Review

Molecular genetic identification of skeletal remains of apartheid activists in South Africa

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The Truth and Reconciliation Commission made significant progress in examining abuses committed during the apartheid era in South Africa. Despite information revealed by the commission, a large number of individuals remained missing when the commission closed its proceedings. This provided the impetus for the establishment of a Missing Persons Task Team within the South African National Prosecuting Authority. The team has used evidence generated by The Truth and Reconciliation Commission as well as their own investigations to identify likely burial sites for activists and has conducted exhumations. The University of the Western Cape (UWC) was privileged to have been able to assist by initiating a DNA testing program to augment the non-DNA evidence gathered by the Missing Person's Task Team. In this review we consider some of the technological developments associated with the testing of skeletal remains and provide an overview of some of the cases analyzed to date.

Key words: Missing people, mass graves, DNA identification, mitochondrial DNA, short tandem repeats, sequence polymorphisms.

INTRODUCTION

Identification of victims in human rights cases, such as those currently under investigation in South Africa, can require the application of innovative biology, technology and genetics. The analysis of non-DNA evidence involving classical physical anthropology will always play an extremely important role in investigations. However, DNA analysis can provide an additional source of evidence which is particularly useful when body parts have been separated from one another or victims are buried in co-mingled graves. The use of dental records still plays an important role in victim identification but this modality obviously requires an intact skull or jawbone along with previously archived dental records.

DNA testing can make a significant contribution because it allows for the identification of victims from dental or dense skeletal elements. It does however require that sufficient DNA has been preserved in the skeletal elements

and that reference samples can be obtained from surviving relatives or personal items containing biological material from the victim. The use of DNA from biological relatives necessitates the added complexity of kinship analysis similar to that employed for paternity or reverse parentage testing.

There are regions of the world that have suffered severely under the hands of ruthless governments. Opponents of these regimes have often been abducted, murdered and buried in unmarked graves. DNA analysis can play an important role in identifying remains of the victims. Many documented examples of identification of victims in mass graves around the world have been resolved with the utilization of DNA technology (Holland et al., 1993; Boles et al., 1995; Corach et al., 1995, 1997). The former Yugoslavia has perhaps the most thoroughly investigated mass grave sites in the world. In that country an estimated 40,000 unidentified bodies are believed to have been buried in mass graves. The International Commission of Missing Persons (ICMP) was created in 1996 to help with identifying human remains in these mass graves. In effect, the ICMP is using DNA

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analysis as a key technology in a process which is mapping a human genocide (Huffine et al., 2001).

South Africa has many documented cases of mass grave sites as a result of human rights abuses under the former apartheid regime; these were revealed at the hearings of The Truth and Reconciliation Commission. Despite the work of the commission, the whereabouts of a number of victims of apartheid violence remain missing. A Missing Persons Task Team has been established within the National Prosecuting Authority to investigate cases of individuals missing as a result of apartheid violence. This process requires the input of various parties including: historians, investigators, physical anthropologists and molecular biologists for cases where DNA tests are required. The Forensic DNA laboratory at the University of the Western Cape (UWC) was privileged to begin a DNA testing programme to assist the Missing Person's Task Team investigations. Although there have been initial successes, there is ongoing research to develop and implement improved molecular genetics methods.

The markers currently applied in our laboratory for identification of human skeletal remains are: mtDNA sequence polymorphisms, Short Tandem Repeats (STRs) and miniSTRs. The latter are a smaller version of STRs that are more efficient in human genetic identification when samples are in poor preservation conditions.

STR MARKERS

Human identity tests focus on Short Tandem Repeat markers (STR markers). STR markers are genetic loci consisting of repeated subunits, 2 - 8 base pairs in length. Discrimination between individuals is possible because the number of subunits present for a given marker varies from person to person. Simultaneous analysis of several STR markers allows for the compilation of a profile, which is almost unique to a given individual.

