles differ by one or more polymorphism [13]. However, due to the presence of heteroplasmy or chance mutation, results can be considered inconclusive if the evidentiary and comparative samples differ by only one polymorphism [13]. If mtDNA sequences of two samples are identical, an exclusion cannot be made. Major weaknesses of mtDNA are the facts its statistical power for determining the 'random match probability' for inclusions is much less than nuclear DNA Short Tandem Repeats (STRs), and that mtDNA cannot discriminate between maternal relatives. Still it is important to point out that the discriminatory power of mtDNA for exclusions is 100% and that for inclusions it is statistically more robust than the

traditional serological approaches that were time honoured before the advent of DNA in forensics and paternity testing.

Case History

The Dottie Cox Saga: A 50 Year Old Mystery

In mid May 1995 human remains were found during the demolition of an auto dealership on Danforth Avenue in Toronto, Ontario [14]. The remains were found approximately 1 metre beneath the cement showroom floor. A human skull and torso remained in situ and infracranial bones were scattered throughout the immediate area. No soft tissue was present although a considerable amount of red hair was present. Artifacts associated with the body included fragments of clothing, a pair of women's shoes and a vulcanite dental plate.

According to city building records, the auto dealership was built in 1949. Forensic odontological analysis by Dr. R. Woods revealed that the vulcanite denture present in the maxilla was popular in the Toronto area during the 1940's. Thus, it is estimated that the inhumation likely occurred between 1940 and1949.

Skeletal analysis was conducted by forensic anthropologist Dr. Jerry Melbye (consultant to the Office of the Chief Coroner). Dr. Melbye determined that the remains were that of a female based on the morphological traits of the hip bones (e.g. ventral arc, lateral recurve, narrow infrasymphyseal border and the presence of parturition scars) and cranium (e.g., slight brow ridge, mastoids, and superior nuchal line). Age at death was determined using several methods including pubic symphysis analysis (Suchey and Brooks Method). It was estimated that the individual was most likely between the ages of 28-40 years old at the time of death. Although race is difficult to assess in skeletal remains, it was hypothesized that the individual is Caucasian on the basis the presence of carabelli's cusp on the maxillary M1s, head hair colour (red) and FORDISC analysis of craniometrics [15]. Using FORDISC 2.0. stature was estimated to be between 5'2.5''-5'8'' [15].

Seven perimortem impact fractures were present in the cranium. The injuries consisted of a series of wounds which all shared similar characteristics (radiating out; beveling in). From the appearance of these blunt force injuries, it was hypothesized that the weapon must have been similar to a hammerhead.

Thus the profile for this individual was that of a 28-40 year old red-headed women of average height, who disappeared during the 1940's. There were no anomalies that could further individuate this individual.

In Toronto, records of missing persons do not go back as far as 1949. Consequently in an attempt to identify this individual, facial reconstruction of the skull was conducted by Bette Clark, formerly of the Toronto Metro Police Department. Though considered 'soft science' in the forensic world it nevertheless was the only other option available in this case. The facial reproduction was published in local newspapers within the Greater Toronto Area (GTA). The investigating officer, Detective J. Crowely, was contacted by Ronald Cox, a Torontonian who claimed that the photo of the facial reconstruction greatly resembled a photograph of his mother, Dottie Cox, who vanished in 1943. He described her as 5'5'' in stature, having red hair and a dental plate. He had also stated that his childhood home was only 2 blocks from the site at which the remains were discovered.

He Provided a Detailed Account of the Night of His Mother's Disappearance

One evening, his mother and father returned from a pub (Lindsmore Hotel) with some friends. He and his brother Melville were awakened by a fight. His father had found his mother in the basement in a compromising position with another male. The guests left and a fight ensued between his mother and father. The next morning when he awoke, his mother was missing. His father said she left because she was humiliated. She was never reported as missing.

