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ORIGINAL RESEARCH

An attempt to extract ancient DNA from the petrous part of the temporal bones and roots of teeth of skeletal remains found in the intermediate climatic zone in Sri Lanka

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Abstract

Ancient DNA (aDNA) is used to explore the genetic landscape of the past and investigate the movement, mixture, and adaptation events that shaped present-day patterns of human genetic diversity. The present study was designed to explore the genetic composition of the ancient people of Sri Lanka. Despite the low amino acid racemization often found in skeletal remains of Sri Lanka, we attempted to extract DNA from skeletal remains of 5 individuals found from several sites in intermediate climatic zone in Sri Lanka by using methodologies particularly designed for highly damaged and degraded DNA. We attempted to extract and sequence DNA from the petrous part of the temporal bones and tooth roots of remains excavated from Sigiriya *Potana, Pellemalala* (4,500 YBP) and *Mini-atheliya* (1,000 – 5,000 YBP) in Sri Lanka. The bone processing, DNA extraction and amplification were performed in three separate rooms with dedicated equipment as accordance to the recommendation for analysis of aDNA. All measures were strictly taken to minimize contamination with modern samples. Using the DNA extracts, we prepared dual-indexed single-stranded libraries treated with uracil-DNA glycosylase (UDG) to reduce the rate of ancient DNA damage. Prior to sequencing, in-solution target hybridization was used to enrich the sequences that overlap the mitochondrial genome and about 1.24 million genome-wide SNPs. Sufficient data passing quality control standards for any of the five individuals studied was not obtained. Coverage of the mitochondrial genome ranged between 0.04-0.45x, with damage rates at the terminal nucleotide that were not indicative of authentic ancient DNA. A range of 453-2,838 SNPs out of ~1.24 million targets were covered across the nuclear genome, translating to a maximum coverage of 0.004x. The damage rates at the terminal nucleotide were not indicative of authentic ancient DNA. The low numbers of SNPs hit on the X and Y chromosomes precluded the confident assessment of genetic sex in all individuals but it is possible that the ancient skeletal sample represented by Sigiriya *Potana* is a female individual. Although the recent published work on using the petrous part of the temporal bone has generated many times more data than other skeletal elements, even this technique did not work to generate aDNA on this set of prehistoric skeletal remains found in the

intermediate climatic zone in Sri Lanka. It is possible that other sites might provide better taphonomic preservation conditions than the sites we analysed, and in addition a priority should be to attempt ancient DNA analysis from skeletal remains found in Sri Lanka's dry zone.

Keywords: ancient DNA, prehistoric skeletal remains, Sri Lanka

Introduction

Ancient DNA (aDNA) is DNA from organisms that lived decades to hundreds of thousands of years ago. It can be used to explore the genetic landscape of the past and investigate the movement, mixture, and adaptation events that shaped present-day patterns of human genetic diversity. Ancient DNA can be recovered from biological remains (including hard and soft tissues) and has also been recovered from sediment as well as from residues on objects used by people; however, it is most common to extract DNA from bones and teeth. Particular elements of the human skeleton – including the petrous part of the temporal bone[15,30], tooth cementum[2,11,18], and the auditory ossicles[34] have been shown to preserve DNA much better than other skeletal elements (particularly elements of the postcranial skeleton but also other elements of the cranium and other parts of the teeth). The most frequently used skeletal element today in aDNA research is the petrous bone, and in particular the cochlea, which has shown to be even more DNA-rich than other parts of the petrous bone[30] (a protocol for obtaining powder from the cochlea for aDNA analysis is published in Pinhasi, et al. 2019.)[29]

While the identification and analysis of parts of the skeleton that have been shown to be particularly DNA-rich has greatly benefitted the field of aDNA research, allowing the recovery of greater amounts of DNA from contexts where bio-molecular preservation is likely to be poor, the DNA recovered from these skeletal elements is still invariably damaged because the enzymatic repair processes that maintained the molecules during life cease to function[24]. At the time of an organism's death, endogenous nucleases begin to break down the nucleotide chain into small pieces that can be millions of times shorter than their original length, while hydrolytic and oxidation reactions fragment the DNA backbone and chemically modify the nucleotide bases[5,19,24,27,28]. Post-mortem DNA damage accumulates at a rate that is influenced by various environmental factors, which include temperature, exposure to moisture, and the pH of the environment in which the remains were deposited or interred[21,25,35]. DNA preserves best in cold and dry environments with stable temperatures, and worst in hot and humid places, particularly those that experience temperature fluctuations[35].

