Pathology and DNA Analysis of Exhumed Human Remains Three-years Post-mortem

Gayvelline C. Calacal[1,*], **Jazelyn M. Salvador**[1], **Minerva S. Sagum**[1],
Raquel D. Fortun[2], and **Maria Corazon A. De Ungria**[1]

[1]DNA Analysis Laboratory, Natural Sciences Research Institute,
University of the Philippines Diliman, Diliman, Quezon City
[2]Department of Pathology, College of Medicine,
University of the Philippines Manila, Ermita, Manila

In the past three decades after the discovery of DNA fingerprinting, there has been a remarkable growth in the use of DNA evidence worldwide. This paper highlights the value of using a scientific approach in assisting courts of law in resolving disputed parentage or kinship issues. This report describes the use of pathology in verifying the identity of a cadaver via examination and comparison with ante-mortem information of the deceased. Subsequent DNA testing of the skeletal remains – exhumed three years post-mortem – was used to confirm the identity of the woman using a living sibling as reference, and to evaluate the relationship of the deceased with a person claiming to be her offspring. Genetic comparisons at 15 autosomal Short Tandem Repeat (aSTR) regions and the mitochondrial hypervariable regions I and II (mtDNA HVR I and HVR II) of the deceased and her brother confirmed that they were siblings. Conversely, the DNA test negated the statements of the person claiming to be the child of the deceased.

Key words: autosomal Short Tandem Repeat (aSTR), exhumed human remains, forensic genetics, maternity analysis, mitochondrial DNA (mtDNA), sibship analysis

INTRODUCTION

DNA typing is the most powerful tool for human identification and for evaluating biological relationships. Depending on the availability of reference and evidentiary samples, Short Tandem Repeat (STR) DNA markers located on autosomes (aSTR), the X (X-STR) and Y (Y-STR) sex chromosomes – as well as single nucleotide polymorphisms (SNPs) on the hypervariable regions (HVR) of mitochondrial DNA (mtDNA) – are commonly used. In routine evaluation of disputed parentage cases, a child's aSTR DNA profile is compared with those of his/her alleged parent. In cases where a putative mother is deceased and biological samples have undergone environmental challenges, the comparative mtDNA sequences of the child

and the alleged mother may be used to complement the data generated from aSTR DNA analysis. Since mtDNA is found in higher copy number per cell than nuclear DNA, it has been an invaluable genetic marker in forensics (Wilson et al. 1995; Holland et al. 1999) and ancient DNA studies (Gill et al. 1994; Nilsson et al. 2010; Kjellström et al. 2012) with samples that are often limited in quantity and are of low quality. Mitochondrial DNA is inherited strictly from the mother, which makes this the marker of choice when identifying persons across a matrilineal line. However, mtDNA analysis is limited to establishing a maternal lineage, but is not conclusive to establish that a woman is the mother of a child.

This study reports the pathological examination and analysis of DNA obtained from skeletal remains of a woman that was exhumed three years post-mortem. DNA

*Corresponding author: gccalacal@up.edu.ph

profiles obtained from genetic testing of human bones were compared with those obtained from a living sibling of the deceased, and a person claiming to be her illegitimate daughter. The paper shows the significance of DNA testing in resolving parentage issues involving a deceased individual. Moreover, the pathological examination of the human remains post-exhumation prevented any claim of "switching" of bodies prior to DNA testing.

METHODS

Gathering of Ante-mortem and Case Information
Ante-mortem data (e.g., age, stature, medical information, pedigree) shown in Figure 1 was gathered from the sibling (S) and the alleged daughter (G) of the deceased (W).

Sample Sources
The remains of W were exhumed for DNA testing following an order of a regional trial court. The remains were contained in a rusty but intact metal casket, which was interred inside an above-ground concrete vault with scant moisture at the bottom. During exhumation, the metal casket was opened and the human remains (now referred to as R) were removed, wrapped in plastic, and sealed with adhesive tape. R was then transported to the UP Manila College of Medicine (UPM-CM) morgue for gross pathological examination. The stature of the deceased was estimated following procedures described previously (Trotter & Gleser 1958).

