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A genetic approach for investigating cannibalism in prehistoric Sweden

Ayça Omrak

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Biology Education Centre and Evolutionary Biology Department, Uppsala University
Supervisors: Anders Götherström and Helena Malmström
External opponent: Carina Schlebusch

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Cover image: A grave from the Ajvide excavation area, Gotland. Courtesy of Niklas Wirsén, 2008

1. Abstract

Cannibalism in prehistoric times has been a much debated subject among scientists for many years. The subject itself is repulsive and emotionally disturbing. However, the damaged, scarred and charred bones found throughout the whole world from different time periods during archaeological excavations suggest that this may actually be true. It is of course not so easy to distinguish by analyzing cut marks on the bones when there are so many other factors in play. However, with new approaches to archaeological remains, new clues are being discovered about cannibalism being a wide spread practice among the ancient people in Paleolithic and Mesolithic Ages. Up to recently Fore people in Papua New Guinea were practicing endo-cannibalism (consuming the flesh of an ally after their death) for religious reasons until approximately 10% of the population perished with kuru, a neurodegenerative disease caused by consuming human flesh. Research that has been carried out after the kuru epidemic in Papua New Guinea showed that a heterozygosity in codon 129 in the responsible gene (PRNP), creates resistance to kuru and prolongs the incubation period of the disease. Therefore, heterozygotes have higher fitness and survival rates, which suggests balancing selection at play.

If our ancestors were cannibals could we trace it back? If they were, did they suffer from kuru like their contemporary successors in Papua New Guinea? In this paper, I investigated these questions by sequencing the coding region of the PRNP gene in Neolithic and Late Mesolithic hunter-gatherers and in Neolithic farmers. Nine Mesolithic and Neolithic human samples were successfully sequenced for 463 basepairs of the protein coding region of PRNP gene on Illumina platform. 25 polymorphisms were detected including codon 129 and a possible ancient polymorphism on codon 169. Tajima's D results yielded no indication of balancing selection in prehistoric Scandinavia. Although, all remains from Late Mesolithic Age were heterozygotes for codon 129, further analysis is needed to confirm the hypothesis of cannibalism in prehistoric Sweden.

2. Introduction

2.1 Prion Diseases

Prion diseases gained the interest of the public in the beginning of 1990's with the Bovine spongiform encephalopathy epidemic in UK. This disease, which is also known as the mad cow disease, clusters in to the prion diseases family. These constitute of fatal neurodegenerative conditions that affect both humans and animals. Although different variants were known independently since the 1930's (vCJD 1930, scrapie 1954, kuru 1959 etc) the only connections made between these different conditions were the “significantly similar light microscopic pathology of the central nervous system” and the cause being a “slow” virus. After the transmission of one prion disease variant to chimpanzees by intra cerebral inoculation it became fairly easy for scientists to fill in the blanks.[1] With the veil of mystery lifted, the revealed picture was rather disturbing: A variation of unconditionally fatal diseases that can occur genetically, sporadically or infectiously. [2] (Table 1.) In addition to that, it was also revealed that all prion diseases occur due to a mutant variation of a protein that the patient's own body produces, contrary to the theories of the cause being a “slow” virus. [1] Genetically inherited types make up to 10-15% of the human prion disease cases. The remaining either occurs sporadically or from exposure to infected prions. Contaminated surgical instruments, injections from cadaveric derived human growth

hormone, corneal transplants (leading to iCJD), blood transfusions or consumption of flesh contaminated with infected prions (leading to vCJD or kuru) are some ways for transmission of acquired prion diseases.

All human prion diseases demonstrate similar symptoms in different combinations including dementia, psychiatric symptoms, motoric incoordination, myoclonus, visual disturbances, seizures, autonomic disturbances, weakness and/or spasticity. [2,3] The outcomes are straightforward: Decreasing control over the body and the mind followed by death. The onset age of the disease depends on many factors including the type of the disease, the amount of exposure they have experienced and patient's genetic susceptibility.

Table 1: Types of prion diseases

Disease	Type	Host
Creutzfeld-Jakob Disease (fCJD)	Familial	Humans
Gerstmann-Sträussler-Sheinker Syndrome (GSS)	Familial	Humans
Fatal Familial Insomnia (FFI)	Familial	Humans
Kuru	**Acquired**	**Humans**
Iatrogenic Creutzfeld-Jakob Disease (iCJD)	Acquired	Humans
Variant Creutzfeld-Jakob Disease (vCJD)	Acquired	Humans
Scrapie	Acquired	Sheep
Feline Spongiform Encephalopathy (FSE)	Acquired	Cats
Bovine Spongiform Encephalopathy (BSE)	Acquired	Cattle
Transmissible Mink Encephalopathy (TME)	Acquired	Mink
Exotic ungulate encephalopathy	Acquired	Greater kudu, nyala, oryx
Sporadic Creutzfeld-Jakob Disease (sCJD)	Sporadic	Humans
Sporadic Fatal Insomnia	Sporadic	Humans

2.2 Kuru

The Fore linguistic group is the general name of the people from North Fore and South Fore in Eastern Highlands of Papua New Guinea who resided in some 65 adjacent villages, with a population of approximately 12,000 people. [4,2] Living in complete isolation they had no contact with modern civilization until the 1950's. According to the oral tradition, at the end of the 19th century it had become customary among the Fore people of Papua New Guinea to consume the body of the deceased during funerals as a way to honor and mourn them. The participants of the rituals were women and youngsters from both sexes (no older than 6-8 years) while men participated very little or not at all. [2,4] Kuru is therefore believed to originate from the consumption of a deceased individual with sporadic CJD, but this must be considered a hypothesis that has yet to be tested. [5] However, the disease spread through the neighboring villages with intermarriages and reached an epidemic level in the 1950's. When

Australian authorities banned cannibalism, kuru significantly regressed but already killed about a thousand people. Although since 1957 an additional 2500 cases were reported, these cases were the result of the long incubation periods of kuru which delays the age onset of the disease.

