MITOCHONDRIAL DNA RECOVERY AND ANALYSIS FROM SPENT CARTRIDGE CASINGS

By

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ABSTRACT

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Firearms, particularly handguns, are frequently used in violent crimes, and thus, spent cartridge casings may be recovered from shooting scenes. Casings have the potential to harbor DNA deposited by the loader of a weapon, which can be used to produce genetic profiles. While short tandem repeat (STR) analysis is the standard method of DNA identification in forensic science today, many researchers have reported limited success obtaining these data from spent casings. The primary goal of this study was to examine if it is possible to generate mitochondrial DNA (mtDNA) profiles from spent cartridge casings. Volunteers loaded cartridges into the magazine of a handgun, the cartridges were fired, and casings were collected and swabbed. DNAs were extracted, mtDNAs were amplified and sequenced, and haplotypes were generated. Two swabbing methods, individual and cumulative, were compared to determine which was most likely to result in mtDNA haplotypes consistent with the loader. Cumulatively swabbing resulted in a greater frequency of consistent haplotypes, as well as a lower mixture frequency than individually swabbing. Furthermore, a larger amount of the first loaded/last fired cartridge casings compared to the first fired cartridges exhibited contamination. Consensus haplotypes, which contained shared polymorphisms from multiple swabs, yielded a greater percentage of consistent haplotypes than the individually swabbed spent casings, but did not outperform cumulatively swabbing. However, none of these differences were statistically significant. Ultimately, mtDNA analysis is a reliable method to generate genetic profiles recovered from spent cartridge casings.

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INTRODUCTION

The Second Amendment to the Constitution of the United States, ratified in 1791, grants citizens the right to own firearms. Almost a century and a half elapsed before startling events involving firearms (*e.g.*, the St. Valentine's Day Massacre in 1929 and the assassination of President John F. Kennedy in 1963) became catalysts for new gun control laws (Bingham, 2012; Goforth, 2013). Despite the implementation of some stringent laws, the 310 million guns possessed both legally and illegally in the US (Krouse, 2012) are not going anywhere. This is because "they last several human lifetimes with minimal maintenance" (Dulan, 2012). Since there are so many firearms, it should be no surprise that they are commonly used weapons in crimes. According to the 2012 Federal Bureau of Investigation (FBI) Uniform Crime Report, 67.8% of the 14,612 murders, 41.3% of the 145,366 robberies, and 21.2% of the 159,240 aggravated assaults in the US in 2011 involved firearms. They were also used as an accessory in forcible rapes (although data on weapons are not collected after such incidences). Furthermore, 19,766 suicides were committed with a firearm in 2011 (Hoyert and Xu, 2012).

Since the early 1990s, crime in the US has steadily declined, with a 17% decrease in the murder rate from 2001 – 2011 (Frieden, 2012). However, the rate of unsolved murders increased from 7% in 1964 (Richardson and Kosa, 2001) to approximately 30% in 2011 (FBI, 2012). This rise is largely due to a higher frequency of stranger-to-stranger homicides as opposed to acquaintance homicides, making it more difficult for investigators to determine a motive and connection to the victim. While acquaintance homicides typically result from a conflict between the victim and offender, felonies, particularly robberies, are committed concurrently with a stranger-to-stranger homicide. Since it is common for perpetrators to plan the robberies, the

chance of investigators discovering physical evidence from a stranger-to-stranger homicide is decreased, as extra care is given by the perpetrators to minimize evidence left behind (Richardson and Kosa, 2001). Perpetrators may clean up after themselves, for example by collecting casings ejected from a firearm. Furthermore, 72.5% of all firearm homicides involve the use of a handgun, despite the fact that they make up only 37% of the firearms in the US (FBI, 2012). This high frequency of handgun usage is attributed to their ease of use and ability to conceal because they do not possess long barrels like rifles and shotguns.

Composition of a Cartridge and Ejection of Cartridge Casings

A cartridge consists of a bullet, propellant powder, primer, and a casing (Figure 1). The role of the primer, which is comprised of an explosive initiation compound, fuel, and oxidizer (Krampen *et al*., 1986), is to start a fire and deflagrate the nitrocellulose-based propellant powder. The primer is contained either in the rim or center of the casing head, which ignites when the firing pin strikes (Westrom *et al*., 2005). The buildup of gases caused by the burning of the propellant expands the casing and forces the bullet out of the cartridge and through the barrel of the firearm. The casing is extracted and ejected from a semi-automatic and automatic firearm by the action mechanism (Sparano, 2000), resulting in a key piece of physical evidence.

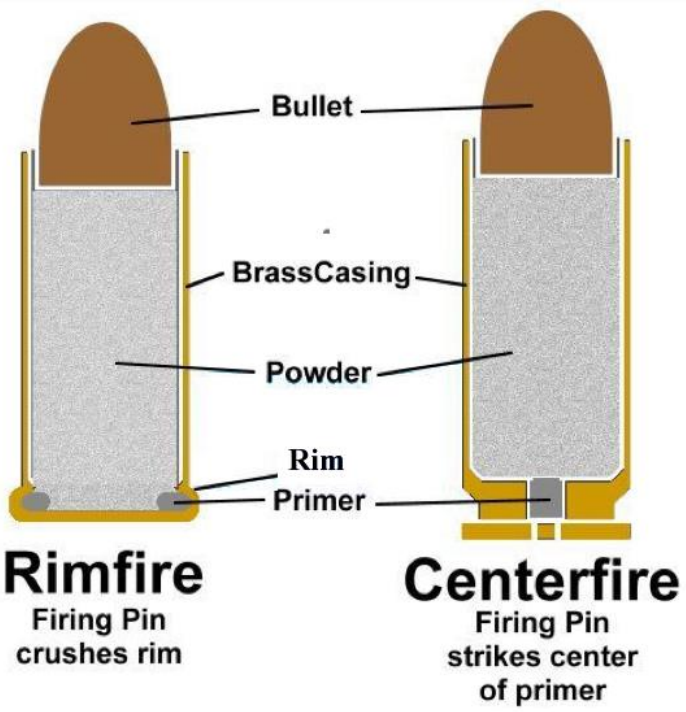


Figure 1. Anatomy of a rimfire and centerfire cartridge. Adopted from http://www.gunclassics.com/ammunition.html. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

Unless the shooter collected the spent cartridge casing(s) or used a firearm that does not eject casings (*e.g.* a revolver), casings will be left behind at the scene of a shooting. The marks made by the firing pin, breechface, extractor, and ejector on the surface of a casing are considered individualizing characteristics that can be used to determine the firearm a casing was ejected from (Cork *et al*., 2010). However, this comparison can only be made if known casings from the firearm are available (Milloy, 2002). Furthermore, this method cannot be used to identify the loader of the firearm; individualizing evidence from a spent casing is contingent on recovering fingerprints and/or ample amounts of DNA left behind. Even if the loader did not pull the trigger, their identification would help with the investigation. For instance, when the identified loader is questioned, the individual may disclose who used or had access to the loaded

firearm. Unfortunately, these identification methods are not always met with success; forensic scientists seldom attempt fingerprint and DNA analyses from the casing surfaces because viable results are rarely obtained (David Arnold and Brandon Good, personal communications). Researchers have examined what happens to identifiable materials left on cartridge casings, with the goal of determining a reliable technique to be used for successful identification. A summary of the major findings from some of the notable studies is outlined in Table 1.

Table 1. Major findings from previous fingerprint and DNA analysis research from spent cartridge casings.

Study	Fingerprint Analysis	DNA Quantification	STR Analysis
Given (1976)	• Gaseous blowback destroys ridge detail	-	-
Bentsen *et al*. (1996)	• Friction from loading and ejection destroy ridge detail	-	-
Spear *et al.* (2005)	• 1 of 24 spent casings with a viable print (bloody)	-	• 1 of 24 spent casings with a partial profile (bloody)
Horsman-Hall *et al.* (2009)	-	• Significantly > DNA recovered using DNA IQ™ vs. organic extraction • Average 0.42 ng from spent casings • DNA recovered from breechface, chamber, and ejection port (surfaces)	• Significantly > alleles using MiniFiler™ vs. PowerPlex® 16 BIO • No amplification using Identifiler® • Loader alleles recovered from surfaces • Individually swabbed (n = 75) Full profiles (1%) Partial profiles (45%) No profiles (54%)
Branch (2010)	-	• Quantified if ≥ 3 correct alleles amplified using MiniFiler™ (n = 47; no distinction of firing status) 11 had ≥ 0.1 ng	• Individually swabbed (n = 251) Full profiles (0%) Partial profiles (8%) No profiles (92%)
Orlando (2012)	-	• No difference between individually (7.67 ± 9.55 pg/μL) and cumulatively (38.23 ± 141.52 pg/μL) swabbed spent casings	• Individually swabbed (n=155) Full profiles (3.2%) Partial profiles (89.7%) No profiles (7.1%) • Cumulatively swabbed (n=31) Full profiles (0.0%) Partial profiles (93.5%) No profiles (6.5%)

- indicates no research conducted

Fingerprints Recovered from Spent Cartridge Casings

Whenever two objects come into contact, an exchange is thought to occur (Locard, 1928). For example, when a person touches the door of a dirty car, fingerprints and cells containing DNA are transferred to the door and dirt from the car is transferred to the person's hand. Therefore, if gloves are not worn, it is probable that fingerprints will be deposited on cartridge casings when an individual loads a firearm, which could subsequently be used to make an identification.

Given (1976) investigated the retention of fingerprints on casings after firing; volunteers picked up 108 brass and 108 nickel-plated .38-caliber cartridges and placed them on a tray. Half the cartridges were fired, casings were dusted, and prints were lifted. Discoloration on a portion of the casing indicated gaseous blowback occurred along the side that is not sealed against the wall of the chamber during firing. As a result of this blowback, fingerprints deteriorated. Bentsen *et al*. (1996) also investigated fingerprint recovery from spent casings by rolling fingerprints on various ammunition and firing with different types of guns (total of 21 combinations). Ridge detail was recovered from 17 casings, only five (23.8%) of which were identifiable. While they did find that gaseous blowback destroyed ridge detail, they concluded that friction from loading the cartridges and during ejection of the spent casings were the main contributors to loss of detail. This conclusion was reached because four of the five identifiable prints came from cartridges that had been gently loaded and extracted from revolvers in a manner that eliminated the added friction encountered with manual firearms. Spear *et al.* (2005) studied the feasibility of recovering fingerprints from spent casings, by placing eccrine, oily, or bloody prints on 48 cartridges (.22 – .45-caliber) made with aluminum, brass, or nickel-plated brass casings. Half of the cartridges were fired and all casings were then stored at room

temperature for several months. Oily and eccrine prints were treated by cyanoacrylate fuming and rhodamine 6G, and bloody prints with amido black. Prints were recovered from five live cartridges (20.8%). The only print recovered from a spent casing was made with blood, which is atypical in that an individual's hands would not normally have blood on them when loading a gun. Furthermore, the Indianapolis Metropolitan Police Department East District processed 201 cartridges and casings for fingerprints and only one viable print was developed (Nunn, 2013). Although it is not mentioned whether the print was from a spent or live cartridge casing, it is indisputable that fingerprints can rarely be obtained from either, both in a controlled laboratory setting and in casework.

Obtaining Nuclear DNA from Touch Samples

In 1997, van Oorschot and Jones reported that nuclear DNA (nDNA) profiles could be generated from briefly handled objects (touch samples). Thus, when an individual loads cartridges either into a magazine or the chamber of a firearm, cells are likely to be deposited on the casings. However, a limitation of touch samples is that a variable number of cells is shed onto a surface—often resulting in less than 0.30 nanogram of DNA (Schulz and Reichert, 2002). In addition, the amount of cells transferred may differ substantially among loaded cartridges. For instance, most cells might be deposited on the first cartridge loaded. In contrast, because an increased amount of force is required to push down on the spring as more cartridges are loaded, the last cartridge might harbor the greatest number of cells. Horsman-Hall *et al.* (2009) recovered an average of 0.54 ± 0.17 ng of DNA from five unfired 7.62 mm X 39 mm cartridges handled by volunteers for 30 s. However, because these rifle cartridges were longer than those

designed for handguns, it is possible that more cells were transferred onto the surface than would be on smaller cartridges.

Based on the finding that ridge detail is lost from blowback during firing (Given, 1976) and also during loading of a cartridge and ejection of a casing (Bentsen *et al*., 1996), it can be rationalized that cells are also lost during these processes. Therefore, it is expected that less DNA would be recovered from spent cartridge casings compared to live cartridge casings. Horsman-Hall *et al.* (2009) did recover a slightly lower average quantity of DNA from five spent casings (0.42 ± 0.10 ng), but the difference was not significant, likely due to the small sample size. In a separate part of the same study, the ejection port, breechface, and chamber of a shotgun were double-swabbed using a wet followed by a dry swab, after which 10 volunteers loaded shotgun shells into the gun (no DNA was recovered from the surfaces prior to firing). After the shotgun shells from the third (set 1), sixth (set 2), and tenth (set 3) volunteers were fired, the surfaces were swabbed again, resulting in an average of 0.07 ± 0.07 ng of DNA obtained from the ejection port, 0.06 ± 0.10 ng from the breechface, and 0.51 ± 0.14 ng from the chamber of the shotgun. These data also indicate that DNA is lost from shells during firing. However, the authors did not investigate whether the order of loading influenced DNA deposition on shells and casings.

Orlando (2012) investigated whether double-swabbing multiple spent casings successively (cumulatively), as opposed to individually, increased the amount of DNA recovered. First, ten cartridges were selected at random out of the manufacturer's box and tested for nDNA; no background DNA was quantified from the cartridge casings. Volunteers then loaded 10 cartridges into the magazine of a gun and the cartridges were fired. DNA was recovered individually from 5 of the 10 spent casings using a double-swab method for each casing. The

remaining five spent casings were cumulatively swabbed with one pair of swabs. An organic extraction was performed followed by DNA concentration and purification with Amicon® and Zymo-Spin™ columns. A flow diagram of sample collection and DNA isolation used by Orlando (2012) is shown in Figure 2. DNAs were then quantified using a Quantifiler™ Human DNA Quantification kit. There was no statistical difference between DNA yields obtained using the two recovery methods.

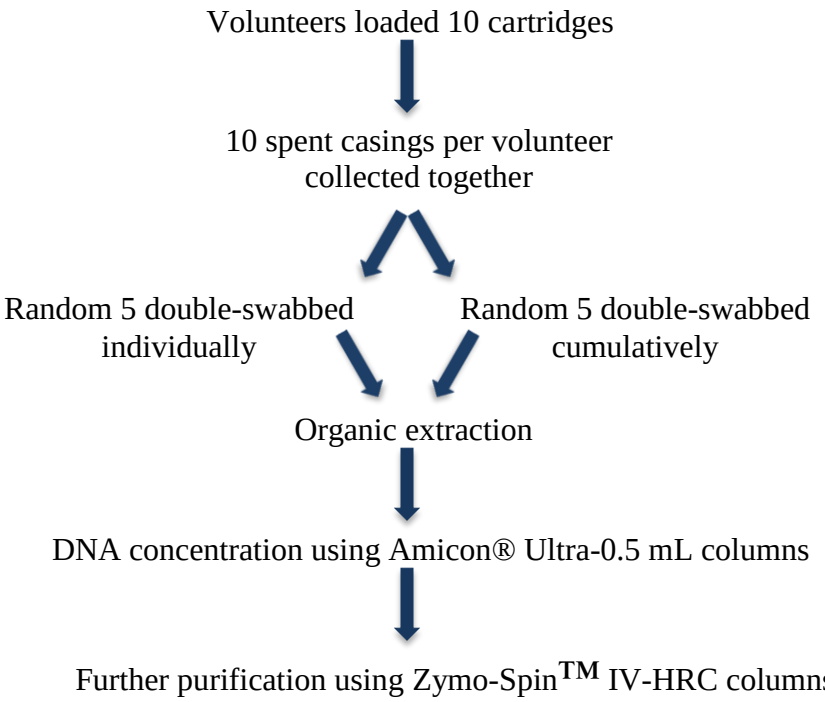


Figure 2. Flow chart of the collection of samples and isolation of DNA used in the Orlando (2012) study.

Horsman-Hall *et al*. (2009) also compared extraction methods of DNA recovered from spent cartridge casings. Unfired cartridges handled for 30 s were double-swabbed and DNAs were digested and extracted using one of four protocols: (1) proteinase K and 20% sarkosyl

digestion and DNA IQ™ extraction; (2) digestion with DNA IQ™ lysis buffer, followed by a DNA IQ™ extraction; (3) proteinase K and sodium dodecyl sulfate (SDS) digestion with SDS added to a DNA IQ™ extraction (final SDS concentrations unknown); and (4) proteinase K and 20% sarkosyl digestion and an organic extraction using Microcon® purification. The organic extraction recovered significantly less DNA than the DNA IQ™ methods, among which there was no significant difference.

Utility of nDNA Recovered from Spent Cartridge Casings for Identification of the Loader

The genetic material deposited on a casing can potentially be used for identification. An identification using nDNA in forensic applications is made by examining short tandem repeats (STRs), which are loci that have a variable number of repeated DNA sequences that are typically 2 to 6 base pairs (bp) long. Individuals have two alleles at every locus: one inherited from each parent. About 0.5 – 1.0 ng of non-degraded DNA is required to obtain a full STR profile (Gill, 2001). If lower quantities of DNA are present, stochastic sampling effects can occur, wherein one allele is unequally amplified over the other, giving false homozygous results.

Regardless of the quantity of DNA recovered from spent cartridge casings, it is important to determine if STR profiles can be generated from them, since full profiles have been obtained from as little as 0.06 ng of nDNA (approximately 10 cells worth) using AmpFℓSTR® MiniFiler™ (Viray, 1998). In addition to examining the utility of fingerprint analysis from live and spent casings, Spear *et al*. (2005) investigated DNA analysis from the casings using a wet swab, extracted DNA organically, and amplified STRs using Profiler™ Plus. Only one spent

casing resulted in STR amplification (4.2%); the profile contained 9 of the 10 loci targeted by the kit.