With this information the case proceeded to test the DNA from the bones with blood samples provided by the sons. The analysis was conducted at Dr. David Sweet's laboratory at the University of British Columbia. He successfully extracted some nuclear DNA from tooth and bone samples of the *Danforth Doe* remains using short tandem repeat (STR) analysis. He noted that although he was successful in obtaining DNA the results were inconclusive, although they were reported in the documentary on this case aired on 'Exhibit A' as indicating an inclusion; the sons DNA matched those recovered from the bone. Not only was an inclusion reported but the documentary stated that the inclusion was based on mtDNA analysis. Since Dr. Sweet's lab does not conduct mtDNA analysis this automatically raised questions among us.

Because of the high copy number characteristic, mtDNA has to be processed in a clean lab environment to reduce the chances of contamination and clearly Dr. Sweet's lab was not constructed for contamination control. Following his conversation with Dr. Sweet it was clear that not only was the documentary in error but that there was a hiatus of information between Dr. Sweet and the forensic investigation. This led to our inquiring about the samples at the Centre of Forensic Science with concomitant permission for mtDNA testing of the bones and sons blood samples that were in storage at the CFS. Fortunately, though the case was assumed solved arrangements for a proper burial had not been carried out!

In order to prevent bias, the results of Dr. Sweet's DNA analysis were withheld from all researchers involved in this study until the results of the mtDNA analysis were completed. This blind approach forces the scientific investigation to emphasize the methodology.

Materials and Methods

Evidentiary and Comparative Samples

A morphologically well preserved left tibia (Figure 1) and blood (filter paper) and buccal samples (FTA paper) from two putative maternal relatives were sent to Lakehead University by one of us (KG) of the Office of the Chief Coroner, Toronto, Ontario. Immediately upon receiving the package (Canada Post-Priority Courier), the CFS was notified VIA electronic mail that the package had been received and its contents verified. The samples were individually contained within barcode labeled clear plastic bags that were sealed and initialed by a signing officer. Both evidentiary and comparative samples were documented, photographed and stored at room temperature in a secure location at Lakehead University's Paleo-DNA laboratory.

Figure 1: A morphologically well preserved tibia received from the Office of the Chief Coroner, Toronto, Ontario.

Contamination Precautions

Stringent precautions were taken to prevent sample contamination. To prevent cross-contamination, evidentiary and modern comparative extractions were carried out in separate facilities on separate days. In addition, pre-PCR and post-PCR activities were carried out in dedicated facilities. Disposable tyvek body suits, sleeves, face masks, hairnets and latex gloves were worn to ensure that foreign DNA was not introduced during pre-PCR procedures. Extraction and PCR preparation hoods, microcentrifuge tubes, trays and tweezers were UV irradiated for a minimum of 20 minutes prior to each use. As well, all laboratory equipment and surfaces were washed with a 10% bleach solution. Extraction and PCR blanks were run in parallel with all experiments to ensure reagent purity. All mtDNA sequences obtained were compared to the mtDNA sequences of laboratory staff.

DNA Extraction

Evidentiary sample: The DNA was extracted from the tibia following a modified protocol of Yang, et al. (1998). The bone was washed with double distilled water to remove surface debris and put under ultra violet irradiation for 12 hours on each side. The outer surface of the bone was then removed and cut into ~1 cm^2 sections using a high speed Dremel tool. The bone sections were ground into a fine powder using an oscillating speed mixer mill (Retsch/Brinkman). Three hundred milligrams of bone powder aliquot was mixed with 1mL of extraction buffer (30mM Tris pH 8, 460nM EDTA and 0.5% SDS) and 50µl of Proteinase K (1mg/mL, QIAGEN). Protein digestion was carried out overnight at 56 °C incubation with moderate agitation (750rpm) in an Eppendorf Thermomixer. DNA was then isolated by boiling at 94°C for 10 minutes and centrifuging for 5 minutes at 12,000 X g. The DNA containing supernatant was transferred to a sterile tube and purified using a QIAquick PCR Purification Kit (QIAGEN) to remove PCR inhibitors [16]. The DNA extract was stored at -20°C. A negative control containing no bone powder was run in parallel throughout the DNA extraction process.

