



Stockholm
University

Bachelor Thesis

Degree Project in
Geoscience 15 hp

Cultural Dedicing Context from Genetic Information

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Stockholm 2022

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Abstract

An experiment has been carried out which shows that it is possible to deduce time and context of an ancient specimen from DNA analysis alone. This is provided that the specimen can be identified with a known population group. The accuracy is best when the specimen can be related to specific events such as population turnovers.

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Introduction

This essay reports from an experiment to evaluate how much contextual information that can be obtained from ancient DNA alone. The objects of the experiment were two femurs and one premolar from concealed ancient archaeological sites in Sweden. This made the experiment into a blind test. The experiment itself consisted of genetic analysis followed by interpretation, see figure 1.

The actual origins of the objects were revealed at the end of the experiment. Then the conclusions from the experiment could be compared with the actual facts. This method is very different from conventional archaeological projects where genetic analysis is carried out on material from known excavations.

After presenting the three objects this essay continues with a description of the peopling of Europe and of Sweden. This is necessary background information and also serves as a survey of important academic papers covering the genetic aspects of this history. Then follows a description of how the genetic analyses and the bioinformatics procedures were done. The essay ends with a presentation of the archaeological sites of the objects and a discussion of the accuracy of the interpretations.

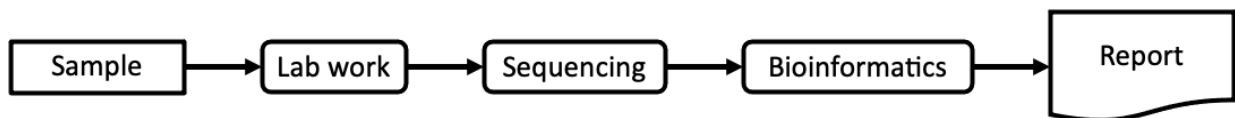


Figure 1. The process of genetic analysis. Rectangles with rounded corners represent activities. Other symbols are objects like databases or documents.

The study objects

The objects assigned for this study were two femurs and one premolar. One femur was broken in two. The objects were photographed still in their plastic bags to keep them from contamination in case they would be used for future ancient DNA studies.



Figure 2. Object L1.

Femur sinister. Composite length ~42.9 cm.

The arrows point to drill holes where samples were taken.



Figure 3. Object L2.

Femur sinister. Length 44.3 cm.

The arrow points to the drill hole where one sample was taken.



Figure 4. Object U1.

Premolar mandibula dexter. Length 2.0 cm.

The drill holes at the root are clearly visible.

There are two formulae for estimating body length from femur length (Maijanen and Niskanen 2010)

- LSQ (*least squares*) $\text{Body} = \text{Femur} \times 2.86 + 37.89$
- RMA (*reduced major axis*) $\text{Body} = \text{Femur} \times 2.94 + 34.23$

Those give an approximate body length for L1 of ~160 cm and for L2 of ~165 cm.

The peopling of Sweden

This chapter describes how Sweden became populated after the Ice Age and which groups participated. The samples must originate from individuals who belonged to those groups.

The Pioneers in Europe – Palaeolithic

Modern humans (*Homo sapiens*) came to Europe ~43,000 BC by way of a land bridge between the Middle East and the Balkans (Fu et al 2016). Europe was in the grip of the ice age but the humans must have chosen this inhospitable place because the hunting was good. The Northern hemisphere was home to a megafauna consisting of mammoths, rhinoceroses, cave bears, bison, Irish giant deer and other imposing animals (Zimov et al 2012, Cooper et al 2015).

In Europe the humans encountered the neandertals (*Homo neanderthalensis*) and there was some mixing between the two species. The proportion of neandertal genes in the human genome dropped from 3-6% ~43,000 BC to today's 2% probably through natural selection (Fu et al 2016). By ~38,000 BC the neandertals were extinct (Nielsen et al 2017).

The glaciation reached its maximum ~18,000 BC when the ice sheet reached as far south as Berlin. The humans had to withdraw to southern refugia along the Mediterranean (Riede 2014). All analysed humans between ~35,000 and ~12,000 BC belonged to one single founder population with no further genetic input during this time. Europeans of today have a trace of these early people (Fu et al 2016).

The climate improved rapidly during the Bølling and Allerød interstadials (Burenhult 1999). Humans and animals began migrating north from the ice age refugia. From this time Europeans have some affinity to people from the Near East indicating a probable migration since it also coincides with a cultural transition (Fu et al 2016).

The earliest traces of human habitation in Sweden are near the Finja lake in Skåne dated ~12,000 BC. This was a hunting camp belonging to the Hamburg culture. At this time the landscape was a tundra and the Hamburg people were reindeer hunters (Riede 2014).

The warming was abruptly halted by the Younger Dryas stadial which lasted from ~10,800 BC to ~9,600 BC. This meant a return to glacial conditions and that the land ice advanced again (Wohlfarth et al 2008). The Younger Dryas stadial caused a population crash in Europe. Several mtDNA lines vanished that had been represented in different places in northern Europe. After the Younger Dryas stadial there were only U5 and minor parts of other U lines present (Posth et al 2016).

The Hunter-Gatherers – Mesolithic

The Younger Dryas stadial ended as suddenly as it had started and the melting continued. The southern Baltic formed a meltwater lake with its outflow at Öresund. As the ice receded the meltwater lake found an outflow to the North sea across mid-Sweden. Huge areas were drained and a land bridge between Denmark and Skåne was established (Wohlfarth et al 2008). This was very important for the migration of plants, animals and humans to southern Sweden. Birch, pine and hazel invaded the former tundra changing its appearance. The reindeers were replaced by aurochs, bison, wild boars and other animals (Liljegren och Lagerås 1993).

The Scandinavian land ice consisted of one huge glacier with its centre approximately at the Baltic coast of northern Sweden. When the ice receded it did so along the edges and an ice free fringe of land opened along the Norwegian coast. When the ice withdrew the land rose and closed the strait across Sweden. A new outflow was found through the Danish straits, see figure 5.

The people who entered Sweden came north from different refugia

- Western Hunting Gatherers (WHG) from continental Europe. They had dark complexion and blue eyes (Svensson and Fraser 2017).
- Eastern Hunting Gatherers (EHG) from Russia who mainly go back on an Ancient North Eurasian population (Damgaard et al 2018). They had lighter complexion and brown eyes (Svensson and Fraser 2017).

The following Swedish individuals from this time have been analysed

- One man from Stora Bjärs on Gotland from ~6,963 – 6,579 BC (Günther et al 2018).
- Three individuals from a cave on the island Great Karlsö near Gotland from ~7,300 – 6,760 BC, younger Maglemose culture (Apel and Storå 2017).
- Six individuals from Motala near lake Vättern (Svensson and Fraser 2017).

Analysis of individuals at this period from the Norwegian coast shows the same admixture but with a higher proportion of EHG. The explanation to this must be that while the WHG had migrated north from the continent, the EHG had wandered west along the coast of the Arctic ocean and then south following the Norwegian coast (Günther et al 2018).

Principal components analysis shows that the Scandinavian Hunter Gatherers (SHG) are between WHG and EHG (Mathieson et al 2015) and are quite close to Latvian Hunter Gatherers (LHG, Günther et al 2018). The admixture of WHG and EHG gave the SHG a high genetic diversity (Günther et al 2018). Analysis has also shown a selection on height which persists today (Mathieson et al 2015).

Chewing gum made from birch bark mastics from Huseby Klev in Western Sweden has been found and dated to ~7,880 – 7,540 BC. Human DNA has been extracted from the mastics and analysed which puts these persons in the same group as those from Motala. The mtDNA haplogroup is U5 (Kashuba et al 2019). The man from Stora Bjärs had Y-DNA haplogroup I2. The mtDNA haplogroups of the individuals from Gotland were U4 or U5 (Svensson and Fraser 2017, table 1). The three Norwegian individuals studied showed Y-DNA haplogroup I2 and mtDNA haplogroup U5 (Günther et al 2018).

