



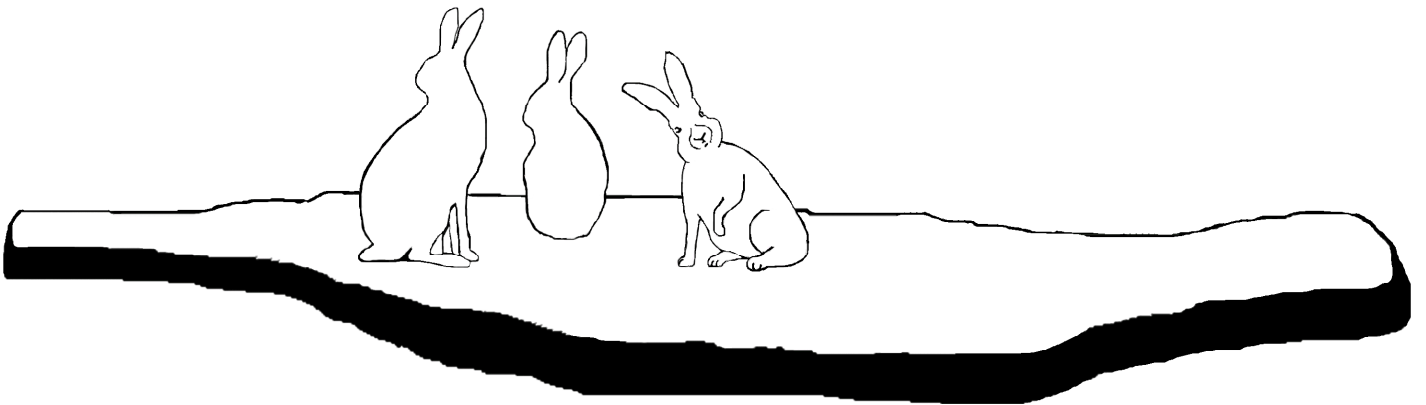
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ON

# THE ORIGIN OF THE MOUNTAIN HARE ON THE ISLAND OF GOTLAND

BY MEANS OF ANCIENT DNA ANALYSIS



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## Abstract

The island of Gotland houses a number of terrestrial mammalian species even though it was covered with ice during the last glacial period. The purpose of this study is to genetically analyse the mountain hare (*Lepus timidus*) to deduce its origin and genetic structure during different time periods, and also to discuss how it reached the island. A 130 base pair sequence of mitochondrial DNA from 38 prehistoric hares was analysed and compared to modern hares from different locations in Europe. The result shows a discrepancy among the samples creating two populations with different origin.

**Keywords:** aDNA, *Lepus timidus*, Gotland, Stora Karlsö, mtDNA, migration, phylogeography

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**Cover illustration:** Mountain hares on their way to Gotland, By H. Ahlgren

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# 1. Introduction

The last glacial period in Europe, the Weichsel, lasted from 110 000 BP until 11500 BP and the ice covered most of Fennoscandia, reaching as far as northern Germany (Liljegren & Lagerås 1993:14pp, Hewitt 2004:184). As the ice slowly retreated northwards, flora and fauna could advance north, possibly via the land bridge that connected Sweden to the continent during some stages (Liljegren & Lagerås 1993:10pp, Hewitt 2004:184). This was a process that progressed for thousands of years (Liljegren & Lagerås 1993:10&26p, Björck 1995:23). It was probably considerably harder to reach Gotland, an island near the middle of the Baltic Sea that has not been linked to the mainland following the glacial period (Liljegren & Lagerås 1993:11, Björck 1995:27p). Although the way in which the island was reached is not elucidated, archaeological evidence points out that humans were already present there sometime between 7500 and 6200 cal. BC (Lindqvist & Possnert 1997:40). The earliest evidence of a terrestrial mammal on the island derives from the mountain hare (*Lepus timidus*). Bones of hares have been found in the lowest and oldest layers, dated to 7420 cal BC, in the cave Stora Förvar on Stora Karlsö, an island about 6 km west of Gotland (Lindqvist & Possnert 1999:78pp).

The number of terrestrial mammalian species on the island is low, but remains from mountain hares have been found at archaeological sites from most parts of Gotland all through prehistory and the island therefore seems to have housed hares since the first colonization. Still, it is not known whether the first mountain hares that were established on the island evidenced in the archaeological materials are the founding population for the later mountain hare populations. There are currently two *Lepus* species on Gotland. In addition to the mountain hare, there is also the brown hare (*Lepus europeaus*), known to have been brought to the island by hunters in the late 19<sup>th</sup> and early 20<sup>th</sup> century and is now the more abundant of the two (Noréhn 1958 D1:116pp, Hedgren 2002:139). As the brown hare and most of the terrestrial fauna now present on Gotland was brought there by people, the question of how and subsequently from where the mountain hare once reached the island still remains.