The incubation period and duration of kuru are highly variable and between five to over 60 years, and three months to two years for the duration of the sickness. It has been shown that these two factors are highly influenced by patient's genetic inheritance. (See sub-section 2.4, PRNP gene) The disease is generally self-diagnosable with symptoms of headaches, joint pains, trouble walking and unsteadiness. The overall duration can be divided in three seperate stages: ambulatory, sedentary and recumbent, in which patient undergoes some serious problems including losing muscle power, losing the ability to walk and sit day by day, suffering from euphoria with inappropriate laughters** and in the end suffering from respiratory failure or pneumonia followed by death. [2]

2.3 Prion Proteins

Prion proteins are the only known cause for prion diseases. While the normal function of the protein remains unknown, some proposed functions are cell signaling and synapse formation. [3,6] Delivering copper to cells is also a proposed function. It has been shown (among many other findings) that the levels of copper are significantly lower in the brains of prion protein lacking transgenic mice. [7] There are two different isoforms of the prion protein where the difference lies not in amino acid sequences but in their biochemical properties: normal, non-pathogenic form (PrP^{c}) and pathogenic form (PrP^{sc}). PrP^{c} consists of alpha helical foldings whereas PrP^{sc} is at least 40% beta plated sheet. This different style of folding in PrP^{sc} makes the protein almost completely resistant to nucleases and insoluble in detergent reagents while alpha helical PrP^{c} is both soluble in detergant and degradable by proteases. [8,3]

All prion diseases are associated with the abnormal accumulation of the misfolded prion protein (PrP^{sc}), working as a template for forcing the other normal prion proteins (PrP^{c}) to misfold themselves. The misfolded proteins (PrP^{sc}) aggregate together and form a plaqoid structure called amyloid plaque which, by damaging the surrounding areas irreversibly, decreases the amount of neurons in the brain. However in contrary to common belief, a number of recent studies have proposed that aggregation may not be the cause of the damage. Instead a neural apoptosis that is ignited either from the lack of normally functioning prion protein (PrP^{c}) or the surplus of physiologically unable prion protein (PrP^{sc}) has been proposed to be the reason behind the damage. [7] Whether aggregation or neural apoptosis, the cause and the outcome of prion diseases remain the same.

2.4 Prion protein gene (PRNP)

PRNP is the short name of the prion protein gene that is responsible for the production of prions and so far, it is the only gene that is known to be related to prion diseases. The PRNP gene is located on the short arm of chromosome 20 and consists of five octapeptide repeats and two exons with an open reading frame of 756 nucleotides located on one of the exons. [9,10]

**Because of the inappropriate laughters generated by euphoria, kuru is known as "Laughing Death" amongst Fore people.*

Point mutations in the coding region cause amino acid substitutions in the prion protein. Several point mutations have been reported for genetically inherited cases (CJD, FFI, GSS). There is also another type of mutation which is the insertion of a whole octapeptide repeat in addition to the five already existing ones. [2] The number of insertions may vary from one to nine, and as the number of inserted octapeptide repeats increase, the incubation period of the disease gets shorter. [3]

There are also a number of polymorphic sites in the PRNP gene including codon 127, codon 219 and codon 129. The codon 127 polymorphism, a recent discovery, is found in a population in New Guinea and is proposed to co-act with codon 219 for increased susceptibility to kuru. [11] The polymorphism in codon 219 is found in low frequencies amongst Japanese population and is proposed to be a protective polymorphism against CJD. [3, 12]

The polymorphism in codon 129 codes for either methionine or valine, with the ancestral allele being methionine. Much research carried out by various scientists indicates that when individuals are homozygous for methionine on codon 129, they are then more susceptible to sporadic and acquired prion diseases. [13, 8] Additionally (to our interest) heterozygosity on codon 129 is known to increase resistance to kuru and prolong the incubation time of kuru. For example, the majority of the survivors studied in kuru epidemics were heterozygous. [12, 1, 15] Further, when a comparison is made for the course of the disease and the age of onset between homozygotes (either MM or VV) and heterozygotes (MV), homozygote individuals have shorter duration for both of these factors. [16]

The issue related to this is to figure out if heterozygosity for this particular polymorphism is favored and if there is balancing selection at play, like we see in the classical sickle cell anemia example (where one homozygote is susceptible for malaria and the other for sickle cell anemia thus the individuals who are more likely to survive are the heterozygotes, which makes the reproductive fitness of the heterozygotes higher than homozygotes, violating Hardy-Weinberg equilibrium). Mead *et al.* were able to provide some answers to this question. [12] Screening the genotype for codon 129 in Fore women aged over 50 and known to have taken part in cannibalistic rituals therefore having been exposed to kuru, 23 of the 30 women were heterozygotes for codon 129 and thus out of Hardy-Weinberg (HW) equilibrium. In contrast, the results for the unexposed Fore population as well as a control group of elderly people in European populations displayed HW equilibrium. The results of the unexposed population suggest the heterozygosity effect is caused by a stress, a selection over the time when the population was exposed to kuru while the results of the European control group eliminate the effect of the age being a factor. Mead and colleagues also screened other populations worldwide (African, South Asian, East Asian, Pacific, European and South American) and Tajima's D statistics indicated that all populations showed signs of being under balancing selection for codon 129. Tajima's D is a statistical method for neutrality for selection, when D value is below zero the results indicate a directional selection and above zero indicates a balancing selection. Additional to Mead *et al.*'s results, Hedrick found out that the signal of selection for codon 129 is stronger than in any other gene in humans. [17]

2.5 What does the ancient people have to do with it? (Aims)

2.5.a Cannibalism in the prehistoric world and Northern Europe

The trail of cannibalism is not easy to track. Postmortem damage from wild animals or any other

external damage over the hundreds of years could lead to confusion. Therefore, scientists have to be careful with their interpretations. The archaeological indicators of cannibalism lie in the untold tales of the remains; butchering techniques that is similar to that used for animals, intentionally broken bones that might indicate marrow extraction, human coprolite (ancient faeces) containing human remains (i.e human proteins like myoglobin) or charred human bones that bear signs of cooking are archaeological indicators of cannibalism. [18] The above mentioned evidence are found in various archaeological sites from various time periods throughout the world. From the agricultural society of Azasari people in Colorado to Lower Pleistocene hominids in Spain, as well as the Neanderthals and Neolithic humans in France all had the indicators of cannibalism. [18, 19, 20, 21, 22]

The indicators of cannibalism are also found in various excavation sites in Sweden and Baltic Sea islands. In Jettböle excavation site on Åland islands, remains from the Pitted Ware Culture, scattered human remains with intentionally broken bones and cutmarks on the bones were observed in a possible sacrifice chamber. [23] There are also more examples of intentionally broken bones and cut marks suggesting marrow extractions reported from Dyrholmen and Jylland Late Mesolithic excavation sites. [24] In remains from Late Mesolithic Age from Stora Förvar at least one human cranium was found with cut marks on it. (Storå J, personal communication)

Finding all above mentioned evidences of cannibalism throughout the prehistoric world, we wanted to investigate if we could trace cannibalism using another perspective. By using the only gene that is responsible for kuru, we could both see the ancient variations in the PRNP gene and examine if our ancestors had suffered from any epidemics as seen in Papua New Guinea.