The warming reached an optimum with temperatures 2-3°C higher than today and Sweden became covered by deciduous forests (Liljegren och Lagerås 1993). The last Mesolithic culture was the Ertebølle coastal culture which was the first to use pottery. Simple pots with no decoration and with pointed bottoms to stand steady in fire or sand (Burenhult 1999).

The Farmers – Neolithic I

Agriculture was created independently in different places around the globe 9,000-3,000 BC. Humanity took the step from hunting and gathering to food production. This depended on the availability of animal and plant species suitable for domestication.

One such place was the Fertile Crescent. The local climate had created cereal species with large seeds which foragers had collected in the wild. There were also sheep, goats, cattle and pigs which are flock animals that can accept being handled by humans. By 8,300 BC crop cultivation had been adopted by a small group of narrow genetic diversity on the central Anatolian plateau. By ~1,500 years later mixed farming and the knowledge of pottery had spread over most of Anatolia. Those people showed much greater genetic variation which implies admixture among local groups and possibly some migration (Kılınç et al 2016).

By ~7,000 BC agriculture had spread to Cyprus, Crete and the Greek mainland. From there it developed into the farming Starčevo–Körös–Criş cultures in the Balkans (Chyleński et al 2017). Early farmers in Europe show close genetic relationship to farmers in western Anatolia (Omrak et al 2016).

From the Balkans ~6,000 BC agriculture advanced in two directions. One was along the Mediterranean coast to Italy and Iberia. The other was northwest along the Danube valley where it formed the Linear Pottery culture (*Linearbandkeramik*, LBK, Olalde et al 2015). The LBK people farmed the loess soil along rivers and streams, lived in longhouses and used pottery with distinct linear decorations. LBK was an inland culture and did not reach out to the coasts.

The farming cultures had a wide variety of mtDNA haplogroups but the main Y-DNA haplogroup was G2 (Szécsényi-Nagy et al 2015). They had light skin but dark hair. The eye colour, however, cannot be decided. The non-Indo-European Basque language might be a remnant from the language spoken by those early farmers (Olalde et al 2015).

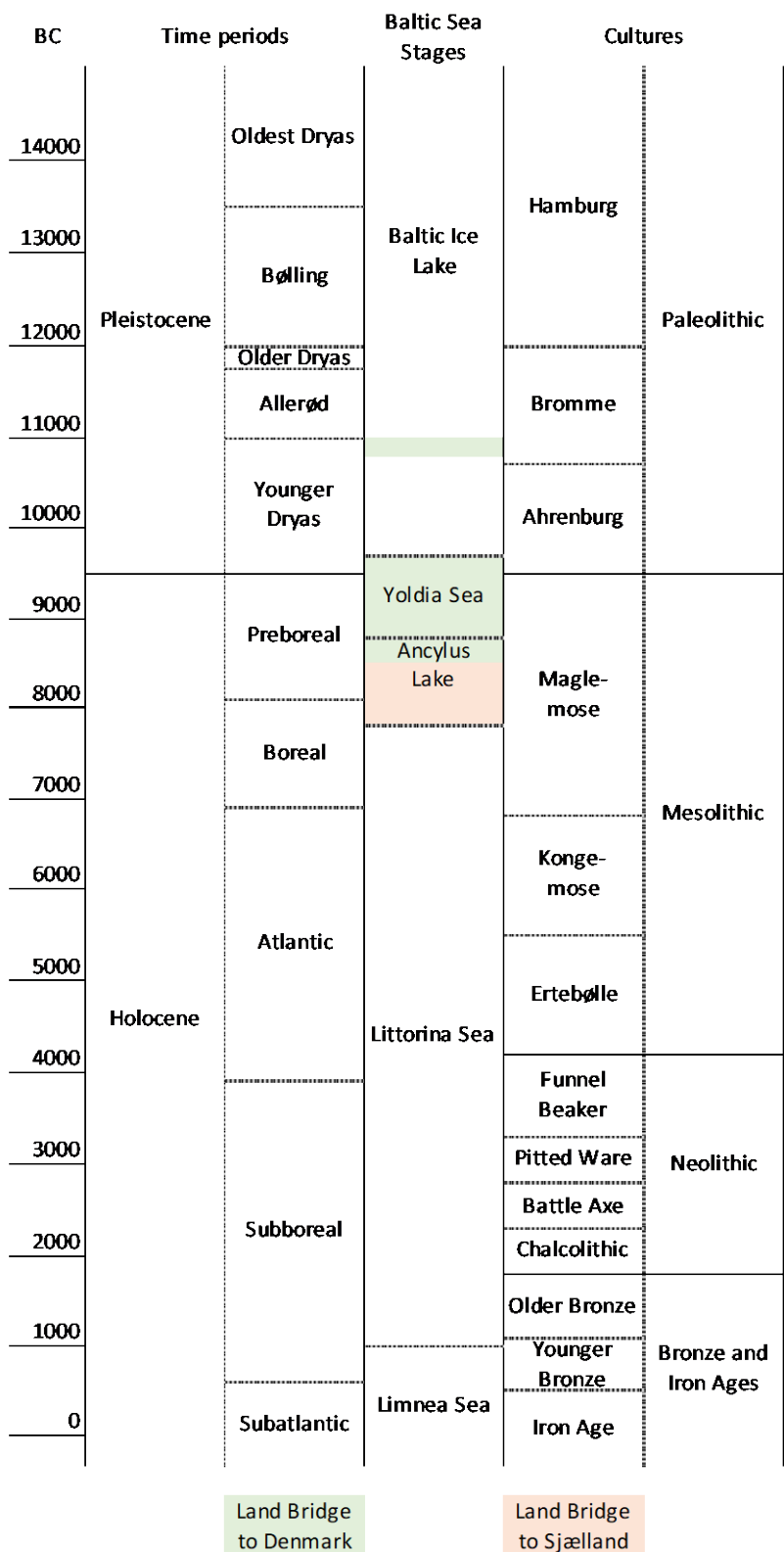


Figure 5.

Overview of Scandinavian geological and archaeological periods from 15,000 BC to year zero.

To the left geological periods and chrons. (Liljegren och Lagerås 1993).

In the middle Baltic sea stages. Land bridges between Denmark and Sweden are indicated (Wohlfarth et al 2008).

To the right archaeological ages and cultures. (Burenhult 1999).

For about one thousand years, 5,500 BC – 4,200 BC, the Mesolithic Ertebølle culture in Denmark and southern Sweden lived side by side with the LBK culture in nearby Germany (Skoglund et al 2014). The hunter-gatherers must have known of agriculture but preferred their own way of life. However, the Ertebølle people picked up the idea of making pottery (Malmström et al 2015). They were probably sedentary beside their fishing grounds and mussel banks.

The farming Funnel beaker culture (*Trichterbecher*, TRB) appeared in Denmark ~4,200 BC as an offshoot of the LBK (Skoglund et al 2012, Fraser et al 2017A). It reached the middle of Sweden after ~500 years. The TRB people lived in small long-houses and practised slash and burn agriculture. They made pottery with incised decorations and funnel shaped necks which are assumed to imitate leather bags. There was an assimilation of hunter-gatherers and there is a small hunter-gatherer component in the LBK and a slightly larger in the TRB (Chyleński et al 2017, Coutinho et al 2020).

The climatic optimum had passed and the climate was getting colder and wetter (Liljegren och Lagerås 1993). This might have made the previous hunter-gathering economy less viable.

A tradition of building megalithic graves (dolmen and passage graves) started ~4,500 BC in southern Brittany and spread both north and south (Schulz Paulsson 2017). It reached western Sweden ~3,600 BC where most megalithic graves can be found in Västergötland. Although this was a cultural diffusion among the farming communities many males buried in these monuments belong to Y-DNA haplogroup I2. Since this is usually related to the hunter-gatherers there must have been an admixture between the two groups (Sánchez-Quinto et al 2019).