The right femur – partially articulated at the pelvis – was removed from the body and was packaged for transport from the UPM-CM morgue to the UP Diliman, Natural Sciences Research Institute, DNA Analysis Laboratory (UPD-NSRI-DAL). The femur was then stored at room temperature for ≤15 h prior to sample processing and DNA extraction.

Blood samples from the decedent's sibling (S) and alleged daughter (G) were collected on Whatman® Flinder's Technology Associates (FTA) cards (GE Healthcare Bio-Sciences Corporation) and were placed in separate brown envelopes and sealed for transport to the UPD-NSRI-DAL.

The appropriate procedures for handling biological samples were observed including the photo-documentation of the entire process (De Ungria et al. 2008). Packages containing the human remains and brown envelopes with the blood cards were sealed and signed over the seals. The appropriate forms to document the chain of custody of all samples were also completed in real-time.

DNA Extraction
The femur bone was cleaned by removing all soft tissue/muscles and was subsequently dried at room temperature. One set of bone fragments was cleaned with Zonrox bleach (Green Cross Incorporated) following the method described by Loreille and colleagues (2007). A second set of bone fragments was washed with a 5% Terg-a-zyme (Alconox Incorporated) detergent solution following methods described previously (Budimlija et al. 2003; Calacal et al. 2015). Four replicates of ~0.1 g bone powder from each set were extracted using an organic extraction procedure described by Budimlija and co-workers (2003) with slight modifications (Calacal et al. 2015).

Blood DNA on Whatman® FTA Cards (GE Healthcare Bio-Sciences Corporation) was purified following manufacturer's instruction.

Genomic DNA Analysis
Estimation of the concentration of DNA extracted from the femur sample was done using a Quantiblot® Human DNA Quantitation Kit following manufacturer's instruction (Thermo Fisher Scientific). Autosomal STR DNA profiling of samples from R, S, and G were performed using the PowerPlex®16 System (Promega Corporation), which simultaneously amplified 15 aSTR markers (D3S1358, TH01, D21S11, D18S51, Penta E, D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D, vWA, D8S1179, TPOX, and FGA) and the Amelogenin marker. Polymerase Chain Reaction (PCR) was carried out using 4.8 µL of undiluted DNA extract in two reactions – a 6.25 µL and 12.5 µL amplification mix on a Perkin Elmer Thermal Cycler 9600 (Thermo Fisher Scientific) following previously published thermal cycling conditions (Krenke et al. 2002). Reagent blanks plus positive and negative PCR controls were analyzed together with the samples to ensure that the reagents used were free from DNA contamination, and that no extraneous DNA was introduced during the preparation and genetic testing of the samples. Amplified fragments were analyzed on an ABI Prism® 310 genetic analyzer (Thermo Fisher Scientific). GeneScan® Analysis and Genotyper® v.3.7 software (Thermo Fisher Scientific) were used to obtain the DNA profiles following recommended parameters (Butler 2014). A method of replicated analyses that was recommended by Taberlet and colleagues (1996) and expanded in 2000 by Gill and colleagues was applied in the assignment of an allele detected at least twice in the consensus profile (Caragine et al. 2009; Benschop et al. 2011; Cowen et al. 2011; Gittelson et al. 2016). Allele recovery was expressed as the percentage of alleles observed in a particular DNA extract compared to the total number of alleles in the consensus profile. Peak height ratios (PHR) of heterozygotes were evaluated by dividing

the peak height of the shorter allele and the peak height of the taller allele.

The mtDNA HVR I (16024-16365 bp) and II (73-340 bp) of R, S, and G were sequenced using primers designed to amplify overlapping fragments (1000, 800, and 400 bp in length) and short 200 bp stretches (Tabbada 2006) that when assembled, covered the entire HVR I and II. Amplicons were sequenced using the Applied Biosystems BigDye® Terminator v3.1 technology on an ABI Prism® 310 genetic analyzer (Thermo Fisher Scientific). Consensus sequences were assembled and aligned with the revised Cambridge Reference Sequence (rCRS) (Andrews et al. 1999) using Sequencher® ver. 5.3 software (Gene Codes Corporation). Analysis of the differences with rCRS reported followed the recommendations of the International Society of Forensic Genetics (ISFG) (Carracedo et al. 2000; Parson et al. 2014) and the Scientific Working Group DNA Analysis Methods (SWGDAM) (2013).