Modern Comparative Samples: DNA was extracted from the buccal and blood samples using a variety of extraction protocols. The buccal swabs on FTA paper were processed according to the manufacturer's recommendations (Whatman ®). The blood smears were processed using two different methods: a Chelex bead protocol and a MO BIO DNA Forensic Kit following the manufacturer's recommendations. Buccal and blood extractions were carried out in triplicate and negative controls were carried out in parallel for each sample.

PCR Amplification of the mtDNA Control Region: The amplification of HV-1 and HV-2, 15996-16401 and 73-340 respectively, was performed in an Eppendorf Mastercycler [9,10]. For each hypervariable region, two overlapping sequences were amplified resulting in a total of 4 PCR reactions per sample. The 25µL reaction volume contained 10X PCR buffer (Invitrogen), 50mM $MgCl_2$ (Invitrogen), 10mM dNTPs (Invitrogen), 0.1 units hot start taq polymerase (Platinum™ *taq polymerase* 5u/µL, Invitrogen) and10µM of forward and reverse primer (Paleo DNA Laboratory). Table 1 lists the primers used and the corresponding DNA sequences and amplicon sizes. One FTA/filter paper punch or 10µL of 1/10 diluted DNA template was added to each 25µL PCR. The amplification was carried out with a hot start of 2 minutes at 95°C followed by 40 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 2 minutes. Five microlitres of PCR product was separated by electrophoresis on a 5% polyacrylamide gel and run in 1X Tris borate EDTA buffer (TBE). A 100bp ladder (MBI Fermentas, Lithuania) size marker was used. After electrophoretic separation the polyacrylamide gel was stained with ethidium bromide and photographed under UV illumination using a Kodak Documentation System. Extraction and PCR negative controls as well as a modern positive control were run in parallel with each PCR series.

Region	Primer Sequence	Product Size	Reference
HV-1	15996 (forward) 5'CTCCACCATTAGCACCCAAAGC 3' 16241 (reverse) 5'TTGATATGTGATAGTTGAGGGTTG3'	245 bp	Paleo DNA laboratory Paleo DNA laboratory
	16241 (reverse) 5'TTGATATGTGATAGTTGAGGGTTG3' 16410 (reverse) 5' TGATTTCACGGAGGATGGTG 3'	191 bp	Pale DNA laboratory Paleo DNA laboratory
HV-2	29 (forward) 5' GGTCTATCACCCTATTAACCAC 3' 274 (reverse) 5' TGTGTGGAAAGTGGCTGTGC 3'	245 bp	Paleo DNA laboratory Paleo DNA laboratory
	247 (forward) 5'GAATGTCTGCACAGCCAC3' 408 (reverse) 5' CTGTTAAAAGTGCATACCGCCA 3'	161 bp	Paleo DNA laboratory Paleo DNA laboratory

Table 1: Primers used for the amplification of HV-I and HV-2 of the mtDNA control region.

Sequencing: PCR product was purified with Performa™ DTR Gel Filtration Cartridges (Edge Biosystems, Gaithersberg, MD). The purified PCR products were sequenced using the ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA)) followed by capillary electrophoresis in an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The 20µL volume sequencing reaction consisted of 2µL Big Dye Terminator reaction mix, 0.5µL primer and 17.5µL of purified PCR product. Forward and reverse reactions were carried out separately using the same primers which were used for initial amplification (Table 1). Sequencing consisted of 40 cycles of the following steps: 30 seconds at 96 °C, 15 seconds at 50 °C, and 4 minutes at 60 °C. Sequencing PCR product was purified by Performa™ DTR gel filtration cartridges to remove unincorporated reagents. The product was then desiccated in a DNA Speed Vac (Savant). Sequences were aligned and compared to the Revised Cambridge Reference Sequence using Sequencher™ 4.0.5 software (Gene Codes Corporation, Ann Arbor, MI) [9,10,17,18].