Ramakrishnan *et al*. [25] have shown the importance of including ancient DNA while investigating for the events that took place in the past populations. Their analysis on various possible bottlenecks with various population sizes demonstrated a statistical increase in accuracy when including ancient DNA data for detecting bottlenecks and selection pressures on a past population. [25] Their results indicate that if we go back to the time where an assumed epidemic caused by cannibalism was taking place, it would be easier to detect the severity of the events with a more accurate perspective. Therefore we wanted to sequence the coding region of the PRNP gene, (particularly codon 129) in our human remains, calculate Tajima's D for each group to investigate if the populations were under balancing selection and check if we could uncover any information about our ancestors' prehistoric cravings.

2.5.b Information about the time period of our samples

The human material that has been studied in this project were remains from Neolithic and Late Mesolithic ages, excavated from various graves and other archaeological sites throughout mainland Sweden and Baltic Sea region. (Figure 1) Mesolithic Age in Scandinavia starts with the end of the Ice Age (8000 BC) and covers the time period until the emergence of farming (4000 BC). According to the eight tons of human and animal remains found in Stora Förvar cave in Stora Karlsö, Mesolithic Age hunter-gatherers lived in the shore lands, hunting gray seal and salmon. Whether Stora Förvar cave was a permanent settlement or a temporary one that is used only in hunting seasons is still under debate. Permanent or temporary, it is known that hunter-gatherers resided in this cave until the end of Late Mesolithic Age and abandoned it for 2000 years, probably due to the worsening weather conditions and came back in the Neolithic Age when the island was habitable again. [26] The broken bone pieces that are found in this cave have created much debate about cannibalism.

The Neolithic Age, or the New Stone Age is a time period covering approximately 6000 years and it is believed to have started in the Middle East gradually spreading to the other parts of the world. Therefore, the time period for different ages varies greatly by region. [27] In Southeast Europe, Neolithic Age covers a time period of 7000 BC to 3000 BC while in Scandinavia, it arrives rather late but spreads rapidly. It starts from 4000 BC and lasts until 1700 BC. This rapid process of Neolithization creates a picture where farmer and hunter-gatherer complexes coexist. The earliest dated farming culture in Scandinavia was the Funnel Beaker Culture (TRB or Tricherbecherkultur) which emerged somewhat before 4000 BC. Pitted Ware Culture (PWC) however, was a hunter-gatherer society from Southern Sweden and the Baltic region, dating back to 3400-2300 BC. A hunter-gatherer culture emerging after a farming culture and continuing its existence together for over a thousand years have obviously created much debate about the origins of the PWC. It has been suggested that the hunter-gatherers were originated from the TRB farming society and took back the old habits of hunting and gathering. [28] Additionally, after coexisting with farmers for approximately 1000 years PWC just disappeared from Scandinavian history, which brings up theories of assimilation and taking up farming habits. However, the osteological and genetic analyses have showed significant differences in between the two cultures. [29, 30] On the other hand, Pitted Ware Culture hunter-gatherers share both cultural and genetic similarities with their contemporary Eastern Baltic region hunter-gatherers. [31]

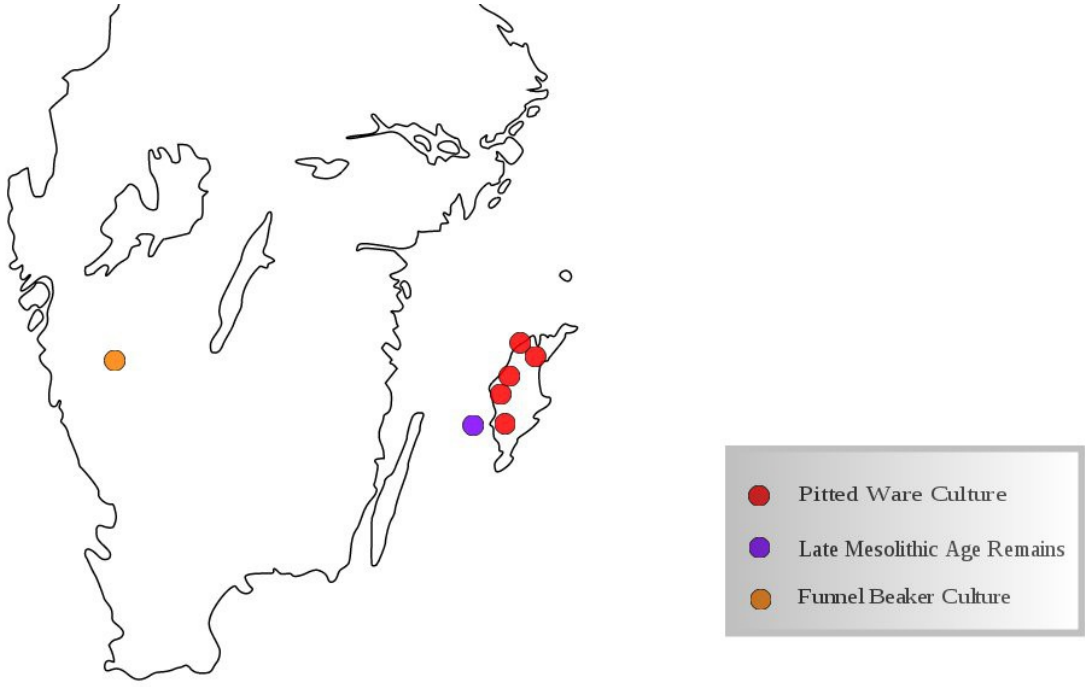

Figure 1: Map of Southern Sweden. Colored markers indicate the locations that the samples are excavated from. (Red: Pitted Ware Culture, Purple: Late Mesolithic Age Remains, Orange: Funnel Beaker Culture)

In our study the majority of the remains belonged to the Pitted Ware Culture (PWC) in Ajvide, Gotland, and dated to 3300-2300 BC. Four of the remains were from the farming society (TRB) in Gökhem, Västra Götaland, dated back to 3100-2900 BC. There were also 2 late Mesolithic samples from Stora

Förvar cave on the island of Stora Karlsö, Gotland, dated to 7500-6000 BC. The reason for focusing on PWC, and also including Mesolithic samples, is because these are the only complexes in the region that has shown morphological indication of cannibalism. The TRB samples were hypothesized to be a population unexposed to cannibalism.

2.6 Working with ancient DNA

There are some obvious peculiarities complicating the work with ancient DNA. First of all, the samples are constantly degrading. The enzymatic repair process of the cells ceased to work when the organism ceased to live and therefore all possible types of degradation will accumulate including damages induced by its own chemical digestion processes and organisms like bacteria, fungi and insects that consumes macromolecules. [32] Deamination is one of the most common outcomes of degradation which is basically caused by hydrolysis in the amino groups of the nucleotides that leads to spontaneous nucleotide loss and/or modifications in DNA sequences. The most common modifications are from C to T and from G to A. By inferring incorrect nucleotides, deamination could lead to serious misinterpretations in sequence divergence and all the results related to it. However, multiple PCR's and cloning before sequencing minimize the risks of misinterpretation.