Short Tandem Repeat markers suitable for forensic analysis occur on both the autosomal chromosomes and the sex chromosomes X and Y. Research on the use of STR markers for identification purposes has focused mainly on autosomal markers. The most obvious reason for this is the fact that they can be used for the identification of individuals from both sexes. The most commonly used STR markers are those which constitute the Combined DNA Index System (CODIS) (Budowle et al., 1998). This system uses DNA profiles generated for 13 core somatic STR loci, with alleles varying in sizes between 115 and 470 bp. In America, data obtained from crime scenes and convicted offenders is stored in the FBI databases to link suspects and repeat offenders to unsolved cases (Butler 2006). US population data is also available in the NIST public database (http://www.cstl.nist.gov/div831/strbase//fbicore.htm). The

establishment of the CODIS loci motivated the development of commercial STR kits that could type these 13 core loci simultaneously. Initially two Applied Biosystems kits, Profiler Plus and COfiler were available which covered the 13 CODIS loci. Subsequent kits were developed which amplified the 13 autosomal CODIS loci along with additional markers: PowerPlex 16 (Promega), and Identifiler 16plex kit (Applied Biosystems). These systems have been successful in identifying individuals from skeletal remains when chromosomal DNA has been present (Alonso et al., 2001; Marjanovic et al., 2007; Milos et al., 2007).

The repositioning of primers as close as possible to the amplicon target, with the aim of obtaining PCR products smaller than 250 bp, originated a new set of markers called miniSTRs, by analogy to their larger counterparts (Butler et al., 2003).

It has been demonstrated that the chances of obtaining a result from a degraded DNA sample is increased when smaller mini-STR fragments of DNA are targeted for detection (Dixon et al., 2006). There have been attempts to standardise the use of mini-STRs internationally by the European Network of Forensic Science Institutes (ENFSI) and the European DNA Profiling Group (EDNAP), which have collectively achieved a standardisation of DNA profiling in Europe (Schneider and Martin, 2001; Martin et al., 2001). Information on the standardization, nomenclature and utilization of miniSTRs is available at the NIST site. (http://www.cstl.nist.gov/div831/strbase/miniSTR.htm).

The STRs contained in the Y chromosome are widely utilized in forensic casework, especially in sexual abuse casework where the Y-chromosome profiles are intended to be separated from the victim's for identification of the crime perpetrators. These markers have also been applied to human identification in mass graves since they can identify gender and paternal lineages (Corach et al., 1997; Definis Gojanović and Sutlović 2007). The design and application of mini-YSTRs would improve the identification of missing people.

Currently the UWC Forensic DNA lab uses the 13 loci constituting CODIS for the profiling of DNA extracted from human skeletal remains. We are also involved in developing and improving the existing STR technology for its application in forensic casework. Our research has focused on the assessment of the value of existing and novel Y-STR markers identified in our lab (Leat et al., 2004, 2006; Ehrenreich et al., 2008) with the aim of identifying markers particularly polymorphic for South African populations and thus more effective in human identification. Part of the population data generated in our lab for South African populations was deposited in the universal database YHRD (www.yhrd.org). Mini-STR technology has not been employed in South Africa; however, we are presently investigating mini-STR markers that are suitable for forensic DNA casework in South Africa. From our studies we are generating a large population

database on the South African populations for different markers; this will allow for a more reliable estimation of matching probabilities in forensic casework and mass disasters (Krakun et al., 2007)

MITOCHONDRIAL DNA

Mitochondrial DNA (mtDNA) is found in the mitochondria, not associated with the nuclear chromosomes. They contain their own small DNA genomes; circular molecules of 16,569 base pairs with variants that can be identified by sequencing. Each cell contains hundreds to thousands of mitochondria. For this reason, a single hair shaft, old bones, or charred remains, which are generally unsuitable for chromosomal DNA analysis, sometimes provide enough intact material for mtDNA analysis. The highest degree of variation in the mtDNA among individuals is found within the non-coding control region in hypervariable regions I and II (HVI and HVII, respectively). For this reason the identification of human skeletal remains often relies on mtDNA sequencing of these two regions.

