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Raman Spectroscopy and STR Analysis of the Elongated Skulls from the Paracas Mummies of Peru

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ABSTRACT

Between 1880-1947, several hundred mummified bodies were excavated from burial sites in the Paracas peninsula of Peru. Each set of recovered remains displayed reduced stature and severe cranial elongation, which led many anthropologists and archaeologists to question the genetic origins of what would be colloquially referred to as the "Cone Head mummies." Since the discovery of these bodies, suggestions have been made that the mummies' cranial deformations point to another human species, an unknown hominid species, or even within some groups, an alien. This project sought to employ commonly used forensic techniques, including but not limited to Raman spectroscopy and DNA fingerprinting, to investigate the hair structure and genetic profile from tissue and hair samples derived from the Paracas skulls. Tissue and hair samples were subjected to comparative hair analysis, Raman spectroscopy, and DNA typing using short tandem repeat (STR) analysis. Comparative microscopic hair analysis and Raman spectroscopy of samples collected from the Paracas skull as well as from humans of varying ethnicities revealed very similar hair structures and characteristics that are associated with human hairs. Following DNA extraction and amplification of tissue samples, STR typing results demonstrated allelic profiles similar and consistent with those DNA profiles observed in modern human populations. Moreover, no foreign DNA or unusual patterns/profiles were observed in any of the samples tested. Data generated to this point strongly suggest that the Paracas mummies are of the same species as humans and not from an unknown hominid or alien society.

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Introduction

Between 1880-1947, several hundred sets of mummified bodies, wrapped in cotton clothes with elaborate patterns, were excavated from burial sites in the Paracas peninsula of Peru. Archaeologists, upon examining the remains, determined that all of the mummified bodies were males, as well as further study of the contents of the burial sites (*i.e.*, intricate wrappings, gifts of notable quality, *etc.*) indicated these males were likely considered to be of high status in their culture. Furthermore, each of the sets of remains possessed significantly shortened stature and elongated skulls.

Well documented throughout the literature is the practice of cranial binding, observed in other "new world" societies, whereby the skull is bound with boards, weights, and/or wrappings until a tall, conical shape is produced [1]. The Paracas mummies, possessing significant cranial elongation, are believed to have been deliberately subjected to cranial binding induced at childbirth using boards and weights [2]. Such shapes are believed to have signified high status in the Paracas society, as well as in other ancient cultures throughout the world. However, since the discovery of these mummies, many anthropologists and archaeologists have not been able to agree on their genetic origins, suggesting that the mummies' cranial deformations or cone-shaped head signify their origins as another human species, an unknown hominid species, or even serves as evidence of an alien species [3,4].

In an early attempt to determine the genetic origin of the Paracas mummies, samples were subjected to DNA testing by an unidentified geneticist in Texas and reported by Brien Foerster in 2014. Foerster was known to believe in ancient astronauts and extraterrestrial origins of humanity, and later endorsed the concept of an alien/human hybrid. Although the preliminary data detailed in that report has not undergone scientific review, it has led to much debate regarding the origins of the Paracas mummies, including claims that samples from the Paracas skulls "...had mtDNA mutations unknown in any human, primate or animal known so far..." Additional statements to the effect that this non-terrestrial life form was unrelated to any of the thirty-five taxonomical phyla of animals have circulated the internet as a result of Foerster's report [3,4]. Although the taxonomical scheme is only a classification system used to group organisms based on morphology/anatomical structure and phylogeny, Foerster's

implications suggest that the Paracas mummies would not suitably be classified into any of these taxonomical categories.

DNA typing has become a powerful tool throughout many biological sciences, including its utility in the identification of individuals in criminal cases and in parentage verification in paternity cases. Specifically, polymerase chain reaction (PCR)-based assays of short tandem repeat (STR) loci using nuclear DNA has become the established method for human identification in the forensic science community. Over time additional methodologies such as mitochondrial DNA (mtDNA) analysis, 18S ribosomal DNA (rDNA) analysis, and PCR using specific human gene primers and sequencing for genotype identification have emerged for the detection of human DNA, providing the forensic science community with a much more diversified approach for identification [5-7].

To better understand the genetic makeup of the Paracas skulls, this project had two areas of focus: hair analysis and STR analysis. The first area of focus examined the Paracas skull's hair structure and characteristics against various types of known human hair samples using comparison light microscopy and Raman spectroscopy. The second area of focus, and ultimately the primary focus of the project was to develop genetic profiles from the tissue of the Paracas skull through STR analysis. Assuming the DNA profiles generated by STR analysis from the examined tissue are comparable with modern human profiles, the data might demonstrate that these skulls are, in fact, of human origin.