To answer these questions, a 130 base pair sequence of mitochondrial DNA from skeletal material from 38 mountain hare bones was amplified and sequenced. The bones derive from different prehistoric sites on Gotland, covering a time period from the Mesolithic, 9500 years BP, to the Early Medieval period 800 years BP, and a large spatial area of the island. A phylogenetic tree was set up to show the genetic relationship between the samples and a statistical parsimony network was built that compared the haplotypes among the samples with 67 haplotypes from hares deriving from different places in Europe, to give an indication on their origin. This study can shed new light on the colonization of Gotland, not only by the mountain hare but by other animals as well as people.

## 1.1 Aims and research questions

The time period of concern for this study is the pioneer phase in the history of Gotland, a period that has not received much attention during the last years. The purpose of this study is to get a further understanding of how the terrestrial mammals colonized the island, with an emphasis on the mountain hare, but also the hunter gather population that dwelled on the island at the time. Since the island is geographically isolated from the mainland by at least 80 kilometres of water, the origin of the mountain hare is unknown. Neither is it known if later populations on the island are its descendants. Therefore this study also aims to see if the first mountain hares on the island survived and left a genetic lineage present during later time periods. One additional aim of this study will be to discuss how the mountain hares and other terrestrial mammals managed to reach Gotland. The aim is not to fully answer this question but to discuss the subject, since there today seems to be an accepted view that the mountain hare probably reached the island by crossing the sea ice (Noréhn 1958 D2:581, Lindqvist 1997:71, Lindqvist & Possnert 1999:68). If the mountain hare was introduced to the island by people, the origin of the hares might provide information of the origin and/or contacts of these people. Based on this overview a number of research questions have been posed.

- Where does the prehistoric mountain hares on Gotland originate from?
- Is there a genetic relationship between the prehistoric mountain hares on Gotland and historic populations?
- How did the mountain hare reach Gotland and can knowledge of this say anything about the early people on the island?

## 1.2 Limitation

The spatial limitation for this study is the island of Gotland. By that, there is a natural limitation of the source material due to a limited availability of hare bones from prehistoric sites on the island. Data will only be compared to recent populations of mountain hares from different parts of Northern Europe. In an ideal situation, bones from Gotland would be compared to bones of similar age from archaeological sites from all over Northern Europe, but this is not possible in this study.

The present population of mountain hares on Gotland is not covered in this study due to the fact that the population has been decimated and replaced by translocations from the mainland several times during the last 200 years (Noréhn 1958:103pp). Hence, the current population of mountain hares on Gotland is not suitable for comparison considering the aim of the study.

## 2. Previous research

### 2.1 DNA

All organisms are composed of cells, which are the smallest functional unit that is alive, and it is responsible for both function and structure in an organism. The genetic material in the cell is composed of the polymer nucleic acid, consisting of nucleotides - a monomer made up of a nitrogenous base, a phosphate group and a five-carbon sugar. The phosphate group and the sugar is the backbone of the polymer and the order in which the bases are located is the genetic code. This code can be described as the blueprint for everything that is produced and occur in the cell, how proteins are made and how a character is expressed. The genetic code consists of a combination of the four bases; Adenine (A) and guanine (G) representing the purines, and cytosine (C) and thymine (T) representing the pyrimidines. The nucleobases form base pairs by binding to their partner, where the base A always binds to T and the base G always binds to C, held together by hydrogen bonds. The hereditary material in the cell nucleus is Deoxyribonucleic Acid (DNA); a double stranded polynucleotide of the four nucleobases. Since the nucleobases only bind to its partner, merely one side is needed to know the complementary side. The DNA helix in the nucleus is wound around proteins called histones and this combination is called chromatin. During some of the stages of the cell cycle the chromatin is coiled into chromosomes (Campbell 2008:98pp, 320p). Nucleic acid has two ends that are distinct from each other and because of that, they are said to have a direction, where the beginning is called 5' and the end is called 3'. The two strands in DNA are antiparallel i.e. they run side by side but in different directions (Campbell 2008:88).

The other nucleic acid in an organism is ribonucleic acid (RNA), which is usually single stranded and composed of the same bases as in DNA, with the exception that the pyrimidine base T is replaced by uracil (U) (Campbell 2008:86pp). RNA is involved in the protein synthesis by transcribing the DNA sequence into messenger-RNA (mRNA) that travels from the nucleus to another organelle, the ribosome, where the translation step is performed. The translation is performed by transfer-RNA (tRNA) that brings an attached amino acid to the ribosome. When the tRNA attach to the complementary chain of mRNA in the ribosome, the amino acid attach to the previous amino acid and a polypeptide is built (Campbell 2008:325pp).