Horsman-Hall *et al*. (2009) also examined STRs from five types of handled ammunition, including two handgun cartridges with different casing metals, two rifle cartridges with different casing metals, and shotgun shells, and compared STR results obtained using AmpFℓSTR® Identifiler®, MiniFiler™, and PowerPlex® 16 BIO kits. The ammunitions were held for 30 s, a gloved firearms examiner loaded them into the chamber of a firearm, and fired them. The spent casings and shells were double-swabbed and since there was no significant difference among the DNA IQ™ extraction methods (detailed above), the authors used the standard protocol of the Virginia Department of Forensic Science to digest and purify the DNAs. DNAs were quantitated using Plexor® HY System, and STRs were amplified. No alleles were obtained using the Identifiler® kit, and there was a significantly greater number of amplified alleles using the MiniFiler™ kit, designed for degraded samples, compared to the PowerPlex® 16 BIO kit. Only 1% of the fired casings generated full profiles with the MiniFiler™ kit, while 54% of the casings had no alleles amplify.

Branch (2010) also investigated the utility of MiniFiler™ and Identifiler® for analyzing DNA from spent casings, but used a robotic extraction method and examined several variables. Ten volunteers washed their hands, immediately handled .22-caliber brass cartridges for 15, 30, or 60 s, and the live cartridges were collected. This protocol was repeated, but cartridges were loaded into a firearm, fired, and spent casings were collected. In phase 2 of the study, five volunteers waited one hour after washing their hands before handling .38-caliber and 9 mm brass, nickel, and aluminum cartridges. The cartridges were handled for the same amount of time and

collected in the same manner as in phase 1, resulting in 420 samples for phase 2. The casings were double-swabbed, a BioRobot®EZ1 robot was used to extract DNA, and STRs were amplified using MiniFiler™. Extracts with a minimum of three amplified alleles consistent with the volunteer were quantified using a Quantifiler™ Human DNA Quantification kit and extracts containing a minimum of 0.1 ng of DNA were amplified using Identifiler®. Caliber, metal type, and handling duration did not have significant effects on the number of alleles amplified. Ninety-two percent of fired casings resulted in no amplified alleles and significantly more alleles were obtained when casings were handled one hour after washing hands. Since the majority of the DNA recovered from live handgun cartridges yielded no profiles (80%), this further supports the theory that an insufficient amount of DNA is recovered from casings for successful STR analysis.

Orlando (2012) used AmpFℓSTR® Identifiler® Plus to generate STR profiles from DNAs recovered from fired casings and compared the number of alleles obtained between individually and cumulatively swabbed casings to determine if it was advantageous to cumulatively swab. The majority of the individually and cumulatively swabbed casings (74.2% vs. 67.7%) had seven or fewer Identifiler® Plus loci amplify, though the frequencies were not dependent on swab method. Consistent with previous studies (Spear *et al.*, 2005; Horsman-Hall *et al*., 2009; Branch, 2010), Orlando (2012) showed that DNA was recoverable from spent cartridge casings, but STR success was low.

Processing of Spent Cartridge Casings by Forensic Science Laboratories

When spent cartridge casings are collected at a crime scene by the Miami-Dade Police Department, each casing is packaged separately in a coin envelope and sent to a latent print

examiner at the Forensic Laboratory, where the examiner visually inspects the casings to determine if any prints can be lifted (Forensic Scientist David Arnold, personal communication). The casings are then transferred to the biology/DNA unit and a DNA analyst processes them only if no other DNA evidence is available for the case, because as noted, STR profiles from spent cartridge casings are rarely obtained. All casings from the same investigation are swabbed with one swab wetted with deionized water, followed by an organic extraction and DNA quantification. A minimum of 0.35 ng of DNA is required to proceed with STR amplification using the Identifiler® kit.

Similarly, Michigan State Police (MSP) routinely package spent casings separately (Forensic Scientist Brandon Good, personal communication). At the MSP Northville Forensic Science Laboratory, the firearms unit examines the casings to determine if they were likely expelled from the same weapon. The latent print and biology units only process the casing(s) if a request, *e.g.* by the prosecutor, has been made to the forensic science division command. When casings are processed, the biology unit individually double-swabs them, unless the firearms unit determines that they were fired from the same gun, in which case they are cumulatively double-swabbed. DNA is extracted organically, purified using Vivacon® 2 columns, and DNA is quantified using Plexor® HY. However, unlike the Miami-Dade Police Department, MSP still proceed to amplify STRs using PowerPlex® 16 BIO, even if no DNA was detected by quantification.

Studies on STR profiling from spent cartridge casings in casework are quite limited. An exception is retrospective research conducted by Dieltjes *et al*. (2011) at the Forensic Laboratory for DNA Research in the Netherlands, where 4,085 live cartridges, bullets, and casings collected from 616 cases were processed. STR profiles with at least one amplified locus were obtained

from 283 of the items, with an average amplification rate of 10.95 loci per item using the PowerPlex® 16 system. Forty-four of these items resulted in a full profile from a single donor. However, there were two major limitations of the study: (1) the type of evidence from which STR data were obtained was not specified and (2) the loader's DNA profile was not known and therefore, it cannot be determined if the amplified alleles from the 48 items were consistent with the loader.

Mitochondrial DNA from Touch Samples

Human mitochondrial DNA (mtDNA), consisting of approximately 16,569 bp, has 37 genes: 22 transfer RNA genes, 13 protein-coding genes, and 2 ribosomal RNA genes (Anderson *et al*., 1981). The control region, depicted at the top of Figure 3, commonly harbors differences among unrelated individuals. Two hypervariable regions (HV1 and HV2) within the control region are sequenced in forensic applications. The sequences are compared to the Cambridge Reference Sequence (Anderson *et al*., 1981) to locate polymorphisms. The possessed polymorphisms comprise an individual's haplotype (mtDNA profile).

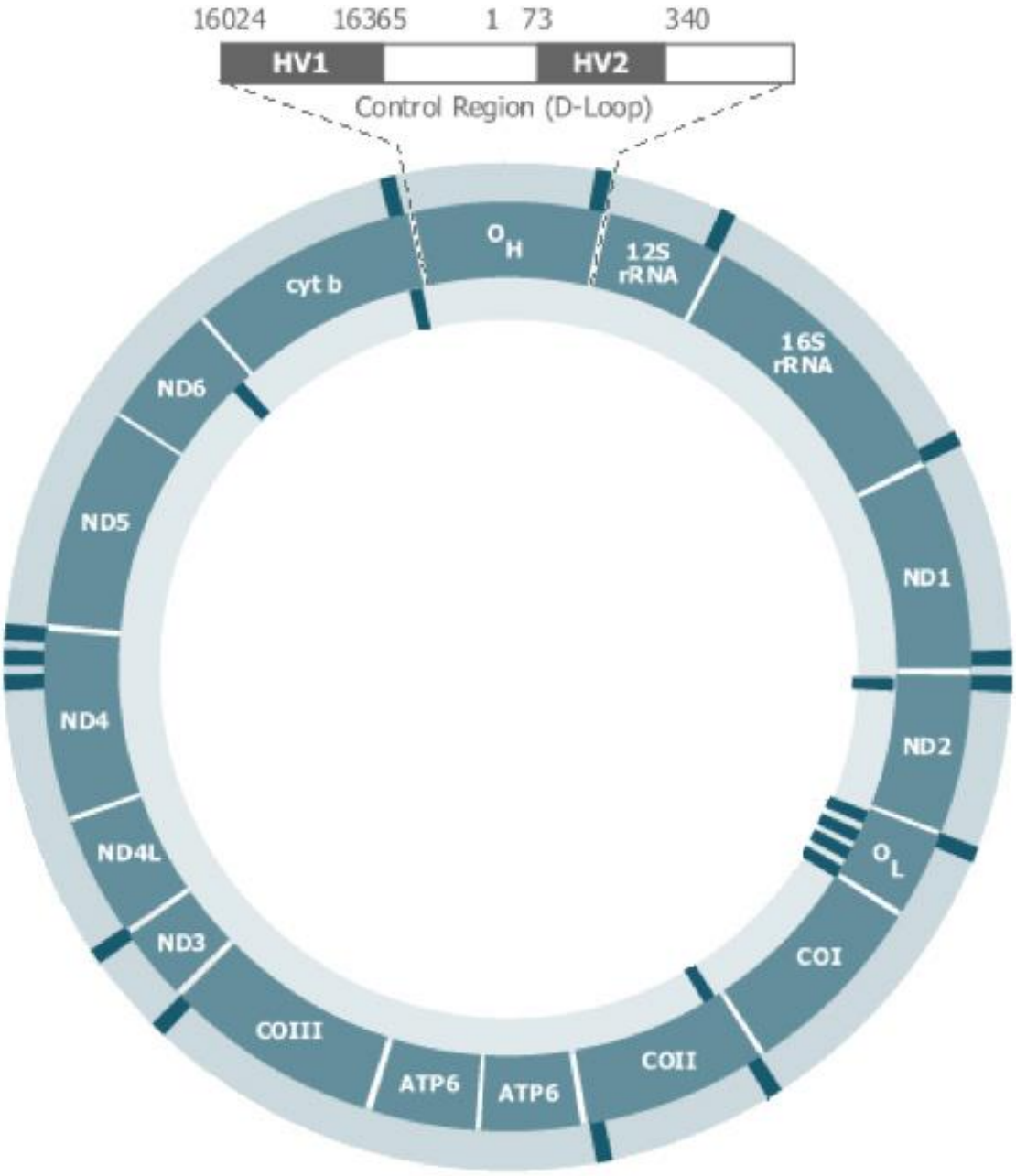


Figure 3. The human mtDNA genome. Taken from National Forensic Science Technology Center. Available at http://www.nfstc.org/pdi/Subject09/pdi_s09_m01_01_b.htm.

There is a greater likelihood of successfully obtaining DNA typing results using mtDNA compared to nDNA (Goodwin *et al*., 1999). Three factors contribute to the higher success rate. First, the high copy numbers of mtDNA in a cell increase the probability of isolating DNA. A

single cell may contain hundreds of mitochondria, each with an average of 4.6 mtDNA molecules (Robin and Wang, 1988; Satoh and Kuroiwa, 1991). Second, the linear structure of nDNA potentially makes it susceptible to degradation by exonucleases, whereas mtDNA's circular structure would make exonuclease attack more difficult. Third, the location of mtDNA molecules in the mitochondrion has been shown to better protect mtDNA compared to DNA in the nucleus (Foran, 2006).

Since mtDNA is maternally inherited, individuals exhibit a high degree of homoplasmy—only one mtDNA type (Monnat and Loeb, 1985; Monnat and Reay, 1986; Bodenteich *et al*., 1991). Therefore, mtDNA sequences are easily interpretable, unless either: (1) a mixture of DNA from multiple individuals exists or (2) heteroplasmy is exhibited. A mixture may result from secondary transfer. For instance, when two people shake hands, an exchange of DNA may occur (van Oorschot and Jones, 1997). When one of the individuals then proceeds to touch a (*e.g*.) pen, it is possible that genetic information from both of them will be deposited onto the surface (Goray *et al*., 2010). A mixture can also result from contamination, which can occur at any step from collection to processing of the evidence. A mtDNA mixture is identified by observing a clean sequence with more than one peak at certain positions, attributed to more than one individual (exemplified in Figure 4).

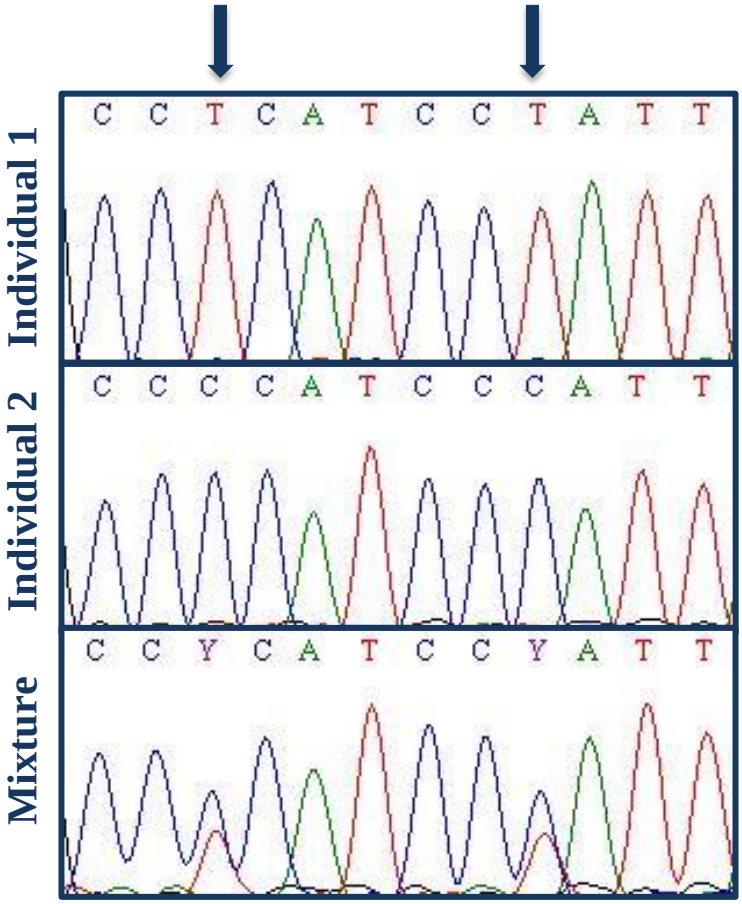


Figure 4. Sequencing electropherograms from buccal swabs of two individuals (top and middle) and a mixture of the mtDNA sequences from the two individuals (bottom). The positions showing mixture are indicated with a 'Y' (pyrimidine) above the peaks.

An estimated 2 – 8% of the human population is heteroplasmic—harboring more than one mtDNA variant (Holland and Parsons, 1999). Four scenarios can result in heteroplasmy: (1) different cell types (*e.g.* blood cells and bone cells) with different mtDNA variants; (2) same cell type with different mtDNA variants; and any cells that contain two variants, either (3) among mitochondria or (4) within mitochondria (Wilson *et al*., 1997). Two peaks at one position of an electropherogram may be indicative of heteroplasmy, such as in Figure 5. The presence of a T and C peak is due to two mtDNA variants at the fourth position shown. Contrary to intuition, the proportion of the bases at a heteroplasmic site depicted in an electropherogram is not always

indicative of the actual ratio because peak heights have been observed to differ between the forward and reverse strands of the same extract (Sigursardottir *et al*., 2000); the same would hold true for mixtures containing multiple sources of DNA. Furthermore, the mtDNA ratio can differ between cells. For example, epithelial cells from a buccal sample may contain a different ratio than the epithelial cells left behind on a cartridge casing. Moreover, one of the epithelial cell origins may contain only one mtDNA variant.

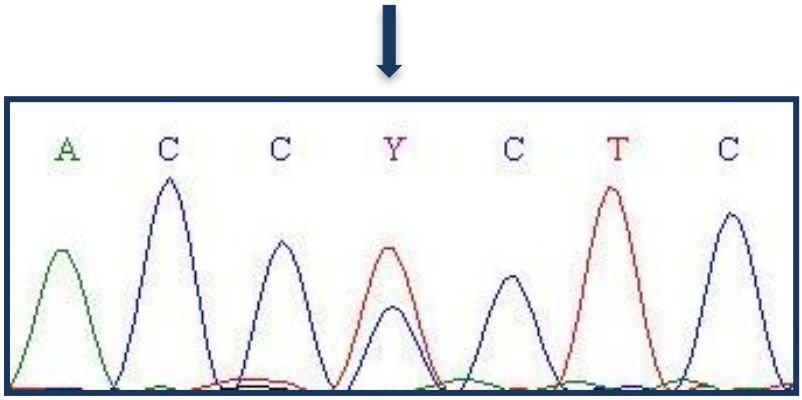


Figure 5. Electropherogram containing a heteroplasmic position indicated with a 'Y' above the peaks. The buccal sample of this individual possessed a T (red) peak and a C (blue) peak.

Goals of This Study

The ability to use DNA recovered from a spent cartridge casing(s) is vital to identifying a criminal, especially in instances when other physical evidence from a crime scene is not available. Despite the fact that scientists have tested various swabbing strategies, DNA extraction techniques, and STR kits, STR typing success remains low. To date, assaying mtDNA from spent cartridge casings has not been presented in the literature or at scientific conferences, most likely at least in part because the majority of forensic science laboratories do not perform mtDNA analysis. However, in 2005, the FBI partnered with the Arizona Department of Public

Safety Central Crime Laboratory, the Connecticut State Police Forensic Science Laboratory, the Minnesota Bureau of Criminal Apprehension Forensic Science Laboratory, and the New Jersey State Police Crime Laboratory to provide a free mtDNA analysis service to local and state law enforcement agencies nationwide (FBI, 2006). Access to mtDNA processing facilities allows analysis of DNA recovered from spent cartridge casings to be a feasible option across the country. Thus, if mtDNA analysis from such samples yields usable information when STR analysis does not, it is a resource that should be taken advantage of.

The primary goal of the research presented here was to thoroughly examine the feasibility of obtaining mtDNA profiles from spent cartridge casings that are consistent with the loader. Results from the first and last loaded cartridges were compared to assess which yields haplotypes consistent with the loader at a higher frequency, coupled with a lower frequency of mixture. This assessment was also made for haplotypes recovered from individually and cumulatively swabbed casings. Finally, a consensus profiling method, combining shared polymorphisms from multiple swabs, was developed to evaluate if this would filter out polymorphisms inconsistent with the loader and subsequently yield more consistent haplotypes.

The experiment was organized into two parts: Part A and B. Orlando (2012) DNA extracts were processed for mtDNA in Part A and new samples were collected and processed for mtDNA in Part B. The following research questions were addressed:

Q1. Can mtDNA haplotypes consistent with the loader be developed from DNA left behind on cartridge casings during loading?

