Proposal to Sample Middle Stone Age Human Remains from Equus Cave for ancient DNA

Background

One of the major questions confronting palaeoanthropologists today is how well the Late Quaternary African human fossil record accords with the results of studies of the modern human genome? An impressive array of evidence from mitochondrial DNA (mtDNA), the Y chromosome, non-coding autosomal microsatellites (short tandem repeats or STRs) and autosomal single nucleotide polymorphisms (SNPs) has been brought to bear on 1) the geographic origin of the species *Homo sapiens*; 2) the age of the divergence of *H. sapiens* from the most recent common ancestor that we shared with our now-extinct sister species; and 3) the phylogeographic structure of Africa populations and lineage divergence times.

In the first instance, the Late Quaternary African hominin fossil record, despite its manifestly incomplete nature, finds at least some consistency with an impressive array of genetic evidence that point to an African origin for our species (Tishkoff et al. 2009). There is currently no question that the geochronologically oldest specimens attributable to *Homo sapiens* derive from sub-Saharan Africa.

In the second instance, haploid mitochondrial DNA (mtDNA) and Y chromosome genetic data indicate a coalescence of lineages to the most recent common ancestor of *Homo sapiens* at between 200 - 100 ka. Poznik et al. (2013) analyzed new mtDNA and Y-chromosome genome data using comparable techniques, and found that both loci produce estimates for the most recent common ancestor (T_{MRCA}) that date to between 150 - 120 ka.

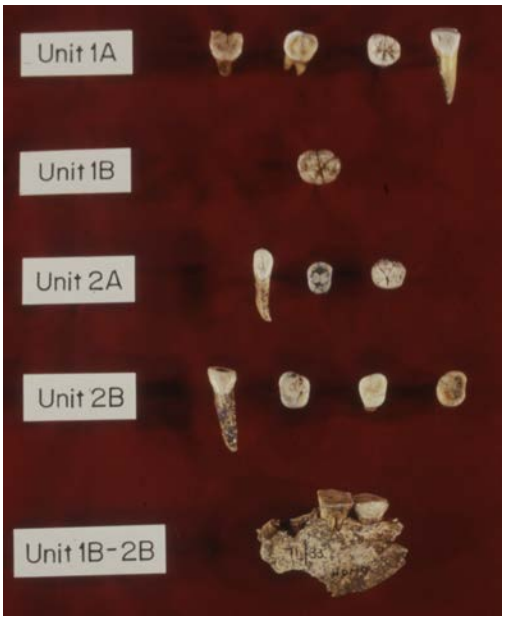
In the third instance, Behar et al. (2008) have shed light on the structure of the mitochondrial DNA (mtDNA) phylogeny by constructing a matrilineal tree composed of 624 complete mtDNA genomes from sub-Saharan Hg L lineages. They paid particular attention to the KhoeSan people of South Africa because other genetic studies have suggested that they carry paternal and maternal lineages belonging to the deepest clades known among modern humans. Their tree (phylogeny), as well as their coalescence calculations suggest that KhoeSan matrilineal ancestry diverged from the rest of the human mtDNA pool some 90,000 – 150,000 years BP, and that at least five additional (currently extant) lineages existed in parallel during this period. Furthermore, they estimate that a number of other lineages flourished in sub-Saharan Africa during the period of modern human dispersal out of Africa (c. 60,000 – 70,000 yrs BP). As aptly noted by Behar et al. (2008), the quest to explain sub-Saharan African demographic history during this period of human evolution has been limited because of the scarce human fossil record from the Middle Stone Age (MSA).

If Behar et al. (2008) are correct in their conclusions, late MSA humans from southern Africa should be expected to express the Hg L0d or L0k mtDNA haplotypes, especially if they are the direct ancestors (or near relatives) to the living KhoeSan inhabitants of the subcontinent. However, to date, there has been no ancient DNA retrieval from southern African human remains. We aim to directly test the mtDNA in MSA samples from Equus cave in order to determine their relationship to contemporary African populations and whether MSA samples support a hypothesis of geographic

continuity for the KhoeSan peoples, potentially determining their ancestral presence in southern Africa for up to 100Kya. Additionally, using recent advances in aDNA technology (such as WISC; see Carpenter et al., 2013 and Orlando et al., 2013) we aim to reconstruct partial genomes from these southern African samples via next generation genome sequencing. Ancient genomes will be compared with full genomes that have already been generated from saliva-derived DNA contributed by contemporary N|u and Nama speaking individuals from the Northern Cape, South Africa (Henn et al. *unpublished data*).