It is well known that the hot and humid environmental conditions in tropical environments promote DNA degradation and oftentimes results in poor DNA recovery from ancient biological materials[3,8,9,31]. Although there is presently no reliable way to evaluate the preservation of DNA without carrying out DNA analysis, amino acid racemization has previously been used as a tool to estimate the

preservation of DNA in bones[8,31]. Reed (2003) reported that the preservation of a DNA is likely to be very poor in Sri Lanka according to records on low amino acid racemization of prehistoric bones found in highland dry caves[32]. Despite the low amino acid racemization found in skeletal remains of Sri Lanka, we attempted to extract DNA from skeletal remains found in intermediate climatic zones in Sri Lanka by using methodologies particularly designed for highly damaged and degraded DNA, as would be expected for remains from Sri Lanka.

On the basis of the annual rainfall pattern, Sri Lanka is classified into three main climatic zones: the wet zone, dry zone and intermediate zone. The wet zone runs over the south-western region of the country which includes the central hill country and receives comparatively high mean annual rainfall of over 2,500 mm. The dry zone runs predominantly over the northern and eastern part of the country and receives a mean annual rainfall of less than 1,750 mm. The intermediate zone lies in between wet and dry zones and receives a mean annual rainfall between 1,750 to 2,500 mm, with a shorter and less-prominent dry season[12].

Prehistoric skeletal remains have been excavated from sites in different climatic zones in Sri Lanka. The sites of *Fa-Hien lena* at Bulathsinghala (34,000 ± 5,400 C^{14} YBP), *Batadomba lena* near Kuruwita (28,500-11,500 C^{14}Y BP), *Beli lena* at Kitulgala (27,000 - 35,000 C^{14} YBP), and Alu lena at Attangoda - Kegalle (10,500 C^{14} YBP) are located in the wet zonal cave sites which have yielded human skeletal remains belonging to Sri Lankan prehistory. *Bellan bandi palassa* at Balangoda (6,500 C^{14} YBP), located in the low country wet zone of the island, has also yielded human skeletal remains belonging to Sri Lankan prehistory[20].

The cave site of Sigiriya Potana, in the Matale district of Sri Lanka, which is situated in the intermediate zone, has yielded human skeletal remains dating to 4,000 YBP1. Pellamalala which is one of the largest shell middens situated at Hambantota district in the Southern province in the intermediate zone has yielded human skeletal remains dating to 4,500 YBP 36 Mini-atheliya, which is a shell midden area at the Hungama, located at Hambantota district, Southern province in the intermediate zone 23 has yielded human skeletal remains dated to 1,000 – 5,000 YBP.

The primary research question of our research was to explore the utility of ancient DNA technology to understand the genetic composition of the ancient people of Sri Lanka and in particular to investigate whether the present-day inhabitants of the Sigiriya region are genetically similar to these ancient people. Therefore, we attempted to extract and sequence DNA from the petrous part of the temporal bones and tooth roots of remains excavated from Sigiriya *Potana, Pellemalala* (4,500 YBP) and *Mini-atheliya* (1,000 – 5,000 YBP) in Sri Lanka. (Fig1.1).