Q2. How do mtDNA results from spent casings compare to STR results?

Q3. Does the order cartridges are loaded/fired influence mtDNA results?

Q4. Are there differences in mtDNA results from individually and cumulatively double-

swabbed spent casings?

Q5. Is it advantageous to use consensus profiling (generating a haplotype comprised of polymorphisms seen the majority of the time from casings handled by the same individual)?

MATERIALS AND METHODS

Part A

Eleven mtDNA control region primer pairs, including F15989 – R16410 (HV1), F15 – R484 (HV2), F15989 – R16322, F15989 – R16251, F16057 – R16322, F155 – R484, F155 – R389, and F256 – R484, were tested on Orlando (2012) DNA extracts 22D and 28A – F. The three primer pairs subsequently used for this study were: F15989 – R16233 (HV1a), F16190 – R16410 (HV1b), and F15 – R285 (HV2a) (Table 2). The entire control region of reference samples was amplified using F15989 – R484. F15989, F16190, R16410, F15, and R285 were developed at the Armed Forces DNA Identification Laboratory (Edson *et al*., 2004) and R16233 was developed by Lee *et al*. (2008).

Table 2. Primers used to amplify mtDNA from cartridge casings and reference samples.

HV1a	
F15989 5’ CCC AAA GCT AAG ATT CTA AT 3’	R16233 5’ TGA TAG TTG AAG GTT GAT TGC TGT 3’
HV1b	
F16190 5’ CCC CAT GCT TAC AAG CAA GT 3’	R16410 5’ GAG GAT GGT GGT CAA GGG AC 3’
HV2a	
F15 5’ CAC CCT ATT AAC CAC TCA CG 3’	R285 5’ GTT ATG ATG TCT GTG TGG AA 3’
Reference Samples	
F15989 5’ CCC AAA GCT AAG ATT CTA AT 3’	R484 5’ TGA GAT TAG TAG TAT GGG AG 3’

HV1a and HV2a amplification reactions were set up for six of the ten unhandled cartridge casings swabbed by Orlando (2012), along with the reagent blank. Furthermore, Zymo-Spin™ IV-HRC columns (Zymo Research, Irvine, CA) were tested for the presence of

mtDNA; a column was added to a collection tube and centrifuged for 3 min at 8,000 rcf to remove the liquid that the matrix comes suspended in. Following UV irradiation for 10 min, 50 µL of TE (10 mM Tris [pH 7.5], 1 mM EDTA) was added to the column, and centrifuged at 8,000 rcf for 1 min (the filtrate was denoted T1). The same protocol was repeated with a new column, but it was exposed to UV for 10 min prior to the first spin and for 5 min after the liquid was centrifuged through (T2). Both filtrates and a negative control were subjected to HV1a and HV2a amplification.

Polymerase chain reaction (PCR) was performed in 30 µL volumes and included: 3 µL of 25 mM $MgCl_2$ (Applied Biosystems, Carlsbad, CA), 3 µL of GeneAmp 10X PCR Buffer II (Applied Biosystems), 3 µL of 2 mM deoxynucleoside 5'-triphosphates, 3 µL of 4 µg/µL bovine serum albumin (BSA; Fisher Scientific, Waltham, MA), 3 µL of 20 µM forward and reverse primers, 1 unit of AmpliTaq Gold® polymerase (Applied Biosystems), 1 µL of template DNA, and 11 µL of water. Six live Orlando (2012) cartridge casing extracts, T1, T2, reagents blanks, and negative controls were amplified in 10 µl volumes with one third the amount of reagents, and 1 µL of extract. PCR conditions were: 94˚C for 10 min, 38 cycles of 94˚C, 60˚C, and 72˚C for 30 s each, and a final extension step of 72˚C for 5 min. Reference samples were amplified using the same PCR conditions, but for 35 cycles. Five microliters of PCR products were electrophoresed on a 4% agarose gel. If no band was present, a new 30 µL reaction was made using 2 µL of template DNA.

Sequencing Genetic Profiles

PCR products of cartridge casing extracts and reference samples were purified using Diffinity RapidTip® (Diffinity Genomics, Inc., West Henrietta, NY) as per manufacturer's

instructions. Ten microliter Sanger sequencing reactions were prepared using 2.5 µL of BigDye® Terminator v3.1 Cycle Sequencing master mix (0.875 µL of BDX64 BigDye® enhancing buffer [MCLAB, San Francisco, CA], 0.125 µL of BigDye® Terminator v3.1 Ready Reaction Mix [Applied Biosystems], 1.5 µL of 5X Sequencing Buffer [Applied Biosystems]), 1 µL of one of the same primers used to amplify the DNA, and 6.5 µL of the purified template. For reference samples, 2 µL of purified template and 4.5 µL of distilled water were added to bring the volume to 10 µL. Sequencing conditions were: 96˚C for 3 min, followed by 30 cycles of 96˚C for 10 s, 50˚C for 5 s, and 60˚C for 2 min. For R16410 sequences, 2.7 µL of BigDye® Terminator v3.1 Ready Reaction Mix, 1.3 µL of 5X Sequencing Buffer, 1 µL of F16410, and 5 µL of amplified DNA from casings or 2 µL of amplified reference sample DNA and 3 µL of distilled water were used. Thermocyling parameters were: 96˚C for 1 min, followed by 25 cycles of 96˚C for 10 s, 50˚C for 5 s, and 60˚C for 4 min.

Two and a half microliters of stop solution (1 µL of 100 mM EDTA [pH 8], 1 µL of 3 M NaOAC, 0.5 µL 20 mg/mL glycogen), the 10 µL of sequenced products, and 35 µL of chilled 95% ethanol were added to 1.5 mL microcentrifuge tubes. Tubes were vortexed for 10 s, centrifuged at full speed for 10 min, and the supernatants were removed without disturbing the pellets. One hundred-eighty microliters of chilled 70% ethanol was added to the tubes, centrifuged at full speed for 4 min, and the supernatants removed. The 70% ethanol wash was repeated twice. DNAs were vacuum-dried for 15 min and resuspended in 10 µL of Hi-Di™ Formamide (Applied Biosystems). DNAs were electrophoresed on an AB 3500 Genetic Analyzer using instrument parameters: oven temperature 60˚C; run time 1020 s; run voltage 19.5 kV; injection time 8 s; injection voltage 1.6 kV; capillary length 50 cm. A runtime of 1400 s was

used for reference samples. Sequences were analyzed using Sequencing Analysis Software v5.4 (Applied Biosystems) and were aligned using BioEdit v7.0.9.0 (Hall, 1999).

Analysis of Genetic Profiles, Consistency of Assignments, and Mixtures

Sequences were compared to the Cambridge Reference Sequence (Anderson *et al*., 1981). If the Sequencing Analysis Software deciphered the same polymorphism in both the forward and reverse sequences, the base call was not altered. However, if differing base calls were made, the electropherograms were analyzed to determine if there was a mixture at that position. The nomenclature used for base calls is summarized in Table 3.

Table 3. Nomenclature used to make base calls for all sequences.

Base(s)	Nomenclature
Adenine	A
Cytosine	C
Guanine	G
Thymine	T
Adenine + Cytosine	M
Adenine + Guanine	R
Cytosine + Guanine	S
Cytosine + Thymine	Y

Haplotypes of the analysts and the seven volunteers comprised the database for Part A. The haplotypes from each extract were randomly assigned a number and a blind study was conducted to determine if the haplotype could be successfully matched back to the loader included in a database. When a mixture was detected, assignments were made for haplotypes with and without the polymorphic base. For example, if the haplotype was a 16291T, 73R, 152C, and 263G, an assignment was attempted for haplotype 16291T, 73A (the reference sequence base),

152C, and 263G, as well as for haplotype 16291T, 73G, 152C, and 263G. The assignments were compared to the loader and assessed using the following rules:

1. Consistent-Single: The haplotype was consistent with a single individual and it was the true loader.
2. Consistent-Multiple: The haplotype was consistent with a group of individuals and the loader was included in this group.
3. Inconsistent: The haplotype was consistent with either a single individual or a group of individuals, but the loader was not one of the individuals.
4. Inconclusive: The haplotype was not consistent with any individual.

The presence of a mixture containing the loader's DNA (loader mixture) was established by identifying more than one peak at any polymorphic base consistent with the loader. In instances when the loader's mtDNA did not contain any polymorphisms, clean regions of the sequences were inspected for any position(s) with a second peak that was larger than the baseline. Extracts with polymorphisms consistent with the primary analyst were noted as 'contaminated'.

Part B

Paper bags and cotton swabs (860-PPC, Puritan Medical Products Co., Guilford, ME) were autoclaved at 135°C for 45 min, followed by a dry cycle of 1 hr. A facemask and two pairs of gloves were worn during sample collection. Lab coat, hair net, sleeves, facemask, and two pairs of gloves were used during all other pre-amplification procedures. Reagents and consumables were exposed to ultraviolet (UV) light for a minimum of 5 min per side (~2.5 J/cm^2) in a Spectrolinker XL-1500 UV Crosslinker (Spectronics Corporation, Westbury, NY)

prior to use; swabs were placed at an angle to the source of UV light and were rotated 180 degrees after 5 min of exposure.

Obtaining and Decontaminating Cartridge Casings

Remington® .40-caliber, brass cartridges were purchased at local retail stores. Since analysis of the Orlando (2012) extracts indicated mtDNA was present on unhandled cartridges, the casings were wiped with ELIMINase® (Decon Laboratories, Inc., Bryn Mawr, PA) as per manufacturer's instructions. Residual ELIMINase® was removed by wiping with deionized water and drying two times. The decontaminated cartridges were separated into sets of six and placed into paper bags labeled 1 through 38.

Loading of Cartridges

The use of human subjects as loaders of cartridges was approved by the Michigan State University (MSU) Committee on Research Involving Human Subjects (IRB# 12-770). Prior to participation, volunteers from MSU and the MSP Sterling Heights (Collection 1) and Northville (Collection 2) Forensic Science Laboratories signed consent forms. One firearm and one magazine were used for Collection 1 and one firearm and five magazines were used for Collection 2. Before Collection 1, the inside of the magazine was wiped with a swab wetted with water. Volunteers then randomly selected a paper bag and loaded the six cartridges into the magazine of a handgun. The cartridges were fired and the casings were collected: for Collection 1, all casings handled by one volunteer fell onto netting around the firing tank and were placed back into their respective paper bags; for Collection 2, they were caught in heat-seal grade plastic bags held by a metal wire next to the ejection port. The second through fifth spent casings

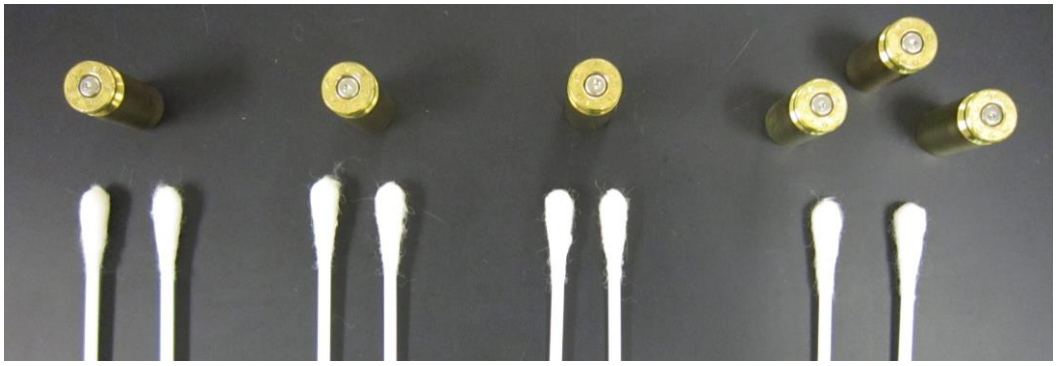
handled by one volunteer were ejected into one bag, while the first and sixth spent casings were caught in individual bags. The plastic bags were then placed into their respective paper bags.

Two buccal swabs obtained from each volunteer as DNA reference samples were placed into a culture tube, and labeled with a letter randomly selected from A – XX. The buccal letter corresponded with the number on the bag containing the cartridges, not the volunteer, thus all DNAs were deidentified. Spent cartridge casings and buccal swabs were stored at -20°C.

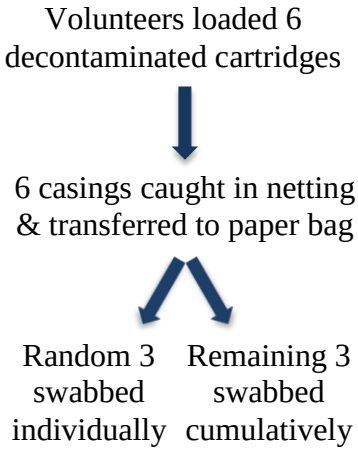
DNA Recovery and Isolation

The casings of three randomly chosen cartridges (L1, L2, and L3) from the manufacturer's box were swabbed using the double-swab technique (Sweet *et al.*, 1997); the first swab used was wetted with approximately 100 μL of digestion buffer (0.1% SDS, 20 mM Tris [pH 7.5], 50 mM EDTA) and then a dry swab was used. Three of the six casings from each Collection 1 volunteer were randomly selected and swabbed with separate pairs of swabs ('individually swabbed') (Figure 6). The remaining three casings were swabbed using a single pair of swabs ('cumulatively swabbed'). The first, sixth, and a random middle casing from Collection 2 were swabbed individually and the other three casings were swabbed cumulatively. Figure 6 also depicts schematics of the design used to collect casings and recover DNAs.

a.



b.



c.

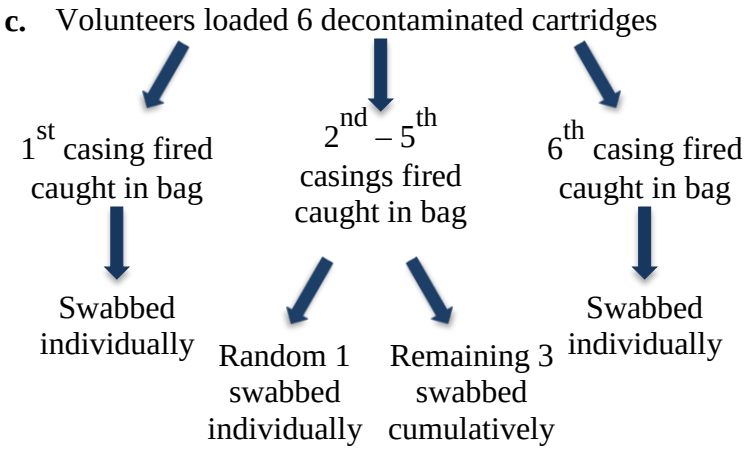


Figure 6. **a**. Cartridge casings individually double-swabbed on the left and cumulatively double-swabbed on the right. **b.** Flow chart of the collection of casings and recovery of cells from Collection 1. **c.** Flow chart of the collection of casings and recovery of cells from Collection 2.

Swab heads were clipped into 1.5 mL microcentrifuge tubes containing 400 µL of digestion buffer and 6 µL of proteinase K (20 mg/mL), the tubes were vortexed for 10 s, and incubated overnight at 55˚C. The swab heads were transferred to spin baskets using forceps and centrifuged at full speed for 4 min. The swab heads were discarded and the flow-throughs were added to the original digestion tubes.

DNAs were extracted by adding 500 µL of phenol to the digestion tubes, which were vortexed for 10 s and centrifuged at full speed for 5 min. The aqueous layers were transferred to tubes containing 500 µL of chloroform, vortexed for 10 s, and centrifuged at full speed for 5 min.

Amicon® Ultra-0.5 mL, 30kDa spin columns (Millipore Corporation, Billerica, MA) were pretreated by adding 1 μL of *Saccharomyces cerevisiae rRNA* (10 mg/mL; Alfa Aesar, Ward Hill, MA) and 499 μL of low TE (10 mM Tris [pH 7.5], 0.1 mM EDTA), centrifuged for 10 min at 14,000 rcf, and the filtrates were discarded. The aqueous layers were transferred to filters, centrifuged for 10 min at 14,000 rcf, and flow-throughs were discarded. The DNAs were washed twice using 300 μL of low TE and centrifuging for 10 min at 14,000 rcf. The filters were inverted into new Amicon® collection tubes and centrifuged for 4 min at 1,000 rcf. Buccal swab DNAs were extracted in the same manner, except three TE (10 mM Tris [pH 7.5], 1 mM EDTA) washes were performed. DNAs were stored at -20°C.

mtDNA Amplification, Sequencing, Analysis of Genetic Profiles, Consistency of Assignments, and Mixtures

Amplification using primer pairs HV1 and HV2 was tested on extract 12C from Collection 1, prior to amplification of all extracts using HV1a, HV1b, and HV2a. PCR, sequencing, analysis of genetic profiles, a blind study, and determination of loader mixtures for all Part B extracts were performed following protocols used in Part A. However, haplotypes of all individuals in the MSU Forensic Biology Laboratory and the 28 volunteers from Collections 1 and 2 were included in the Part B database. Sequences for inconsistent and inconclusive assignments from Collections 1 and 2 were re-analyzed in the Sequencing Analysis Software by activating the mixed base identification (MBI) option set to 30% peak height as recommended by Nickerson *et al*. (1997). A blind study was re-performed using the MBI haplotypes.

Construction of Consensus Haplotypes

Consensus haplotypes for Collections 1 and 2 were generated using data from the three individually swabbed casings (Indiv-C), as well as the individually and cumulatively swabbed casings (All-C). The haplotypes developed with MBI were used for initial inconsistent and inconclusive assignments. A polymorphism that was present in at least n – 1 of the extracts from each loader was included in the consensus profile. Since there were only two individually swabbed casings from bag 33, polymorphisms that were present in both were included in the Indiv-C haplotype. When a polymorphic position used in the consensus profile contained a mixture for all of the extracts, the nomenclature for the mixture of bases was kept; if one or more extracts contained a clean polymorphism, the corresponding non-mixture nomenclature was given. Haplotypes were then reassigned.