It is not only the hydrolysis processes that the organisms are exposed to. Furthermore, Background radiation, oxidation of bases and deoxyribose residues will damage the DNA and cause fragmentation. Crosslinking with proteins and DNA, and various processes such as maillard reactions is other alterations that limit the accessibility of ancient DNA for polymerase enzymes. Therefore the amount of base pairs that could be amplified with one primer pair is limited to 50-200 bp. Moreover, if the remains were preserved in a climate warmer than permafrost, due to increasing decomposition rates with temperature, targeting fragments longer than 100 base pairs could decrease the chances of authentic DNA yield. Therefore, when designing primers, it is important to design short and overlapping fragments in order to successfully amplify authentic aDNA. [32, 33]

Last, but not the least, from the death of an organism until the time it is handled in the lab, the remains are constantly exposed to contamination from the outside world. Contamination is a big threat especially when handling human samples due to high levels of similarity and shared haplotypes between the sample and the sample handlers. In order to minimize contamination in the handling process all related procedures should take place in a designated laboratory facility, isolated from all post-PCR work, cleaned regularly with bleach and UV irradiated and accessible by only authorized personnel wearing protective clothes, gloves and face masks. [32] Taking all these precautions might not be enough since the material might be contaminated during excavating, handling, storing etc. However, it is shown that treating the material with diluted bleach before extraction may significantly decrease the amount of extraneous DNA (However also damaging authentic aDNA). [34, 35]

Given all the factors listed above, it should be clear that it is a difficult procedure to retrieve aDNA, particularly from human remains. Even if authentic DNA has survived in the remains for retrieval, it is not easy to distinguish the authentic human remains from its contaminant. However, it has been proposed that by conducting a comparative analysis between the fragmentation levels of the retrieved DNA fragments, it could be possible to separate the authentic aDNA from extraneous, contemporary DNA. Since fragmentations accumulate over time it is expected that the authentic remains would

contain more short fragments than the contemporary DNA. Therefore, using molecular approaches for comparison of the degradation rate, one could identify the authentic DNA in the retrieved fragments. [36, 32, 35]

2.7 Illumina Sequencing

With the innovation of next generation sequencing, whole genome approaches and retrieval of sequences from challenging materials became fairly easy. With the developing technology, it is now possible to get up to billions of megabases in just a few hours. The Illumina platform is one of the next generation sequencing platforms which have an enormous reading capability of two gigabase a day for comparably low cost. It is a synthesis based sequencing where the materials to be sequenced are immobilized on a slide-like flow cell.

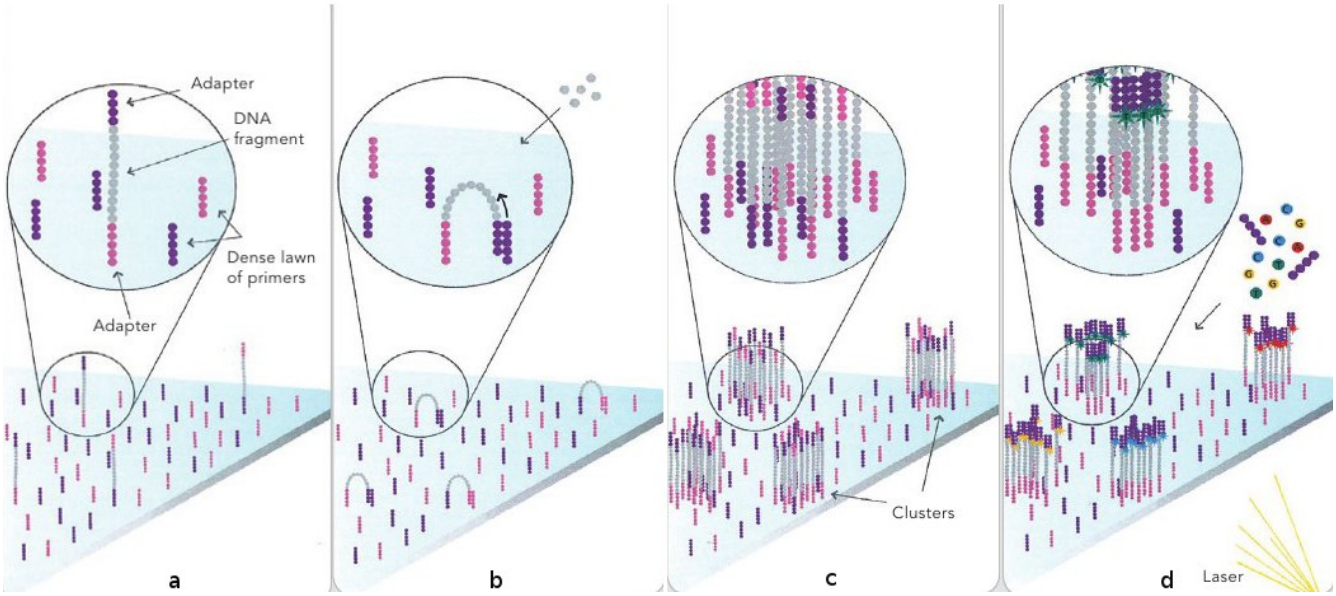

Figure 2: Steps of Illumina sequencing.(a) Denaturated DNA attaches randomly to the slide. (b) The adaptors attached to the DNA fragments finds its comlementary adaptor sequence and creates a replication process. (c) Re-denaturated DNAs form a cluster on the surface of the slide. (d) After the addition of all nucleotides at the same time, a laser detects the light of the incorporated nucleotides (Image modified from Technology Spotlight: Illumina Sequencing brochure, 2008) [37]

The process starts with breaking the double stranded DNA into fragments but when working with aDNA, this step is usually skipped as aDNA fragments are short from the beginning. These fragments are then attached with the designated primers that work as “adaptors” in the next steps. The DNA fragments are then put on to the slide where the adaptors randomly attach themselves to their complementary adaptors that lie on the surface of the slide. (Figure 2.a) This movement creates a bridge like bend (Figure 2.b) that triggers a replication process where a complementary strand is made and replicated by using the same process. After repeated amplifications double stranded DNA is denatured which causes the creation of clusters of the same fragment in random spots on the slide. (Figure 2.c) When the nucleotides and all other necessary reagents (polymerase etc.) are added, a computer monitors the frequencies of light that are radiated from every incorporated nucleotide in

every cluster and therefore prints the sequences of interest. (Figure 2.d)

With the effective amplification process, Illumina platform can sequence billions of reads from a challenging PCR product with low quality. Additionally, the rather short reading frame of Illumina makes it one of the best platforms for ancient DNA sequences.