The Seal Hunters – Neolithic II

The Pitted Ware culture (PWC) appeared ~3,300 BC along the east coast of Sweden. This was a hunter-gatherer culture based on seal hunting. The PWC people made characteristic pottery with little pit marks. The PWC spread from south-eastern Sweden along the Baltic coast both north and southwest. Principal components analysis shows that PWC hunters from Ajvide on Gotland cluster together with Mesolithic hunter-gatherers from a cave on the Stora Karlsö island (Skoglund et al 2014). One PWC male had Y-DNA haplogroup I2 which was common among hunter-gatherers (Malmström 2017). The PWC belonged genetically to the north European hunter-gatherers and are closest related to hunter-gatherers in the east Baltic countries (Coutinho et al 2020). They must have invented new hunting methods or there were plentiful seals to be hunted (Malmström et al 2009).

TRB and PWC settlements coexisted on the isle of Gotland for at least half a millennium, 3,200-2,300 BC. Isotope analysis shows that the TRB people had a terrestrial diet with some added fish whereas the PWC people had a strictly marine diet (Fraser 2017B). Probably Gotland was sparsely populated and the two cultures could coexist because their different economies did not compete with each other. Any gene flow between these two groups seems to have been in the direction from hunter-gatherers to farmers (Skoglund et al 2014).

On many archaeological sites PWC has overlain TRB. This has been interpreted as that farmers have returned to a hunter-gathering lifestyle but the genetics shows that this is not correct. (Burenhult 1999).

The Pastoralists – Neolithic III

Horses were domesticated by people of the Botai culture ~3,500 BC (Damgaard et al 2018). They were hunter-gatherers living on the steppes north of present Kazakhstan. Horse domestication was picked up by the Yamna (or Yamnaya) culture on the Pontic steppe north of the Black and Caspian seas. The Yamna descended from EHG and Caucasian hunter-gatherers (Damgaard et al 2018). The Yamna people developed a herding culture and began expanding both west and east.

One offshoot of the Yamna culture was the Corded Ware culture (CWC) in northern continental Europe from France to Russia from ~2,900 BC (Krzewińska et al 2018, Juras et al 2018). The CWC got its name from its typical ceramics decorations made with the help of strings. The CWC appeared in Scandinavia ~2,800 BC as the Battle Axe culture (BAC, Malmström et al 2019). The CWC is also known as the “single grave” culture since people were buried singly and not communally as in the megalithic graves (Burenhult 1999).

The Yamna culture spread over Europe in just a couple of hundred years due to its mobility with wagons and horses. Genetic studies indicate that it was mainly men on the move, similar to the Viking raids several thousand years later (Goldberg et al 2017). The Yamna will have practised exogamy (Kristiansen et al 2017).

The spread of the Yamna culture may have been aided by a drastic reduction among the existing European population (Haak et al 2015). In Britain there was almost a total population turnover as the previous people nearly disappeared (Olalde et al 2018). The explanation was probably that the bacterium, *Yersinia pestis*, which causes the plague, had developed a virulent strain which struck the close-knit farming communities badly (Rascovan et al 2019). The plague may have hit the Yamna people earlier so that they had developed some immunity.

An approximation of the ancestries of people in Germany during the CWC is Yamna 79%, Neolithic 17% and WHG 4%. The main Yamna Y-DNA haplogroups were R1a and R1b which are dominant in Europe today (Haak et al 2015).

The Yamna migrants spoke a proto-Indo-European language (Kristiansen et al 2017) which split into dialects which turned into proto-Germanic, proto-Celtic and other languages. By studying words common in existing languages some of the vocabulary of proto-Indo-European can be constructed. It turns out to contain words dealing with horses, wagons, sheep, dairy production but not for many common crops. Such words in today's languages may be loan words from the languages spoken by the LBK, TRB and other Neolithic farmers (Iversen and Kroonen 2017).

For some time there existed three genetically different cultures in parallel in Sweden, the farming TRB, the seal hunting PWC and the pastoral BAC (Coutinho et al 2020).

By this time beeches from the south and fir trees from the north west had arrived to Scandinavia and spread. The fir trees would change the forest landscape completely. Scandinavia became the westernmost outpost of the Siberian taiga (Liljegren och Lagerås 1993).

The Late Neolithic

The different cultures amalgamated by ~2,300 BC in the Late Neolithic period. This is also known as the Chalcolithic or the Copper stone age. Their ceramics was without decorations. Pot decorations must have lost their symbolic meaning (Burenhult 1999).

A new megalithic tradition, cists, appeared. Cists were used for communal burials even into the early Bronze age. Individuals buried in cists in Gotland have been analysed and the maternal ancestry is distributed as follows, 55% BAC, 40% TRB and 5% PWC (Fraser et al 2018).

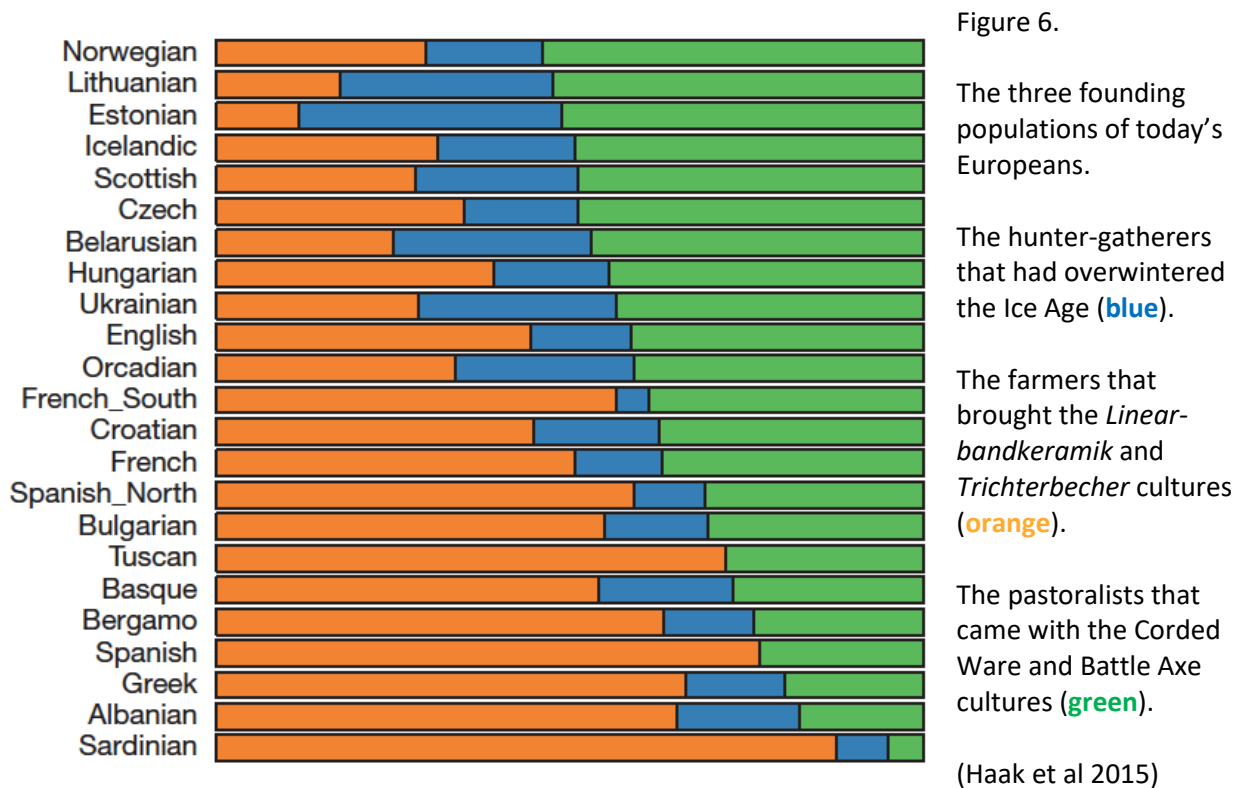
Knowledge of metals spread. The Tyrolean Iceman ~3.300 BC had an axe made of nearly 100% pure copper and was of TRB ancestry (Sikora et al 2014).