Statistical analysis of aSTR DNA profiles and likelihood ratio (LR) calculations were performed following methods described by Buckleton and colleagues (2005) and using the Philippine population reference database (Maiquilla et al. 2011; Rodriguez et al. 2015). MtDNA haplotype frequency was estimated using the Clopper-Pearson (1934) formula and the Philippine mtDNA data (Tabbada 2006; Tabbada et al. 2010).

The mtDNA haplogroup was determined using HaploGrep2 using Phylo Tree build 17 (haplogrep.uibk.ac.at).

RESULTS AND DISCUSSION

Ante-mortem Information
The woman (W) died of natural causes at the age of 75 years old. During her lifetime, W took in a young girl (G) and treated her as her own daughter. After she died, G claimed the entire estate for herself alleging that W was her biological mother. The siblings of W denied G's claims and filed a case in court (Figure 1).

Post-mortem Information
The cranium, mandible, cervical vertebrae (from 1st to 5th), bilateral clavicles, all the bones of the upper extremities, and all the bones of the lower extremities distal to all femurs were found to be skeletalised. The trunk including the vertebral column from the 6th cervical vertebra, bilateral scapulae, sternum, all the ribs, the innominate bones, and femur were still articulated in a mummified state. Clumps of dark brown hair were found near the skull.

No perimortem injury was detected. The cranium showed female features such as gracile mastoid processes, small rounded chin, and non-prominent nuchal ridges. Focal osteophytic spurs in the bones examined also indicated degenerative changes consistent with the advanced age of the person prior to death.

Dentition was completely absent with the maxillary and mandibular ridges showing severe bone resorption with fused and sharp alveolar ridges. Estimated stature from measurements of both femurs indicated a height ranging from 5'3" to 5'6".
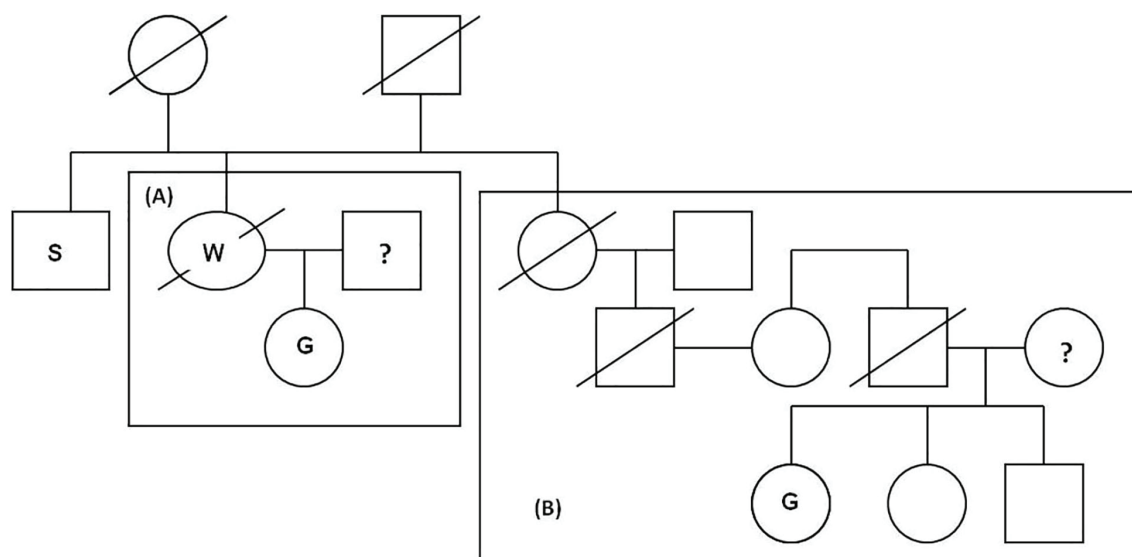

Figure 1. Pedigree figure pertaining to the disputed parentage case. A: the scenario as presented by the child claiming that she is an illegitimate daughter of the deceased B: the scenario as presented by the surviving kin of the deceased. DNA analysis was conducted on the exhumed remains of the deceased (W), the living sibling (S), and the child (G).