Materials and Methods

Sample Collection

Hair and tissue samples were provided by Dr. Dennis Swift (Creation Science Ministries, Beaverton, Oregon, USA). The samples were specifically taken from a cone-shaped skull that was unearthed in 2016 in Palpa ICA, Peru. To serve as a comparison for microscopic hair analysis and Raman spectroscopy, known human hair samples were collected from various ethnicities (*e.g*., African American, Hispanic, Caucasian, *etc*.).

Microscopic Hair Analysis

To prepare specimens for microscopic examination, clear fingernail polish was swatched on a microscope slide, and individual hairs from both the known human hair samples and the Paracas skull were placed in the wet polish and allowed to harden. All hair samples, Paracas and known reference samples, were compared and microscopically analyzed using an Accu-scope™ forensic comparison microscope (Model 3002-CB, Commack, NY). Hair diameter and other morphological structures (*e.g*., cuticle, cortex, and medulla) were measured using an Exelis HD microscopy camera with Captavision™ imaging software (Accu-scope™, Commack, NY).

Raman Spectroscopy

To prepare samples for spectroscopic analysis, all hair samples were separated from each other and placed in separate glass vials. Prior to testing, the portable Raman spectrometer (First Defender RM, ThermoFisher Scientific Inc., Waltham, MA) was calibrated against known compounds such as polystyrene, ethanol, and sodium bicarbonate. Such calibration was routinely performed throughout the analysis of the hair samples. Once the reference spectra were searched against the digital database and all samples correctly identified, Paracas hair samples were subsequently examined using the portable Raman spectrometer.

DNA Extraction, Quantitation, Amplification, and STR Analysis

Tissue samples were subjected to robotic extraction using the AutoMate Express™ Nucleic Acid Extraction System with PrepFiler™ lysis buffer (ThermoFisher Scientific Inc., Waltham, MA). Additional samples were also extracted using the DNeasy Blood and Tissue Kit™ with the Qiagen Lyse and Spin Basket Kit™ (Qiagen, Germantown, MD). Positive and negative controls were extracted in parallel with the samples collected from the Paracas skull. Following extraction, the concentration of DNA in each sample was determined using a NanoDrop 2000™ spectrophotometer (ThermoFisher Scientific Inc., Waltham, MA) and the quality and quantity of the DNA analyzed via agarose gel electrophoresis. Following quantitation, 0.5 ng of DNA was amplified at 24 loci (GlobalFiler™ Reagents Kit, Applied Biosystems, ThermoFisher Scientific, Pittsburg, PA) in a Bio-Rad Cl000™ Touch thermocycler (Bio-Rad, Laboratories, Hercules, CA). Amplicons were separated and detected using the Applied Biosystems Hitachi Genetic Analyzer 3500™ (ThermoFisher Scientific Inc., Pittsburg, PA) and the results analyzed via GeneMapper ID-X™ (Applied Biosystems, ThermoFisher Scientific Inc., Pittsburg, PA).

Results

Microscopic Hair Analysis

Known human hair samples from various ethnicities (*e.g*., African American, Hispanic, Caucasian, *etc*.) were compared to hair samples collected from the Paracas skull using a forensic comparison microscope (Figure 1). Hair diameter and other morphological structures (*e.g*., cuticle, cortex and medulla) were measured and compared between the known hair samples and the hair from the Paracas skull.

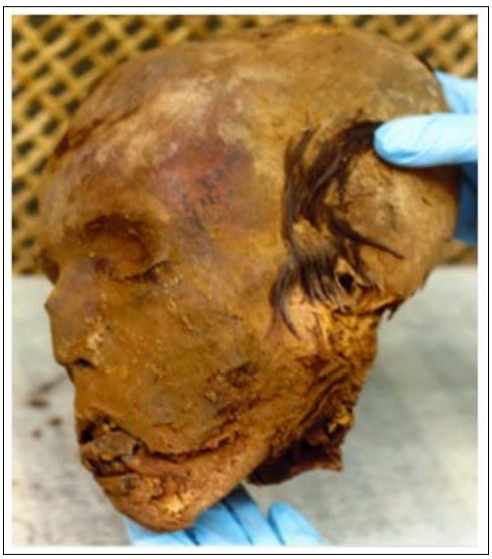


Figure 1: A Paracas skull (*i.e*., Cone Head skull) that was excavated in 2016 in Palpa ICA, Peru. Hair and tissue samples, tested in this project, were taken directly from the skull depicted above.

When examining the Paracas skull hair, the cuticle or the outer coating was a light red in color and did not consist of overlapping scales. Similar findings were observed with known human hair samples from the various ethnicities. Characteristics of the cuticle may be important in distinguishing between animal hairs of different species but are often not useful in distinguishing between different people.