#### 2.1.1 Mitochondrial DNA

The mitochondrion is the organelle responsible for most of the energy production in the cell (Campbell 2008:109p). This organelle has its own DNA, a circular molecule considerably shorter than the nuclear DNA molecule. In humans it is comprised of 16569 base pairs distributed on a number of genes, primarily coding for the ATP synthesis in the cell (Avisé *et al.* 1987:493, Freeland 2005:32, Campbell 2008:301p). There are also non-coding regions called control regions, characterized by a high degree of polymorphism (Hummel 2003:20pp). In this study, the mitochondria d-loop control region is used since it is the most variable part (Freeland 2005:33). Mitochondrial DNA is a haploid molecule and it is uniparentally inherited from the mother, which means that they have the same haplotype i.e. a specific gene

sequence. This is because the few mitochondria present in the sperm are located at the tail, which generally do not enter the ovum. If they do, the individual can have several different mitochondria in the cell and this is called heteroplasmy, mtDNA typing of such an individual would show extensive polymorphism (Hummel 2003:20p, Freeland 2005:34). Heteroplasmy can also be caused by mutations, however, both causes of heteroplasmy are unusual (Avisé *et al.* 1987:493) Since mtDNA supposedly do not go through recombination as it is passed down to the offspring, the mtDNA is identical to its mother if no mutation has occurred. This simplifies the trace of a genetic lineage and it can be followed back in time (Avisé *et al.* 1987:493, Hummel 2003:20pp, Freeland 2005:32pp). As a result of being uniparentally inherited and haploid, the effective population size will be small, 25% of the population size compared to when biparentally inherited markers are used (Freeland 2005:33). Hence, identifying events such as migration is easier, but can be a problem in other studies, such as genetic variation in a population, since the result might be biased (*ibid*). In such cases, a complementary analysis of nuclear DNA might be needed.

Mitochondrial DNA has some advantageous characteristics compared to DNA from the nucleus, when used as a genetic marker on low quality DNA such as ancient DNA. One is that there are 1000-10000 copies of the mitochondrion in every eukaryotic cell as compared to the nucleus, which has only one copy per cell (Brown 2001:303, Freeland 2005:32). This increases the chance of finding a preserved sequence in highly degraded samples. Essential for this study is the fact that extensive research has been performed on mtDNA, and this means that sequences from modern samples can easily be gathered from the NCBI genebank for comparison.

## 2.2 Ancient DNA

Studies of DNA from ancient material have been carried out since the beginning of the 1980s. Although the field is still fairly young, the history of aDNA has been eventful, with several scientific breakthroughs but also setbacks that have shaken the entire field.

### 2.2.1 Historic overview

The history of aDNA-analysis began in China in the beginning of the 1980s, when a research group from the Hunan Medical College showed that DNA was preserved in human remains from the Han dynasty (Hummel 2003:1). In 1984, a 229 bp DNA sequence was extracted from a sample of dried muscle from the extinct species Quagga (*Equus quagga*) from a museum specimen (Higuchi *et al.* 1984:282pp). In 1985, Svante Pääbo performed DNA analysis on Egyptian mummies and extracted and cloned fragments of DNA from a 2400 year old mummy (Pääbo 1984:213pp, 1985:644p). In the same year, DNA extracted from mammoth tissue was isolated and compared to DNA from modern elephants (Johnson *et al.* 1985:1045pp). Successful extractions were also performed on plant tissues from seed and embryos during the same year (Rogers *et al.* 1985:69pp). These studies were all groundbreaking work that led the way for future research in aDNA. However, the real breakthrough for the field occurred when Kary Mullis presented the PCR method, allowing small amounts of DNA to be amplified exponentially, creating amounts of DNA large enough to work with for further analysis (Mullis & Fallona 1987:335pp, Hummel 2003:1, Freeland

2005:16pp, Campbell 2008:403pp). In 1989 a breakthrough of special importance for archaeology occurred, when a research group managed to amplify DNA from bone material (Hagelberg *et al.* 1989:485). This opened up opportunities for archaeology since bones are often all that remains from ancient humans and animals. This breakthrough also proved to be applicable in other disciplines. In 1994, skeletal remains of the Russian royal family Romanov were identified using DNA analysis (Gill *et al.* 1994:130pp).

The optimistic spirit of the new research area continued through the 1990s, and in 1994 a research group claimed to have succeeded the extraction of DNA from 80-million year old bones (Woodward *et al.* 1994:4541pp). The following year, researches claimed to have accomplished to retrieve and revive bacterial spores from a bee trapped in amber, 25- 40 million years ago. The DNA in the bacterium was identified and compared to modern bacteria (Cano *et al.* 1995:1060pp). These studies later proved to be either human contamination or impossible to replicate (Austin *et al.* 1995:303pp, Zischler *et al.* 1995:1192p).

### 2.2.2 Applications

During the relatively short era of this research area, aDNA has proved to be applicable to answer a wide range of research questions.

#### **Kinship studies**

As in the example with the Romanov family, aDNA can be used to determine the genetic relationship between buried people, although morphological traits in rare cases can indicate relatedness between people, this method has proven to be fallible (During 1996:33, Brown 2001:308, Hummel 2003:183pp). In archaeology, kinship studies by means of aDNA analysis is used to deduce the relationship between people in a grave field or between people buried in the same grave (Brown 2001:308, Hummel 2003:183pp).