3. Material & Methods

3.1. DNA extraction from the ancient human remains

DNA was extracted from 18 human samples from various Swedish sites and various time periods where the majority were from the Ajvide site on the Baltic island Gotland. 5 seal samples *(Phoca groenlandica)* also from the Ajvide site, and 16 extraction blanks were added to the batch. (see Table 2)

Prior to extraction, bone samples were UV-irradiated on both sides and the outer layer of the bones were removed with a drill, enabling only interior parts which have not been exposed to modern contaminating DNA to the same extent as the outer parts. Approximately 40-70 milligrams of bone powder was collected from each sample and thereafter treated with diluted bleach. Treating samples with bleach is known to decrease the amount of authentic DNA. However, this treatment also reduces the amount of external contamination to a much higher rate, which makes the odds affordable. The extraction process was performed in a designated ancient DNA laboratory, isolated from all post PCR and modern pre-PCR work areas. In addition, handlers also wore protective paper suits, face masks and two pairs of sterile latex and nitrile gloves to minimize the risk for contamination in extraction process. Yang's extraction protocol was used since it has provided satisfying DNA yield from a variety of archaeological remains. The protocol was modified and Urea was added in the extraction buffer as in Svensson *et al.* 2007. [38] Bone powders were incubated in one ml extraction buffer (900 µl EDTA (0.5M, pH 8) and 100 µl Urea (1M)) with 10µl 100µg/ml proteinase K at 55°C over night. The next day, incubated samples were centrifuged at 2000 rpm for five minutes and the supernatant was transferred to a 15 ml Amicon filter. (Amicon® Ultra-4 Centrifugal Filter Unit, Millipore) They were then centrifuged again for 10-15 minutes in 4000 g. When approximately 100 µl of sample mix was left in the filter, it was transferred to QiaGen spin column with 500 µl of PB buffer and centrifuged for one minute at 13000 rpm and the flowthrough was discarded. When all the PB+DNA mix was passed through the filter, 750 µl of PE buffer was added onto the column and the centrifuge step was repeated until no liquid was left in the spin column. Moving the column in to a clean 1.5 ml eppendorf tube, 50 µl of Elution Buffer was added and incubated for one minute. After centrifuging in 13000 rpm for a minute, the step is repeated and extraction was finished with a total volume of 100 µl of DNA in each sample.

Table 2: Information about the investigated samples

Sample Name	Species	Region	Parish	Era	Additional Information
Vis 17	Human	Visby	Visby parish, Gotland	Pitted Ware Culture	N/A
Jet 1	Human	Jettböle	Jettböle, Åland	Pitted Ware Culture	N/A
Fri 28	Human	Fridtorp	Västerhejde parish, Gotland	Pitted Ware Culture	Female, 40-50 years old
Fri 22	Human	Fridtrop	Västerhejde parish, Gotland	Pitted Ware Culture	N/A
Ire 3	Human	Ire	Hangvar parish, Gotland	Pitted Ware Culture	Female, 25-30 years old
Ire 7	Human	Ire	Hangvar parish, Gotland	Pitted Ware Culture	N/A
Ajv 5	Human	Ajvide	Ajvide parish, Gotland	Pitted Ware Culture	N/A
Ajv 29A	Human	Ajvide	Ajvide parish, Gotland	Pitted Ware Culture	Female, 20-30 years old
Ajv 52A	Human	Ajvide	Ajvide parish, Gotland	Pitted Ware Culture	Child, 7 years old
Ajv 54	Human	Ajvide	Ajvide parish, Gotland	Pitted Ware Culture	Male, 25-35 years old
Ajv 66	Human	Ajvide	Ajvide parish, Gotland	Pitted Ware Culture	N/A
Ste 7	Human	Gökhem	Frälsegården, Västra Götaland	Funnel Beaker Culture	N/A
Gök 4	Human	Gökhem	Frälsegården, Västra Götaland	Funnel Beaker Culture	Female, 20 years old
Gök 5	Human	Gökhem	Frälsegården, Västra Götaland	Funnel Beaker Culture	N/A
Gök 7	Human	Gökhem	Frälsegården, Västra Götaland	Funnel Beaker Culture	N/A
F13	Human	Stora Förvar	Stora Karlsö, Öland	Late Mesolithic Age	N/A
G9	Human	Stora Förvar	Stora Karlsö, Öland	Late Mesolithic Age	N/A
GS Ir 5ab	Seal	Ire	Hangvar parish, Gotland	Pitted Ware Culture	N/A
GsIr1	Seal	Ire	Hangvar parish, Gotland	Pitted Ware Culture	N/A
GsAj5	Seal	Ajvide	Ajvide parish, Gotland	Pitted Ware Culture	N/A
GsFrAj 1	Seal	Ajvide	Ajvide parish, Gotland	Pitted Ware Culture	N/A
GsVi5	Seal	Visby	Visby parish, Gotland	Pitted Ware Culture	N/A
GsVi1ab	Seal	Visby	Visby parish, Gotland	Pitted Ware Culture	N/A

Even though all selected samples had previously yielded high quality mitochondrial DNA data [31] the success rate for amplifying nuclear DNA was largely unknown, although a few of the samples had previously yielded nuclear SNPs. [30] Thus, 18 human samples were extracted and tested for nuclear DNA yield by using the previously optimized primer systems.

Eight out of these 18 yielded nuclear DNA (Vis 17, Fri 28, Ire 3b, Ajv 29A, Ajv 52A, Gök4, Gök7, G9). Although some of the lesser preserved samples were used as well to increase chances of success, the main effort were on these eight samples.

3.2 Designing the primers

14 primate specific primer pairs were designed with short overlapping fragments, for amplifying

approximately 400 base pairs of the PRNP gene by using PSQ Assay & Design Software™.(Biotage, Uppsala) *(designing of the primers were done by Helena Malmström)* (Table 3) As mentioned in the section 1.7, designing short, overlapping fragments increase the chance to amplify the targeted authentic DNA.