The medulla or central core, which may be absent, was observed in Paracas hair samples and compared to reference human hair samples. The medulla "pattern" from the Paracas skull hair was found to vary between hair samples in such that the medulla from some hairs was a dark, red-orange color and classified as containing an interrupted medulla, while other hairs did not appear to contain a medulla. In the reference human hair samples, the medulla was also found to be absent. Furthermore, the width of the medulla from the Paracas hair was found to be less than one-third the diameter of the hair which is consistent with human hair. It should be noted that for animals, the medulla is generally equal to or greater than one-half the diameter of the hair. Like the cuticle, the medulla can be important for distinguishing between hairs of different animal species, but often does not lend much information to the differentiation between hairs from different people.

The cortex can vary in thickness, texture, and color and distribution of pigments. The cortex from the Paracas skull hair was also a light orangish-red in color and was similar to the pigment distribution observed in human hair. Of all the hair samples analyzed no significant differences were observed in the hair diameter and/or morphological structure between the known human hair samples and the hair from the Paracas skull (Figure 2).

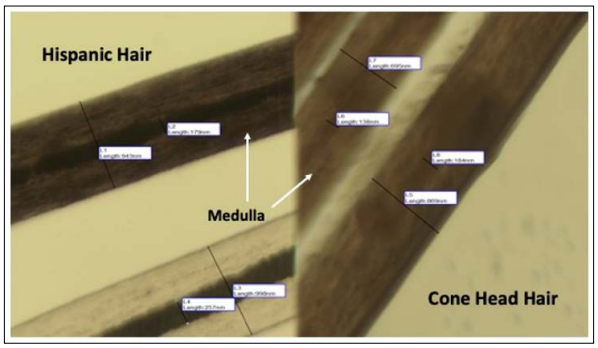
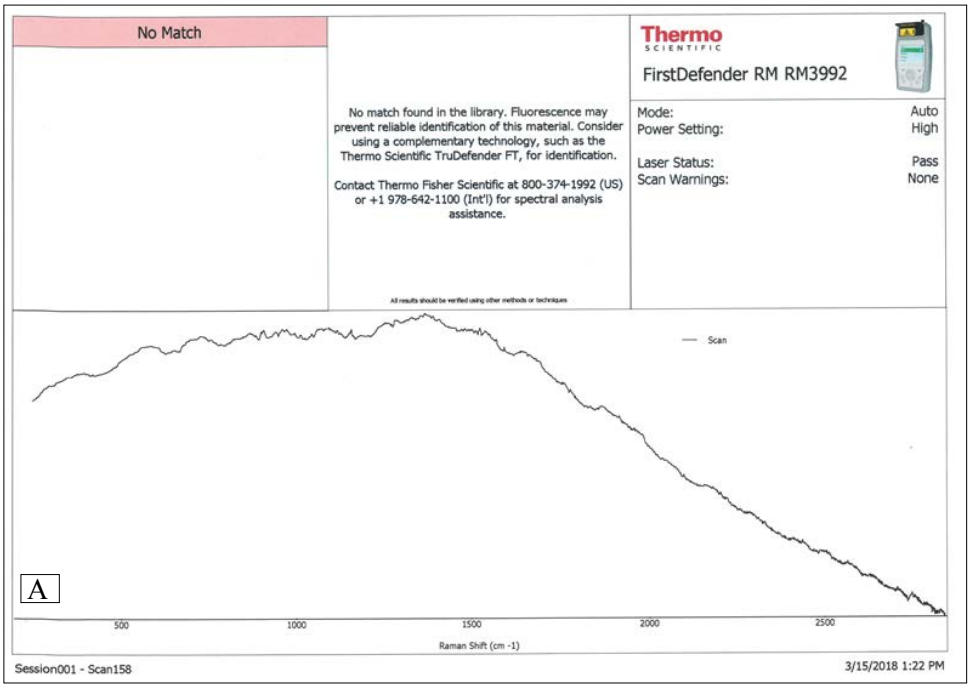
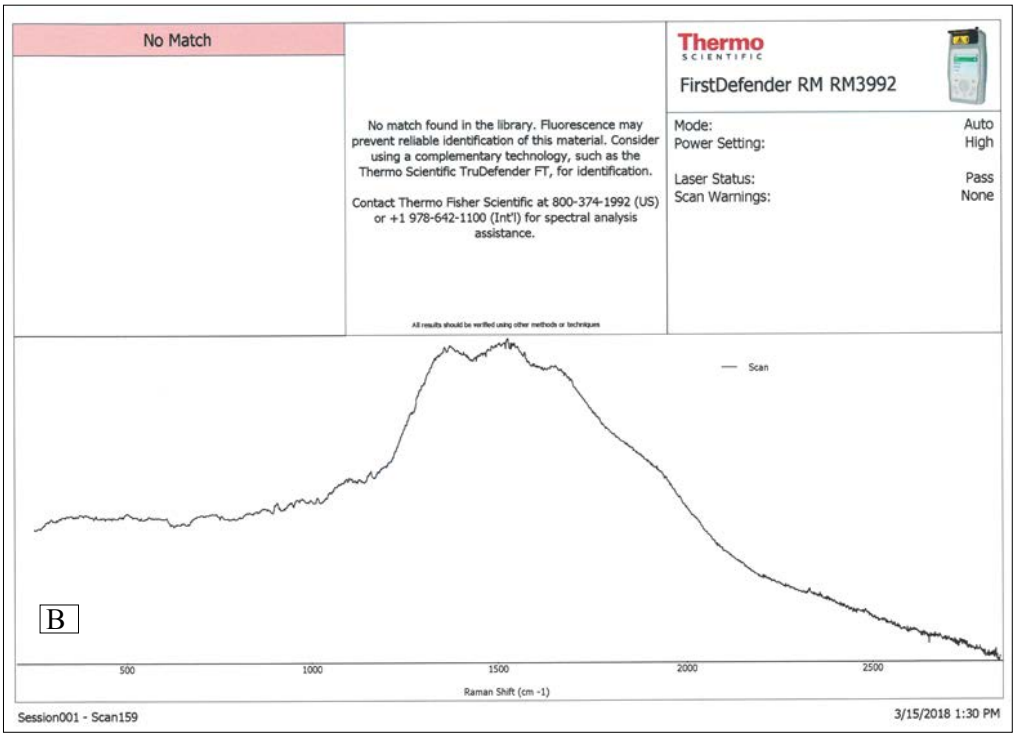