MtDNA is maternally inherited and does not undergo recombination. These features mean that all maternal relatives, barring mutation, will share the same haplotype. This limits the degree to which mtDNA analysis can discriminate between individuals. However the high copy number is useful when analyzing degraded samples containing low concentrations of DNA (Butler, 2001; Carracedo et al., 2000; Jehaes et al., 2001).

Mitochondrial DNA analysis is an established area of forensic testing in many countries. At present, the two most important mtDNA databases for forensic use are the Scientific Working Group of DNA Analysis Methods (SWGDAM) of the FBI (www.fbi.gov/hq/lab/fsc/backissu/april2002/miller1.htm) and EMPOP (www.empop.org) (Parsons and Dür, 2007) control region databases.

MtDNA haplotypes are currently employed mainly to establish a relationship of sample material among family members in the absence of the individual suspected of leaving the sample. They may also be used to provide information for group identification and were used to identify some of the bodies of the Russian royal family, the Romanovs (Gill et al., 1994). Many law enforcement agencies, crime scene investigators, defense and prosecuting attorneys have now begun to appreciate that this form of testing, while not as discriminating as nuclear DNA testing, may successfully advance the investigation and prosecution or lead to exoneration of cases with limited biological evidence, such as hairs and degraded skeletal remains. Mitochondrial DNA often is the only source of successful DNA recovery from bones that have been in the ground for many years (Holland et al., 1993; Andelinović et al,, 2005), and although its discriminatory power is much lower than that of STRs, mtDNA provides the additional advantage of allowing for identification of more distant (maternal) relatives (Palo et al., 2007).

At present the South African Police use the Profiler Plus (AmpFℓSTR® Profiler Plus®, Applied Biosystems) DNA-based human identification system in forensic analysis, which consists of 9 of the 13 CODIS somatic markers and the amelogenin locus, but they do not use mtDNA analysis. The Profiler Plus DNA-based human identification system works well on fresh samples and has been used with great success around the world and in South Africa. However it is not suitable for DNA extracted from old bones or charred remains because the system relies on chromosomal DNA being present, which is not the case in these sorts of samples.

At the UWC Forensic DNA lab we are currently investigating polymorphisms in mtDNA HV1 and HVII in South African ethnic groups with a view to their use in forensic casework. The efficacy of the systems will be tested on ancient and burnt bone samples. We also plan to establish the capacity to analyse large numbers of skeletal remains using technology suitable to this purpose. The systems we develop will be routinely challenged by actual casework samples we are currently receiving from the National Prosecuting Authority on human rights cases in South Africa. Our refinement of mtDNA identification systems could also help the South Africa Police Service in their future investigations in instances where minute samples of hair, teeth or bones are found at crime scenes.

SNPs (SINGLE NUCLEOTIDE POLYMORPHISMS)

An alternative method for analysing degraded DNA is with single nucleotide polymorphism (SNPs) analysis. SNPs account for approximately 85% of the genetic variation in humans. At present, over the 3 billion bases of the human genome, 3 million SNPs have been identified (International HapMap Consortium, 2007), this information is available from the HapMap site (http://snp.cshl.org). Since SNP analysis involves the amplification of very small products (40 - 70bp) highly degraded DNA samples can be typed. Both mitochondrial and chromosomal DNA present sequence variations at punctual sites, and they usually show binary allelic polymorphism. As with microsatellites, the variation is not only present at the individual level but also at the ethnic level. These polymorphisms in the non-recombining portion of our genome (mtDNA and Y-chromosome) are both used in human evolutionary studies and forensics (Brion et al., 2005; Kline et al., 2005; Hammer et al., 2006; Niederstätter et al., 2006; Onofri et al., 2006). The inter population variation at somatic chromosomes SNPs can also provide information on ethnic background (Turakulov and Easteal, 2003, Paktsis et al., 2007; Pakstis et al, 2007, 2008). Notwithstanding efforts have been made to select SNPs and implement these systems