Figure 2. Loci present in the PowerPlex®16 are plotted across the X-axis; wash solution (volume of PCR reaction) and aSTR DNA typing results of each extract is shown on the Y-axis. Green box correspond to concordant allele/s with peak height ratio ≥ 50% in heterozygotes; yellow box correspond to concordant allele/s with peak height ratio ≤ 50% in heterozygotes; red box correspond to absence of an allele at a homozygous locus or 2 alleles at a heterozygous locus; black box correspond to the possibility of allele drop-in, contamination or elevated stutter at a locus.

Overall examination of the skeletalised human remains (R) was consistent with the information about the deceased that was provided by S and G. The casket contained the body of a female person that was clothed in the same dress worn by the deceased when she was buried. The known height of W was 5'6" and she died at the age of 75 that was consistent with the observations on her bones and her estimated stature, respectively. Based on the gross examination, the researchers concluded that the human body R was that of W.

DNA Analysis of Bone Sample
The identity of the exhumed remains (R) was verified using DNA technology. To evaluate the best conditions for processing bone samples interred under this condition,

samples were washed with bleach or detergent to remove contaminants prior to DNA testing, and two volumes of PCR mix (6.25 µL or 12.5 µL) were used to generate the DNA profile of R.

Using the Quantiblot® Human DNA Quantitation Kit, no visible band was observed for bleach-washed and detergent-washed bone DNA, indicating that the DNA concentration of these samples was <0.125 ng/µL, which was the detection limit for this kit. The use of two PCR volumes allowed the researchers to maximize amplification of trace amounts of DNA and to reduce the effect of inhibitors, if any, in solution.

Although none of the samples yielded detectable quantities of DNA, full to partial concordant aSTR profiles were generated from the bone sample except for 1 DNA extract (Figure 2). Estimates of DNA concentration was limited by the technology available at that time which can now be overcome with the development of more sensitive qualitative and quantitative real time PCR-based (qPCR) assays (Ewing et al. 2016; Holt et al. 2016).

Using two-way ANOVA with 0.05 level of significance, neither type of washing method (p=0.38) nor volume of PCR mix (p=0.10) significantly affected allele recovery. However, it seems that the type of washing agent significantly affected the balance of heterozygous alleles (p=0.03). The detergent-washed DNA extracts amplified in 12.5 µL PCR reaction volume showed a higher allele recovery with relatively balance peaks (Figure 2). Loss of heterozygosity and imbalance peaks (PHR≤50%) were frequently observed in samples washed with bleach and in larger DNA markers HUMCSFIPO (321-357 bases) and Penta E (379-474 bases). PCR inhibitors and DNA fragmentation may lead to the loss of signal or reduced detection sensitivity of larger PCR products (Butler 2010). Amplification of larger markers (i.e., HUMCSF1PO and Penta E) was less efficient in bleach-washed samples and reduced volume PCRs that may be attributed to the presence of inhibitors. In contrast, detergents are surfactants that lower water tension interacting more effectively with a wider range of contaminating substances (e.g., soil, formaldehyde, fatty deposits). The Terg-a-zyme detergent used in this study also has protein-degrading enzymes that enhance its ability to remove proteins on biological samples.

Analysis of 1 DNA extract in bleach-washed bone sample amplified in a 6.25 µL PCR reaction mix showed the presence of more than two alleles. No peak signal was detected in the reagent blanks and negative controls after DNA amplification. The extraneous alleles were spurious and were not replicated in other extracts, which indicate that these peaks may be attributed to stochastic effects inherent to low quantity and quality DNA extracts. With the amplification of low level target DNA (<0.125 ng), there is a higher degree of stochastic effects such as locus and allelic drop-out. Gross peak imbalance (PHR≤ 50%) was more frequently observed in DNA extracts amplified in reduced PCR reaction mix volumes (6.25 µL). Except for the result of amplification of one DNA extract where five extraneous alleles were observed, a consensus bone DNA profile from replicate analyses was generated using the biological method of performing replicate analyses combined with a consensus interpretation to deal with the uncertainty produced by stochastic effects (Caragine et al. 2009; Benschop et al. 2011; Cowen et al. 2011; Kokshoorn & Blankers 2013; Gittelson et al. 2016).