Figure 2: Photomicrographs using a forensic comparison microscope. A) A photomicrograph of hair collected from an individual of Hispanic origin and analyzed using a forensic comparison microscope at a total magnification of 400X. B) A photomicrograph of hair from the Paracas skull at 400X. Measurements of the diameter of the cortex from all samples revealed morphological similarities.

Raman Spectroscopy

Known human hair samples from various ethnicities were compared to hair collected from the Paracas skull using Raman spectroscopy. Of the known human hair samples analyzed, the spectra from a piece of dark Caucasian hair, a blond Caucasian hair, and an Asian hair were very similar, if not identical, to the spectra generated from hair from the Paracas skull (Figure 3 A-D). This is based on the results displayed in the "fingerprint region" or the wavenumbers below 1500 cm^{-1}. This area (1600 – 1000 cm^{-1}) is the most critical portion of the spectrum for identification. When comparing the spectra or specific footprint from each hair sample the frequencies (x – axis) and intensities (y – axis) from each sample are very similar. In fact, there were no significant differences observed in the intensity or wavenumber shifts between the various ethnicities and the Paracas skull hair, demonstrating through Raman spectroscopy that the chemical characteristics of the hair samples are practically identical. In fact, the hair from the Paracas skull was found to be, in general, consistent with all the reference human hairs examined.