for individual identification (Kidd et al., 2005; Dixon et al., 2006), the wide application of autosomal SNPs in individual identification has been questioned due to statistical problems (Amorim and Pereira 2005), and their lower power of discrimination than that of microsatellite markers (Gill et al., 2004). Although these markers would probably not replace microsatellites in individual identification, SNPs can be particularly useful in cases of highly degraded DNA such as mass graves and mass disasters (Gill et al., 2004; Dixon et el., 2005). Methodological approaches to score SNP data was reviewed in Sobrino et al. (2005).

Our intention is to implement SNP genotyping as an additional and complementary technique to the current DNA sequencing and STR genotyping protocols currently employed. This technology will be developed for both chromosomal DNA and mtDNA and will initially be implemented by using well known published polymorphisms and SNaPshot (Applied Biosystems) protocols.

OVERVIEW OF UWC PROTOCOLS FOR THE ANALYSIS OF DEGRADED SKELETAL MATERIAL

Isolation of pre and post-PCR environments: The pre-PCR laboratory is located in a separate building from the post-PCR laboratory. Disposable face masks, hair caps and lab coats are used in the pre-PCR lab and dedicated equipment is stored in the lab. Surfaces are bleached before use and key areas of the lab are subject to nightly UV irradiation. Laboratory members are not allowed to move from the post-PCR lab to the pre-PCR lab on the same day.

Specimen cleaning bleaching and milling: For bone specimens 3 g sections are sampled using a dremel tool fitted with an emery cutting disk. Surface material is removed using a dremel tool fitted with an abrasive head. The bone fragment is cut into sections small enough to be loaded into the vials of a Spex 6750 liquid nitrogen mill. When processing teeth, attached debris is removed with a toothbrush or scraped off with a blade. Where necessary 3.5% (w/v) sodium hypochlorite is used to rinse off debris. Water is not used to rinse bone or tooth specimens.

All specimens are bleached prior to milling. The bleaching protocol was implemented after considering the recommendations of Kemp et al. (2005). The authors suggest immersion in sodium hypochlorite at a concentration greater than 3% (w/v) for at least 15 min. In the laboratory, specimens are soaked for 1 h in 3.5% (w/v) sodium hypochlorite. After bleaching, specimens are washed four times with sterile water and twice with ethanol. Samples are then dried for 1 h in a laminar flow hood, under UV irradiation. The bone fragments are milled using Spex 6750 liquid nitrogen freezer mill.

DNA extraction: A classical phenol chloroform based DNA extraction protocol is used. Up to 3 g of milled powder is resuspended in 10 ml 0.5 M EDTA containing *N*-lauroyl sarcosine and proteinase K. Samples are incubated overnight at 37°C and then subject to several phenol, phenol/chloroform and chloroform extractions. The aqueous phase is concentrated down to around 100 µl by dialysis centrifugation using Centriplus 30 tubes (Millipore). The extract is washed at least twice with sterile water and stored at -20ºC before being used in PCR reactions.

An extraction blank is included to test for lab based contamination. The extraction blank is handled in exactly the same way as the bone extractions, with the exception that no milled bone powder is introduced. The concentrate from the extraction blank is subject to MtDNA and STR analysis in the same way as bone extracts.

Mitochondrial DNA analysis: HVI and HVII fragments are amplified in uniplex PCR reactions. At present, fairly large products are amplified (HVI - 454bp and HVII - 422bp). Amplifying smaller products may improve results for samples.