Statistical Analyses

An Analysis of Variance (ANOVA) was performed on STR profiles from Orlando (2012) extracts, excluding contaminated samples, to determine if there was a difference in the number of inconsistent alleles among the mtDNA assignments. This was repeated using the frequency of consistent alleles. Residuals were extracted, and the Shapiro-Wilk test was used to assess normality. Homogeneity of variances for each group of mtDNA assignments was compared using Levene's test. If the assumptions of normality and variance were valid, the parametric ANOVA was used to compare the means of the groups, whereas if the assumptions were violated, the non-parametric Kruskal-Wallis test was conducted to compare the medians of the groups. A Pearson's chi-square test for count data or a Fisher’s exact test was performed to determine whether the swabbing method influenced the consistency of assignments and to establish if there

were significant differences between the assignments made. In instances when there were no differences in assignments between the swab methods and Collections, the data were combined. A Pearson's chi-square test was also used to determine if there was a difference in the amount of loader mixtures among the four swabbing categories used for Collection 2. Significance was identified for all tests at $p < 0.05$. XLSTAT (Addinsoft, 1995 – 2013) was used for the Kruskal-Wallis test, while all other statistical tests were performed in R (www.r-project.org).

RESULTS

Part A

Degradation of DNA Recovered from Spent Cartridge Casings

There was substantial degradation of DNAs isolated from the fired casings at both the nuclear and mitochondrial levels. Table 4 lists the frequency of STR alleles consistent with the loader for the 46 Orlando (2012) DNA extracts that were sequenced in this research. The smallest amplicons (D8, D3, D19, and amelogenin) had the highest frequency of amplification, followed by a decreasing trend as the loci became larger, with the CSF locus being the sole exception.

Table 4. Frequency of consistent alleles amplified at each locus illustrating DNA degradation of DNAs from Orlando (2012) extracts that underwent mtDNA analysis. Amplicons are arranged from smallest to largest for each dye. The smaller amplicons, on average, had higher frequencies of amplification compared to the larger amplicons.

Locus	Frequency of Consistent Alleles Per Locus
Blue Dye	
D8	2.48%
D21	0.47%
D7	0.14%
CSF	1.09%
Green Dye	
D3	3.26%
Tho	2.03%
D13	1.09%
D16	1.09%
D2	0.16%
Yellow Dye	
D19	3.19%
vWA	1.67%
TPOX	1.51%
D18	0.67%

Table 4 (cont'd)

Red Dye	
Amelogenin	6.16%
D5	2.31%
FGA	0.43%

The levels of mtDNA degradation from fired cartridge casings are exemplified using Orlando (2012) extracts 22D and 28C (Figure 7). The DNAs amplified using HV1b (F16190 – R16410; 221 bp) and HV2a (F15 – R285; 271 bp). However, they had lower band intensities using HV1 (F15989 – R16410; 422 bp) and showed no amplification using HV2 (F15 – R484; 470 bp).

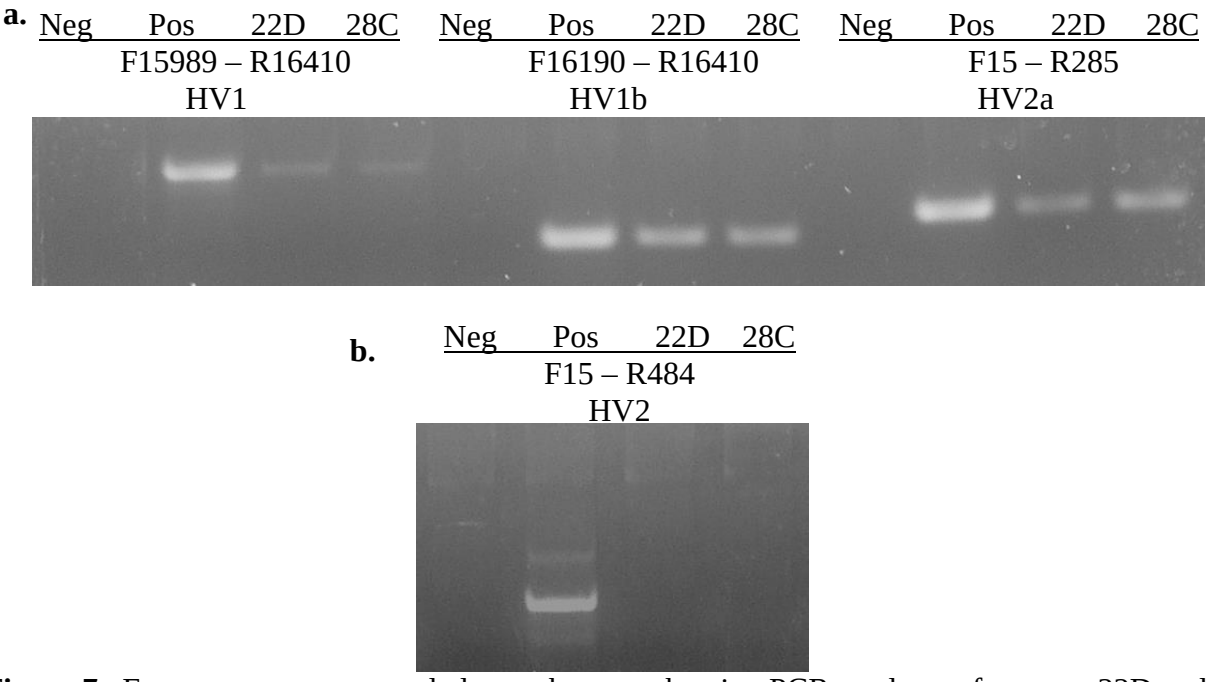


Figure 7. Four percent agarose gel electrophoreses showing PCR products of extracts 22D and 28C from Orlando (2012). Primer pairs were: **a.** HV1 (left), HV1b (center), HV2a (right), and **b.** HV2. No bands were present in the negative controls (Neg), and were in the positive controls (Pos). The extracts amplified using HV1b and HV2a, had decreased amplification using HV1, and no amplification using HV2.

Contamination of Zymo-Spin™ IV-HRC Columns and Cartridges

mtDNA from Zymo-Spin™ IV-HRC column extract T1 amplified using primer pairs HV1a and HV2a (Figure 8). In contrast, T2 did not result in amplification. Three of the six live cartridge casings swabbed by Orlando (2012) prior to handling resulted in mtDNA amplification using both HV1a and HV2a, as did the reagent blank.

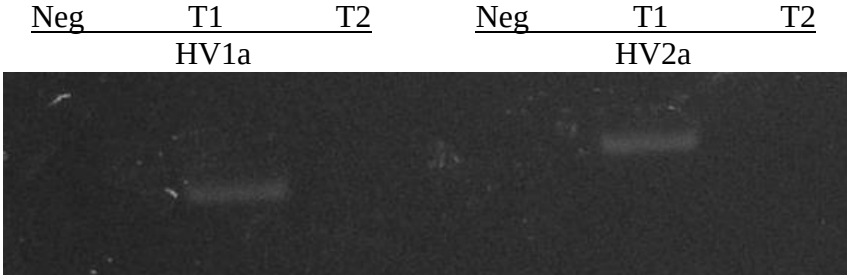


Figure 8. Four percent agarose gel electrophoresis showing PCR results from the Zymo-Spin™ IV-HRC column contamination test. No bands were present in the negative controls and filtrate T2 using HV1a and HV2a, but there were bands for T1.

mtDNA Amplification, Sequencing, and Assignments of Orlando (2012) DNAs

The average amplification rate on the first try of Orlando (2012) extracts was 97.7% (HV1a, 95.8%; HV1b, 100.0%; HV2a, 98.1%). Forty-two of the 46 extracts had evidence of mtDNA mixture. Eight individually and one cumulatively swabbed casing DNAs contained all polymorphisms consistent with the primary investigator from the Orlando (2012) study and thus were deemed contaminated and were eliminated from the pool of data. Figure 9a shows the assignments made for the 30 individually swabbed casings; 11 of the assignments were consistent-single (36.7%), 3 were inconsistent (10.0%), and 16 were inconclusive (53.3%), while no consistent-multiple assignments were made. From the seven cumulatively swabbed casings, three were consistent-single (42.9%) and the remaining four were inconclusive (57.1%; Figure

9b). Overall, the swabbing method had no significant effect on assignment categories (p = 0.680). There were significantly more consistent-single than inconsistent assignments as well as inconclusive than inconsistent assignments when data from individually and cumulatively swabbed casings were combined (p = 0.008 and p = 3.93E-4, respectively; Table 5). However, there was not a significant difference between the number of consistent-single and inconclusive assignments (p = 0.304).

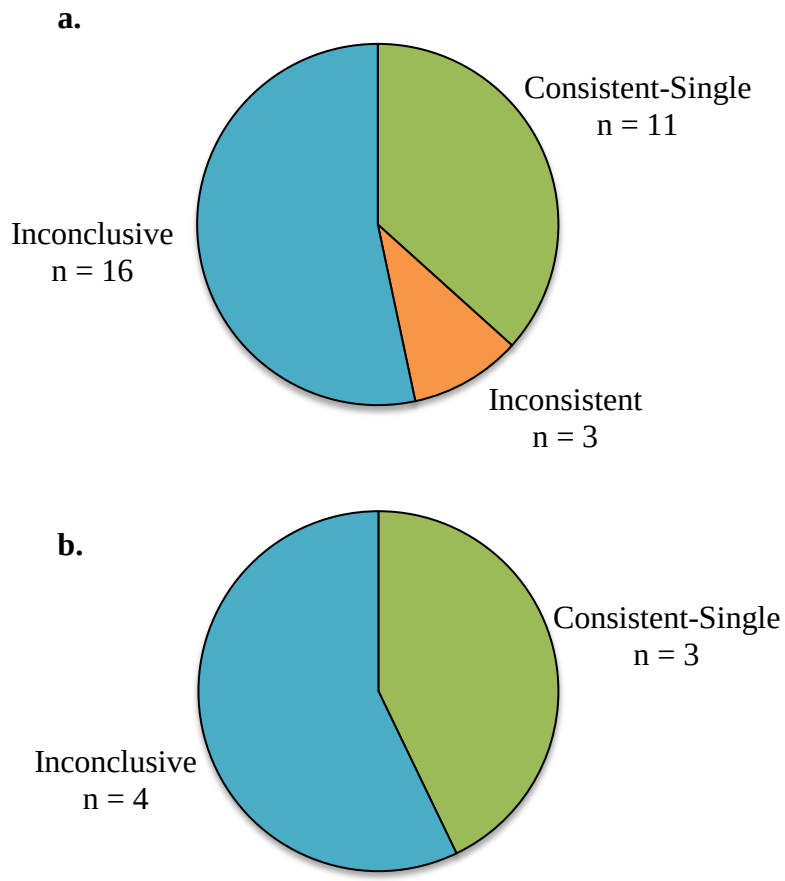


Figure 9. Assignments made for Orlando (2012) DNA extracts: **a.** individually swabbed and **b.** cumulatively swabbed spent cartridge casings. The majority of assignments for both swab methods were inconclusive.

Table 5. Pearson's chi-square p-values comparing assignments for Part A individually and cumulatively swabbed spent cartridge casings after they were combined. The assignment with the higher frequency is listed first. There were significantly more consistent-single than inconsistent, and inconclusive than inconsistent assignments (p = 0.008 and 3.93E-4, respectively).

Assignment Comparison	p-value
Consistent-Single vs. Inconsistent	0.008
Inconclusive vs. Consistent-Single	0.304
Inconclusive vs. Inconsistent	3.93E-4

Table 6 shows that consistent-single mtDNA assignments had, on average, amplification of 17.3% of STR alleles consistent with the loader, while inconsistent assignments had a frequency of 15.3%, and inconclusive assignments had 10.4%. Also listed in Table 6 is the number of inconsistent STR alleles for each DNA extract; consistent-single assignments had an average of 1.1 inconsistent alleles, inconsistent mtDNA assignments had 2.0, and inconclusive assignments had 3.8. Although consistent-single mtDNA assignments had the highest frequency of consistent STR alleles and fewest inconsistent STR alleles, the average frequencies of consistent alleles and number of inconsistent alleles from the three groups of mtDNA assignments did not differ significantly (ANOVA, p = 0.175 and Kruskal-Wallis, p = 0.101, respectively).

Table 6. The percentage of STR alleles consistent with the loader, number of alleles inconsistent with the loader, and mtDNA assignments for Orlando (2012) DNA extracts. Extract numbers followed by an A indicate cumulatively and letters B – F indicate individually swabbed casings. Extracts with a consistent-single mtDNA assignment had a higher average frequency of consistent alleles, compared to inconsistent assignments (17.3% vs. 15.3%, respectively).

Extract	Alleles Consistent with Loader	Alleles Inconsistent with Loader	mtDNA Assignment
2A	35.7%	3	Consistent-Single
2B	35.7%	1	Consistent-Single
2D	21.4%	0	Consistent-Single
6A	28.6%	1	Consistent-Single
6B	25.0%	0	Consistent-Single
6C	17.9%	3	Consistent-Single
6D	10.7%	1	Consistent-Single
6E	25.0%	2	Consistent-Single
22C	3.4%	1	Consistent-Single
22D	17.2%	0	Consistent-Single
22E	0.0%	1	Consistent-Single
22F	3.4%	0	Consistent-Single
28A	18.5%	1	Consistent-Single
28E	0.0%	1	Consistent-Single
2C	17.9%	0	Inconsistent
8B	20.7%	6	Inconsistent
28F	7.4%	0	Inconsistent
3A	22.2%	7	Inconclusive
3B	7.4%	4	Inconclusive
3C	0.0%	1	Inconclusive
3D	33.3%	17	Inconclusive
3E	11.1%	1	Inconclusive
3F	7.4%	4	Inconclusive
5A	18.5%	0	Inconclusive
5B	0.0%	2	Inconclusive
5C	0.0%	0	Inconclusive
5D	0.0%	3	Inconclusive
5E	11.1%	3	Inconclusive
5F	11.1%	6	Inconclusive
7C	28.6%	14	Inconclusive
7D	0.0%	0	Inconclusive
8A	13.8%	0	Inconclusive
22A	10.3%	6	Inconclusive
22B	10.3%	2	Inconclusive
28B	7.4%	4	Inconclusive
28C	14.8%	0	Inconclusive
28D	0.0%	2	Inconclusive

Table 6 (cont'd)

2E	7.1%	0	Contaminated
2F	0.0%	1	Contaminated
6F	39.3%	8	Contaminated
7A	17.9%	1	Contaminated
7B	10.7%	4	Contaminated
7E	17.9%	3	Contaminated
7F	7.1%	2	Contaminated
8C	24.1%	8	Contaminated
8D	13.8%	5	Contaminated
Average	17.3%	1.1	Consistent-Single
Average	15.3%	2.0	Inconsistent
Average	10.4%	3.8	Inconclusive

Part B

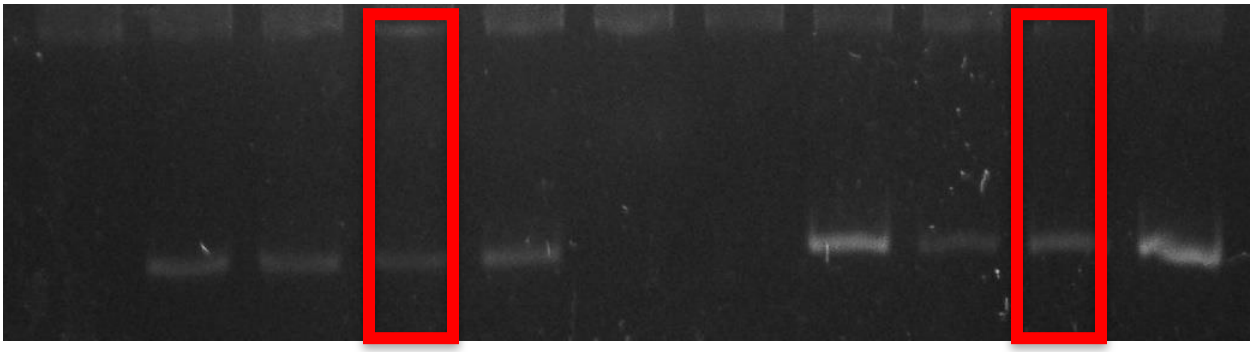
Degradation of DNA Recovered from Spent Cartridge Casings

PCR results from Collection 1 extract 12C exhibited DNA degradation (Figure 10). There was mtDNA amplification using primer pairs HV1a and HV2a, no amplification using HV1, and weak amplification using HV2. Given these results, the smaller amplicons were assayed in subsequent experiments. The average amplification rate on the first attempt was 97.9% (HV1a, 97.3%; HV1b, 99.1%; HV2a, 97.3%). A second PCR with double the amount of input DNA yielded successful amplification in all other instances.

a.

Neg 12A 12B 12C 12D Neg RB 12A 12B 12C 12D

HV1a HV2a



b. Neg 12C Neg 12C

HV1 HV2

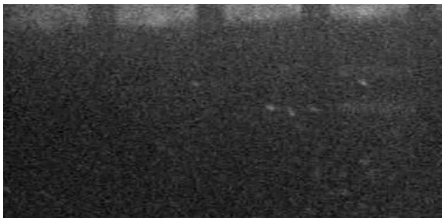


Figure 10. a. Four percent agarose gel electrophoreses showing PCR results of Collection 1 extract 12C using primer pairs HV1a, HV2a, **b**. HV1, and HV2. In **8a.**, PCR results for extracts 12A – D are shown; 12C extracts are boxed in red. No bands were present in negative controls and the reagent blank (RB). Extract 12C amplified using HV1a and HV2a, had a lower level of amplification using HV2, and no amplification using HV1.