The Late Neolithic period in Scandinavia was parallel with the Únětice culture in central Europe which was the beginning of the Bronze age. The Elder Bronze age started in Sweden ~1,800 BC. Use of bronze required a large trading network since copper and tin are only found in certain places. This must have been facilitated if people all over Europe spoke dialects of Indo-European.

The Elder Bronze age was a brilliant time in Scandinavia with monumental grave mounds for the elite and exquisite bronze craft. Bronze seems to have been used for swords, shields and special objects. For everyday purposes the stone age lived on (Burenhult 1999).

The population of Europe became more or less set with the migrations leading up to the Bronze Age (Lazaridis et al 2014, Allentoft et al 2015, Mathieson et al 2015). Later movements like the German *Völkerwanderungen* AD 300-500 made less impact because of growing population numbers (Günther and Jakobsson 2016).

The femurs and the premolar should relate back to these three founding populations of contemporary Europeans, the hunter-gatherers, the farmers and the pastoralists, see figure 6.



Lab work

This work was performed in a clean room laboratory where everything is kept meticulously clean and all workers wear a covering overall, plastic gloves and mouthguard.

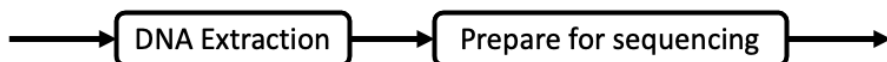


Figure 7. The lab work is done in two steps.

DNA Extraction

This work followed a modified version of the protocol described by Yang et al (1998), see detailed descriptions in Appendix 1.

Sampling

The first action was to take samples from the objects using a dentist's drill to produce bone powder. Each object was first UV irradiated on both sides using a crosslinker. Then the selected areas of the objects were polished with the drill to clean off dirt. The actual samples were obtained by drilling a hole or a depression and the powder was collected in tubes and weighed. The drill holes can easily be seen on the bones in figures 2, 3 and 4 above.

Two samples were taken from each of the femurs, both from the diaphysis. One proximale anterior linea pectinea, the other distale posterior labium mediale. From the premolar only one sample was taken by drilling on the surface of the root. There was also a sixth sample which was a blank to check for contamination.

Object	Sample	Weight
L1 femur	L1A prox ant	0.078 g
	L1B dist post	0.070 g
L2 femur	L2A prox ant	0.081 g
	L2B dist post	0.094 g
U1 tooth	U1 root	0.061 g

Table 1. The samples.

DNA Release

In order to extract and isolate the DNA the bone powder was put in a solution with the enzyme Proteinase K and left to incubate overnight. The enzyme breaks down the cell and nucleus walls thus releasing the DNA in the solution. Then the tubes were spun down whereby undissolved grains and solids collected as pellets at the bottom of the tubes. See appendix 1, step 1-3.

The liquid above the pellets, the supernatant, was filtered and centrifuged until there was only 100 μ l left. See appendix 1, step 4-5.

Purification and Elution

The technique of purifying the DNA in the solution is to use filters with a silica membrane, see figure 8. The liquid was mixed with a binding buffer, incubated for 10 min and spun down. The binding buffer bound the DNA to the silica while fats, residue from the bore and other matter passed through. See appendix 1, step 6-12.



Figure 8. Tube with silica filter (Qiagen 2008 figure 2).

Twice washing buffer was added to the filters to remove unwanted substances and spun down. The washing buffer consisted mostly of ethanol. This was followed by a dry run with no buffer to get rid of the ethanol. Finally the DNA was released from the silica using an elution buffer. This was added twice and the mixture was incubated and spun down. See appendix 1, step 13-16.

Preparation for Sequencing

Ancient DNA consists only of many short sequences as fragments from the chromosomes. Longer DNA sequences would be recent contamination. The DNA fragments were prepared for sequencing by attaching predefined DNA sequences called adapters to the ends and amplifying the DNA molecules into millions of copies by *polymerase chain reaction*, PCR. This work followed a modified version of the protocol described by Meyer and Kircher (2010), see detailed descriptions in appendix 2.

Blunt End Repair

The DNA fragments often have blunt (frayed) ends which had to be repaired. This was done by mixing the DNA in a solution containing nucleoside triphosphate, dNTPs, the building blocks for creating nucleotides, and the enzyme T4 Polynucleotide Kinase. This solution was incubated in a thermal cycler for 15 min at 25°C followed by 5 min at 12°C. The enzyme acted as a catalyst for a reaction to complete the frayed ends. The actions of enzymes are controlled by temperature. See appendix 2 section 1.

Blunt end repair left many substances in the solution which had to be washed away. This was done by purification and elution as described above. See appendix 2 section 2.

Adapter Ligation

The adapters were added in two steps, first ligation, then fill in. The adapters were partly single stranded DNA sequences provided by suppliers. The DNA was put in a solution containing adapters and the enzyme T4 DNA ligase. The ligation was carried out by incubation at 22°C for 30 min in a thermal cycler. See appendix 2 section 3. This action was also followed by purification and elution as described above. Purification is rather time consuming. See appendix 2 section 4.

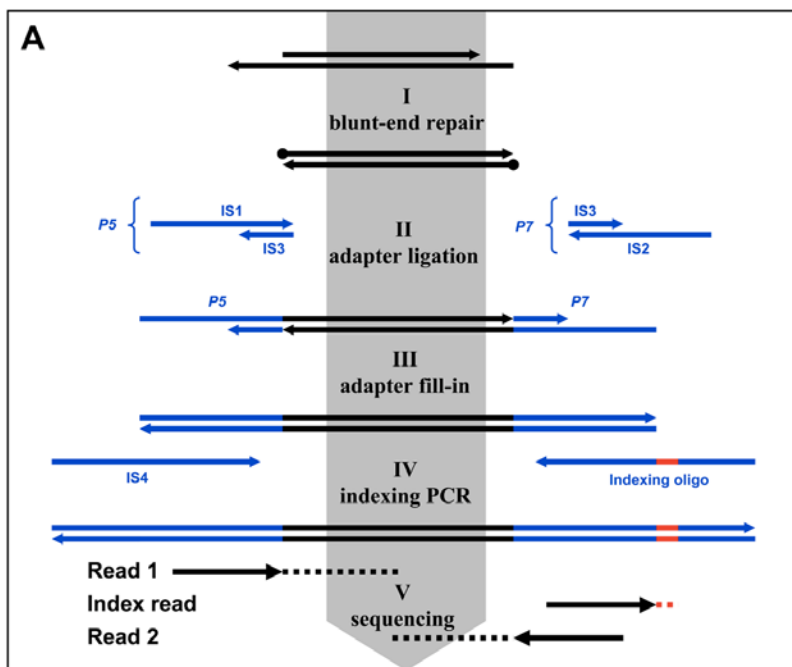


Figure 9. Adding adapters to the DNA fragments: blunt end repair, adapter ligation and adapter fill-in.

Furthest down is shown how the fragment is read during sequencing, first in one direction, then in the other.

(Meyer and Kircher 2010 figure 1).

Adapter Fill In

The adapters had to be filled in to be double stranded. This work was similar to blunt end repair. The DNA was put in a solution of dNTPs and the enzyme Bst polymerase. The reaction was carried out by incubation at 37°C for 20 min followed by heat kill at 80°C for 20 min. See appendix 2 section 5.

Test Library with qPCR

The DNA sequences with adapters formed a “library” ready for sequencing except that they had to be multiplied by PCR. Before that a qPCR test run is carried out. This gave an estimate of how many cycles that will be required in the final PCR to achieve samples of a reasonable concentration. The results are shown in table 2. An empty blank should properly require 22-25 cycles. That only 14 cycles would suffice was an indication of contamination. See appendix 2 section 6.

Sample	Number of PCR cycles
L1A	10
L1B	8
L2A	10
L2B	10
U1	14
Blank	14

Table 2. Results from qPCR.

Library Index PCR

PCR was applied to produce multiple clones or copies of the DNA sequences. This used the enzyme *polymerase* which cells apply to duplicate DNA in mitosis. The same procedure was run several times in a thermal cycler according to the results from the qPCR run. See appendix 2 section 7.