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Contact Thermo Fisher Scientific at 800-374-1992 (US) or +1 978-642-1100 (Int'l) for spectral analysis assistance.
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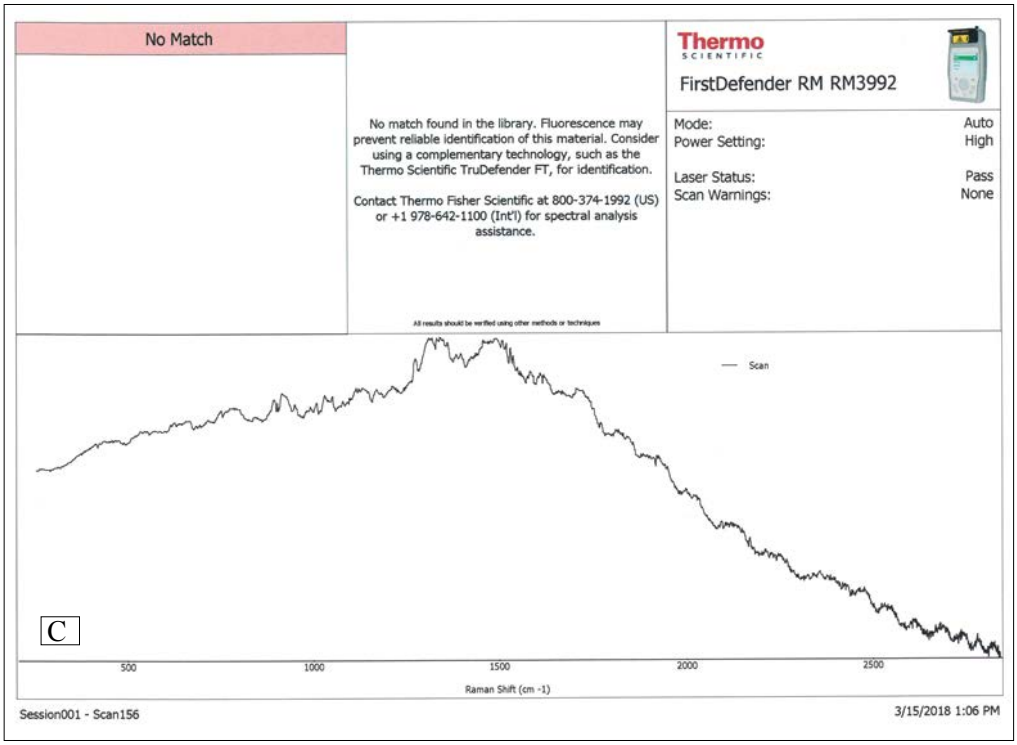
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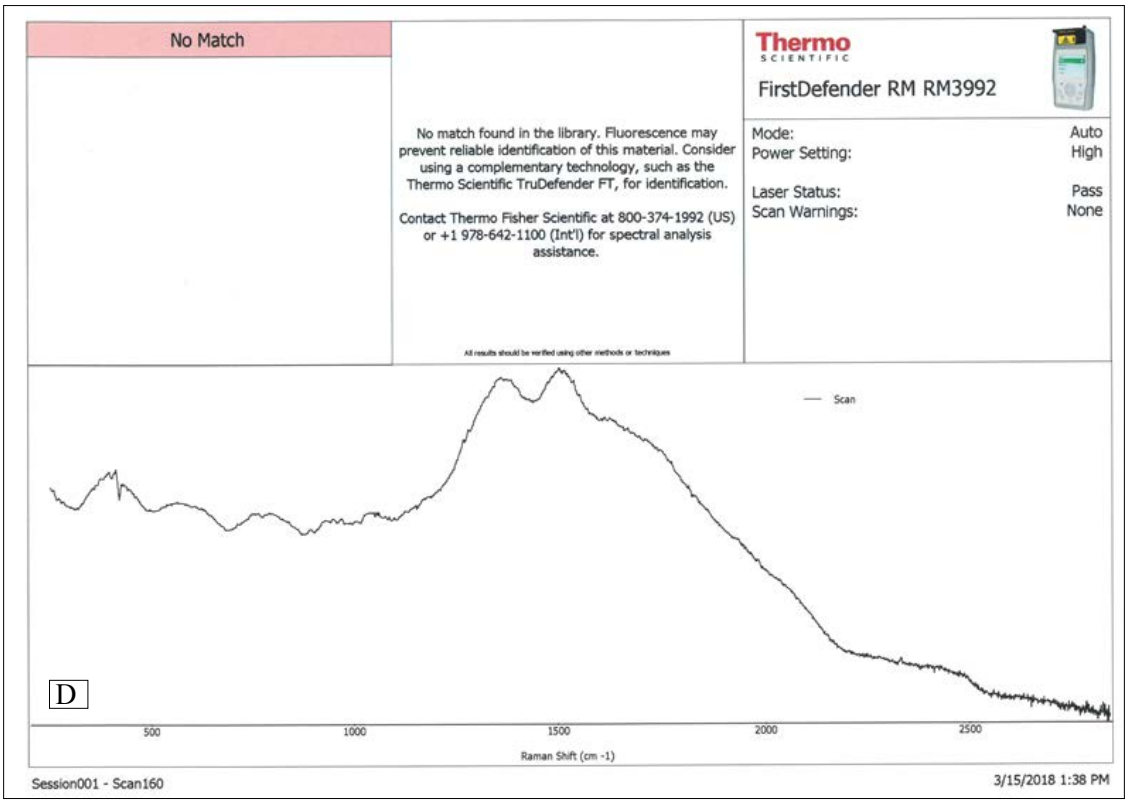

Figure 3: Known human hair samples from various ethnicities were compared to hair collected from the Paracas skull using Raman spectroscopy. Raman spectra of A) Caucasian hair, B) a blond hair from a Caucasian, C) Asian hair, and D) hair from the Paracas skull. The X-axis represents frequencies and the y-axis represents intensities (wavenumber/cm^{-1}).