Contamination of Cartridges and the Magazine

mtDNA from the three live cartridge casings tested in Part B amplified using primer pairs HV1a and HV2a (L1 – 3; Figure 11). The mtDNA recovered from the magazine prior to Collection 1 yielded a haplotype of 16126C, 73G, and 263G using the same primer pairs.

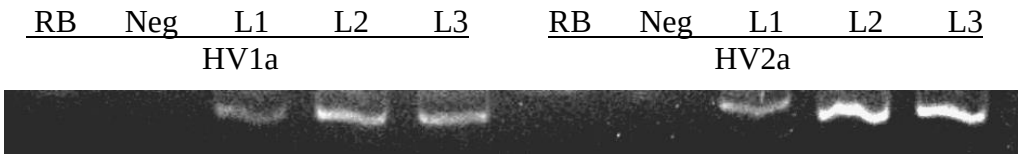


Figure 11. Four percent gel electrophoresis showing PCR results from live cartridges L1 – 3 using primer pairs HV1a and HV2a. L1 – 3 amplified, while no bands were present in the reagent blanks or negative controls.

mtDNA Haplotypes and Loader Assignation for Collections 1 and 2

Three volunteers (SS, TT, and XX) from Collection 2 expressed heteroplasmy. Furthermore, the only difference between volunteer VV (16069T, 16126C, 73G, 185A, 228A, 263G) and XX (16069T, 16126Y, 73G, 185A, 228A, 263G) was a heteroplasmic site at position 16126 for volunteer XX. Therefore, all extracts with a C at position 16126 in addition to the remaining five polymorphisms were assigned to both volunteers.

Figure 12 illustrates that 7 of 12 (58.3%) individually swabbed casings and 3 of 4 (75.0%) cumulatively swabbed casings from Collection 1 yielded consistent-single mtDNA assignments. The remaining five assignments for individually swabbed casings were inconclusive (41.7%) and the fourth cumulatively swabbed extract yielded an inconsistent assignment (25.0%). The mtDNA assignments were not significantly different between the two swab methods ($p = 0.091$).

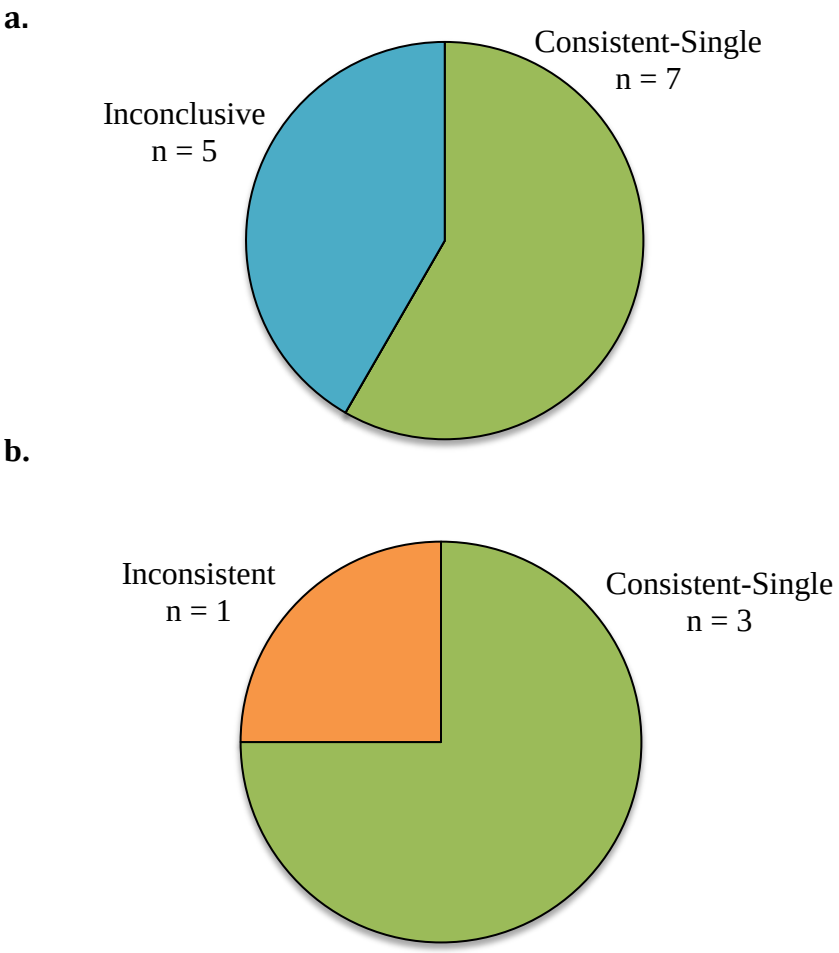


Figure 12. Assignments made for Collection 1: **a.** individually swabbed and **b.** cumulatively swabbed spent cartridge casings. The majority of assignments for both swab methods were consistent-single.

One cartridge from bag 33 was missing, resulting in one less individually swabbed sixth ejected casing for Collection 2 (23 versus 24 for the other categories). Figure 13 shows that consistent-single assignments comprised 12 (50.0%) of the first, 9 (37.5%) of the random middle, 10 (43.5%) of the sixth, and 12 (50.0%) of the cumulatively swabbed spent casings from Collection 2. Consistent-multiple assignments amounted to 5 (20.8%) of the first, 3 (12.5%) of the random middle, 4 (17.4%) of the sixth, and 5 (20.8%) of the cumulatively swabbed casings.

Inconsistent assignments were obtained from 2 (8.3%) of the first, 2 (8.3%) of the random middle, 3 (13.0%) of the sixth, and 1 (4.2%) of the cumulatively swabbed casings. Inconclusive assignments included 5 (20.8%), 10 (41.7%), 6 (26.1%), and 6 (25.0%), respectively. The assignments were independent of the swab method ($p = 0.883$), and remained so after data from all individually swabbed cartridge casings (first, random middle, and sixth) were combined ($p = 0.768$). There was no significant difference between assignments made for individually swabbed casings between Collections 1 and 2 ($p = 0.243$). Similarly, the assignments made for cumulatively swabbed casings between the Collections were not significantly different ($p = 0.230$). The individually swabbed casings from both Collections were combined, and compared to the combined cumulatively swabbed casings, which also resulted in no difference between swabbing methods ($p = 0.758$). Finally, there was no difference when the consistent-single and consistent-multiple assignments were combined and assignments were compared between the combined individual and cumulative swab methods ($p = 0.557$).

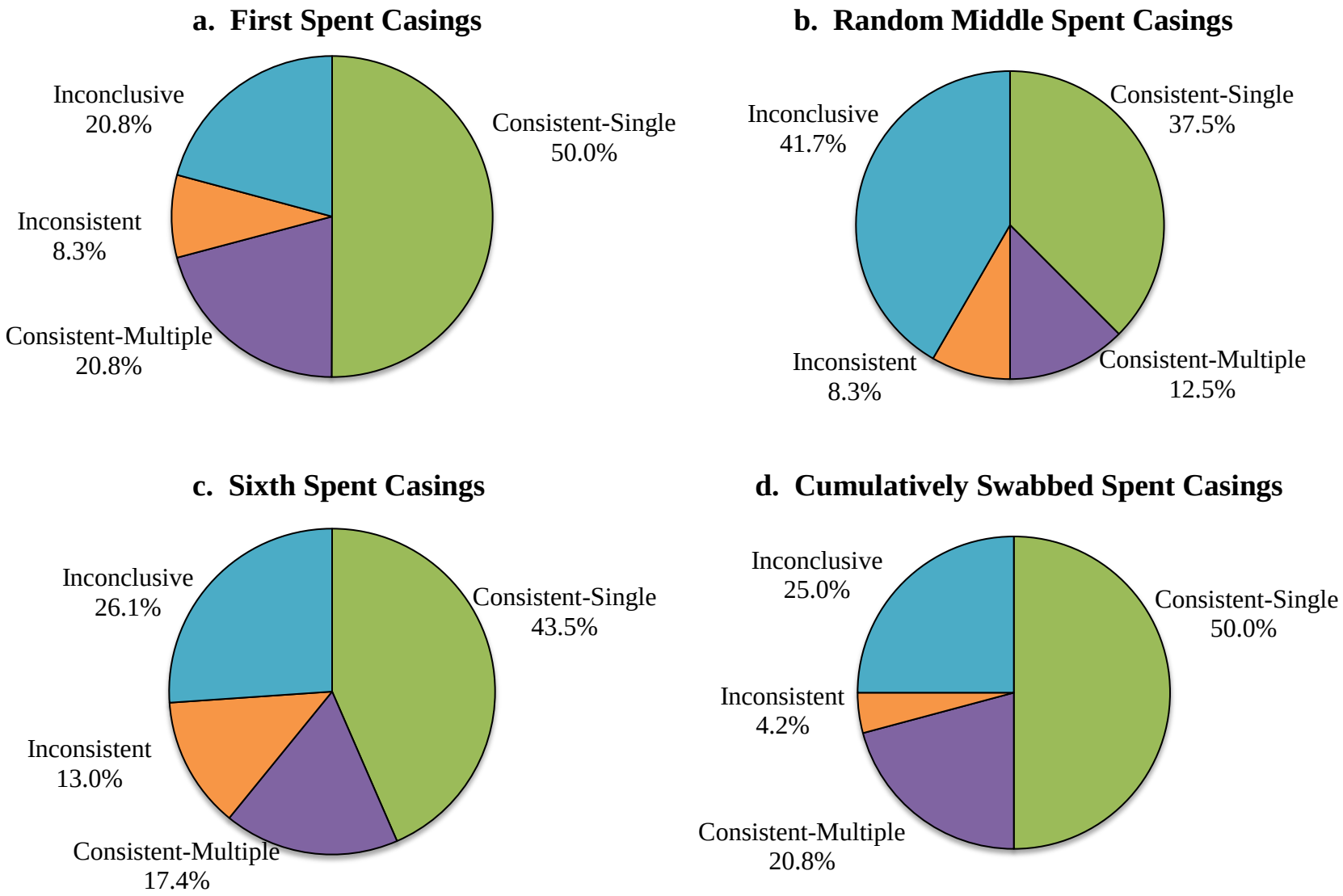


Figure 13. Assignments made for spent casings from Collection 2: **a.** the first (n = 24), **b.** a random middle (n = 24), **c.** the sixth (n = 23), and **d.** three cumulatively swabbed spent cartridge casings (n = 24). When consistent-single and consistent-multiple assignments were combined, they comprised 50.0% or more of assignments for the four categories.

The four assignment categories were not equally obtained (p = 8.51E-9) after data from individually and cumulatively swabbed casings were combined from Collections 1 and 2. This was also true when consistent-single and consistent-multiple assignments were combined (p = 7.26E-12). Figure 14 shows the percentage of assignments made for the 111 extracts from Collections 1 and 2; 70 (63.1%) of the extracts resulted in consistent assignments, 9 (8.1%) were inconsistent assignments, and 32 (28.8%) were inconclusive. Results displayed in Table 7 indicate there was a significantly greater number of consistent-combined assignments than inconsistent assignments (p = 6.74E-12), consistent-combined than inconclusive assignments (p = 1.68E-4), and inconclusive than inconsistent assignments (p = 3.28E-4).

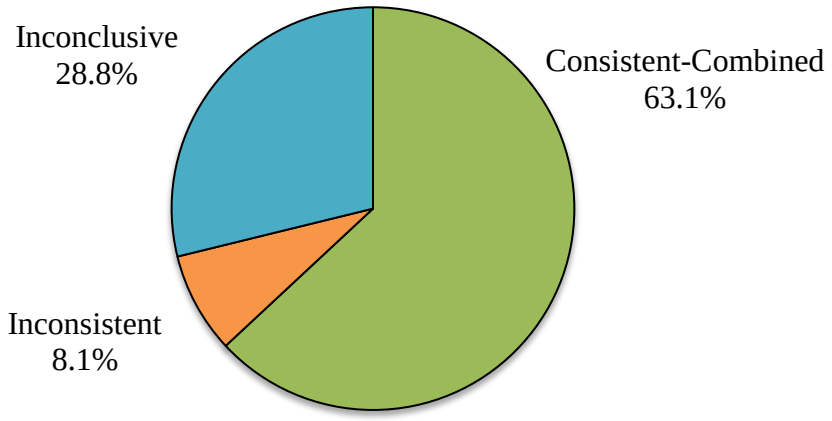


Figure 14. Assignments made for all profiles from Collections 1 and 2; consistent-single and consistent-multiple assignments were combined. The majority of assignments for all Part B extracts were consistent-combined (63.1%).

Table 7. Pearson's chi-square p-values obtained when comparing assignments made for combined individually and cumulatively swabbed spent cartridge casings from Collections 1 and 2. The assignment with the higher frequency is listed first. There were significantly more consistent-combined than inconsistent and inconclusive assignments (6.74E-12 and 1.68E-4, respectively). There were more inconclusive than inconsistent assignments (3.28E-4).

Assignment Comparison	p-value
Consistent-Combined vs. Inconsistent	6.74E-12
Consistent-Combined vs. Inconclusive	1.68E-4
Inconclusive vs. Inconsistent	3.28E-4

Detection of Mixtures from Collections 1 and 2

Table 8 shows that one of the seven consistent individually swabbed and two of the three cumulatively swabbed casings from Collection 1 contained mixtures. The assignments made with and without MBI, which did not resolve any inconsistent or inconclusive assignments made for Collection 1, are also compared. The inconsistent assignment and two of the five (40.0%) inconclusive assignments had a mixture with a haplotype consistent with the loader. Seven of the 16 (43.8%; 1A, 1B, 1C, 1D, 7B, 7CD, and 13C) extracts contained at least one polymorphism that could not have originated from any of the loaders (Appendix B).

Table 8. mtDNA assignments made for haplotypes developed with and without MBI for Collection 1. The fourth column indicates if the extracts contained a mixture that included a haplotype consistent with the loader (loader mixture). Extract numbers followed by an A indicate cumulatively swabbed and letters B – D represent individually swabbed spent casings. MBI did not alter any inconsistent or inconclusive assignments. Six of the 16 extracts contained a loader mixture.

Extract	mtDNA Assignment without MBI	mtDNA Assignment with MBI	Loader Mixture
7A	Consistent-Single		Yes
7C	Consistent-Single		Yes
12A	Consistent-Single		N/A
12B	Consistent-Single		N/A
12C	Consistent-Single		N/A
12D	Consistent-Single		N/A
13A	Consistent-Single		Yes
13B	Consistent-Single		N/A
13C	Consistent-Single		Indeterminate*
13D	Consistent-Single		N/A
1A	Inconsistent	Inconsistent	Yes
1B	Inconclusive	Inconclusive	Yes
1C	Inconclusive	Inconclusive	Yes
1D	Inconclusive	Inconclusive	No
7B	Inconclusive	Inconclusive	No
7D	Inconclusive	Inconclusive	No

N/A = not applicable
*the six polymorphic positions consistent with the loader did not contain a mixture, but two additional mixture positions were detected

Eight of the 17 (47.1%) consistent-combined first from Collection 2, 5 of the 12 (41.7%) random middle, 11 of the 14 (78.6%) sixth, and 8 of the 17 (47.1%) cumulatively swabbed cartridge casings contained loader mixtures (Table 9). MBI for the 35 inconsistent and inconclusive assignment from Collection 2 yielded 17 new assignments (bolded). Nine of these became consistent-multiple. However, seven inconclusive assignments were converted to inconsistent assignments and one inconsistent assignment became inconclusive. Four (50.0%) of the extracts with an initial inconsistent assignment and 17 (63.0%) with inconclusive assignments contained a mixture consistent with the loader, whereas it was not possible to

determine if the remaining two extracts contained a loader mixture. Two of the 95 (2.1%; 11C and 20C) extracts from Collection 2 had at least one polymorphism that could not have originated from any of the loaders (Appendix B).

Table 9. mtDNA assignments made for haplotypes developed with and without MBI for Collection 2. The fourth column indicates if the extracts contained a loader mixture. Extract numbers followed by an A represent cumulatively swabbed spent casings, and individually swabbed spent casings are designated by B (first), C (random middle), and D (sixth). MBI altered 17 of the 35 inconsistent and inconclusive assignments. Fifty-three of the 95 extracts contained a loader mixture.