#### **Species identification**

Well preserved bone remains can generally be morphologically identified by an osteologist. However, when bones are highly fragmented or when morphologically similar animals are to be identified, i.e., bones of sheep (*Ovis aries*) and goat (*Capra hircus*) or red fox (*Vulpes vulpes*) and dog (*Canis lupus familiaris*), aDNA analysis can be used (Hummel 2003:165pp).

#### **Sex determination**

When bones are highly fragmented or originate from juveniles prior to puberty, sex determination can be challenging even for a skilled osteologist. In these occasions, DNA analysis might be needed to make a certain determination (Brown 2001:307, Hummel 2003:165pp).

#### **Paleopathology**

Paleopathology has two main applications in archaeology. It can be used to analyse if an individual carries a gene that can be linked to a genetically inherited disease, an application made possible due to the mapping of the human genome through the project HUGO (Human



Genome Project) (Brown 2001:308p, Nuorala 2004). Ancient DNA can also be used to study infectious diseases back in time to see how they have spread around the world and how they have evolved, e.g. diseases like leprosy, tuberculosis, malaria or syphilis (ibid).

### **Population genetics**

In this application, DNA from archaeological or historical sources can be extracted and compared to a modern source material to study population alterations e.g., changes in genetic diversity through time to find population bottlenecks (Brown 2001:309p, Wandeler *et al.* 2007:634). Ancient DNA can also be used to study origin and movement of people and things, a method further described below (Brown 2001:309p).

#### **2.2.3 The use of human remains to trace human movement and origin**

In this research area, aDNA is being regularly used, both to see how people have moved around the world and where they originate from. DNA analyses on human remains to deduce their origin are rather straightforward as compared to using non-human remains to answer the same questions, but it also has its disadvantages. Since the DNA in archaeological bone material is highly degraded, it is more susceptible to contamination than a well preserved, recent material (Hummel 2003:131pp). This means that it can be difficult to prove that the result are authentic and not an artefact of contamination. This can be a severe problem when working with human archaeological material since there are more sources for contamination, see (2.3.2). However, this type of study has been undertaken at several occasions and following are a few examples.

Gilbert *et al.* (2008) showed that DNA extracted from coprolites deriving from a cave in northern America, were evidence of human presence deriving from a period long before when humans previously were believed to have dwelled on that continent. The DNA matched haplogroups from Native Americans (Gilbert *et al.* 2008). In another study, human bone remains have been used to elucidate the origin of an early cultural group in Japan (Adachi *et al.* 2009). Another study analysed the genetic relationship of current Europeans to Paleolithic hunter-gatherers and later farmer populations that settled in Europe 7500 years ago, to see whom their ancestors were (Haak *et al.* 2005). The same kind of study has also been performed on a material from Sweden, where the genetic relationship between the Pitted Ware culture and the Funnel Beaker culture has been analysed and compared to current human populations around the Baltic region (Linderholm *et al.* 2008a).

#### **2.2.4 The use of non-human remains to trace human movement and origin**

Since they first left Africa for around 100 000 years ago, modern humans have moved around the world, colonizing new areas (Boyd & Silk 2009:358p). On their journeys, they brought animals and plants which sometimes can be traced by DNA analysis. The method of using non-human material to trace human migration has gained some interest in recent years since it has several advantages compared to human aDNA. For one thing, animal bones are often more abundant on archaeological sites and are better preserved than human bones (During 1996:24). Another reason why animal material is preferably used is simply because it is easier to work with in terms of contamination. However, the method of tracing human movement by

using non human remains also has some disadvantages. It can be a problem to prove that the animal was actually brought to a place by people and did not get there on its own, especially if the animals are not domesticated.

Although these kinds of studies are mainly performed on species considered to be domesticated, other examples exist. Studies have been done on the colonization of Remote Oceania by the Lapita culture, using DNA from the Pacific rat (*Rattus exulans*), pig (*Sus*) and chicken (*Gallus gallus*) (Matisoo-Smith & Robins 2004, Larson *et al.* 2007b, Storey *et al.* 2010). Other studies have also shown on contact between Polynesia and the Americas, using bone material from chicken but also plants like coconut (*Cocos nucifera* L.) (Storey *et al.* 2007; Baudouin & Lebrun 2009)

In Europe, non-human remains have been used to look for human contacts between the Iberian Peninsula and North Africa by analysing extracted DNA from cattle bones found at archaeological sites (Anderung *et al.* 2005). In another study, the introduction of pigs in Europe was studied to identify routes of introduction (Larson *et al.* 2007a). This method is best suited for geographically isolated areas such as islands, something that can explain its popularity in Polynesia. Gotland is suitable for this kind of study and DNA analyses of the Gotlandic hedgehog (*Erinaceus europaeus*) has been performed to elucidate where it originated from and the result has shown a western origin (Fraser 2006). Since hedgehogs are hibernating during the winter and the distance to the mainland is probably too far for them to swim, they were most likely brought there by people (*ibid.*).