STR Analysis

Two different extraction and purification methods were utilized to ensure sufficient DNA was isolated from the tissue of the Paracas skull. Both extraction and purifications techniques yielded sufficient DNA (11.2 ng/ml – 12.0 ng/ml) to generate STR profiles. The STR profiles generated from the Paracas skull was consistent with a single source male contributor. This finding is consistent with the sex (*e.g*., male) identified when the remains were originally excavated. Of the twenty-four (24) loci analyzed, eleven (11) loci showed either one (homozygosity) or two (heterozygosity) peaks which is consistent with allelic profiles obtained from humans. Two loci (*e.g*., D8S1179 and D21S11) displayed more than two peaks which would suggest that the sample was consistent with a mixture containing more than one contributor. It should be emphasized that these minor peaks observed at the D8S1179 locus (*i.e*., 9, 11, and 14) and the D21S11 locus (*i.e*., 26.2, 27, 31, and 31.2) represent DNA from a minor contributor or from a contaminating source, possibly from handling, and are only slightly above the allelic threshold established by forensic laboratories.

An issue that is a common concern when analyzing samples from the past that have been exposed to the environment is degradation. The electropherograms, observed in Figure 4 (A-C) clearly demonstrate the effect of the natural environmental condition after hundreds or thousands of years. Specifically, the downward slope from left to right observed with the allelic profile in the electropherograms indicates that degradation of the DNA in the analyzed samples had occurred. Also, it should be noted that some of the loci (*e.g*., D5S818, SE33, D18S51, and DYS391) did not generate any allelic response suggesting that the DNA concentration was low or that the sample was degraded beyond the ability to produce an allelic response.

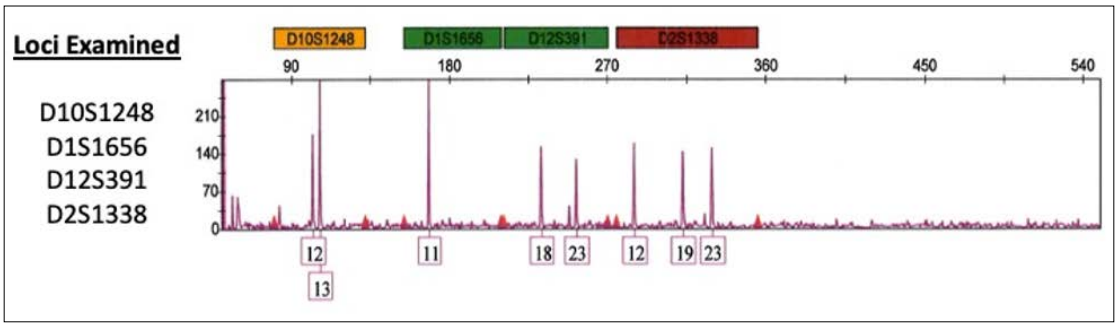

Figure 4A: An electropherogram revealing the allelic profiles of DNA samples from the Paracas skull. Loci examined: D10S1248, D1S1656, D12S391, and D2S1338. The numerical values in the red boxes represent the individual alleles at each locus. The x-axis represents the relative fluorescent units (RFUs).

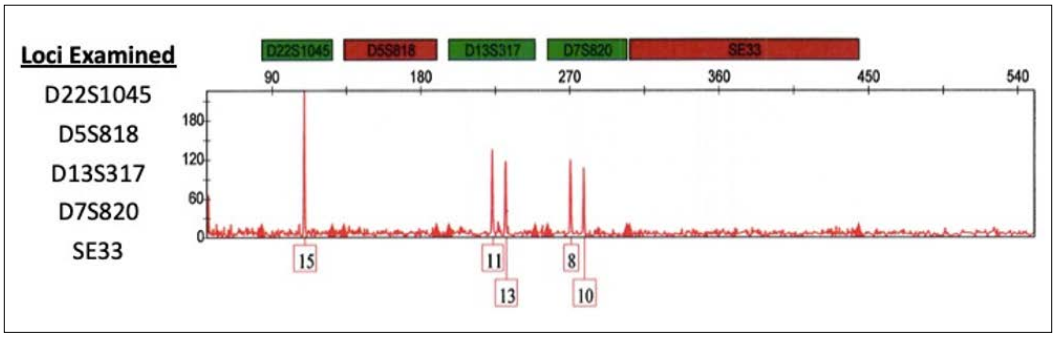

Figure 4B: An electropherogram revealing the allelic profiles of DNA samples from the Paracas skull. Loci examined: D22S1045, D5S818, D13S317, D7S820, and SE33. The numerical values in the red boxes represent the individual alleles at each locus. The x-axis represents the relative fluorescent units (RFUs).