Extract	mtDNA Assignment without MBI	mtDNA Assignment with MBI	Loader Mixture
8B	Consistent-Single		Yes
8D	Consistent-Single		Yes
9A	Consistent-Multiple		N/A
9B	Consistent-Multiple		N/A
9C	Consistent-Multiple		N/A
9D	Consistent-Multiple		Yes
11A	Consistent-Single		N/A
11B	Consistent-Single		N/A
11D	Consistent-Single		N/A
15A	Consistent-Multiple		Yes
16A	Consistent-Single		Yes
16B	Consistent-Single		N/A
16C	Consistent-Single		N/A
16D	Consistent-Single		N/A
17A	Consistent-Single		N/A
17B	Consistent-Single		N/A
17C	Consistent-Single		N/A
17D	Consistent-Single		Yes
18A	Consistent-Multiple		N/A
18B	Consistent-Multiple		Yes
18D	Consistent-Multiple		Yes
19A	Consistent-Single		N/A
19B	Consistent-Single		Yes
19C	Consistent-Single		Yes

Table 9 (cont'd)

19D	Consistent-Single		Yes
20A	Consistent-Multiple		N/A
20B	Consistent-Multiple		N/A
20D	Consistent-Multiple		Yes
21A	Consistent-Single		Yes
21B	Consistent-Single		Yes
21C	Consistent-Single		Yes
21D	Consistent-Single		Yes
24A	Consistent-Single		Yes
24C	Consistent-Single		Yes
25B	Consistent-Multiple		Yes
25C	Consistent-Multiple		Yes
25D	Consistent-Multiple		Yes
27A	Consistent-Single		Yes
30B	Consistent-Single		Yes
30C	Consistent-Single		N/A
30D	Consistent-Single		Yes
31A	Consistent-Single		Yes
32A	Consistent-Single		Yes
32B	Consistent-Single		N/A
32C	Consistent-Single		Yes
32D	Consistent-Single		Yes
33A	Consistent-Single		N/A
33B	Consistent-Single		N/A
34A	Consistent-Single		N/A
34B	Consistent-Single		N/A
34C	Consistent-Single		N/A
34D	Consistent-Single		Yes
35B	Consistent-Single		Yes
36A	Consistent-Multiple		Yes
36B	Consistent-Multiple		Yes
36C	Consistent-Multiple		N/A
37A	Consistent-Single		N/A
37B	Consistent-Single		N/A
37C	Consistent-Single		N/A
37D	Consistent-Single		N/A
15C	Inconsistent	Inconsistent	No
15D	Inconsistent	**Consistent-Multiple**	Yes

Table 9 (cont'd)

27B	Inconsistent	Inconsistent	No
27C	Inconsistent	**Consistent-Multiple**	Yes
27D	Inconsistent	Inconsistent	Yes
31B	Inconsistent	**Consistent-Multiple**	Yes
31D	Inconsistent	**Inconclusive**	Indeterminate*
38A	Inconsistent	**Inconsistent**	No
8A	Inconclusive	Inconclusive	No
8C	Inconclusive	Inconclusive	No
11C	Inconclusive	Inconclusive	Yes
15B	Inconclusive	**Inconsistent**	No
18C	Inconclusive	Inconclusive	Yes
20C	Inconclusive	Inconclusive	Yes
22A	Inconclusive	Inconclusive	No
22B	Inconclusive	**Inconsistent**	No
22C	Inconclusive	Inconclusive	No
22D	Inconclusive	**Inconsistent**	No
23A	Inconclusive	Inconclusive	Yes
23B	Inconclusive	**Consistent-Multiple**	Yes
23C	Inconclusive	Inconclusive	Yes
23D	Inconclusive	Inconclusive	Yes
24B	Inconclusive	Inconclusive	Yes
24D	Inconclusive	**Consistent-Multiple**	Yes
25A	Inconclusive	Inconclusive	No
30A	Inconclusive	**Inconsistent**	Yes
31C	Inconclusive	**Consistent-Multiple**	Yes
33C	Inconclusive	**Consistent-Multiple**	Yes
35A	Inconclusive	**Consistent-Multiple**	Yes
35C	Inconclusive	Inconclusive	Indeterminate**
35D	Inconclusive	**Consistent-Multiple**	Yes
36D	Inconclusive	**Inconsistent**	Yes
38B	Inconclusive	**Inconsistent**	Yes
38C	Inconclusive	Inconclusive	Yes
38D	Inconclusive	**Inconsistent**	No

N/A = not applicable

*not possible to determine if one of the polymorphisms consistent with the loader was above background

**contained four of five polymorphisms consistent with the loader

Table 10 summarizes the mixture frequencies of consistent-combined assignments from Collection 2, as well as the percentage of extracts containing a loader mixture: 8 of the 17 (47.1%) first, 5 of the 12 (41.7%) random middle, 11 of the 14 (78.6%) sixth, and 8 of the 17 (47.1%) cumulatively swabbed casings had a mixture. Adding in inconclusive and inconsistent assignments, 12 of the 24 (50.0%) first, 13 of the 24 (54.2%) random middle, 17 of the 23 (73.9%) sixth, and 11 of the 24 (45.8%) cumulatively swabbed casings contained a mtDNA mixture consistent with the corresponding loader's DNA. The frequency of loader mixture was not statistically different among the four groups ($p = 0.223$).

Table 10. Percentage of consistent-combined assignments, inconsistent and inconclusive assignments, mixture, and loader mixture made for the four Collection 2 categories. The second and fourth columns summarize the percentage of consistent-combined, and inconsistent and inconclusive assignemnts made, respectively. The third and fifth columns indicate the percentage of mixture and loader mixture, respectively, for the two combined assignment types. The sixth column contains the percentage of the four categories with a loader mixture. The sixth spent casings had the highest frequency of loader mixture (73.9%), while cumulatively swabbed casings had the lowest (45.8%).

	Consistent-Combined		**Inconsistent & Inconclusive**		**Loader**
	Total	**Mixture**	**Total**	**Loader Mixture**	**Mixture**
First	70.8%	47.1%	29.2%	57.1%	50.0%
Random Middle	50.0%	41.7%	50.0%	66.7%	54.2%
Sixth	60.9%	78.6%	39.1%	66.7%	73.9%
Cumulatively	70.8%	47.1%	29.2%	42.9%	45.8%

Assignments for Collections 1 and 2

A summary of the assignments made for individually, cumulatively, and all casings from Collections 1 and 2, as well as the percentage of assignments obtained with the consensus methods (All-C and Indiv-C) are shown in Table 11. Of the 83 individually swabbed casings, 50 (60.2%) yielded consistent-combined assignments, 7 (8.4%) were inconsistent, and 26 (31.3%)

were inconclusive. From the 28 cumulatively swabbed casings, 20 (71.4), 2 (7.1%), and 6 (21.4%) respective assignments were made. The 28 Indiv-C haplotypes yielded 19 (67.9%) consistent-combined, 4 (14.3%) inconsistent, and 5 (17.9%) inconclusive assignments. All-C resulted in 19 (67.9%) consistent-combined assignments, 3 (10.7%) inconsistent, and 6 (21.4%) inconclusive. The assignments were not significantly different among the five methods ($p = 0.858$).

Table 11. Percentage of consistent-combined, inconsistent, and inconclusive assignments made from individually, cumulatively, and all swabbed spent casings, as well as All-C and Indiv-C from Part B. Cumulatively swabbed casings had the highest frequency of consistent-combined assignments (71.4%), while individually swabbed casings had the lowest frequency (60.2%). Both Indiv-C and All-C resulted in 67.9% consistent-combined assignments.

	Consistent-Combined	**Inconsistent**	**Inconclusive**
Individually Swabbed	60.2%	8.4%	31.3%
Cumulatively Swabbed	71.4%	7.1%	21.4%
All Swabbed	63.1%	8.1%	28.8%
Indiv-C	67.9%	14.3%	17.9%
All-C	67.9%	10.7%	21.4%

DISCUSSION

Spent cartridge casings found at a crime scene may harbor valuable genetic information that can be used by law enforcement to connect a perpetrator to a crime. STR analysis has become the method of choice for DNA identification in forensic science because the genotypic information obtained has exceptional discriminating capabilities. However, STR analysis of touch DNA and compromised biological samples is not as successful as analysis of high molecular weight DNA. Despite attempts to overcome challenges associated with touch DNA recovered from spent casings, such analysis has met with minimal success, even in controlled environments (Spear *et al.*, 2005; Horsman-Hall *et al*., 2009; Branch, 2010; Dieltjes *et al*., 2011; Orlando, 2012). In contrast, mtDNA analysis from spent cartridge casings has not been previously investigated; thus the primary objective of the research presented here was to determine if the unique traits of mtDNA would allow forensic biologists to successfully develop the haplotype of a loader.

Humans shed many thousands of skin cells a day (Roberts and Marks, 1980) and as a result, DNA is deposited through physical contact during the normal use of objects (van Oorshot and Jones, 1997). DNA has been recovered from items such as steering wheels (Pizzamiglio *et al*., 2004; Brevnov *et al*., 2009), drinking containers (Abaz *et al*., 2002; Brevnov *et al*., 2009), various types of handles (Ladd *et al*., 1999), firearms (Horsman-Hall *et al*., 2009; Richert, 2011; Nunn, 2013), backpacks (Hoffmann *et al*., 2012), and improvised explosive devices (Esslinger *et al*., 2004). Horsman-Hall *et al*. (2009) hypothesized that full STR profiles were unlikely to be obtained from DNA left on spent cartridge casings due to a combination of low levels of deposition, PCR inhibition from gunshot residue, and DNA degradation caused by temperatures

reaching upwards of 1800°C in the chamber of the firearm. Similar to the aforementioned touch DNA studies, the authors recovered DNA from live cartridge casings handled for 30 s (more than the time needed to load a cartridge). However, like other handled items, the amount of DNA deposited onto cartridge casings during the simulated loading process was low—14.4 pg/μL—less than the concentration recommended for STR analysis.

PCR inhibitors, likely in gunshot residue, co-extract with DNA, providing an added challenge in analyzing recovered DNAs from spent casings. Horsman-Hall *et al*. (2009) detected PCR inhibition from 11% of shotgun shells and Orlando (2012) encountered inhibition during DNA quantification prior to additional purification using Zymo-Spin™ IV-HRC columns. Owing to this, BSA was added to PCR assays in this study. BSA has been shown to help overcome PCR inhibition (Kreader, 1996; Eilert and Foran, 2009), likely due to inhibitor binding capabilities (Dufour and Dangles, 2005; Fasano *et al*., 2005). BSA is a common additive in some STR kits, such as Identifiler®, Identifiler® Plus, MiniFiler™ (Applied Biosystems, 2012), and PowerPlex® 16 System (Promega, 2013) and is included in the standard operating procedure for amplifications of degraded skeletal remains at The Armed Forces DNA Identification Laboratory (AFDIL), which uses 0.625 μg/μL of BSA in PCR (Edson *et al*., 2004). The mtDNA PCR containing BSA in this study did not appear to be inhibited.

DNA degradation is another obstacle faced by forensic biologists. Various environmental factors, such as sunlight, humidity, and heat lead to the breakdown of DNA. The barrel of an M16A1 rifle after firing 180 rounds over the span of 45 s has been documented to reach 889°C (Elbe, 1975), although this is only half the 1800°C value purported by Horsman-Hall *et al*. (2009). DNA begins to degrade at 130°C under arid conditions, with full degradation documented at 190°C (Karni *et al*., 2013). Using a thermal imaging camera, Gashi *et al*. (2010)

reported the temperature of 9 mm brass cartridge casings reaches a maximum of 63°C during firing. Therefore, the outer surfaces of casings may not get hot enough to degrade the DNA, particularly the first few that are fired. DNA is also degraded during keratinization (Kita *et al*., 2008), thus it is expected that DNA from cells shed onto touched items is degraded. The lack of amplification of loader alleles at the larger loci in Part A indicated the samples were degraded (Bender *et al.*, 2004). Furthermore, the MiniFiler™ STR kit, specifically designed to target small amplicons (70 – 283 bp in length) (Applied Biosystems, 2012c), has been tested on DNA recovered from spent casings, but full STR profiles were rarely obtained (Horsman-Hall *et al*., 2009; Branch, 2010). mtDNA degradation was also encountered in this study; larger mtDNA amplicons (~450 bp) either resulted in minimal or no amplification, whereas ~250 bp amplicons almost always amplified. As a result, overlapping mtDNA amplicons were successfully used to generate mtDNA profiles. Since forensic laboratories strive to maximize casework turn-around rates, it would be less time consuming to initially target smaller overlapping amplicons that amplify more reliably (Gabriel *et al.*, 2001), as opposed to first attempting to amplify larger amplicons and later resorting to smaller amplicons after amplification failure.

Prior to this study, it was not known whether mtDNA results could be obtained from spent casings. Therefore, Orlando (2012) DNA extracts, which were available at the MSU Forensic Biology Laboratory, were processed in Part A to answer the primary question: *can mtDNA haplotypes consistent with the loader be developed from DNA left behind on cartridge casings during loading?* It was hypothesized that loaders' cells would be deposited onto a cartridge casings during loading, and as a result, their mtDNA profiles would be obtained from the spent casings. This was the case for 14 of the 37 haplotypes developed from DNAs recovered in Part A. Only one of the four extracts that did not contain a mtDNA mixture

resulted in a haplotype consistent with the loader, indicating the DNA extractions from three of the casings had become contaminated. The Zymo-Spin™ IV-HRC columns used to remove inhibitors were not UV irradiated prior to use (Orlando, 2012), and given mtDNA was recovered from a column, the possibility that the extracts were contaminated via the columns cannot be ruled out. However, the haplotypes potentially resulting from contamination were not consistent, which is contrary to a single contamination source. In this regard it is possible that there were multiple sources of DNA that contaminated the Zymo™ columns. To determine if the columns did in fact contribute to the contamination, mtDNAs recovered from several columns would need to be sequenced and haplotypes compared to the casing extracts.

The second question in this study, *how do mtDNA results from spent casings compare to STR results?*, was next examined by comparing mtDNA data to the Orlando (2012) Identifiler® Plus data. mtDNA haplotypes consistent with the loader were generated for 14 of the extracts, whereas none yielded full STR profiles. Furthermore, less than a fifth of the alleles from the loader amplified. These differences likely resulted from the heightened sensitivity of mtDNA analysis. In addition, possible correlations between mtDNA mixtures and non-loader STR alleles were examined. Since almost all of the mtDNA profiles were mixtures, it is not surprising that STR alleles inconsistent with the loader also amplified. However, 11 of the extracts that contained a mtDNA mixture did not have any inconsistent STR alleles. Therefore, the heightened sensitivity of mtDNA analysis resulted in the detection of non-loader mtDNA from some extracts, while STR analysis did not. Nevertheless, it would still be beneficial to perform mtDNA analysis when casings are recovered from crime scenes, since haplotypes were developed from all casings.

Data collected at the MSU Forensic Biology Laboratory subsequent to the Orlando (2012) study demonstrated an increase in DNA yields when Amicon® columns were pretreated with RNA. Consequently, the loss of the loaders' DNAs during purification might have caused a decrease in the recovery of loader alleles. Furthermore, as discussed above, contamination from the Zymo^{TM} columns could have resulted in inconsistent alleles. Owing to the amount of DNA contamination in the Orlando (2012) DNA extracts, a new round of firing was undertaken (Part B) and precautions were made to minimize the risk of contamination: the Zymo^{TM} columns were not used, the primary analyst wore a hairnet, and gloves were periodically wiped with 70% ethanol during pre-PCR steps to remove any DNAs that may have been on the gloves.

Despite the increased precautions taken to avoid contamination, the netting used to catch the fired casings still represented a possible source of contamination in Collection 1 of Part B. If exogenous cells on the netting contaminated the casings, a lower frequency of consistent assignments and/or more mixtures would be expected from Collection 1 compared to Collection 2 when the netting was not used. However, Collections 1 and 2 had similar levels of consistent assignments (62.5% and 63.2%, respectively), while a greater percentage of DNA extracts from Collection 2 contained a loader mixture (37.5% vs. 55.8%). The lower frequency of loader mixture may be due to chance, given that the sample size was small (n = 16). Interestingly, 43.8% of Collection 1 DNAs contained at least one mtDNA polymorphism that could not have been contributed by any of the loaders, compared to only 2.1% from Collection 2, which suggests that exogenous cells on the netting likely contributed to contamination. However, this number may be skewed given that Collection 2 contained many more loader haplotypes, thus any contaminating mtDNA polymorphisms would be less likely to be inconsistent with all loaders. Ultimately, since there were similar levels of consistent assignments between the Collections and

a greater percentage of loader mixture from Collection 2 extracts, the plastic bags did not appear to provide an advantage in preventing contamination, and the source of contamination was likely not due to the way the casings were caught. Of course, since casings ejected at a crime scene fall onto less than pristine surfaces, the use of the nets likely comes closer to simulating casework conditions than collecting casings in individual bags.

Horsman-Hall *et al*. (2009) found that alleles consistent with previous loaders (average of 3 alleles) amplified using MiniFiler**TM**, when post-firing swabs of the breechface, ejection port, and chamber of firearms were tested. If DNA is already present on the surfaces of the firearms, it is possible that casings could become contaminated when they come into contact with them, resulting in mixtures. Moreover, gun sharing is becoming more common among gang members (Freund, 2012), and the possibility of mixtures is likely increasing. Therefore, the use of one firearm for each Collection in this study mimicked a scenario increasingly encountered in firearm crimes. If contamination of the casings from the surfaces of a firearm does occur, haplotypes consistent with the previous loader(s) would be detected when sharing guns. Taking note of the sequence that cartridges are loaded into multiple magazines and are fired would allow researchers to examine any such correlation in more detail; in an effort to maintain anonymity in this study, however, the order that the magazines were used was not recorded.

van Oorschot and Jones (1997) reported that DNA can be transferred from one individual to another, for example when shaking hands. Secondary transfer of exogenous DNA on one's hand to a touched item has been documented (Lowe *et al*., 2002; Goray *et al*., 2010), and could also have contributed to contamination in this study. For example, Daly *et al*. (2012) autoclaved 300 wood, glass, and fabric items, followed by UV irradiation, and had volunteers firmly hold them for 60 s. While the majority of handled items did not contain a STR mixture,

approximately 10% did, which indicated exogenous DNA(s) that was present on some of the volunteers' hands was transferred onto the items. Volunteers in the present study were not asked to wash their hands prior to loading the cartridges, thus foreign DNA picked up immediately before their participation could have been transferred onto the casings. Furthermore, a large majority of the volunteers from Collection 2 were married and DNA from intimate partners has been found to persist on individuals for longer periods of time, likely because there is a greater quantity of DNA deposited (Matte *et al*., 2012). Therefore, the DNA from an intimate partner could also have been deposited during loading of the cartridges. In future studies, it may be useful to have volunteers submit buccal swabs from their intimate partners and their haplotypes compared to those recovered from the casings.