Table 3: Primers used in this study

Forward	5`3` Sequence	Reverse	5`3` Sequence	Temp.	Bp.
n0F	ACCCACAGTCAGTGGAACAAG	n0R	AGCTRCTGCAGCACCA	56.8	72
n1F	AAAAACCAACATGAAGCACAT	n1R	AAGGCCCCCCACCACT	59.3	61
n2F	ACATGGCTGGTGCTGCA	n2R	CTGCTCATGGCACTTCCC	57.0	76
n3F	AGTGGTGGGGGGCCTTG	n3R	AGTCACTGCCGAAATGTATGATG	58.0	75
4F	AAGTGCCATGAGCAGGC	4R	CGGTGCATGTTTTCACGA	54.0	75
5F	GCAGTGACTAKGAGGACCG	5R	TCCATGGGCCTGTAGTACACT	52.0	76
6F	AAACATGCACCGTTACCC	6R	TGCACAAAGTTGTTCTGG	52.0	75
7F	TACAGGCCCATGGATGA	7R	GCTGCTTGATTGYGATATTG	52.0	73
8F	CCAGAACAACTTTGTGCA	8R	CTTGGTGGTTGTGGTGAC	48.6	70
9F	ATATCRCAATCAAGCAGC	9R	CATCATCTTAACGTCGGTC	50.3	77
10F	CACCAAGGGGGAGAACT	10R	GGGTGATACACATCTGCTC	52.1	74
11F	CGACGTTAAGATGATGGAGC	11R	GGTAATAGGCCTGAGATTCCC	53.1	77
12F	TTGAGCAGATGTGTATCACC	12R	TGCTCGATCCTCTCTGGTA	54.7	66
13F	AGAGGGAATCTCAGGCC	13R	AGATCAGGAGGATCACAGGT	54.3	78

3.3 Optimizing the primers

3.3.a Testing with modern DNA

For the optimization of the primer pairs, several different approaches were followed. First, the primers were tested with modern DNA. Sampling was made from mouth swaps of 8 different individuals, incubated in 200 µl Lysis Buffer (Gene-Mole, Norway) with 2µl of 2 mg/ml Proteinase K at 55°C for 2 hours. Extraction was further continued on Gene-Mole extraction robot following the manufacturers instructions. Different annealing temperatures and $MgCl_2$ concentrations were tested, and the most effective conditions were further tested on ancient DNA remains.

3.3.b Testing with ancient DNA

Optimization of some of the primers was successful and these primers were then later used for testing the nuclear DNA yield in the samples of interest. However, for problematic primers another approach with ancient DNA samples from different time periods were used. DNA was extracted from a total of 24 human remains; ten Neolithic human remains, (nine from Sweden, one from the Baltic Sea region,

one Early Bronze Age from Portalón, Spain and twelve Viking Age human remains from Norway and twelve extraction blanks. The previously mentioned ancient DNA protocols were used. The samples were extracted at least twice (sometimes up to twelve times). Primary DNA tests were conducted with different sets of primers that are known to work under complicated conditions. Additional to three recently optimized PRNP primer pairs, (PRNP 5, PRNP 6, PRNP 7) an already designed biotynlated primer pair, [39] which is 70 bp long, and is able to amplify both human and cattle mtDNA by targeting an A/T substitution in the 16S rRNA gene was used to check for amount of preserved human DNA. [39] (The substitution occur as A in humans.) With its biotynlated structure, this primer pair was used for pyrosequencing. Water blanks were included in the PCR tests. Test samples were visualized on a GelRed (Biotium) stained 3% Agarose gel and also pyrosequenced (only for 70 bp) to verify the quality of the amplified fragments. Only the ones which showed both *(1)* one band in the desirable length in agarose gel, and *(2)* contained human DNA in pyrosequencing were selected. Out of 24 samples, 14 fulfilled the above mentioned requirements.

The optimization of the 14 primer pairs were carried out on these 14 samples. For optimization of the annealing temperatures, a program with 5-6°C below and above the approximate annealing temperature of each primer was created on a gradient PCR machine. In order to get the precise annealing temperature for each primer, the temperature deltas between every block in the PCR machine was limited to 0.2 to 0.4 °C. Performances under different $MgCl_2$ (1.5 – 2.0 – 2.5) concentrations were also tested as well as the presence and the amount of particular reagents like RSA (Rat Serum Albumin) and SSB (Single Stranded Binding Protein, New England Biolabs). Several conditions were tested individually for each primer. There were no positive results in the extraction and PCR blanks.

3.4 Amplification of the PRNP gene

The complete protein coding region of the PRNP gene was amplified in 14 fragments from 18 Neolithic human samples, each fragment's length ranging from 61 to 78 bp. All samples were amplified in parallel in batches with five seals *(Phoca groenlandica)* , five extraction blanks and four PCR blanks. 3 µl DNA extract was used for a 25 µl reactions using 1x PCR buffer (Smart-Taq 10x PCR buffer, Naxo) 2mM $MgCl_2$ (Smart-Taq $MgCl_2$ 25 mM Solution, Naxo), 0.2 mM dNTP, 300 nM of each primer, 2U of SmartTaq (Naxo) 50U of SSB (Single Stranded Binding Protein, New England Biolabs) and 10 µg/mol of RSA (Invitro) with the thermal cycling conditions of 95°C 15 min and 47 cycles of 94°C 30 sec, 49.9°C to 60°C (annealing temperature for each primer are shown in Table.3) 30sec, 72°C for 30 sec., 72°C for 15 minutes.

The amplicons were visualized on GelRed (Biotium) stained 3% Agarose gel on 150V for 45 minutes. The long runtime on the agarose gel electrophoresis step enabled us to separate the short sized amplicons from primer dimers. The amplified fragments of each sample were then pooled together in an Amicon filter (Amicon® Ultra-4 Centrifugal Filter Unit, Millipore) and purified by centrifuging for 25 minutes, washing with 1 ml of ddH_2O and then centrifuging for 25 more minutes.

Purified PCR products were then quantified using Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE) (with a positive control of contemporary DNA whose results were already calculated) and sequenced on the Illumina platform. Sequencing was performed at Natural History Museum of Denmark by Eske Willerslev's team as described in Skoglund *et al.* [40]

Sequenced data was mapped and aligned to the reference sequence with the accession number DQ408531 using BWA (Burrow-Wheeler Aligner) with default parameters. SAMtools (The Sequence Alignment/Map) [41] were used further for consensus calling with default parameters. The data was authenticated by a comparison of C-T polymorphisms to overall polymorphism rate by randomly selecting 100 000 sequences from each individual. The consensus files were then visualized and edited in Bioedit for further alignment to the reference sequence. DNAsp [42] was used to phase the data, analyze the polymorphic sites and for Tajima's D calculations.

4. Results

We managed to amplify the complete protein coding region of the PRNP gene from nine out of 18 samples. Out of eight thoroughly selected samples six were successfully amplified additional to the three samples from the lesser preserved remains. According to the quantification results, the amount of double stranded DNA in each pooled sample varied between 79.6 and 220.3 ng/µl (Table 4). None of the negative controls, either blank controls, animal controls, extraction and PCR-controls, displayed any human DNA contamination.