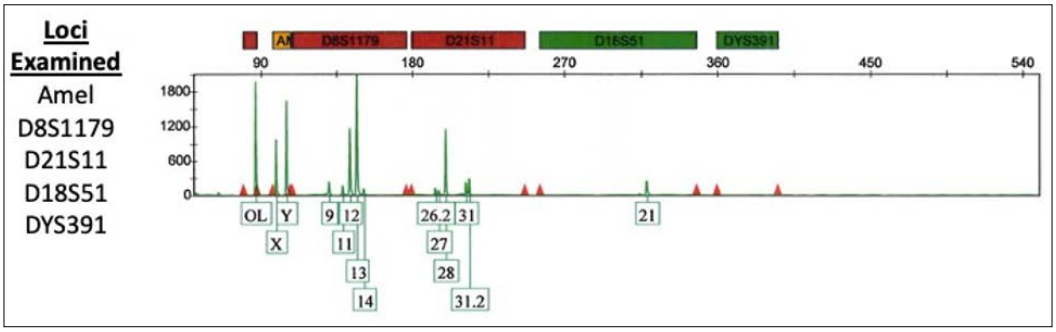

Figure 4C: An electropherogram revealing the allelic profiles of DNA samples from the Paracas skull. Loci examined: the amelogenin locus (a gender specific locus), D8S1179, D21S11, D18S51, and DYS1391 (a male specific locus). The numerical values in the red boxes represent the individual alleles at each locus. The x-axis represents the relative fluorescent units (RFUs).

Discussion

Microscopic Hair Analysis

Hair samples from several different ethnicities were analyzed and compared to the hair samples collected from the Paracas skull using light microscopy. The hair from the Paracas skull was dark and almost reddish in color, supporting the earlier observation that the Paracas mummies were comprised mostly, if not entirely, of redheads (Dr. Dennis Swift, personal communication). However, following death hair color of buried bodies can change [8]. During decomposition, hair outlasts most other soft tissues due to the insoluble and stable physical structure of keratin. Furthermore, the chemical composition of keratin makes it difficult for proteolytic enzymes to degrade or break down hair which contains a mixture of black-brown-yellow eumelanin and red pheomelanin pigments. Pheomelanin tends to be stable in the hair even under extreme conditions; however, eumelanin is less chemically stable than pheomelanin and breaks down much more rapidly when oxidized. Even in wet oxidizing climates, the eumelanin in the hair is lost over extended periods of time, leaving behind the red pigment, pheomelanin. Thus, the color of hair changes faster under extreme conditions and will often turn a reddish color. Consequently, it is difficult, if not speculative, to assume that the Paracas society was a population of redheads [9,10].

When the diameter and morphological structures (*e.g.*, cuticle, cortex, and medulla) of the hairs from the Paracas skull were analyzed using microscopic analysis and compared to the human reference hair samples of varying ethnicities, no significant differences were observed. Although hair comparisons are generally performed using a forensic comparison microscope, this approach or technique cannot provide information such as age and/or gender without DNA testing. However, hair evidence can provide important investigative leads in cases by determining the racial origin. The morphological characteristics and structures observed in this instance strongly suggest that the hairs from the Paracas skull are indistinguishable from human hair.

Typically, light microscopy is the exclusive means by which hair is currently analyzed. Although light microscopy has proven to be invaluable, there are some disadvantages. First, subjectivity is inherent whenever an analyst performs qualitative assessments. This unintentional bias can affect the results and influence any conclusions made. Recently it has been demonstrated that conclusions made by FBI hair examiners based on microscopy were determined to be incorrect or overstated, after obtaining a DNA profile from the hair samples in question. Additionally, no set of standards exists for how many characteristics are needed between two hairs in order to confirm they originated from the same source. Because of these limitations, a hair examiner can only state that two hairs are either consistent or inconsistent with each other, based on various physical attributes (*e.g.*, color, morphology, *etc*.). This strongly suggests that using microscopy as the only technique for drawing conclusions in hair analysis should be reconsidered. Consequently, Raman spectroscopy was utilized in this study to further characterize and identify the hairs from the Paracas skull.

Raman Spectroscopy

Edwards *et al*. used Raman spectroscopy to analyze a tissue sample found inside a human skull that was 2500 years old [10]. In the present study, a portable Raman spectrometer was used to collect spectra from the hair samples (*i.e.*, known human hair and hairs from the Paracas skull) without any preparation delivering spectral results in real time. The authors noted that this particular trait was valuable because sample preparation can be destructive, and if possible, must be avoided. As such, Raman spectroscopy

has been shown to be a powerful analytical tool in the study of biological material [12]. Due to its non-destructive sample preparation and its ability to provide a detailed "fingerprint" that can be used for sample identification. The resulting Raman spectra have proven to be specific, and unknown materials are able to be identified in most instances by searching a digital database [13]. In all instances, data and/or observations developed from both microscopic and spectroscopic techniques showed that the hairs from the Paracas skull were consistent with the hairs collected from the different ethnicities.