## 2.3 Contamination

In living organisms, DNA has its own repair system that steps in when a mutation has happened or when a transcription has gone wrong but this repair system is lost when an organism dies (Lindahl, T. 1993:709p, Brown 2001:305). As the DNA molecule degrades, it will be reduced both in length and number, which means that modern contamination easily outnumbers the aDNA in a sample, making it more likely that the contamination will be amplified during PCR (Götherström & Lidén 1998:56, Hummel 2003:131, Yang & Watt, 2005:332). Examples where this has happened are when million year old DNA-samples were thought to have been successfully amplified, when in reality it was contamination. It is difficult and most often impossible to know who has been in contact with a bone material originating from an archaeological excavation, in particular if the excavation took place a long time ago. Even though historic contaminations cannot be prevented, the damage can be minimized if a certain procedure and attitude is used throughout the analysis. Follows is a description of the degradation of DNA and the sources of contamination.

### 2.3.1 Degradation and preservation

When the organism dies, the DNA is exposed to degradation caused by the organisms own microorganisms and enzymes (Pääbo *et al.* 2004:646). The effect of environmental conditions for the preservation of DNA in bones has been shown to be of uttermost importance. Key factors for preservation are pH, temperature, humidity, the amount of microorganisms and how the bones have been stored post-excavation (Burger *et al.* 1999:1725p). Ideal conditions

for bones are places with a low and constant temperature, neutral to alkaline pH-value, with no microorganisms and the soil humidity should be low (Burger *et al.* 1999:1725p). When bones are removed from the ground, the environmental conditions drastically change and this can negatively affect the DNA preservation (Pruvost *et al.* 2007:739). Few studies have analysed the subject, but there are indications that storing samples in room temperature over long period of time is destructive for the DNA molecules (Burger *et al.* 1999 1726p, Pruvost *et al.* 2007:739), a fact that might have implications for this study. The degradation of DNA leads to strand breaks on the polynucleotide, making the fragments smaller than contemporary DNA, hence amplification of aDNA sequences of more than 200-300 BP are rarely successful (Hummel 2003:73p&102p). There are also several types of chemical degradation such as hydrolytic damages that can be divided into; depurination which can cause loss of a purine, and deamination when the nucleobase C is converted to a U, read as a T during PCR and subsequently causing a misreading of the sequence (Lindahl, T. 1993:709p, Pääbo *et al.* 2004:646pp). Damage to DNA can also be caused by free radicals that attack the bonds between both the base pairs and the sugar ring and hamper the PCR, an occurrence called oxidation (Lindahl 1993:709p, Pääbo *et al.* 2004:646pp).

### 2.3.2 Contamination sources

There are several categories of contamination and different procedures are used to avoid and detect them. When analyses are carried out on human material from an archaeological excavation, the sources of contamination can be the excavator or the osteologist handling the material during and after excavation and also the researcher performing the work in the laboratory (Götherström & Lidén 1998:56p, Hummel 2003:131pp, Yang & Watt 2005:332, Linderholm *et al.* 2008b:5). Contamination can also originate from the manufacturer of lab consumables (*ibid*). These sources of contamination are less of a risk on studies of faunal material (Hummel 2003:134). Contamination has been showed to come from the soil, both as contamination from other species and from microorganisms that can live in the bone and be more abundant than the aDNA (Götherström & Lidén 1998:56, Yang & Watt 2005:332). Of greater risk is the fact that the bones in the study may be contaminated by a recent animal bone material, used for comparison by an osteologist during identification of the bone (*ibid*). There can also be a cross-contamination between the samples as some of the bones have been kept in the same plastic bags during storage, or between samples in the aDNA-lab. One serious source of contamination is PCR products from earlier PCR runs. In some cases, the chemicals can be contaminated by previous or contemporary researchers working in the lab (Götherström & Lidén 1998:56, Hummel 2003:131pp, Yang & Watt 2005:332).

## 2.4 Post-glacial period

Post-glacial events had an immense affect on the Baltic Sea and the overview below describes its general stages.

### 2.4.1 The history of the Baltic Sea

The stages of the Baltic Sea are complex and difficult to reconstruct since parallel events affected the sea levels on both a global scale (eustasy) and in the Baltic Sea basin at the same time. The global sea level was considerably lower than today since large amounts of water

were locked in the ice sheet (Liljegren & Lagerås 1993:14p&28). As the ice cover melted, land that had been depressed for thousands of years started to rebound by isostatic uplift. The melting ice also caused large areas to be covered in water.