Bone DNA vs. Reference DNA

Initial analysis involved the comparison of the aSTR profile of R and S. A likelihood ratio was computed to test the hypothesis that R and S were genetically related versus the presumption that R and S were unrelated individuals. Likelihood ratio (LR) calculations of the aSTR DNA profiles of R and S was equal to 76,055. This means that the probability that the bones were from a sibling was 76,055 more likely than if the bones were from a random person.

In addition, the mtDNA profiles of R, S, and G were obtained. Short regions within mtDNA HVRI (208 bp) and HVRII (270 bp) regions were generated for the bone sample. The sequences of R and S at these regions were identical confirming their matrilineal relationship. The mtDNA haplotype of R and S was not observed in the Philippine mtDNA population database. Their Likelihood Ratio is equal 42 (LR=42), supporting the proposition that R and S are maternally related versus the presumption that they are unrelated. The mtDNA haplogroup identified for the maternal lineage is B4a1a. This haplogroup is one of the most common types found in the Philippines (Tabbada et al. 2010; Delfin et al. 2014) and is the immediate precursor to the Polynesian motif (Pierson et al. 2006). In contrast, the mtDNA sequence of G differed from the mitotypes of R and S at eight (8) nucleotide positions, namely: 16217, 16223, 16261, 16293, 16311, 16362, 146, and 152 (Table 1). The presence of eight mismatching sites is sufficient to show that G is not maternally related to R and S. The specific mtDNA haplogroup of G could not be assigned since the mtDNA sequence coverage was insufficient at this time. Autosomal STR DNA profiling of R and G revealed three mismatches out of the fifteen

Table 1. Sequence analysis of the mtDNA HVRI (208 bp) and HVRII (270 bp) regions of the bone sample (R) compared to the sibling (S) and alleged child (G).

Revised Cambridge Sequence (rCRS) at certain nucleotide positions	Bone (R)	Sibling (S)	Alleged Child (G)
T16217	C	C	T
C16223	C	C	T
C16261	T	T	C
A16293	G	G	A
T16311	T	T	C
T16362	T	T	C
A73	G	G	G
T146	C	C	T
T152	T	T	C
A263	G	G	G

aSTR DNA markers that were tested (Table 2), which was sufficient to exclude W from being the biological mother of G (Poetsch et al. 2006).

Table 2. Autosomal STR typing results at three genetic markers showing non-matching alleles of the alleged child (G) and bone sample (R).

STR markers	Bone (R)	Alleged Child (G)
D21S11	29, 31	28, 32.2
Penta E	11, 15	14, 19
HUMFGA	19, 26	21, 28

CONCLUSION

A key step for any forensic analysis is the identification of evidence. If evidence is not properly documented, collected, packaged, and processed, it will likely not be admitted in court. In this particular case, the identity of the human remains as those of W was consistent based on ante-mortem information, post-mortem examination, and DNA evidence.

The researchers were able to recover and generate DNA profiles from human remains three years post-mortem that were exposed to conditions of embalming, internment, and exhumation. Full to partial concordant aSTR profiles were generated from the femur bone sample. There was less efficient amplification of bleach-washed bone samples and use of reduced volume PCRs that may be attributed to the presence of inhibitors. Detergent washing of bone samples prior to extraction appears to be a more effective treatment for removing bone contaminants prior to DNA analysis. Utility of two DNA typing techniques – namely aSTR DNA analysis and mtDNA sequencing – both supported biological relationship of the deceased and her brother and conversely negated the claims of the alleged child, which aided in the resolution of this case.

Under the Philippine Law, a child of the deceased would exclude relatives like siblings, nephews, and nieces from inheritance. The DNA test provided an objective evidence that aided our Courts of Law resolved issues of biological relationships involving the deceased individual. Thus, the Court resolved to grant the petition in favor of the sibling of the deceased woman after the DNA test conclusively established that the alleged child is not a biological child and that the sibling is the next of kin, therefore qualified to administer the estate of the deceased. With the development of forensic DNA technology in the Philippines, the potential of including DNA evidence to resolve issues of biological relationships in order to expedite these cases in Philippine courts should be realized.

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