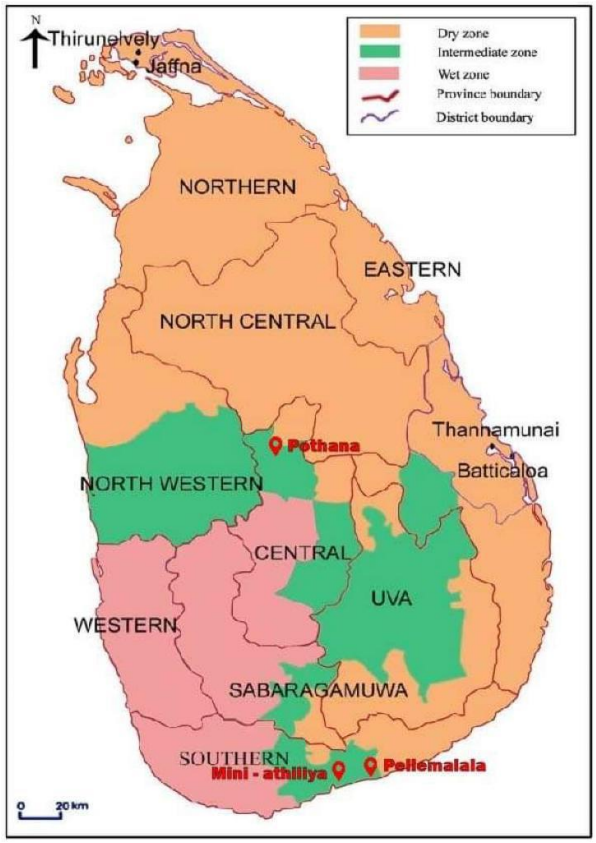

Fig 1.1 Location of the prehistoric sites from which remains were studied – *Potana*, *Pellemalala* and *Mini –ethiliya*. These sites are located in the intermediate zone in Sri Lanka.

Study Design, Materials and Methods

The present study was designed to extract and sequence DNA from human skeletal remains of five ancient individuals from sites within the intermediate zone in Sri Lanka and was carried out as a collaboration of researchers in Sri Lanka and the United States (Harvard Medical School - HMS; Boston, Massachusetts, USA). Included in the study were three individuals from the site of *Pallemalala* and one individual from the site of Sigiriya *Potana* who were curated at the Postgraduate Institute of Archaeology at University of Kelaniya, and one individual from *Mini-athiliya*, *Hungama*, *Hambanthota* who was curated at the Star Fort Archaeological Museum at *Matara*. Ethical approval for this work was granted by the Ethics Review Committee of Faculty of Medical Sciences, University Sri Jayewardenepura, Sri Lanka. Two teeth that included tooth roots from *Pellemalala* and three petrous parts of skull fragments were transported to HMS for DNA analysis after obtaining official approval from the Director General of Archaeology in Sri Lanka for export of the remains and for their scientific analysis. The processing of skeletal remains and the extraction, amplification, sequencing, and analysis of DNA data was done at HMS.

The selection of particularly DNA-rich bone elements for analysis followed Pinhasi et al. 2015 and Hansen et al. 2017[18,30]. The processing of bone material was carried out in a dedicated ancient DNA 'clean room' in order to minimize the impact of modern contamination. The powder for DNA extraction was produced from petrous samples (n=3) following the protocol described in Pinhasi (2019)[29] by combining the use of a Dremel disk saw and a fine sandblaster (Renfert Classic Basic). A drilling technique was used to create powder from the tooth roots (n=2). After processing the bone, DNA was extracted from ~50mg bone powder by dissolving it in a DNA extraction solution following published protocols[10,22,33]. All bone processing was carried out by trained technicians wearing full cover suits, double gloves, hair nets, and face masks. All non-disposal equipment and work surfaces were sterilized through chemical cleaning as well as the use of UV-irradiation.

From the DNA extracts, we prepared dual-indexed single-stranded libraries[16] treated with uracil-DNA glycosylase (UDG) to reduce the rate of ancient DNA damage[4]. Prior to sequencing, we used in-solution target hybridization to enrich for sequences that overlap the mitochondrial genome and about 1.24 million genome-wide SNPs[13,14,17,26]. We adopted this approach because we expected that these ancient samples would likely have low percentages of human DNA, making a strategy to enrich for informative positions more plausible than a shotgun sequencing approach. DNA libraries were sequenced using Illumina sequencing technology.