94°C	12 min
94°C	30 sec
60°C	30 sec
72°C	45 sec
72°C	10 min
12°C	indefinitely

} Repeated X times
|

Table 3. PCR Thermal profile. The value of X depended on the results from the qPCR test.

Gel Electrophoresis

3 µl from each of the samples were taken and used for electrophoresis, i.e. they were put in a gel and subjected to a magnetic current. This resulted in a separation since smaller molecules move further than larger molecules, see figure 10. Unfortunately there seemed to be some DNA also in the blank sample which would be an indication of contamination.

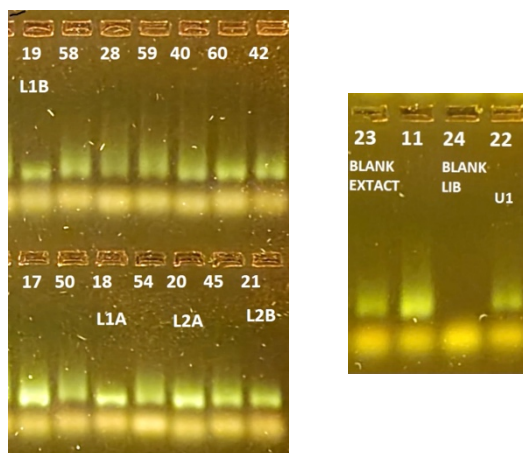


Figure 10. Results from gel electrophoresis. Photos provided by Petter Larsson.

Purification and Elution

This time microscopic magnetic beads were used instead of tubes with a silica membrane. The beads are tiny paramagnetic grains which were put in the solution together with the DNA sequences. Using a suitable buffer DNA fragments greater than 120 base pairs bound to the beads whereas smaller DNA fragments remained in solution. Those included tiny fragments with no adapters and as well as loose adapters. By placing the tubes in a magnetic rack the beads were attracted in one direction and the remaining fluid could be siphoned off. This process was repeated using a washing buffer. Finally an elution buffer was used to release the DNA from the beads.

Quantification of Concentrations (using Bioanalyzer 2100)

A quantification of the amount of DNA sequences of various lengths was done using a Bioanalyzer. The purpose of this is was to assess the DNA concentration of DNA of the samples.

Pooling in Equimolar Ratios

By adding distilled water the concentrations of the different samples in the batch were adjusted to be equal in molarity for the sequencing.

Sequencing and Bioinformatics

The sequencing was carried out at SciLifeLab (<https://www.scilifelab.se/>). Their Illumina Novaseq™ sequencers (Illumina 2021) use Next Generation Sequencing techniques which imply massively parallel processing. The actual processes are described in documents and videos available on the internet, such as <https://www.youtube.com/watch?v=CZeN-IgjYCo>.

Sequencing is a costly process and has to be done in large batches with samples from many projects. The batch with the six samples from this project also included samples from Neanderthals and ancient Kazakhs.

BAM File Statistics

The results from the sequencing is presented as FASTQ files, see figure 11.

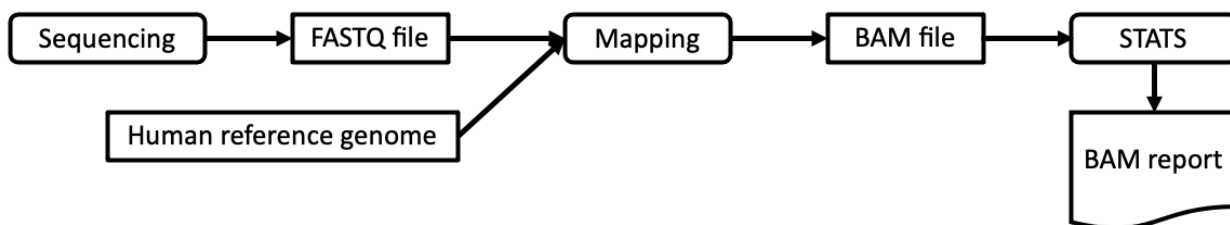


Figure 11. Bioinformatics, the first steps.

In this project there was only one FASTQ file for each sample. A FASTQ file contains all decoded DNA sequences in the order that they were found. The sequences are in text form like GATAACTG with a quality score attached.

The next step, which was carried out at the department of Organismal Biology at Uppsala University, was to map the FASTQ files against a human reference genome. The result were BAM files containing human genomes with DNA sequences in the right order. BAM files are in binary format to keep the volume down.

These computations were carried out at the UPPMAX (<https://www.uppmax.uu.se/>) supercomputer centre in Uppsala. The computers run under the Linux operating system and operations are carried out using Linux commands. Most programs to manipulate the files are in the SAM tools (<http://www.htslib.org/>) library available over Github.

STATS is a script which invokes various programs to extract information from BAM files. Applying STATS to the BAM files of the five samples resulted in the statistics shown in table 4A, 4B, 4C.

Sample	Library	Comment	File	Seq_date	Merged_seqs	Human_seq
lkg001	lkg001-b1e1l1p1	81	lkg001-b1e1l1p1_CATGCTC-AGA	2015-12-20	12563111	4637
lkg001	lkg001-b1e1l1p1	81	lkg001-b1e1l1p1_GAATCTC-ATG	2015-12-20	28629498	3375
lkg002	lkg002-b1e1l1p1	81	lkg002-b1e1l1p1_ACGCAAC-GAC	2015-12-20	38086283	24670
lkg002	lkg002-b1e1l1p1	81	lkg002-b1e1l1p1_GCATTGG-ATC	2015-12-20	24339314	124926
utt201	utt201-b1e1l1p1	81	utt201-b1e1l1p1_GATCTCG-GCC	2015-12-20	13153183	573829

Table 4A. Library = N/A. Comment = N/A. File = N/A. Seq_date = N/A. Merged_seqs = Number of sequences read twice and merged. Human_seq = Number of sequences identified as human.

Sample	Prop_human	Avg_read_length	Clonality	Too_short	Genome_cov
lkg001	0,00265531364006892	59	24,8029017656404	67,2202404148805	0,0000495994
lkg001	0,00287685798752042	64	27,5390648713621	70,7392882726467	0,0000287931
lkg002	0,00159682161685350	66	22,7732377460249	52,1301609747274	0,0003188510
lkg002	0,00692316143339126	93	26,5291831102934	15,3496928874514	0,0028891600
utt201	0,05616305954231760	78	34,4360470704174	22,9842579694960	0,0116650000
					Total: 0.0032864035

Table 4B. Prop_human = Proportion human DNA. Avg_read_length = Average length of readings. Clonality = Clonality. Too_short = Number of sequences that are too short to be located in the human genome. Genome_cov = Coverage.

Sample	Mt_cov	Mt_seq	X_seq	Y_seq	Biological_sex	5_damage	3_damage	Mt_haplo
lkg001	0,00000000	1	166	36	consistent with XY but not XX	0,2314990	0,2490770	N
lkg001	0,00621643	2	132	25	consistent with XY but not XX	0,1625440	0,1724140	N
lkg002	0,05848270	19	954	181	Not Assigned	0,0925083	0,0915387	N
lkg002	0,29156900	86	3848	1020	consistent with XY but not XX	0,0738813	0,0768022	N
utt201	107882,0	388	15946	4329	Not Assigned	0,1173470	0,1161900	T2a1a1
Total: 0.35626813								

Table 4C. Mt_cov = Mitochondrial coverage. Mt_seq = Number of sequences identified as human mitochondrial sequence. X_seq = Number of sequences identified as X chromosome sequence. Y_seq = Number of sequences identified as Y chromosome sequence. Biological_sex = XX female and XY male. 5_damage and 3_damage = Amount of damage to the of DNA sequences. Mt_haplo = Mitochondrial haplotype.

Prop_human indicates the proportion of human DNA in the sample. Any non-human DNA would be viruses, contamination or any other DNA. For the premolar it was 5.6% but for the femurs it was less than 1%. **This meant that only the premolar sample was of sufficient quality for continued analysis.** The number of sequences identified as human (Human_seq) was also largest for the premolar.