This gradually created the first stage in the post-glacial history of the Baltic Sea, called the Baltic ice lake 16 000–11 600 BP, a freshwater lake above the global sea level (Liljegren & Lagerås 1993:19, E-source 7). No remains from aquatic animals have been found in the sediment from this time (*ibid*). An outflow of the Baltic ice lake was created in Öresund, south of present Sweden causing erosion down to the bedrock. This flow was gradually closed due to the uplift of land (Björck 1995:21f, E-source 7). A region of lowland was uncovered in the middle of Sweden caused by the melting ice, creating a new outflow between the Baltic ice lake and the ocean (*ibid*). This marks the beginning of the next stage called the Yoldia Sea, a stage with brackish water that lasted between 11 600-10 700 BP (E-source 8). The southern coastlines on the Yoldia Sea were lower than the current ones and a land bridge connected Sweden to the continent during some phases during this time (Björck 1995:23). Traces of this are still visible as tree stumps below the present sea surface (Liljegren & Lagerås 1993:25pp). The island of Bornholm was also connected to the continent during this time whereas the islands of Gotland and Öland were not (Liljegren & Lagerås 1993:11, Björck 1995:27p). Remains from seal and fish have been found in the sediment from the Yoldia Sea, in contrast to the sediments of the Baltic ice lake (Liljegren & Lagerås 1993:25pp, Björck 1995:27, E-source 8). At the end of this stage, the land rise in the middle of Sweden gradually closed the inflow of salt water and a stage with fresh water begun - the Ancylus Lake that lasted between 10 700-8 500 BP (Liljegren & Lagerås 1993:27, E-source 9). In its initial phase, the outflow of water from the lake continued in the middle of Sweden, but as the isostatic uplift continued in the north and the water level rose, a new outflow was created in the Great Belt south of Sweden (Liljegren & Lagerås 1993:27, Björck 1995:29p, Schmölcke *et al.* 2006:425, E-source 9).

The Ancylus Lake caused a transgression phase in the south and the traces from this, called the Ancylus wall, can still be seen (*ibid*). As the ice cover gradually melted, the global sea level rose and connected the two basins once more, creating a new stage with salt water, called the Littorina Sea, 8 500-3 000 BP (E-source 10). This stage is characterized by fluctuations of the sea level and traces from the Littorina transgression, the Littorina wall, is still visible around the Baltic Sea (Schmölcke *et al.* 2006:428). The Littorina wall is found at variable altitudes due to variation in the speed of the isostatic uplift, exemplified by the Littorina wall on Gotland; 27 meters above the present sea level in the northern parts of the island and 15 meters above sea level in the southern parts (E-source 10). It has been proposed that the salinity in the Littorina Sea was higher than the current due to a greater inflow of water from the ocean. An estimation of the fauna in the Littorina Sea has been done based on bone remains from coastal settlements and sediments from that time and it is rather similar to the present (Liljegren & Lagerås 1993:35, Schmölcke *et al.* 2006:429p, E-source 10). Although not generally accepted, a fifth stage called the Limnea Sea 3000-500 BP is proposed, characterized by lower salinity than that of previous stages (Liljegren & Lagerås 1993:37pp, E-source 11).

#### 2.4.2 Climatic oscillations

The climate has varied continuously since the last glacial maximum, and based on climate change, the period has been divided into different stages which will be briefly described.

**Bölling period**, 13 000-12 000 BP, during this period, the ice cover in southern Sweden melted and flora and fauna entered the new domain and a steppe landscape formed, the climate is described as temperate/subarctic (Liljegren & Lagerås 1993:19, E-source 17 )

**Older Dryas**, 12 000-11 800 BP, this period is characterized by a cold climate (Liljegren & Lagerås 1993:19pp, E-source 18)

**Alleröd Period**, 11 800-11 000 BP; characterized by a temperate climate. During this phase, deciduous forest advanced in southern Sweden (Liljegren & Lagerås 1993:19pp, E-source 19).

**Younger Dryas**, 11 000-10 000 BP, characterized by a shift to a colder climate, but this gradually changed towards the end of this phase (Liljegren & Lagerås 1993:19pp, E-source 20).

**Pre-boreal Period**, 10 000-9000 BP, much of the flora and fauna retracted far south during the previous phase and during the Preboreal phase, they colonized the land once more. The climate changed to be warmer (e-source 21)

**Boreal period**, 9000-8000 BP, the warm climate continued during this phase but it was fluctuating. The last ice cover melted in northern Sweden (Liljegren & Lagerås 1993:24pp, E-source 22).

**Atlantic period** 8000-5000 BP, this is described as a warmth period where the temperature was 2-4° C warmer than today. The climate is described as maritime with dense forests of heat-demanding trees which spread further north than their current extension (Liljegren & Lagerås 1993:32).

**Sub-boreal period**, 5000-2500 BP, this period is characterized by a substantial climate change towards colder and dryer conditions (Liljegren & Lagerås 1993:37p). The forests that had extended during the last period retracted.

**Sub-Atlantic period**, 2500 BP-present, characterized by fluctuating weather, with a period of colder climate in the beginning of the period (Liljegren & Lagerås 1993:41).