Double-stranded sequencing is conducted for each PCR product. The heavy and light chain sequences are aligned to identify discrepancies. The discrepancies are resolved by consulting the relevant electropherograms. A consensus sequence is then compared with the Cambridge Reference Sequence (CRS). Sequencing beyond homopolymeric regions can become difficult. In these cases the heavy and light chain sequences either side of the homopolymeric region are compared directly with the CRS. Discrepancies with the CRS are verified against the relevant electropherogram. We have assigned mtDNA haplotypes over HVI and HVII from sites 16024 to 16365 and from sites 73 to 340 respectively. Quality control of the obtained data was performed following the procedures of Bandelt el al. (2002) and Salas et al. (2005).

STR analysis: We have implemented the CODIS STR typing system and have supplemented this with the mini-STR typing systems developed by Butler et al. (2003). The mini-STR systems have been demonstrated to be more efficient than commercial systems when analyzing degraded DNA from skeletal remains (Opel et al., 2006).

BRIEF OVERVIEW OF THREE CASES

Case 1: Skeletal remains of four individuals buried in a co-mingled grave

Our success in implementing this technology is best described by outlining recent cases we have worked on. This first case involved the identification of four anti-apartheid

activists who were abducted in 1987, tortured and their bodies dismembered with explosives. The non DNA evidence suggested that their fragmented remains had been buried in a commingled grave in a cemetery north of Pretoria. The Missing Person's Task Team conducted exhumations in the cemetery and identified a grave with the commingled remains of at least 4 individuals. DNA analysis was requested to assist with the identification of the individuals.

Four fractured left hip bones were provided for analysis to our laboratory at UWC. In each case two fragments were removed from each hip bone and analyzed independently. DNA was extracted and mitochondrial DNA and autosomal STR analysis was conducted. MtDNA analysis involved the amplification and sequencing of HVI and HVII, while STR analysis involved the amplification and typing of the 13 autosomal loci constituting the Combined DNA Indexing System (CODIS). Complete mtDNA sequences could be generated for three specimens and STR profiles for two.

The authenticity of the results was partly established by the fact that all extraction blanks were clear and that consistent mtDNA sequences and STR profiles were generated for the duplicated samples.

Samples were obtained from relatives of the victims. These samples included maternal relatives as well as the wives and children of the victims. Each of the mtDNA sequences obtained from the bone specimens matched the sequence from a maternal relative. Where STR genotypes were generated the results were entirely consistent with the paternal relationship with specimens provided from wives and children.

The STR data provided a probability of paternal relationship of 0.99 assisting the identification of two of the missing activists. We were not able to generate STR evidence for the third individual but did generate mtDNA data which matched mtDNA sequences for maternal relatives of the third victim. We did not however conduct a statistical analysis to determine the probability or random match of the mtDNA result.

Case 2: Looksmart Ngudle

This case involved the identification of Looksmart Ngudle who in 1961 became the Western Cape commander of the African National Congress's military wing uMkhonto weSizwe (MK). Ngudle was detained by police soon after the introduction of the 90 day detention law in 1963. This act of parliament provided the security police and military intelligence with greater opportunity to interrogate and torture suspects. Abuse was common because the act allowed for the solitary confinement of detainees without charge or trial for renewable periods, and without access to lawyers.

Ngudle went into hiding when the high command of the

African National Congress's military wing was captured just months after the introduction of the detention act. However information from an informer led to him being arrested on August 19 1963. Shortly after his arrest he was found hanged in his cell at Pretoria North police station. An inquest concluded his death was the result of suicide. In spite of searching for over 30 years his family were unable to locate his place of burial.

As a result of evidence at The Truth and Reconciliation Commission hearings, the Missing Person's Task Team located Ngudle's grave and exhumed his remains. A femur bone from these remains was sent to our laboratory at UWC for DNA genotyping along with three family reference samples and three unrelated controls. The UWC Forensic DNA laboratory typed all samples using CODIS mini STR systems. The STR data was consistent with a parent-child relationship between the bone specimen and two family reference samples. These family reference samples were later confirmed as coming from the sons of Looksmart Ngudle.