Results and Discussion

We did not obtain sufficient amounts of data passing quality control standards for any of the five individuals studied. Coverage of the mitochondrial genome ranged between 0.04-0.45x, with damage rates at the terminal nucleotide that were not indicative of authentic ancient DNA. A range of 453-2,838 SNPs out of ~1.24 million targets were covered across the nuclear genome, translating to a maximum coverage of 0.004x. For this small amount of DNA, the damage rates at the terminal nucleotide were not high enough to be indicative of authentic ancient DNA.

Although low numbers of SNPs hit on the X and Y chromosomes precluded the confident assessment of genetic sex in all individuals, the sequences from the extracted DNA from the ancient skeletal sample represented by Sigiriya *Potana* are consistent with being a female. Individual sex identification from skeletal material has become a complimentary analysis to morphological assessment, the latter requiring at least some level of skeletal preservation and limited to adult individuals or sub-adults with soft tissue preservation. Sex determination is critical for investigating the demography of a population, such as sex-based population movements, marriage practices, settlement practices, and infanticide, among other topics. The female genetic sex of the Potana individual is consistent with morphological assessment by Chandimal et al. (2018)7. However, since there is no clear damage associated with the sequences, it is possible that the female DNA we have sequenced is not that of the ancient individual, but instead of a contaminating individual. An important aspect of ancient DNA research is to record the sex of the ancient individuals studied whenever possible in order to obtain greater insight into social structures or sex-biased practices in the past.

A likely reason that we failed to recover authentic aDNA from the five ancient individuals from Sri Lanka is that the bio-molecular preservation of these individuals was extremely poor due to the climatic features of Sri Lanka's intermediate zone. In addition, after removal from the archaeological site, samples are frequently stored in archives or museums, and the process of DNA degradation continues. Long term storage of archaeological specimens outside a lab freezer and prolonged exposure to UV irradiation can function to reduce the amount and the quality of DNA in ancient specimens[6]. This may be another factor contributing to the poor DNA preservation from of the individuals studied as part of this project. The skeletal samples of *Potana* and

Pellamalala were excavated about ten years prior to their attempted DNA analysis, and are presently stored in boxes at room temperature at the museum and the osteology laboratory of the Postgraduate Institute of Archaeology, located in Sri Lankan wet zone where the environmental conditions are not conducive to the preservation of DNA.

As aridity, low temperature, and neutral or slightly alkaline pH are conditions that promote the preservation of DNA[6,19], a next step of this research project could be to attempt to recover DNA from skeletal remains found in Sri Lankan dry zone, such as Mantai, Jaffna, and Anuradhapura (among other sites), and also to attempt analysis of DNA from fresh excavations. Before attempting DNA analysis on such unique prehistoric bones, it would be informative to attempt DNA analysis on historic bones from the same or similar context, as it is evident that the DNA preservation in tropics is not ideal. Although the recent published work on using the petrous part of the temporal bone has generated many times more data than other skeletal elements, even this technique did not work out to generate aDNA on prehistoric skeletal remains found in intermediate climatic zone in Sri Lanka; however, given that the superior DNA preservation of this bone is evident, we believe that it should continue to be targeted in future ancient DNA work in Sri Lanka.

With the very limited availability of prehistoric skeletal remains it is of utmost importance that even negative results are published, and that researchers minimize the damage caused to each bone, recognizing that there is a non-trivial chance that their analyses will not yield usable data based on current methods. Whenever possible, an antimere should be preserved untouched for future research that may utilize more powerful methods than are available today. In addition, we encourage archaeologists to store human remains in temperature-controlled settings following excavation to reduce further degradation to aDNA within the skeletal remains.

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Conflict of Interest

None declared

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