Results, Discussion, Conclusion

The mtDNA profiles obtained from the blood and buccal samples of Ronald and Melville Cox were identical, as they shared four polymorphic differences from the Cambridge Reference Sequence (Table 2). MtDNA was successfully extracted, amplified and sequenced from the *Danforth Doe* skeleton (Table 3). These results were independently replicated with forward and reverse primers to address ambiguous transversions and transitions. Moreover, the results were independently run at Molecular World Inc., a private mtDNA testing facility in Thunder Bay, by Arlene Lahti and Curtis Hildebrandt.

The mtDNA profile of *Danforth Doe* does not match or share any polymorphisms with the mtDNA profile of Ronald or Melville Cox. The profile of mtDNA extracted from the bone showed 5 polymorphic sites, none of which were found in the brothers. The mtDNA profiles of Ronald and Melville Cox and the *Danforth Doe* also do not match mtDNA profiles of any of the researchers involved in this project. It is clear that Ronald and Melville Cox are maternally related but not to the Danforth Doe skeleton.

mtDNA Region	Polymorphic Site	Cambridge	Melville Cox Ronald Cox
HV-1(15996-16401)	16129	G	A A
	16304	T	C C
HV-2 (15-408)	263	G	A A
	315.1	C	C C

Table 2: MtDNA sequencing data of comparative samples.

mtDNA Region	Polymorphic Site	Anderson	*Danforth Doe*
HV-1(15996-16401)	16256	C	T
	16270	C	T
	16278	C	T
	16399	A	G
HV-2 (15-408)	147	T	C

Table 3: MtDNA sequencing data of the Danforth Doe.

Following these somewhat unexpected mtDNA results the report from Dr. Sweet's lab was opened among a group of researchers at the forensic anthropology lab at Lakehead University. Dr. David Sweet's results using STRs also support the interpretation that *Danforth Doe* is not biologically related to Melville and Ronald Cox. As only four nuclear STR loci (amelogenin, D3S1358, HUMvWA, HUMFGA) were tested Dr. Sweet suggested that the results were inconclusive, although no alleles were shared between the skeletal remains and Melville Cox. However, in conjunction with Dr. Sweet's STR results, we are confident that *Danforth Doe* is not the biological mother of Melville and Ronald Cox. Given the strength of the circumstantial evidence (i.e., hair colour, height, location, dental appliance, acrimony between spouses) supporting the interpretation that *Danforth Doe* is Dottie Cox, in contrast to the DNA results supporting the alternative hypothesis what other interpretive options could explain this paradox.

One possibility is that these remains really do represent another women who disappeared in the 1940s and was not reported missing. As the records from the time period under consideration have clearly been exhausted for other persons this option can no longer be investigated. Another possibility is that Melville and Ronald were adopted and, as is often the case, were not told. Withholding adoption information was common in the past and only recently has legislation been passed into law defining the rights of adoptees in terms of knowing their exact parentage. Unfortunately adoption records were not formalized with the government back in the 1930s and 40s so the scent for this trail would have to be pursued by interviewing possible family members that may have this knowledge. However this raises another issue, namely that the surviving son, Melville Cox, has had closure on this case and agreed to have the documentary aired.

This demonstrates that even with cold cases that are removed enough in time to be outside the 'forensic realm' in terms of pending charges etc. that the responsibilities of the forensic scientist do not change. How a mtDNA match reported erroneously on the documentary could have ever transpired is beyond belief! Forensic scientists are trained to deal objectively with the media, usually through an investigative point person. Information flow to the media usually travels from the forensic scientist to this designated person often through the coroner. In the case of Danforth Doe it is clear that the chain of continuity was broken and this is unfortunate, since we are left with unidentified human remains and a son who is still unaware that his closure is open.

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