The absence of fossils from the Late Pleistocene with identifiable KhoeSan affinities led Morris (2002) to suggest that the KhoeSan morphotype arose relatively late in South Africa. He has hypothesized that the ancestors of recent KhoeSan populations underwent a population (and hence phenotypic) bottleneck associated with the Last Glacial Maximum of MIS 2 at ca. 29 - 14 ka. Although this is an interesting suggestion, there is, unfortunately, no genetic evidence for it.

Why Equus Cave?



There are comparatively few human fossil remains from sub-Saharan Africa that date to this critical period and that might profitably be tested for the remnants of ancient DNA (aDNA). Among those that date to the period during which African populations begin to diverge are the remains from the Middle Stone Age deposits of Equus Cave (27°37'S, 24°38'E). These remains were recovered in a series of excavations led by P. Beaumont and M. Shackley in 1978 and 1982.

Four human teeth derive from the Holocene Unit 1A. The age of unit 1A has been determined by 14C dates of 2,390 and 7,480 years BP obtained at mid-level and its base (Beaumont et al., 1984).

Eight human teeth come from the MSA units 1B - 2B; these levels almost certainly date to between 32,700 and 94,000 years BP (Grine & Klein, 1985). A fragment of mandible of uncertain provenance likely derives from one of the MSA units (Grine & Klein, 1985).

All of the human remains from Equus Cave have been described in detail, with accompanying photographic documentation, by Grine & Klein (1985).

Specimen Choice

Tooth Root

Two of the isolated human permanent teeth recovered from the Middle Stone Age deposits (levels 1B - 2B) at the archaeological site of Equus Cave (27°37'S, 24°38'E) will be chosen for analysis. There are four specimens with complete or nearly complete roots from these units. All have been described in detail, with photographs by Grine & Klein

(1985), and some have also been examined already by micro-CT (Smith et al., 2006). Three of the four specimens possess a root that is reasonably well-preserved.

Choice of the teeth for aDNA sampling will be based on the state of preservation of the root, examined by us first hand at the McGregor Museum. The four specimens from the Middle Stone Age units are listed below. Of these four specimens, EQ-H 12 possesses the most poorly preserved root. Specimens EQ-H 6 and EQ-H 9 are illustrated here. It is proposed that the root from both be sampled for aDNA.

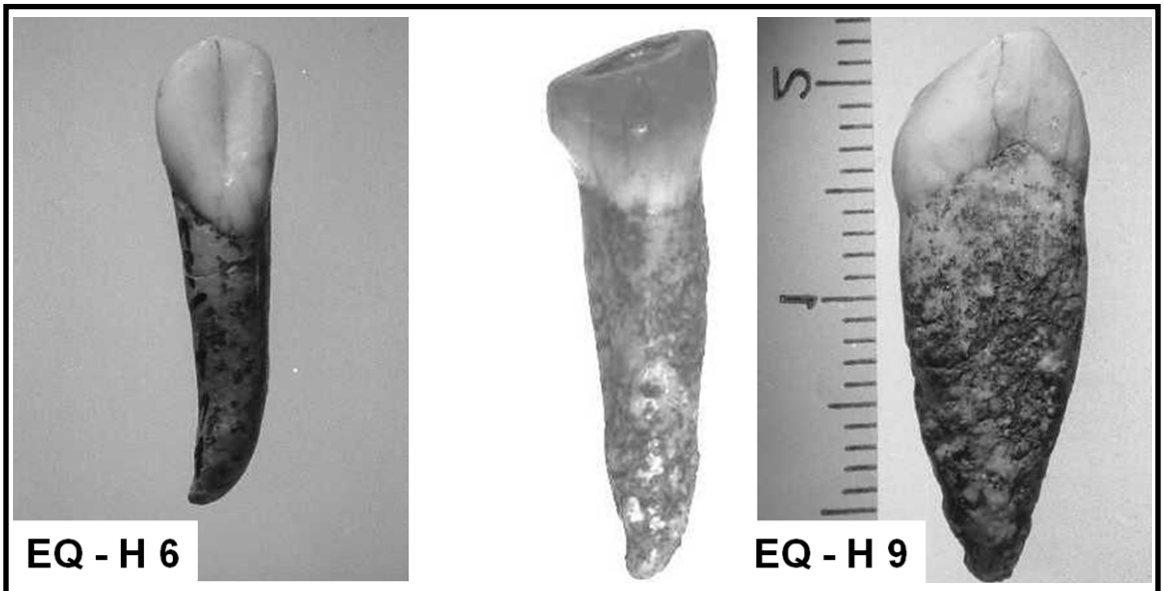
It is crucial to sample multiple specimens for aDNA analysis as microenvironments can affect DNA preservation across samples within the same archaeological site or even within a single sample (Rasmussen et al., 2014).