The average length of readings (Avg_read_length) is also interesting. Ancient DNA always only consists of fragmentary sequences. If they are too short (Too_short) they cannot be identified and used but if they are too long they must be the result of recent contamination. 5_damage and 3_damage give an indication of the amount of damage at the end of the sequences. This is also an indication whether the sequences are ancient or not. Clonality is an indication of how many sequences that are duplicated.

Mt_haplo gives the mitochondria haplogroups. This is N for the femurs. However, that is a supergroup which encompasses almost all other haplogroups so it is almost the same as undefined. The mitochondria haplogroup for the premolar is stated as T2a1a1 with a large uncertainty. The haplogroup T2 is a minor haplogroup in Europe with the largest concentrations down on the continent, see figure 12.

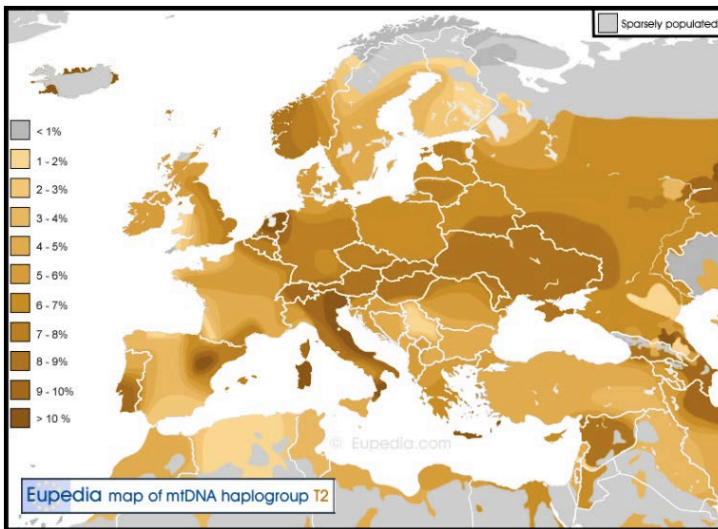


Figure 12. Distribution of the mtDNA haplogroup T2 in Europe, North Africa and the Middle East.

From <https://www.eupedia.com/europe>

Principal Components Analysis

A dataset of two variables can be illustrated by a 2-dimensional x-y-graph but this is not possible when there are three or more variables. *Principal Components Analysis*, PCA, is a statistical technique to display a dataset of many variables on a 2-dimensional graph by reducing the many variables to a few principal components. When displayed on a graph, elements of large similarity will be placed close to each other whereas dissimilar elements will be spread out (Jobling et al 2014 p 179-182).

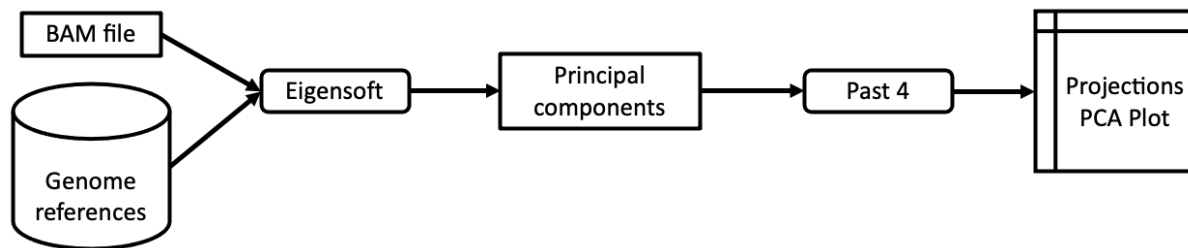


Figure 13. Bioinformatics, Principal Components Analysis.

Eigensoft is a program to compute principal components for BAM files of a large number of specimens (Eigensoft 2013). The BAM files can then be plotted as points on a graph which is easy to survey and where related specimens are grouped together.

The purpose of doing a PCA for the premolar sample was to find out how it was related to other previously known samples. This was done by computing the PCA for its BAM file together with the BAM files for a selected reference group. The project first attempted at building a specialized reference group based on published Swedish specimens but in the end the AADR was used instead. This is a publicly available database of genetic information from more than 12,000 ancient and contemporary individuals (AADR 2021). It is a compilation of practically all published research into aDNA including 189 ancient Swedes.

The Eigensoft computation took a couple of hours as a background job at UPPMAX. On the other hand the resulting PCA was not larger than it could be held as an Excel file. Graph plotting was done on a laptop using the PAST4 software package (Hammer 2021).

The most important plotting was to project the premolar onto ancient Europeans in the AADR, see figure 14. This plot clearly indicated that the premolar belongs to the pastoral gene pool. Since those arrived in Europe ~2,800 BC at the earliest, the premolar cannot be older than that. However, it could well be younger, for example from the Bronze or the Viking Age.

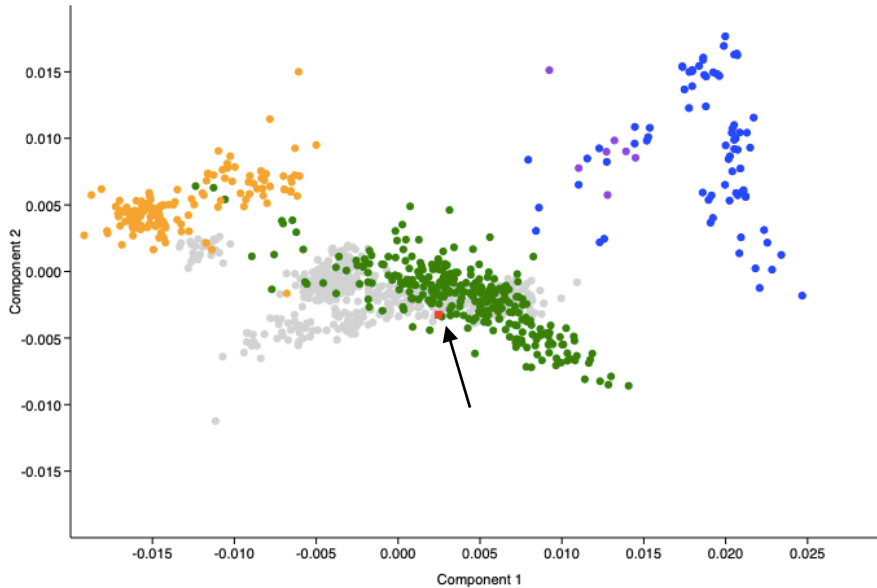


Figure 14. Principal Components Analysis. The premolar sample and Mesolithic and Neolithic European individuals from the AADR database. The figure follows the same colour scheme as figure 6 above.

Legend

<p>Orange dots = Farmers (141) Funnel Beaker, Globular Amphorae, Impressa, Megalithic, LBK (<i>Linearbandkeramik</i>), Starčevo, TRB (<i>Trichterbecherkultur</i>) and Vinča</p>	<p>Blue dots = Hunter Gatherers (75) Bichon, Comb Ceramic, HG (<i>Hunter Gatherer</i>), Loschbour, Magdalenian, Mesolithic and Villabruna</p>
<p>Green dots = Pastoralists (296) BAC (<i>Battle Axe Culture</i>), Bell Beaker, Corded Ware and Yamnaya</p>	<p>Violet dots = Pitware (7) PWC (<i>Pitted Ware Culture</i>)</p>
<p>Grey dots = Contemporary Europeans (541) Albanian, Basque, Bulgarian, Croatian, Czech, English, Estonian, Finnish, French, Greek, Hungarian, Icelandic, Lithuanian, Moldavian, Norwegian, Orcadian, Romanian, Russian, Sardinian, Scottish, Sicilian and Spanish</p>	<p>Red square = Premolar sample (1)</p>

The next plotting was to project the premolar onto Europeans of today, see figure 15. Remarkably enough it seemed most related to today's central Europeans like Czechs or Hungarians.