Table 4: Quantification results of the successfully retrieved samples

Sample Name	260/280	260/230	ng/µL
Ajv29	1.37	0.52	105.2
Ajv54	1.17	0.41	79.6
Ajv52	1.16	0.37	146.5
Gök7	1.06	0.31	124.3
Gök4	1.44	0.56	86.1
G9	1.00	0.35	220.3
Ire3	1.06	0.34	135.9
Fri28	1.11	0.36	104.4
F13	1.35	0.46	108.04

Post-sequencing, raw data was aligned against the PRNP gene sequence as reference (DQ408531) using BWA with default parameters. BWA is known to be successful at aligning short sequences to long reference files, and SAMtools generates a native format where post alignment processes like indexing, alignment visualizing, variant and consensus calling is made possible. [40] Mapping and aligning enabled us to remove the irrelevant data and the number of sequences dropped drastically after alignment and mapping procedures. (Table 5)

Table 5: A comparison of the number of sequences obtained after sequencing and after mapping

Sample Name	Number of sequences in raw data	Number of sequences after mapping
Ajv29	57 482 508	3 200 616
Ajv54	15 943 115	961 733
Ajv52	13 464 876	787412
Gök7	42 132 523	2 446 209
Gök4	19 298 488	1 118 243
G9	27 253 495	1 573 171
Ire3	22 490 593	1 302 756
Fri28	16 200 798	973 666
F13	43 219 997	2 455 076

The duplicates were not removed and all sequences from different flowcells were merged in a single BAM file per individual. Consensus calling were made using the pileup command of SAMtools and the consensus sequences from each sample reads were further processed as single-end reads.

When the polymorphisms for authentication were compared, all our individuals yielded approximately 2 times more C to T changes than C to A and C to G changes which supports the existence of ancient DNA in our samples (Figure 3). In G9 and Gök4 the overall rate of polymorphism was significantly lower than the rest of the individuals. (0.6%, 0%, 0.1% and 1.3%, 0%, 0.1% respectively) Additionally, positions of C to T changes when compared with the protein sequence of the PRNP gene showed that 50% of the C to T changes were observed in the first or second codon of the amino acids which wouldn't be observed at such a high frequency in a contemporary organism's DNA sequence.

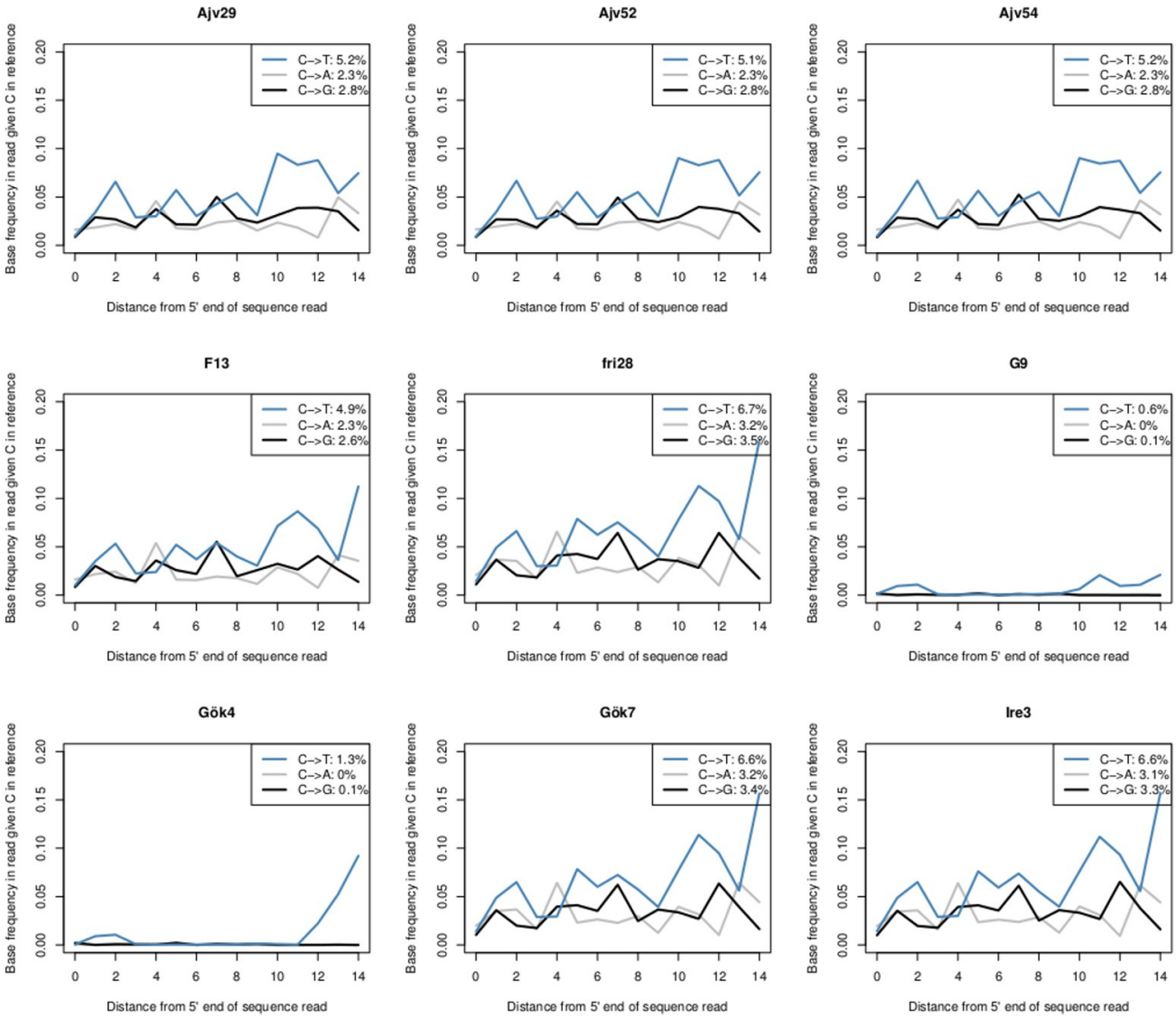

Figure 3: The rates of C substitutions per individual, Overall, C >T changes are twice as common than C>A and C>G changes.

After aligning the nine consensus sequences against the reference sequence by using BioEdit, heterozygosity for codon 129 was observed in two Mesolithic samples (F13 and G9) (Table 6). The remaining samples were homozygotes for the A allele except for sample Ire3 which was a GG homozygote. Additionally all individuals presented a unique C to T polymorphism in codon 169, the first codon position coding for Histidine. All the samples displayed homozygosity for allele T, which codes for Tyrosine, instead the reference allele C. Additional 23 more polymorphisms were observed and will be investigated in the future.