Individual bags were used to separately collect the first and sixth spent casings in Collection 2. Results from these casings were used to answer the third study question: *does the order cartridges are loaded/fired influence mtDNA results?* There is immense variation in the amount of DNA deposited onto touch samples among individuals (Alessandrini *et al*., 2003; Raymond *et al*., 2009; Daly *et al*., 2012), as well as in the quantity an individual deposits each time he/she touches an item (Thomasma and Foran, 2013). Goray *et al.* (2010) found that an increase in applied pressure correlated with an increase in the amount of DNA transferred. Therefore, it was hypothesized that in this study, the greatest amount of the loader's DNA would be deposited onto the last loaded cartridges (first spent casings), which require the most force to load into the magazine. However, no significant difference between the assignments made for the first and last loaded cartridges was found, although the first loaded (last fired) contained a lower percentage of consistent assignments (60.9% vs. 70.8%). Even though there was no statistical difference in the percentage of loader mixture among the four Collection 2 categories

($p = 0.223$), a higher frequency of loader mixture resulted from the first loaded cartridges compared to the random middle and sixth loaded (73.9%, 54.2%, and 50.0%, respectively), indicating contamination decreased as cartridges were loaded. It is possible that most of the exogenous cells on the loaders' hands were deposited onto the first cartridge loaded, and as a result, there was decreasing contamination for subsequent cartridge casings. Furthermore, even though the haplotype recovered from the magazine used for Collection 1 was not detected in the cartridge swabbing extracts, a magazine cannot be ruled out as a source of DNA contamination. Approximately the top fifth of the magazine used for Collection 1 was swabbed, whereas the first loaded cartridge in a series of six is pushed further down into the magazine. Since this cartridge is exposed to the most surface area of the magazine, it is possible that cells from a previous loader may be picked up at a greater frequency compared to the other cartridges. However, due to the variability of touch DNA, it is important to note that with the data obtained in this study, no definitive conclusions can be drawn regarding the source(s) of contamination.

An additional goal of this study was to identify a DNA recovery method that would yield the most consistent mtDNA profiles. Two swabbing methods were compared, which addressed the fourth study question*: are there differences in mtDNA results from individually and cumulatively double-swabbed spent casings?* The double-swab method (Sweet *et al*., 1997) is an efficient way to recover DNA from surfaces; the wet swab is used to rehydrate the forensic sample, which likely loosens the cells from the substrate and causes the cells to cling to the swab. When a dry swab is swirled over the same surface, more of the rehydrated cells are collected. What has not been studied is whether presumably related evidence should be double-swabbed individually or cumulatively, as there are potential advantages and disadvantages to both. Orlando (2012) found that cumulatively swabbing cartridge casings increased the average

quantity of DNA recovered. In addition, it is more cost and time effective because reagents and supplies are only needed for one extract. However, DNA loss has been documented with cumulatively swabbing, wherein cells that adhered onto the swab from one surface can be deposited onto the next swabbed surface (Hebda *et al*., in press). Furthermore, if multiple sources of DNA are present on the different surfaces, there is a greater chance that mixtures will result. Similar to Orlando (2012), a greater percentage of profiles from cumulatively swabbed casings yielded haplotypes consistent with the loader (71.4% vs. 60.2% for individual swabbings), though the difference was not statistically significant. Cumulatively swabbing also had a slightly lower percentage of loader mixtures (50.0% vs. 53.6%). Melton *et al*. (2012) suggested that a polymorphic variant comprising less than 10% of the total mtDNA in a mixture could go undetected. While there was a higher possibility of picking up DNAs that originated from multiple sources when cumulatively swabbing cartridge casings, it seems that 90% or more of the DNA recovered from 20 of 28 swabs originated from the loader. As a result, higher loader DNA recovery using cumulatively swabbing likely swamped out any contaminant DNA.

There were instances of mixture where the ratios of the peaks at a polymorphic position were dissimilar in the forward and reverse sequences from the same PCR product. For example, extract 13C from Collection 1 had a 3:1 ratio of A:G at position 153 in the F15 sequence, but a 2:3 ratio in the R285 sequence. Since the ratios were not consistent, mixture nomenclature (*e.g.* R for a A/G mixture) was used. Inconsistently and inconclusively assigned haplotypes were re-analyzed using MBI to determine if the analysis method would aid in detecting the loader's haplotype when the loader's DNA was not as abundant as other DNA variants. While 25.7% of MBI profiles from Collection 2 resulted in consistent-multiple assignments ('best-case scenario'*)*, supporting the theory that the loaders' mtDNAs were not as abundant as the contaminant

mtDNA(s), seven of the inconclusive haplotypes (25.9%) resulted in inconsistent assignments ('worst-case scenario'). Until larger sample sizes are obtained, it is not recommended to use MBI when analyzing DNA recovered from casings. Electropherograms of consistent and inconsistent assignments were also examined manually to determine if they contained loader mixtures, to identify differences between Collections 1 and 2, and to examine the order cartridges were loaded. Investigating the possibility of a suspect's inclusion when there were inconsistent assignments could result in useful information that may otherwise be overlooked. However, this may only be useful for investigative purposes because statistics that attempt to put an inclusion interpreted from a mtDNA mixture are currently not admissible in court (Melton *et al*., 2012).

The last question addressed in this study was: *is it advantageous to use consensus profiling?* Consensus profiling has the potential to filter out inconsistent polymorphisms based on the premise that the loaders' mtDNAs should be recovered from more spent casings than would contaminating DNA. In other words, seeing the same polymorphism multiple times develops confidence that it originated from the loader. Both consensus methods (Indiv-C and All-C) yielded more consistent-combined assignments than individually swabbing (67.9% and 67.9% vs. 60.2%), but neither was as effective as cumulatively swabbing (71.4%; though these differences were not significantly different). Consensus profiling did not outperform cumulatively swabbing, partly because three sets of individually swabbed cartridge casings (15, 18, and 31; Appendix B) exhibited shared non-loader polymorphisms that were not detected from the corresponding cumulatively swabbed casings. Since a polymorphism that showed up two out of three times for Indiv-C and three out of four for All-C haplotypes were included in the consensus profile, the detection of the contaminant DNA from the individual extracts influenced

overall results. However, because non-loader polymorphisms were detected more frequently in a minority of the individually swabbed casings, consensus profiling can be used to 'weed out' some of the inconsistencies when multiple casings are individually swabbed.

CONCLUSION

The results of this study establish the viability of mtDNA analysis as a method to generate valuable genetic information from spent cartridge casings. Despite the degraded nature of the DNA, it was possible to develop haplotypes with the use of overlapping amplicons. Owing to the sensitivity of mtDNA analysis, haplotypes were obtained from all individually and cumulatively double-swabbed casings, approximately two-thirds of which were consistent with the loader. However, mixtures were also obtained, which were most prevalent from the first loaded cartridges and the least from the cumulatively swabbed casings. Consensus profiles were used to filter out non-recurring polymorphisms, and resulted in a larger percentage of consistent haplotypes compared to the individually swabbed spent casings. Nonetheless, cumulative swabbing remained the superior DNA recovery method, as it yielded the highest frequency of consistent haplotypes.

The research presented leads to several other sets of experiments that could be performed. It would be valuable to analyze mtDNA recovered from various calibers and casing metals to establish if a similar level of consistency exists with the different ammunition. Since it is possible that the use of a single gun for each Collection contributed to contamination, it would also be helpful to examine if firing cartridges from a gun handled and loaded by the owner results in fewer mixtures. As more sensitive DNA analysis techniques are developed, it would also be worthwhile to investigate various DNA recovery and extraction method combinations, coupled with STR amplification using different kits. This would aid in optimizing a strategy for STR analysis from spent cartridge casings.

mtDNA haplotypes consistent with the loader were successfully recovered from casings

that were caught in a non-decontaminated net following firing. Since the loaders' mtDNA profiles were obtained when using non-cleaned firearms and magazines, it seems likely that the loaders' mtDNA profiles could be successfully developed from casework samples. Furthermore, the ability to generate mtDNA haplotypes from individual casings provides an added benefit when only one casing is recovered at a crime scene or when investigators want to compare the DNA profiles obtained from multiple cartridge casings. However, when possible, fired casings should be cumulatively swabbed to maximize the probability of recovering the loaders' haplotypes without the detection of mixture.

APPENDICES

APPENDIX A

mtDNA Profiles from Orlando (2012)

Tables of mtDNA profiles recovered from Orlando (2012) spent cartridge casings;
Extract A = cumulatively swabbed spent casings;
Extracts B – F = individually swabbed spent casings;
Ø = no polymorphisms;
N/A = sequencing not attempted;
Yellow highlight = polymorphism consistent with the loader;
Note: all haplotypes contain a 263G.

Table A1. mtDNA profiles obtained from spent cartridge casings loaded by volunteer H.

Extract	HV1a	HV1b	HV2a	mtDNA Assignment
H	16162G, 16189C, 16209C	Ø	73G	
3A	16192T	16256T, 16270T, 16272G	73G	Inconclusive
3B	16124C, 16148C	16304C, 16309G	73G	Inconclusive
3C	16129A	16362C	Ø	Inconclusive
3D	16189C	16311C	73G, 199C, 203R, 204C, 250C	Inconclusive
3E	Ø	16362C	73G, 146C, 200G	Inconclusive
3F	16093C	16234T, 16271C, 16362C	73G, 146C, 152C	Inconclusive

Table A2. mtDNA profiles obtained from spent cartridge casings loaded by volunteer K.

Extract	HV1a	HV1b	HV2a	mtDNA Assignment
K	16069T, 16126C, 16145A, 16172C	16261T	73G, 242T	
28A	16069T, 16126C, 16145A, 16172C	16261T	73G, 242T	Consistent-Single
28B	Ø	16311Y	73G, 146C, 152C	Inconclusive
28C	Ø	16311Y	73G, 146C, 152C	Inconclusive
28D	Ø	16311C	73G, 152Y	Inconclusive
28E	16069Y, 16126C, 16145A, 16172Y	16261T	73G, 146Y, 242T	Consistent-Single
28F	Ø	Ø	Ø	Inconsistent

Table A3. mtDNA profiles obtained from spent cartridge casings loaded by volunteer O.

Extract	HV1a	HV1b	HV2a	mtDNA Assignment
O	16126C	16256T, 16292T, 16294T	73G, 146C, 152C	
22A	Ø	16256T, 16270T	73G	Inconclusive
22B	16126C	Ø	73G, 152C	Inconclusive
22C	16126C	N/A	73G, 146C, 152C	Consistent-Single
22D	N/A	16256T, 16292C, 16294T	73G, 146Y, 152Y	Consistent-Single
22E	16126C	16256N/A, 16292C, 16294T	73G, 146C, 152C	Consistent-Single
22F	16126C	16256N/A, 16292C, 16294T	73G, 146C, 152C	Consistent-Single

Table A4. mtDNA profiles obtained from spent cartridge casings loaded by volunteer P.

Extract	HV1a	HV1b	HV2a	mtDNA Assignment
P	16196A	Ø	Ø	
5A	Ø	N/A	73R, 152C, 200A	Inconclusive
5B	Ø	N/A	73G, 146C, 152C, 200A	Inconclusive
5C	N/A	N/A	200A	Inconclusive
5D	Ø	N/A	73G, 146C, 152C, 200A	Inconclusive
5E	Ø	N/A	73G, 146Y, 152C, 200A	Inconclusive
5F	16094C	N/A	N/A	Inconclusive

Table A5. mtDNA profiles obtained from spent cartridge casings loaded by volunteer R.

Extract	HV1a	HV1b	HV2a	mtDNA Assignment
R	Ø	Ø	146C, 195C	
8A	Ø	N/A	73G	Inconclusive
8B	Ø	N/A	Ø	Inconsistent
8C	Ø	N/A	73G, 146C, 152C	Contaminated
8D	Ø	N/A	73G, 146C, 152C	Contaminated

Table A6. mtDNA profiles obtained from spent cartridge casings loaded by volunteer T.

Extract	HV1a	HV1b	HV2a	mtDNA Assignment
T	16092C	Ø	Ø	
2A	16092C	Ø	Ø	Consistent-Single
2B	16092C	Ø	Ø	Consistent-Single
2C	Ø	Ø	Ø	Inconsistent
2D	16092C	Ø	Ø	Consistent-Single
2E	Ø	16311C, 16362C	73G, 146C, 152C	Contaminated
2F	16092C	16311Y, 16362C	73G, 146C, 152C	Contaminated

Table A7. mtDNA profiles obtained from spent cartridge casings loaded by volunteer U.

Extract	HV1a	HV1b	HV2a	mtDNA Assignment
U	Ø	Ø	Ø	
7A	Ø	N/A	73R, 146C, 152C	Contaminated
7B	Ø	N/A	73G, 146C, 152C	Contaminated
7C	16410C, 16189C	N/A	73G, 210G	Inconclusive
7D	16140C, 16189C	N/A	146C	Inconclusive
7E	Ø	N/A	73G, 146C, 152C	Contaminated
7F	Ø	N/A	73G, 146C, 152C	Contaminated

Table A8. mtDNA profiles obtained from spent cartridge casings loaded by volunteer DD.

Extract	HV1a	HV1b	HV2a	mtDNA Assignment
DD	Ø	16216G	72C	
6A	Ø	N/A	72C	Consistent-Single
6B	Ø	N/A	72C	Consistent-Single
6C	Ø	N/A	72C	Consistent-Single
6D	Ø	N/A	72C	Consistent-Single
6E	Ø	N/A	72C	Consistent-Single
6F	Ø	N/A	73G, 146C, 152C	Contaminated

APPENDIX B

mtDNA Profiles from Collections 1 and 2

Tables of mtDNA profiles recovered from Collections 1 and 2 spent cartridge casings;
Extract A = cumulatively swabbed spent casings;
Extract B = individually swabbed first spent casing;
Extract C = individually swabbed random middle spent casing;
Extract D = individually swabbed sixth spent casing;
Ø = no polymorphisms;
Yellow highlight = polymorphism consistent with the loader;
MBI = mixed base identification: haplotypes developed with the mixed base identification option set to 30% peak height during analysis;
All-C = consensus haplotype developed from individually and cumulatively swabbed spent cartridge casings;
Indiv-C = consensus haplotype developed from individually swabbed spent cartridge casings;
Note: all haplotypes contain a 263G.

Table B1. mtDNA profiles obtained from spent cartridge casings loaded by volunteer M during Collection 1.

Extract	Haplotype	mtDNA Assignment
M	16126C, 16294T, 16304C, 73G	
12A	16126C, 16294T, 16304C, 73G	Consistent-Single
12B	16126C, 16294T, 16304C, 73G	Consistent-Single
12C	16126C, 16294T, 16304C, 73G	Consistent-Single
12D	16126C, 16294T, 16304C, 73G	Consistent-Single
All-C	16126C, 16294T, 16304C, 73G	Consistent-Single
Indiv-C	16126C, 16294T, 16304C, 73G	Consistent-Single

Table B2. mtDNA profiles obtained from spent cartridge casings loaded by volunteer S during Collection 1.

Extract	Haplotype	mtDNA Assignment
S	16126C, 16294T, 16296T, 16304C, 73G, 103A	
13A	16126C, 16294T, 16296T, 16304C, 73G, 103A	Consistent-Single
13B	16126C, 16294T, 16296T, 16304C, 73G, 103A	Consistent-Single
13C	16126C, 16294T, 16296T, 16304C, 73G, 103R, 153R, 195Y	Consistent-Single
13D	16126C, 16294T, 16296T, 16304C, 73G, 103A	Consistent-Single
All-C	16126C, 16294T, 16296T, 16304C, 73G, 103A	Consistent-Single
Indiv-C	16126C, 16294T, 16296T, 16304C, 73G, 103A	Consistent-Single

Table B3. mtDNA profiles obtained from spent cartridge casings loaded by volunteer HH during Collection 1.

Extract	Haplotype	mtDNA Assignment
HH	16291T, 16304C, 146C	
7A	16291T, 16304C, 146C	Consistent-Single
7B	16270T, 16296T, 146Y, 150Y, 200R	Inconclusive
7B-MBI	16270T, 16296Y, 73R, 146Y, 150Y, 200R, 236Y	Inconclusive
7C	16291Y, 16294Y, 16304Y, 146C	Consistent-Single
7D	16126C, 73G, 152C	Inconclusive
7D-MBI	16126C, 16278Y, 16296Y, 73G, 152Y	Inconclusive
All-C	146C	Inconclusive
Indiv-C	146C	Inconclusive

Table B4. mtDNA profiles obtained from spent cartridge casings loaded by volunteer OO during Collection 1.

Extract	Haplotype	mtDNA Assignment
OO	16093C, 16224C, 16311C, 73G	
1A	152Y	Inconsistent
1A-MBI	16189Y, 16311Y, 73R, 152Y	Inconsistent
1B	16051G, 16129C, 16183C, 16189C, 73G, 152C	Inconclusive
1B-MBI	16051G, 16129S, 16183C, 16189C, 16278Y, 73G, 152C	Inconclusive
1C	16126C, 16187T, 16189C, 16264T, 16270T, 16278T, 16293G, 16311C, 73G, 152C, 185T, 189G, 195C, 247A	Inconclusive
1C-MBI	16126C, 16187T, 16189C, 16264T, 16270T, 16278T, 16293G, 16311C, 73G, 152C, 185T, 189G, 195C, 247A	Inconclusive
1D	16093C, 16278T, 16294T, 16309G, 16311Y, 73G, 152C, 195Y	Inconclusive
1D-MBI	16093Y, 16278Y, 16294Y, 16309R, 16311Y, 16368Y, 73G, 146Y, 152C, 195Y	Inconclusive
All-C	16189C, 16278T, 16311C, 73G, 152C	Inconclusive
Indiv-C	16189C, 16278T, 16311C, 73G, 152C	Inconclusive

Table B5. mtDNA profiles obtained from spent cartridge casings loaded by volunteer B during Collection 2.

Extract	Haplotype	mtDNA Assignment
B	16126C, 16294T, 16296T, 16304C, 16362C, 73G	
24A	16126C, 16294T, 16296T, 16304C, 16362C, 73G	Consistent-Single
24B	16126C, 73R	Inconclusive
24B-MBI	16126C, 16294Y, 73R	Inconclusive
24C	16126C, 16294T, 16296T, 16304C, 16362C, 73G	Consistent-Single
24D	16126C, 16294T, 16296T, 16304C, 16362C, 73G, 228A	Inconclusive
24D-MBI	16069Y, 16126C, 16294Y, 16296Y, 16304C, 16362Y, 73G, 185R, 228R	Consistent-Multiple
All-C	16126C, 16294T, 16296T, 16304C, 16362C, 73G	Consistent-Single
Indiv-C	16126C, 16294T, 16296T, 16304C, 16362C, 73G	Consistent-Single

Table B6. mtDNA profiles obtained from spent cartridge casings loaded by volunteer C during Collection 2.