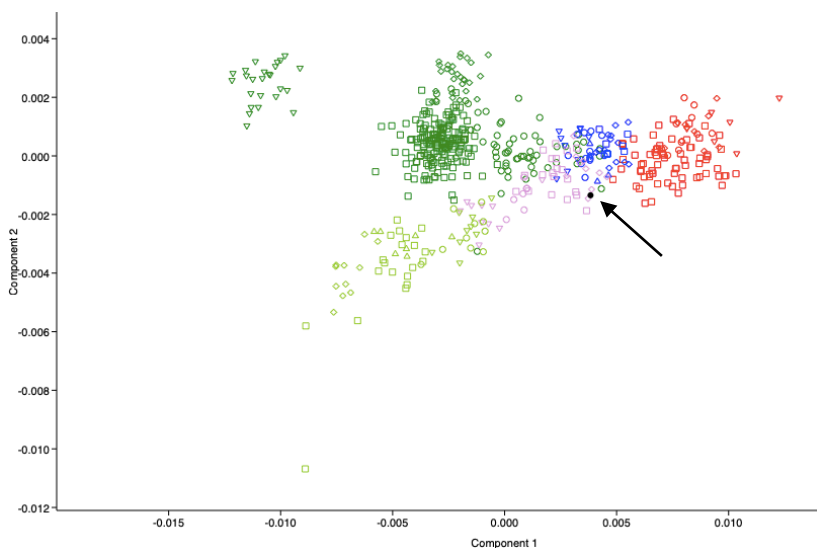


Figure 15. Principal Components Analysis. The premolar sample and contemporary Europeans from the AADR database.

Legend

<p>Green</p> <ul style="list-style-type: none"> □ Spanish (72) ○ French (162) ◇ Basque (29) ▽ Sardinian (25) 	<p>Blue</p> <ul style="list-style-type: none"> □ Norwegian (11) ○ Orcadian (13) ◇ Icelandic (12) ▽ English (10) △ Scottish (4) 	<p>Red</p> <ul style="list-style-type: none"> □ Russian (71) ○ Lithuanian (10) ◇ Estonian (10) ▽ Finnish (6)
<p>Light green</p> <ul style="list-style-type: none"> □ Greek (20) ○ Bulgarian (10) ◇ Sicilian (11) ▽ Moldavian (9) △ Albanian (7) 	<p>Plum colour</p> <ul style="list-style-type: none"> □ Hungarian (20) ○ Croatian (10) ◇ Czech (10) ▽ Romanian (10) 	<p>Black</p> <ul style="list-style-type: none"> ● Premolar sample (1)

Clustering

The PCA data could also be used for clustering. This grouped similar individuals pairwise followed by pairing groups etc. until a tree diagram is formed. Applying clustering to the premolar together with the 189 ancient Swedes in the AADR database grouped the premolar together with an individual who lived during the Viking age and was buried at Kopparsvik on Gotland (Margaryan et al 2020).

This individual was closest to Lithuanians than to any other population and belonged to mitochondria haplogroup X2_G225A. This is rare but has a wide distribution (by mail communication from Ricardo Rodriguez Varela, researcher at the Centre for Palaeogenetics).

Both PCA and clustering indicate that the premolar probably belonged to someone related to continental Europeans. If the premolar was found in Sweden he/she might have originated from continental Europe.

The facts revealed

At this point of the project the true background of the objects was revealed in a mail from Jan Storå, professor at the Osteoarchaeological Research Laboratory at Stockholm university.

Both femurs came from a passage grave at the Locke farm in Slöta parish in Västergötland, registry number L1961:4621. Passage graves belong to the Middle Neolithic farming tradition but such graves could also be used for secondary burials during the Late Neolithic and the Bronze Age. The finds are listed under the inventory number SHM 3166 at the Swedish History Museum and includes 11 pieces of bone. Among the artefacts are pieces of flint, a whetstone and a pearl.

The premolar came from a cist in the village of Utterstad in the parish of Appuna in western Östergötland, registry number L2012:783. Cists appeared during the Late Neolithic and this cist is hidden in a mound which has the registry number L2012:1159. The mound was excavated by county officials in 1869 who also wrote a report. The mound has a diameter of 20 meters and is 2 meters high with a rather flat top. Western Östergötland around the lake Tåkern must have been a very rich Bronze age district and there remain several large mounds (Nerman 1936).

The archaeological site at Utterstad has the inventory number SHM 4240 at the Swedish History Museum. This lists some beautiful artefacts including a sword and a razor both made in bronze. There are also nine crania which have been deposited at the museum of Karolinska Institutet. The premolar comes from the mandibula from one of those. The web pages of the Swedish History Museum only states Bronze age as date but current research points to an interval of 1600-1400 BC.

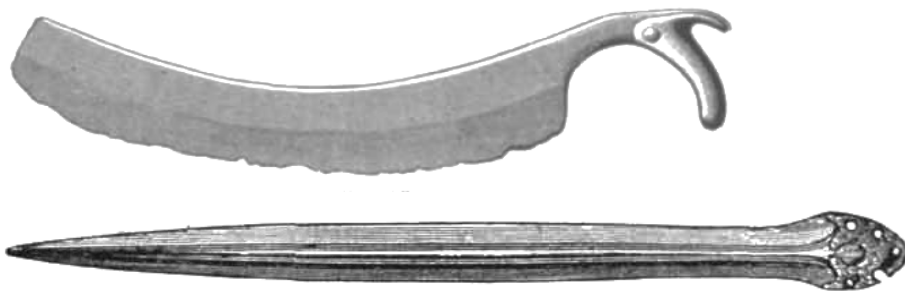


Figure 16. Bronze razor and sword from the mound at Utterstad (Nerman 1936, figure 14 and 15). Not to scale.

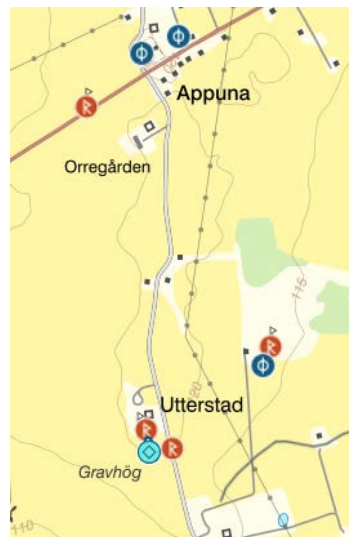


Figure 17. Location of Utterstad in Östergötland (Nerman 1936, figure 18) and of the mound at Utterstad (Fornsök <https://app.raa.se/open/fornsok/>).

Conclusions

Conclusions from this experiment

1. The experiment was a success. The genetic analysis showed that the specimen must be younger than ~2,800 BC and the correct date is ~1,600 BC.
2. Ancient DNA can be used for dating and for identifying cultural context. This is done by associating specimens with known population groups from databases like the AADR.
3. Teeth are better at preserving ancient DNA than diaphyses of long bones.
4. Experiments with ancient human material can easily fail. In this project two out of three specimens did not make it to the end.

However, it is difficult to get a high degree of accuracy. Ancient DNA analysis for dating relies on points in time when distinct changes occur like population turnovers. In this project two such points were identified, ~4,200 BC with the introduction of the Funnel beaker culture and ~2,800 BC with the start of the Battle Axe culture. The individual who had carried the premolar is associated with the Battle Axe gene pool but lived one thousand years later during the early Bronze age.

Ancient DNA analysis can point out probable areas of birth but people may move to completely different locations. In this experiment an individual who probably came from continental Europe ended up in Östergötland.

Acknowledgements

Acknowledgements are due above all to my supervisors Anders Götherström, Professor of Molecular Archaeology, and Barbara Wohlfarth, Professor of Quaternary Geology, for allowing me to do this task as a bachelor project and to Jan Storå, Professor of Osteoarchaeology, who proposed the theme for the project and also provided the three test objects that were used.

I am deeply indebted to Anna Linderholm who took over as supervisor when Barbara Wohlfarth became pensioner, to Vendela Kempe Lagerholm and Petter Larsson who helped me with the lab work and, indeed, carried out most of the delicate work that it would be risky to hand over to a novice enthusiast and to Ricardo Rodriguez Varela, Violeta Anca Prado, Zoé Pochon and Maja Krzewińska who helped me with the Bioinformatics. Indeed, I have been guided every step on the way by kind people at the Centre for Palaeogenetics.