Tajima's D was calculated in DNAsp [42] for each sequence set divided according to their time period. Neolithic hunter-gatherer, farmer and late Mesolithic hunter-gatherers yielded no significant results for Tajima's D (-0.95680, 0.67384, 1.00055 respectively)

*Table 6: Observed polymorphic positions in relation to the reference sequence in PRNP gene coding region. The two important polymorphisms are located on nucleotide 385 for codon 129 and nucleotide 505 for codon 169. (**PWC:** Pitted Ware Culture hunter-gatherers, **Mes:** Mesolithic Age hunter-gatherers, FB: Funnel Beaker Culture farmers, **A**: Adenine, **T**: Thymine, **C**: Cytosine, **G**: Guanine, **R**: Adenine or Guanine, **K**: Guanine or Thymine)*

		279	280	281	282	313	333	358	385	387	388	418	423	426	435	505	532	536	540	547	555	607	618	681	683	696
Era	**Reference**	**T**	**G**	**G**	**C**	**C**	**C**	**G**	**A**	**G**	**C**	**C**	**C**	**C**	**T**	**C**	**G**	**G**	**C**	**A**	**G**	**G**	**G**	**G**	**C**	**G**
PWC	**Ajv29**	.	.	.	.	.	.	.	.	R	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
PWC	**Ajv52**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	R	.	A
PWC	**Ajv54**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
Mes	**F13**	A	T	C	T	.	.	.	R	.	.	.	.	K	.	T	.	.	.	R	.	.	.	.	.	.
PWC	**Fri28**	A	T	C	T	.	.	R	.	.	.	.	.	K	.	T	.	.	.	R	.	.	.	.	.	.
Mes	**G9**	.	.	.	.	T	.	.	R	.	.	.	.	.	.	T	.	.	Y	.	.	.	.	.	.	.
FB	**Gök4**	A	T	C	.	.	Y	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
FB	**Gök7**	A	.	.	Y	.	.	.	.	.	.	.	.	K	.	T	.	.	.	R	.	.	.	.	.	.
PWC	**Ire3**	.	T	C	Y	.	.	.	G	Y	Y	Y	Y	.	.	T	R	R	.	.	R	R	R	.	Y	.

5. Discussion

We had limited bone material, which led to limited DNA extractions. The samples that were not sequenced were already amplified for more than 8 fragments before running out of DNA. So, if scarcity was not a problem it would be probable to have more samples that were sequenced for the desired PRNP region. Targeting short fragments of DNA due to fragmentation and degradation of the human remains makes it challenging to distinguish the primer dimers from the original sequence of interest in agarose gel electrophoresis. However, the long runtime on the agarose gel electrophorase step enabled us to separate the short sized amplicons from primer dimers. The lower results observed in quantification step is another side effect of working with ancient DNA. Generally when working with aDNA, expected quantification results are never as high as in modern DNA due to all problems listed in section 2.6, but our samples fulfilled the amount of DNA that is necessary for further sequencing (Table 4).

There were no contamination in negative controls during the laboratory procedures which was the first indicator of our data being authentic. We tried a further approach for authentication. As previously mentioned, ancient DNA have deaminations due to degradation with C to T changes being the most common. The C to T changes could occur in random positions and naturally would be expected to be more in quantity than all actual polymorphisms one could observe in an individual. Therefore if a

comparison is made for overall C-T polymorphisms to all other changes from C observed in an individual, the outcome would be that C-T polymorphisms are observed much more often than the latter. The results we got is agreeing with the proposed theory, leading us to believe all our samples were authentic ancient DNA. The observed lower rate of polymorphism in G9 and Gök4 could be explained by low quality DNA yield during PCR amplification process which effected the sequencing process by only providing one or two types of molecules that are very much similar to each other. This, we suggest, probably decreased the variation and caused the low levels of polymorphism rates observed in G9 and Gök4.

A C to T polymorphism in nucleotide 505 (codon 169) was observed in all individuals. The reference sequence was histidine homozygote in codon 169 while ancient samples were tyrosine homozygotes. This is a polymorphism that has never been reported or mentioned in any articles before. C to T changes are commonly pronounced as damage in ancient DNA. However, C to T damages occur most likely randomly in different parts of the gene for each organism. On the contrary, in our case all individuals presented the same polymorphism in codon 169 which could suggest this as a unique ancient polymorphism. A comparison with large number of modern human data set is planned to confirm the theory.

Heterozygosity that is observed in F13 and G9 combined with the cut marked skull observed from the same cave could suggest that cannibalism might be practiced in late Mesolithic ages and came to a halt in PWC hunter-gatherers and TRB farmers since none of those individuals were heterozygotes for codon 129. On the contrary, Ire3 was homozygotes for valine, which is reported as the most susceptible type for kuru. [16] If they had a kuru epidemic, that individual could not survive too long. However, assuming these were all healthy individuals is an archaeological paradox since they are all dead.

Tajima's D analysis showed no significant results for balancing selection. Naturally, the number of individuals analyzed were too low and therefore it was not possible to observe any significance in the analysis. However, balancing selection for codon 129 as mentioned in Mead *et al.* might be an overreaction to begin with. There are different ways to explain the outcomes of that study. One way is that heterozygosity in codon 129 actually provides resistance to acquired prion diseases like kuru due to a widespread prehistoric endocannibalism. [43] Another way is that the positive results in Tajima's D is nothing but an outcome of narrowed research focus rather than a sign of endocannibalism throughout the ancient world. [44] Kreitman *et al.* proposes a solid case, suggesting that since Mead *et al.* focused on only one polymorphism instead of looking for the changes and effects of all polymorphisms throughout the whole gene, the outcome of the study was biased. Therefore, expecting a balancing selection on codon 129 even with enough individuals for a significant Tajima's D could have given insignificant results.

Another theory could be that cannibalism never took place in Sweden and the observed indicators of cannibalism found in old settlements and caves were a part of a religious ritual for handling the deceased. Even though the act of cannibalism has been supported with both osteological and biochemical analyses in many of the other sites mentioned above in section 2.5.a [45, 46, 18, 19], a big debate is still going on, either suggesting alternative explanations for damaged pieces or to figure out the motives behind cannibalism. [19, 47] Whether it was famine, hunger or religious beliefs that led our ancestors performing marrow extractions or burning the dead, remain unknown. To sum up,

heterozygosity in codon 129 for two late Mesolithic samples could mean selection in relation to cannibalism. Unfortunately, limited material makes it impossible to draw conclusions based on concrete scientific facts. In the future, a broadened research with more individuals would give a bigger picture of what really happened in the prehistoric Sweden.

Further analysis is planned on mapping the sequences against the entire human genome, phylogenetic reconstruction of the samples, comparison of the polymorphisms with modern data, Hardy-Weinberg equilibrium test, and a re-calculation for Tajima's D test.

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