Interpretation and Analysis of the STR DNA Profiles

After samples were subjected to two different extraction and purification methods, the tissue samples from the Paracas skull had sufficient DNA to generate allelic profiles, as indicated by the quantitation data. The electropherograms of these Paracas samples demonstrated alleles across multiple loci with only one locus displaying an off-ladder (OL) peak, possibly suggesting sequence variation between STR alleles when compared to commonly observed human alleles. Most of the samples analyzed showed either homozygosity or heterozygosity, with which is consistent with samples containing a single contributor of DNA (Figure 4). It should be noted that the allelic profiles that were observed were not consistent with any member of the laboratory performing the analyses.

The additional alleles (*i.e*., more than two alleles at any given locus) observed at the D8S1179 locus (*i.e*., 9, 11, and 14) and the D21S11 locus (*i.e*., 26.2, 27, 31, and 31.2) may be attributed to several factors. The first is that the response may have been an artifact and below the designated threshold level recommended by the manufacturer. On the other hand, the presumed allelic response could, in fact, have been an allele from another contributor. In this latter scenario, allelic pairing, or lack thereof, may suggest more than one contributor. For example, at the D8S1179, the peak height for the 12 and 13 alleles is significantly greater than the height of the 9, 11, and 14 alleles. Moreover, the peak height for allele 11, relative to the 12 allele, excludes stutter, a small peak proceeding the true allele (*i.e*., 12) produced during the amplification process. At the D21S11 locus, five (5) allelic responses (*i.e*., 26.2, 27, 28, 31, and 31.2) were observed. However, when these alleles are paired (*i.e*., 26.2 and 27 or 31 and 31.2 or any combination of these alleles) based on peak height, the presence of a "major" allele (*i.e*., 28) suggests either another contributor or that the response is an artifact detected slightly above the threshold level.

A common concern when analyzing samples from the past is that those samples have been constantly exposed to potentially harsh environmental conditions, and thus, the DNA would likely be degraded. Cellular material can easily lyse over time and thus, release and expose the DNA to the natural environmental conditions, such as heat, humidity, enzymatic activity, *etc*., which lead to degradation. The STR profiles that were observed at some loci (D12S391, D2S1338, D13S317, D7S820, D21S11, and DS18S51) revealed degradation as demonstrated by the downward slope (*e.g*., left to right) of the alleles at the examined loci. This is not surprising since the Paracas mummies are thought to be hundreds or thousands of years old. Consequently, the potential for allelic drop-out (*i.e*., the absence of alleles at the larger loci) could be due to degradation or to low levels of available DNA for typing. One locus near the amelogenin site, displayed an OL peak, where a peak labeled as OL may be a true allele simply not represented in the allelic ladder or a migration artifact. The OL allele or microvariant may be larger or smaller than the alleles spanning the allelic ladder, or this OL response may fall between the known alleles in the ladder. In this scenario, the peak was labeled as an OL allele and was smaller than both amelogenin alleles.

Conclusions

The application of Raman spectroscopy as a versatile analytical tool for use in forensic science is constantly evolving. Raman spectroscopy is very selective with the ability to provide confirmatory identification in a non-destructive and rapid manner. It has been shown here that the application of Raman spectroscopy for hair analysis can prove to be a worthwhile technique. Although the ideal situation is to couple the analysis with another technique such as light microscopy, Raman spectroscopy can provide additional information that light microscopy cannot and has demonstrated its potential to stand on its own as a useful tool in forensic investigations.

DNA typing using STR analysis demonstrated allelic profiles or responses similar and consistent to those DNA profiles observed in humans. Moreover, no foreign DNA or unusual profiles were observed in any of the Paracas samples tested. In addition, the DNA profiling identified the remains as a male, with both X and Y alleles at the amelogenin locus. Additional genetic analyses, including but not limited to mtDNA testing as well as developmental gene analysis, will only provide further support as to the characterization of these elongated skulls from Peru [14].

During this project, PCR-based studies were initiated to determine the presence and/or absence of specific human developmental genes. Previous studies have identified certain genes that are involved in human growth and development (*i.e*., TP53 and FOXP2). The TP53 gene encodes the p53 protein which is involved in cell division and death. Studies have shown that p53 could be involved in the development of the meninges, the thick membrane that surrounds the brain under the skull. In humans, the forkhead box P2 (FOXP2) gene is specifically involved in speech and language development [3,5]. Both genes are essential for normal human growth and development and could provide further genetic information regarding the characterization of the skeletal deformations observed in the Paracas population.

To discriminate between modern human and Paracas DNA, PCR primers were designed for the FOXP2 gene and for exons 1, 5, 6, 10, and 11 of the TP53 gene. The expected PCR product lengths for exons 1, 5, 6, 10, and 11 were 517 bp, 472 bp, 396 bp, 421 bp, and 380 bp, respectively. Unfortunately, the expected PCR products were not generated and, with limited amount of Paracas skull tissue remaining, that portion of the project was abandoned. It is hoped that in the future we will be able to acquire additional tissue samples to continue this analysis.

Acknowledgements

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