Extract	Haplotype	mtDNA Assignment
C	16274A, 152C	
27A	16126Y, 16274A, 16294Y, 152C	Consistent-Single
27B	16126C, 16294T, 16304C, 73G, 183G	Inconsistent
27B-MBI	16126C, 16294T, 16304C, 73G, 183R	Inconsistent
27C	73R, 152Y	Inconsistent
27C-MBI	16274R, 16294Y, 73R, 152Y	Consistent-Multiple
27D	73R, 152Y	Inconsistent
27D-MBI	16291Y, 16294Y, 73R, 152Y	Inconsistent
All-C	16294T, 73G, 152C	Inconclusive
Indiv-C	16294T, 73G, 152Y	Inconclusive

Table B7. mtDNA profiles obtained from spent cartridge casings loaded by volunteer E during Collection 2.

Extract	Haplotype	mtDNA Assignment
E	16192T, 16256T, 16270T, 73G	
8A	16192T, 73G	Inconclusive
8A-MBI	16192T, 16294Y, 73G	Inconclusive
8B	16192T, 16256T, 16270T, 73G	Consistent Single
8C	16126Y, 16163R, 16186Y, 16189Y, 16192Y, 73G	Inconclusive
8C-MBI	16126Y, 16163R, 16186Y, 16189Y, 16192Y, 73G	Inconclusive
8D	16192T, 16256T, 16270T, 73G	Consistent-Single
All-C	16192T, 73G	Inconclusive
Indiv-C	16192T, 16256T, 16270T, 73G	Consistent-Single

Table B8. mtDNA profiles obtained from spent cartridge casings loaded by volunteer H during Collection 2.

Extract	Haplotype	mtDNA Assignment
H	16145A, 16188T, 16189C, 16193T, 16193.1C, 16256T, 16270T, 16311C, 73G, 195C	
32A	16145A, 16188T, 16189C, 16193T, 16193.1C, 16256T, 16270T, 16311C, 73G, 195C	Consistent-Single
32B	16145A, 16188T, 16189C, 16193T, 16193.1C, 16256T, 16270T, 16311C, 73G, 195C	Consistent-Single
32C	16145R, 16188T, 16189C, 16193Y, 16193.1C, 16256Y, 16270T, 16311C, 73G, 195Y	Consistent-Single
32D	16145A, 16188T, 16189C, 16193T, 16193.1C, 16256T, 16270T, 16311C, 73G, 195C	Consistent-Single
All-C	16145A, 16188T, 16189C, 16193T, 16193.1C, 16256T, 16270T, 16311C, 73G, 195C	Consistent-Single
Indiv-C	16145A, 16188T, 16189C, 16193T, 16193.1C, 16256T, 16270T, 16311C, 73G, 195C	Consistent-Single

Table B9. mtDNA profiles obtained from spent cartridge casings loaded by volunteer I during Collection 2.

Extract	Haplotype	mtDNA Assignment
I	16222T, 16235G, 16291T	
11A	16222T, 16235G, 16291T	Consistent-Single
11B	16222T, 16235G, 16291T	Consistent-Single
11C	16222T, 16343G, 73G, 150T	Inconclusive
11C-MBI	16222Y, 16235R, 16291Y, 16343R, 73G, 150T, 156R, 160R	Inconclusive
11D	16222T, 16235G, 16291T	Consistent-Single
All-C	16222T, 16235G, 16291T	Consistent-Single
Indiv-C	16222T, 16235G, 16291T	Consistent-Single

Table B10. mtDNA profiles obtained from spent cartridge casings loaded by volunteer J during Collection 2.

Extract	Haplotype	mtDNA Assignment
J	16357C	
21A	16357C	Consistent-Single
21B	16357C	Consistent-Single
21C	16357C	Consistent-Single
21D	16357C	Consistent-Single
All-C	16357C	Consistent-Single
Indiv-C	16357C	Consistent-Single

Table B11. mtDNA profiles obtained from spent cartridge casings loaded by volunteer L during Collection 2.

Extract	Haplotype	mtDNA Assignment
L	16189C, 16362C, 152C	
22A	16179T, 16294T, 73G	Inconclusive
22A-MBI	16179Y, 16294T, 73G	Inconclusive
22B	16069Y, 16126C, 16362Y, 73G	Inconclusive
22B-MBI	16069Y, 16126Y, 16189Y, 16362Y, 73G, 185R, 228R	Inconsistent
22C	16069T, 16126C, 73G	Inconclusive
22C-MBI	16069T, 16126C, 16186Y, 73G	Inconclusive
22D	16126C, 73G	Inconclusive
22D-MBI	16069Y, 16126Y, 16189Y, 16362Y, 73G, 185R, 228R	Inconsistent
All-C	16069T, 16126C, 73G	Inconclusive
Indiv-C	16069T, 16126C, 16189Y, 16362Y, 73G, 185R, 228R	Inconsistent

Table B12. mtDNA profiles obtained from spent cartridge casings loaded by volunteer N during Collection 2.

Extract	Haplotype	mtDNA Assignment
N	Ø	
25A	16235G, 16291T	Inconclusive
25A-MBI	16235G, 16291T, 73Y, 195Y	Inconclusive
25B	Ø	Consistent-Multiple
25C	Ø	Consistent-Multiple
25D	Ø	Consistent-Multiple
All-C	Ø	Consistent-Multiple
Indiv-C	Ø	Consistent-Multiple

Table B13. mtDNA profiles obtained from spent cartridge casings loaded by volunteer T during Collection 2.

Extract	Haplotype	mtDNA Assignment
T	16224C, 93G	
15A	16186Y, 16189Y, 16224Y, 93R	Consistent-Multiple
15B	16294T, 73R	Inconclusive
15B-MBI	16294Y, 73R	Inconsistent
15C	Ø	Inconsistent
15C-MBI	Ø	Inconsistent
15D	16069T, 73G, 185R, 228R	Inconsistent
15D-MBI	16069Y, 16126Y, 16224Y, 73R, 93R, 185R, 228R	Consistent-Multiple
All-C	Ø	Inconsistent
Indiv-C	73R	Inconsistent

Table B14. mtDNA profiles obtained from spent cartridge casings loaded by volunteer BB during Collection 2.

Extract	Haplotype	mtDNA Assignment
BB	16298C, 16318C, 72C	
31A	16298C, 16318M, 72C	Consistent-Single
31B	16189Y, 73Y, 151Y, 152Y	Inconsistent
31B-MBI	16189Y, 16256Y, 16298Y, 16311Y, 16318M, 16362Y, 72Y, 73R, 151Y, 152Y	Consistent-Multiple
31C	16298Y, 72C	Inconclusive
31C-MBI	16189Y, 16256Y, 16270Y, 16298Y, 16311Y, 16318M, 16362Y, 72Y, 73R	Consistent-Multiple
31D	16093Y, 16298Y, 195Y	Inconsistent
31D-MBI	16093Y, 16256Y, 16270Y, 16298Y, 16311Y, 16318M, 16362Y, 195C	Inconclusive
All-C	16298C, 16318M, 72C	Consistent-Single
Indiv-C	16189Y, 16256Y, 16270Y, 16298Y, 16311Y, 16318M, 72Y, 73R	Consistent-Multiple

Table B15. mtDNA profiles obtained from spent cartridge casings loaded by volunteer EE during Collection 2.

Extract	Haplotype	mtDNA Assignment
EE	Ø	
18A	Ø	Consistent-Multiple
18B	73R	Consistent-Multiple
18C	73G	Inconclusive
18C-MBI	73G	Inconclusive
18D	Ø	Consistent-Multiple
All-C	Ø	Consistent-Multiple
Indiv-C	73G	Inconclusive

Table B16. mtDNA profiles obtained from spent cartridge casings loaded by volunteer FF during Collection 2.

Extract	Haplotype	mtDNA Assignment
FF	16093C, 16189C, 16270T, 73G, 150T	
35A	16189Y, 16270T, 73G, 150Y	Inconclusive
35A-MBI	16093Y, 16189Y, 16270Y, 16291Y, 73R, 150Y	Consistent-Multiple
35B	16093Y, 16189Y, 16270T, 73G, 150T	Consistent-Single
35C	16235G, 16291T, 73G	Inconclusive
35C-MBI	16093Y, 16189Y, 16235R, 16270Y, 16291Y, 73G, 183R	Inconclusive
35D	16270Y, 150Y	Inconclusive
35D-MBI	16093Y, 16189Y, 16270Y, 16291Y, 73R, 150Y	Consistent-Multiple
All-C	16093Y, 16189Y, 16270T, 16291Y, 73G, 150T	Consistent-Single
Indiv-C	16093Y, 16189Y, 16270T, 16291Y, 73G, 150T	Consistent-Single

Table B17. mtDNA profiles obtained from spent cartridge casings loaded by volunteer GG during Collection 2.

Extract	Haplotype	mtDNA Assignment
GG	152C	
16A	152C	Consistent-Single
16B	152C	Consistent-Single
16C	152C	Consistent-Single
16D	152C	Consistent-Single
All-C	152C	Consistent-Single
Indiv-C	152C	Consistent-Single

Table B18. mtDNA profiles obtained from spent cartridge casings loaded by volunteer II during Collection 2.

Extract	Haplotype	mtDNA Assignment
II	16278T, 16311C, 146C	
34A	16278T, 16311C, 146C	Consistent-Single
34B	16278T, 16311C, 146C	Consistent-Single
34C	16278T, 16311C, 146C	Consistent-Single
34D	16278T, 16311C, 146Y	Consistent-Single
All-C	16278T, 16311C, 146C	Consistent-Single
Indiv-C	16278T, 16311C, 146C	Consistent-Single

Table B19. mtDNA profiles obtained from spent cartridge casings loaded by volunteer JJ during Collection 2.

Extract	Haplotype	mtDNA Assignment
JJ	Ø	
9A	Ø	Consistent-Multiple
9B	Ø	Consistent-Multiple
9C	Ø	Consistent-Multiple
9D	Ø	Consistent-Multiple
All-C	Ø	Consistent-Multiple
Indiv-C	Ø	Consistent-Multiple

Table B20. mtDNA profiles obtained from spent cartridge casings loaded by volunteer NN during Collection 2.

Extract	Haplotype	mtDNA Assignment
NN	16069T, 16126C, 16145A, 16172C, 16222T, 16261T, 73G, 242T	
37A	16069T, 16126C, 16145A, 16172C, 16222T, 16261T, 73G, 242T	Consistent-Single
37B	16069T, 16126C, 16145A, 16172C, 16222T, 16261T, 73G, 242T	Consistent-Single
37C	16069T, 16126C, 16145A, 16172C, 16222T, 16261T, 73G, 242T	Consistent-Single
37D	16069T, 16126C, 16145A, 16172C, 16222T, 16261T, 73G, 242T	Consistent-Single
All-C	16069T, 16126C, 16145A, 16172C, 16222T, 16261T, 73G, 242T	Consistent-Single
Indiv-C	16069T, 16126C, 16145A, 16172C, 16222T, 16261T, 73G, 242T	Consistent-Single

Table B21. mtDNA profiles obtained from spent cartridge casings loaded by volunteer PP during Collection 2.

Extract	Haplotype	mtDNA Assignment
PP	16311C, 152C	
17A	16311C, 152C	Consistent-Single
17B	16311C, 152C	Consistent-Single
17C	16311C, 152C	Consistent-Single
17D	16311C, 152C	Consistent-Single
All-C	16311C, 152C	Consistent-Single
Indiv-C	16311C, 152C	Consistent-Single

Table B22. mtDNA profiles obtained from spent cartridge casings loaded by volunteer RR during Collection 2.

Extract	Haplotype	mtDNA Assignment
RR	16224C, 16311C, 73G, 199C	
23A	16311C	Inconclusive
23A-MBI	16189Y, 16224*, 16261Y, 16311Y, 73R, 103R	Inconclusive
23B	16311C, 73R	Inconclusive
23B-MBI	16224Y, 16311Y, 73R, 199Y	Consistent-Multiple
23C	16224Y, 16311C, 73R	Inconclusive
23C-MBI	16224C, 16261Y, 16270Y, 16311C, 73R, 152Y	Inconclusive
23D	73G, 183G	Inconclusive
23D-MBI	16294Y, 16311Y, 73G, 183G	Inconclusive
All-C	16224N/A, 16311C, 73G	Inconclusive
Indiv-C	16224C, 16311C, 73G	Inconclusive

*base was not identifiable from the electropherograms

Table B23. mtDNA profiles obtained from spent cartridge casings loaded by volunteer SS during Collection 2.

Extract	Haplotype	mtDNA Assignment
SS	16126C, 16294T, 16304C, 73G, 183R	
33A	16126C, 16294T, 16304C, 73G, 183G	Consistent-Single
33B	16126C, 16294T, 16304C, 73G	Consistent-Single
33C	16126C, 16294T, 16304C	Inconclusive
33C-MBI	16126C, 16294T, 16304C, 73R, 150Y	Consistent-Single
All-C	16126C, 16294T, 16304C, 73G	Inconsistent
Indiv-C	16126C, 16294T, 16304C, 73G	Inconsistent

Table B24. mtDNA profiles obtained from spent cartridge casings loaded by volunteer TT during Collection 2.

Extract	Haplotype	mtDNA Assignment
TT	16224C, 16261Y, 16270T, 16311C, 73G, 146C, 152C	
19A	16224C, 16261T, 16270T, 16311C, 73G, 146C, 152C	Consistent-Single
19B	16224C, 16261T, 16270T, 16311C, 73G, 146C, 152C	Consistent-Single
19C	16224C, 16261Y, 16270T, 16311C, 73G, 146C, 152C	Consistent-Single
19D	16224C, 16270T, 16311C, 73G, 146C, 152C	Consistent-Single
All-C	16224C, 16261T, 16270T, 16311C, 73G, 146C, 152C	Consistent-Single
Indiv-C	16224C, 16261T, 16270T, 16311C, 73G, 146C, 152C	Consistent-Single

Table B25. mtDNA profiles obtained from spent cartridge casings loaded by volunteer UU during Collection 2.

Extract	Haplotype	mtDNA Assignment
UU	16129A, 16311C, 16316G	
38A	16126C, 16294T, 16304C, 73G, 185R, 228R	Inconsistent
38A-MBI	16126Y, 16294Y, 16304Y, 73G, 183R, 185R, 228R	Inconsistent
38B	16126C, 16294Y, 73G	Inconclusive
38B-MBI	16126Y, 16294Y, 16304Y, 16311Y, 16316R, 73R, 150Y	Inconsistent
38C	16126C, 16189Y, 16294T, 73G	Inconclusive
38C-MBI	16126C, 16163R, 16186Y, 16189Y, 16294Y, 73R, 183R	Inconclusive
38D	16126C, 16294T, 73G	Inconclusive
38D-MBI	16126C, 16294Y, 16304Y, 73R, 183R	Inconsistent
All-C	16126C, 16294Y, 16304Y, 73G, 183R	Inconsistent
Indiv-C	16126C, 16294Y, 16304Y, 73R, 183R	Inconsistent

Table B26. mtDNA profiles obtained from spent cartridge casings loaded by volunteer VV during Collection 2.

Extract	Haplotype	mtDNA Assignment
VV	16069T, 16126C, 73G, 185A, 228A	
20A	16069T, 16126C, 73G, 185A, 228A	Consistent-Multiple
20B	16069T, 16126C, 73G, 185A, 228A	Consistent-Multiple
20C	16069T, 16126C, 73G, 185R	Inconclusive
20C-MBI	16069T, 16126C, 16146R, 73G, 185R	Inconclusive
20D	16069T, 16126C, 73G, 185A, 228A	Consistent-Multiple
All-C	16069T, 16126C, 73G, 185A, 228A	Consistent-Multiple
Indiv-C	16069T, 16126C, 73G, 185A, 228A	Consistent-Multiple

Table B27. mtDNA profiles obtained from spent cartridge casings loaded by volunteer WW during Collection 2.

Extract	Haplotype	mtDNA Assignment
WW	16189C, 16192T, 16256T, 16270T, 16311C, 16362C, 73G, 151T, 152C	
30A	16256Y, 16270T, 16311Y, 16362C	Inconclusive
30A-MBI	16256Y, 16270T, 16311Y, 16362C, 73Y, 151Y, 152Y	Inconsistent
30B	16189C, 16192Y, 16256T, 16270T, 16311C, 16362C, 73G, 151T, 152C	Consistent-Single
30C	16189C, 16192T, 16256T, 16270T, 16311C, 16362C, 73G, 151T, 152C	Consistent-Single
30D	16126Y, 16189C, 16192Y, 16256T, 16270T, 16311C, 16363C, 73G, 151T, 152C	Consistent-Single
All-C	16189C, 16192T, 16256T, 16270T, 16311C, 16362C, 73G, 151T, 152C	Consistent-Single
Indiv-C	16189C, 16192T, 16256T, 16270T, 16311C, 16362C, 73G, 151T, 152C	Consistent-Single

Table B28. mtDNA profiles obtained from spent cartridge casings loaded by volunteer XX during Collection 2.

Extract	Haplotype	mtDNA Assignment
XX	16069T, 16126Y, 73G, 185A, 228A	
36A	16069T, 16126C, 73G, 185A, 228A	Consistent-Multiple
36B	16069T, 16126C, 73G, 185A, 228A	Consistent-Multiple
36C	16069T, 16126C, 73G, 185A, 228A	Consistent-Multiple
36D	16126C, 16163R, 16189Y, 73G, 152Y, 185R, 228A	Inconclusive
36D-MBI	16126Y, 16163R, 16186Y, 16189Y, 73R, 152Y, 178R, 185R, 228R	Inconsistent
All-C	16069T, 16126C, 73G, 185A, 228A	Consistent-Multiple
Indiv-C	16069T, 16126C, 73G, 185A, 228A	Consistent-Multiple

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