In addition to reference samples being provided by Ngudle's sons, a sample was also provided by his brother. Although mini-STR analysis can confirm parent-child relationships, it is unable to confirm sibling relationships. However by using the Y-STR testing system developed in our laboratory (D'Amato et al., unpublished data), full Y-STR profiles for all reference samples and the bone specimen were obtained. The samples from Looksmart Ngudle and his alleged brother were confirmed as sharing the same paternal lineage. Following the confirmation that the bones exhumed were those of Looksmart Ngudle, they were returned to his family for burial in May 2007.

Case 3: The case of ten missing African National Congress activists

This is one of the most high profile cases analysed by the UWC Forensic DNA laboratory. It was also a case involving highly degraded samples because of the circumstances of the deaths of the individuals concerned. In this case ten young activists from a township in Pretoria were lured into a trap by members of the Northern Transvaal Security Police. The Security Police sent in a police informant, who pretended to be an MK member who had come to take the group to Botswana for military training with the African National Congress. On 26 June 1986, the group was driven in a minibus towards Botswana. Once past Zeerust, the minibus drove into the bushes and was surrounded by security forces in camouflage uniforms. The group was made to lie on the ground where they were injected with an unknown substance which rendered them unconscious. They were then placed in another minibus and an accident was faked by driving the minibus off the road into a tree. The

vehicle was set alight with petrol and a limpet mine (which later exploded due to the fire) and two AK47s were placed in the vehicle as well.

The burning vehicle was later found by local police and the severely burnt remains were taken to the George Stegman Hospital, after which they were removed to the Ga-Rankuwa state mortuary. They were given paupers burials by Maupa Undertakers on 31 July 1986 in the Winterveld cemetery. Ten years ago the exact grave sites, which were pointed out to investigators, were no longer certain because the markers had been removed in intervening years.

A series of graves were subsequently exhumed by the Missing Person's Task Team yielding the burned remains of ten individuals thought to be those of ten activists abducted by the Northern Transvaal Security Police in 1986. The remains had been thoroughly burned and were extremely poorly preserved. Given the highly degraded nature of the remains, two laboratories were asked to conduct DNA tests: one being the UWC laboratory and the other being the BODE forensic laboratory in the United States. At UWC full mitochondrial and partial STR profiles were generated from bone and teeth samples for four of the ten skeletons (Figure 1). Matches were conducted with living relatives and the overall conclusion was that the DNA evidence supported the hypothesis that the remains were those of the missing ten activists

FUTURE PERSPECTIVES

The maintenance of power by unrepresentative governments is often associated with substantial human rights abuses. There has been an increasing trend over the last few decades to investigate human rights violations committed by such governments as well as abuses committed by non-state forces in circumstances of political violence. Human rights abuses occur in contexts where perpetrators are often able to hide evidence and prevent investigations for decades. Such evidence that is left behind such as human remains is often highly degraded. Analysis of skeletal remains, whilst extremely difficult, often provides crucial forensic evidence which is able to determine circumstances of death and identification of the deceased.

There can be little doubt regarding the growing need for human rights forensic DNA capacity on the African continent, given the ongoing conflict situation and the almost complete absence of specialized DNA facilities on the continent as a whole. Since its foundation in 2004 the UWC Forensic DNA Laboratory has hitherto successfully identified twenty apartheid victims from exhumations carried out by the National Prosecuting Authority Missing Persons Task Team. The UWC laboratory is currently upgrading its technical and human resources capacity to enable it to extend its capacity to the analysis of human

Figure 1. Examples of teeth and bone specimens sent to the UWC Forensic DNA laboratory for genetic identification.

remains on the African continent. In general, most of the skeletal remains received by the UWC Forensic DNA laboratory are at least fifteen to twenty years old, with others dating back as long ago as the 1960s. Many of these remains have been damaged by peri-mortem injuries (such as burning and explosions), which affect the quality of the bone material available for DNA testing. The UWC laboratory will continue to focus its research activities on the development and improvement of technologies suitable for the genotyping of such highly compromised skeletal material

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