Unit 2A

EQ-H 6	maxillary LI2	(Grine & Klein, 1985, pp. 70-72, fig. 5a)

Unit 2B

EQ-H 9	mandibular LC	(Grine & Klein, 1985, p. 73, fig. 6a)
EQ-H 11	maxillary RM3	(Grine & Klein, 1985, pp. 75-76, fig. 6c)
EQ-H 12	maxillary RM3	(Grine & Klein, 1985, pp. 76-78, fig. 6d)



(Left) Lingual view of the EQ - H 6 left upper I2. (Middle and Right) Lingual and mesial views of the EQ - H 9 left lower canine.

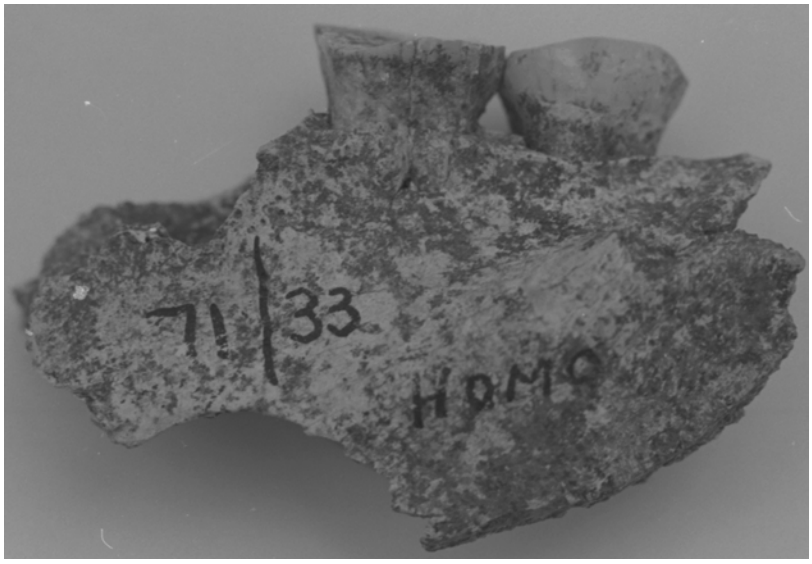
Mandibular Bone

In addition, there is a fragment of mandible containing two molar teeth from the site. As noted by Grine & Klein (1985), the stratigraphic provenance of this fragment is uncertain, but its state of preservation strongly suggests that it derived from one of the three lower units (i.e., MSA units 1B, 2A, or 2B) rather than the Holocene unit 1A. The mandibular fragment was found by C.K. Brain when he and K.W. Butzer visited the site

in 1971. At that time, the quarry road had cut through the deposit, and bone-bearing sediments were being eroded down the cutting. The mandibular fragment was among the bones recovered by Brain from the scree, and Brain has stated that he had no doubt at the time that it had come from the Equus Cave deposit.

Bone traditionally presents a better opportunity for preservation of aDNA, because the bone cells (osteocytes) containing genetic material are "trapped" within the confines of the tissue itself. We therefore propose to sample the bone preserved in the mandibular fragment. It is proposed to remove a small piece of bone from the anterior lateral aspect, or the basal aspect of this fragment. The mandibular fragment is illustrated below. The proposed sampling areas are situated on the left side and at the bottom of the fragment.