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Appendix 1

DNA extraction from human materials

Yang with urea (Amicon filters)

- Wipe samples with bleach and ddH₂O if dirty.
- UV irradiate on both sides for 6J/cm² (what the crosslinker is already set for).
- UV weighing boats/tin foil.
- Drill and weigh powder.
- Clean drill bits with DNAway, ddH₂O, EtOH, and UV.

Lysis buffer (final conc):

0.5M EDTA pH 8 }
1M Urea } Yang-Urea buffer
100 µg/mL Proteinase K

Mix **3.02 g Urea** (8M) with **50 ml EDTA** (0.5M, pH 8) in a Falcon tube and UV irradiate.
(Also UV the amount of PE, EB and LB needed for extraction)

Tubes for post-lysis steps (per sample)

Amicon tubes	1
1.5 LoBind tubes	3
MinElute tubes	1
Collection tubes	3
50ml tube	1

1. Add 1.5 ml Yang-Urea buffer to bone sample + at least 15 µl Proteinase K (10mg/ml).
Vortex
2. Incubate at 38-55°C over-night. If powder is fully dissolved, continue extraction. If not, add 10 µl Proteinase K and incubate for at least a few more hours at 50 °C.
3. Spin down at 3 000 rpm for 5 min
4. Transfer supernatant to Amicon filters.
5. Centrifuge at 5000 rpm (=max on AFL centrifuge) until there is ca 100 ul liquid left.
Start with 10 min spin time.
6. Transfer Amicon supernatants to 1,5ml LoBind tubes with 5X PB buffer (ca 500 ul), vortex gently
7. Transfer the PB-mix to MinElute filter, incubate 10 min, and spin down at 13 000 rpm for 1 min

8. Pour off flow through. Take new collection tubes
9. Add 710 ul PE buffer to MinElute filter. Spin at 13 000 rpm for 1 min
10. Pour off flow through. Take new collection tubes
11. Add 710 ul PE buffer to MinElute filter. Spin at 13 000 rpm for 1 min (*i.e.* 2 PE washes in total)
12. Pour off flow through. Take new collection tubes. Spin again at 13 000 rpm for 1 min (dry the column)
13. Transfer column to a 1.5 ml LoBind tube with removed lid. Leave to dry 5min
14. Add 35 ul EB buffer. Incubate **10 min at 37°C**. Spin at 13 000 rpm for 1 min
15. Add another 35 ul EB buffer, incubate, and spin down as above
16. Transfer the 70 ul DNA extract to a new LoBind tube and store in the freezer

Appendix 2

BLUNT END ILLUMINA LIBRARIES (MEYER oligos and NEBNext E6070L kit)

0. Preparation of oligo hybridisation buffer (200 rxns) (Meyer and Kircher supplementary?)

500 mM NaCl
10 mM Tris-Cl pH 8 (Trizma from Sigma)
1 mM EDTA pH 8

- Preparation of adapter mix (100 µl for 200 rxns) (after Meyer & Kircher)
- Make hybridization mix for adapter P5 and adapter P7 in PCR tubes

Hybridization mix for adapter P5 (200 uM)

40 µl of 500 uM IS1_adapter_P5.F
40 µl of 500 uM IS3_adapter_P5+P7.R
10 µl of 10X Oligo hybridization buffer
10 µl ddH₂O

Hybridization mix adapter P7 (200 uM)

40 µl of 500 uM IS2_adapter_P7.F
40 µl of 500 uM IS3_adapter_P5+P7.R
10 µl of 10X Oligo hybridization buffer
10 µl ddH₂O

- Mix and incubate the reactions for 10 sec at 95°C followed by a ramp from 95°C to 12°C at a rate of 0.1°C/sec.
- Combine both reactions to get a ready-to-use mix (with 100 uM of each adapter).

1. Blunt End Repair (40µl final volume/Rx)

Tango Buffer	4 µl	-----	36 µl
dNTPs	0.16 µl	x 9	-----1,44 µl
ATP	0.4 µl	-----	3,6 µl
T4 PNK	2 µl	-----	18 µl
T4 DNA Pol	0.8 µl	-----	7,2 µl
H ₂ O	12.64 µl	-----	113,76 µl
DNA	20 µl		

- Mix and incubate in a thermal cycler for 15 min at 25°C followed by 5 min at 12°C.

2. Purify with MiniElute

- Apply 200 μ l of PBI Buffer to 40 μ l of the repaired DNA to column (mix first), spin 13000 rpm for 1 min.
- Discard waste and wipe with bleach cloth.
- Add 700 μ l PE Buffer to column, spin 13000 rpm for 1 min and repeat this PE wash one more time (2 washes in total)
- Discard waste and wipe with bleach cloth.
- Spin 13000 rpm for 1 min.
- Change to new 1.5 ml tube.
- Add 22 μ l EB Buffer to column and incubate at 37°C for 5 min.
- Elute DNA by spinning it down for 1 min at 13000 rpm.

3. Adapter Ligation (40 μ l final volume/rx)

Prepare a master mix for the required number of ligation reactions as shown below. If white precipitate is present in the 10X DNA ligase buffer after thawing, warm the buffer to 37°C and vortex until the precipitate has dissolved. Since PEG is highly viscous, vortex the master mix before adding T4 DNA ligase and mix gently thereafter. Add the adaptors and the DNA before the ligase to avoid chimera.

H ₂ O	10 μ l-----x 9-----	90 μ l
T4 DNA ligase Buffer (10x)	4 μ l-----x 9-----	36 μ l
PEG-4000 (50%)	4 μ l-----x 9-----	36 μ l
Adapter mix	1 μ l -----x 9-----	9 μ l
<small>(1μl of a 1:10 dilution in TE of the stock = 10pmol in final volume)</small>		
T4 DNA ligase (5 U/ μ l)	1 μ l-----x 9-----	9 μ l
DNA		20 μ l

- Incubate for 30 min at 22°C

4. Purify with MiniElute

- Apply 200 μ l of PBI Buffer to 40 μ l of the repaired DNA to column (mix first), spin 13000 rpm for 1 min.
- Discard waste and wipe with bleach cloth.
- Add 700 μ l PE Buffer to column, spin 13000 rpm for 1 min and repeat this PE wash one more time (2 washes in total)
- Discard waste and wipe with bleach cloth.
- Spin 13000 rpm for 1 min.

- Change to new 1.5 ml tube.
- Add 22 μ l EB Buffer to column and incubate at 37°C for 5 min.
- Elute DNA by spinning it down for 1 min at 13000 rpm.

5. Adapter fill in

H ₂ O	14.1 μ l	-----x 9-----	126,9 μ l
Thermopol buffer 10x	4.0 μ l	-----x 9-----	36 μ l
dNTPs (25uM each)	0.4 μ l	-----x 9-----	3,6 μ l
<i>Bst</i> polymerase, LF (8 U/ μ L)	1.5 μ l	-----x 9-----	13,5 μ l
DNA		20 μ l	

- Incubate for 20 min at 37°C and heatkill for 20 min at 80°C

6. Test Library with qPCR

7. Library PCR1

Set up 4 reactions per DNA library in 50 μ l reactions containing:

ddH ₂ O	31.5 μ l	x4-----	126 μ l
10X TaqGold Buffer	5 μ l	x4-----	20 μ l
25mM MgCl ₂	5 μ l	x4-----	20 μ l
25mM dNTPs	0.5 μ l	x4-----	2 μ l
Index Primer Mix	2 μ l	x4-----	8 μ l
AmpliTaQ Gold	1 μ l	x4-----	4 μ l
DNA	5 μ l	x4-----	20 μ l

- Mix and spin down and use the following thermal cycler program

Initial denaturation	94°C	12 min		
Denaturation	94°C	30]	
Annealing	60°C	30	>	10 cycles
Elongation	72°C	45]	
Final extension	72°C	10 min		
Hold	4°C	forever		

- AmPure bead purification and elution in 20 μ l EB Buffer + 0.05% Tween 20



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