Again, it is crucial to sample multiple specimens for aDNA analysis as microenvironments can affect DNA preservation across samples within the same archaeological site or even within a single sample (Rasmussen et al., 2014).



Lateral view of the EQ - H 71/33 mandibular fragment

Sampling and Analysis

There are two possible sampling strategies available to us. The same analytical procedures will be employed in either case.

Sampling Strategy One

The first sampling strategy involves the removal of the apical two-thirds of the two tooth roots, and the removal of cortical bone from the anterior and/or basal portion of the jaw fragment by F.E. Grine at the McGregor Museum. The pieces will be removed

using a battery-powered Dremmel affixed with a thin wafering blade to minimize tissue loss. Each piece will be wrapped first in plastic wrap and then placed in sealable plastic bags. The pieces will be hand-carried to Professor Eske Willerslev's lab in Copenhagen, Denmark for analysis. Any material that remains un-sampled (see *Analysis* below) will be returned to the McGregor Museum (hand-carried by Grine or Willerslev) for re-attachment to the tooth or jaw.

Sampling Strategy Two

In the second sampling strategy, the two teeth and the entire mandibular fragment will be borrowed by F.E. Grine and hand carried from the McGregor Museum to Professor Eske Willerslev's lab in Copenhagen, Denmark for analysis. Any material that remains un-sampled (see *Analysis* below) will be re-attached to the tooth crowns and the jaw fragment, and the specimens will be returned to the McGregor Museum (hand-carried by Grine or Willerslev). If the tooth roots and pieces of bones from the jaw fragment are sampled in their entirety, the remaining tooth crowns and the remaining (large) part of the jaw fragment will be returned to the McGregor Museum (hand-carried by Grine or Willerslev).

The Lab

Professor Eske Willerslev's lab in Copenhagen, Denmark is at the forefront of ancient DNA analysis. Professor Willerslev is Director of the Centre of Excellence in GeoGenetics and the National CryoBank and Sequencing Facility, situated at the National History Museum and the Biological Institute, University of Copenhagen. Willerslev is an internationally recognized research leader in the field of ancient DNA and DNA degradation, and he and his lab group (some 70 individuals!) have pioneered many of the advances in the extraction and amplification of DNA from ancient skeletons. Over the past few years they have sequenced ancient DNA from hair, coprolites, soil samples and bone. His group recently obtained ancient DNA from an Upper Paleolithic skeleton that revealed dual ancestry for Native Americans, and aDNA from a human skeleton associated with the ancient Clovis culture in North America. Both of these studies have been published within the last month in the journal *Nature*.

Analysis

The analysis proposed here will proceed in two stages. In the first stage, around 10 mg of dentine will be removed from the root neck of each tooth root and 10 mg of mandibular bone will be removed using a small drill. These samples will be used for time-of-flight secondary ion mass spectrometry (TOF-SIMS) and for high-resolution tandem-mass spectrometry sequencing of proteins. Both are indicators of whether it is likely that putative DNA molecules will be obtained (Orlando et al., 2013).

If these initial analyses do not suggest likely DNA preservation, then the project will be terminated for the dental specimens and the mandibular bone, and the un-sampled tooth roots and mandibular bone will be re-attached and returned to the museum, or returned to the museum for re-attachment (depending upon the sampling strategy adopted).

On the other hand, if these analyses suggest likely DNA preservation, then a larger sample (ca. 400 mg) will be removed from the same area(s) from which the first

sample(s) was (were) taken. The details of sampling (i.e., amount of tissue loss) will depend on the particular specimen.

From the sample(s) thus collected, DNA can then be extracted and mtDNA and nuclear DNA can be amplified, including controls for contamination. The success rates of samples that look promising from the standpoint of protein, although Griehaber et al. (2008) found that exposure of modern bone to radiation from X-rays and CT scans results in DNA fragmentation, which thereby decreases the amount that is available for amplification, it is not clear what effect such exposure (and, more particularly, what levels of exposure) have on ancient, fossilized bone and dentine.

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