#### 2.4.3 Post-glacial colonization routes

Within the research field intraspecific phylogeography, historic events such as glacial periods or spatial separation that might have affected the distribution of species in the past, are used to explain the current distribution and genetic variation within a species (Avise *et al.* 1987:489pp, Jaarola *et al.* 1999:114, Knowles & Maddison 2002:2623, Freeland 2005:155pp). Fennoscandia was covered with ice during the last glacial period and this makes it a unique place to study phylogeography. Three different ways of colonization have been proposed for terrestrial mammals in Fennoscandia. Colonization from the south by the land bridge that was present for some periods, colonization from the north-east through Finland and southwards, and colonization using both routes (Fedorov *et al.* 1996:557pp, Jaarola *et al.* 1999:117pp). The area where species using the different routes meet, is called a suture zone, and this zone is similar for many Scandinavian species i.e. it is located at somewhat the same

location in northern Sweden, making a similar history of colonization plausible (Jaarola *et al.* 1999:121pp, Hewitt 2004:184). The northern and southern routes have been used by species deriving from different refugia where they dwelled during the last Ice Age, and the difference in origin is visible as intraspecific genetic differentiation (Jaarola *et al.* 1999:118pp). For European species, refugia have been proposed in the Iberian peninsula, Italy, Balkan and in the Caucasus region (Hewitt 2004:184p). The area between the ice sheet and the refugia in the south was a tundra landscape with permafrost (Hewitt 1999:89pp). If the rate of colonization was rapid, a loss of genetic diversity is expected due to repeated founder effects from the refugia, however, if colonization processed slowly, this loss would not have the same effect (Hewitt 1999:91p, Hewitt 2004:184p).

## 2.5 The island of Gotland

Gotland is an island in the middle of the Baltic Sea that was completely covered in ice during the last glacial period, but at around 12 000 years BP the ice cover had retracted (Björck 1995:23). The distance to the mainland is, 80 km to Sweden 150 km to the Baltic countries, and c. 230 km to the continent in the south (Fig. 1) (Lindqvist & Possnert 1999:65).



Figure 1. The Baltic Sea with Gotland marked revised from (Björck 1995).

The shortest distance to reach Gotland from the mainland today is via the island of Öland, east of the Swedish mainland and then to the island of Stora Karlsö 6 km from the main island, a route of 50 km (fig. 2) (Österholm 1997:161pp). Gotland has calcareous bedrock (E-source 16), which is good for the preservation of skeletal remains (Burger *et al.* 1999:1726). The island has been populated since the Mesolithic and onwards and its position in the middle of

the Baltic Sea has given it particular importance. Following is an overview of the islands post-glacial history.

### 2.5.1 Human colonization

The first people in Scandinavia were hunter gatherers that gradually colonized the new land where the ice had retreated (Larsson 1990:275pp, Eriksen 2002:35pp, Riede ms.). Their food utilisation varies between different sites, but deer hunting and fishing were important sources of food (Larsson 1990:290, Riede ms.). Sources of plant-foods were used but remains are usually seldom found in the archaeological material other than nutshells (Larsson 1990:292p).

Traces from the first settlers on Gotland found in the cave Stora Förvar on Stora Karlsö have been radiocarbon dated to between 7500 and 6200 cal. BC (Lindqvist & Possnert 1997:40). The remains from animals in the earliest layers from Stora Förvar give an idea of the fauna on the island at the time when people settled and also for food preferences. Marine mammals such as grey and ringed seal were predominately hunted, but also animals such as birds, fish and mountain hare. Stora Förvar cave is described in more detail under 3.1. There are a number of Mesolithic sites on the island of which three are covered in this study.



Figure 2. The shortest distance to the mainland, view from Stora Karlsö heading west. Photograph by H. Ahlgren

In an archaeological experiment, the shortest distance from Gotland to the mainland, i.e. via Öland, was travelled in a dugout canoe, supposedly similar to what the first colonizers on Gotland used. The trip between Stora Karlsö and Öland (fig. 2) took about 13 hours to paddle (Österholm 1997:161pp), and the experiment also made it clear that no land was visible from the canoe for a couple of hours out on open sea (Österholm 1997:169). As seen in figures 3 a & b, Cumulus clouds can form above islands and these hovering cloud formations can be



visible from a long distance, and this could possibly have helped the first pioneers on Gotland to find the island, long before the island itself was visible.



Figures 3 a, b. An example of clouds that have formed above the islands of Stora Karlsö (left) and Lilla Karlsö (right). Photographs by H. Ahlgren

### 2.5.2 The fauna of Gotland

Much of what is known about the prehistoric terrestrial mammals on the island derives from animal remains found at archaeological sites and bogs. Most spectacular are the remains found in the cave of Stora Förvar, with continuous layers from the first colonization until the archaeological excavation at the end of the 19<sup>th</sup> century, only with an interruption encompassing 2000 years during the Littorina transgression (Lindqvist & Possnert 1999:80). Although the archaeological material can contribute to invaluable information that otherwise would be lost, it is not infallible. Archaeological bones do not necessarily represent the fauna at a site (During 1996:93). Bones from certain species may be lacking due to various excavation methods used by archaeologists. It is also problematic to use a few animal remains to deduce whether Gotland actually housed a living population of a certain species at a site. The animal might just as well have been dead on arrival, brought as food or been used for artefacts. They may even have floated ashore as has happened in historic time (Noréhn 1958 D1:45, Liljegren & Lagerås 1993:4pp). A review of the prehistoric mammalian fauna on Gotland is difficult, since archaeological bone materials discussed in the literature sometimes have been lost and in other cases one has to rely on hearsay. The number of species of the current terrestrial mammalian fauna on Gotland is low, consisting only of:

- **Mountain hare**, earliest dating on the island is from 7420 BC (Lindqvist & Possnert 1997:79). Skeletal remains have been found from all archaeological periods, with a decline after the Mesolithic period (Lindqvist & Possnert 1997:40). It was heavily decimated due to diseases in the beginning of the 2000<sup>th</sup> century, saved only by translocations from the mainland (Noréhn 1958 D1:103p, Lindqvist & Possnert 1999:79). They are not very abundant today according to game bags (E-source 4).
- **Red fox**, skeletal remains from the red fox have been found on Gotland at sites dating to the Mesolithic and onwards (Lindqvist & Possnert 1997:43f, e-source 4). The earliest radiocarbon dating of the red fox is 5500 cal BC (ibid).
- **Yellow-necked Mouse** (*Apodemus flavicollis*), has been found in Mesolithic and Neolithic contexts (Lindqvist & Possnert 1997:44 & 52).



- **Hedgehog**, this species is found in the archaeological material from the Neolithic and onwards. Since hedgehogs hibernate and therefore could not have walked across the ice, this species has been proposed to be brought to Gotland by humans (Noréhn 1958 D1:107, Lindqvist & Possnert 1997:69). DNA-analyses performed on skeletal remains from hedgehogs conclude that it has deduced from a western subspecies (Fraser 2006:19).
- **Squirrel** (*Sciurus vulgaris*), was important during the medieval trade (Noréhn 1958 D1:102). A paragraph that regulates the hunting on squirrels is written in Gotalagen, a law book concerning Gotland from the 14<sup>th</sup> century (Wessén & Holmbäck 1943:102p Noréhn 1958 D1:102).
- **Brown hare** was introduced in the early 20<sup>th</sup> century and is currently the more abundant of the two species of hare on the island (Noréhn 1958 D1:116pp, Hedgren 2002:139).
- **Rabbit** (*Oryctolagus cuniculus*), was brought to Gotland in 1907 and the population grew rapidly until a project to control the population was launched (Noréhn 1958 D1:113pp).
- **Mink** (*Mustela vison*), the population of mink on the island are descendants of farmed minks that became feral in the 20<sup>th</sup> and 21<sup>th</sup> century (Noréhn 1958:D1:119p Noréhn 1958 D2:569pp, Hedgren 2002:139).
- **Roe deer** (*Capreolus capreolus*), brought to Gotland in the middle of the 19<sup>th</sup> century and repeatedly on several occasions since (Hedgren 2002:139). One bone from the Mesolithic site Gisslause has been interpreted as a bone from roe deer. However, this bone is nowhere to be found and was in very poor condition according to the excavator, so no big conclusions should be drawn from this find (Hansson & Munthe 1930:269, Noréhn 1958 D1:46&78p, Lindqvist & Possnert 1997:69).
- **Brown rat** (*Rattus norvegicus*), probably brought to the island in the beginning of the 1900<sup>th</sup> century (Noréhn 1958:D1:99).
- **Bat** (*Chiroptera*), 11 species of bats are present on the island (Hedgren 2002:138). Skeletal remains from bat have been found in archaeological contexts, but the dating is uncertain (Lindqvist & Possnert 1997:72&76).
- Present in the current fauna is also the **Wood mouse** *Apodemus sylvaticus* and the **House mouse** (*Mus musculus*) (Hedgren 2002:138).

### Bones or antlers found from animals no longer present on the island

- **Elk**, (*Alces alces*) Antlers and skeletal remains from elk have been found on Neolithic, as well as Bronze Age/Iron Age sites on Gotland (Noréhn 1958 D1:44pp, Sten 2004:90p). Despite these finds, it is not considered that a population of elk has ever lived on the island. This is mainly based on the small amounts of finds and that the elk is missing on Mesolithic sites (Lindqvist & Possnert 1997:69). A bog find of several bone elements from elk has been found in Mällingsmyr on Gotland, and this has been interpreted as an elk that had crossed the ice and drowned in the bog. It has been

radiocarbon dated to  $3735 \pm 80$  BP (Noréhn 1958 D1:45, Lindqvist. & Possnert 1997:65&69).

- **Red deer**, (*Cervus elaphus*), antlers from the red deer have been found in a grave from the Mesolithic site Stora Bjärs, radiocarbon dated to 5700 cal BC (Lindqvist & Possnert 1997:69). One find of an artefact, possibly made from red deer antler was found in Stora Förvar (Lindqvist & Possnert 1997:69). There are also finds from red deer on some Neolithic and Medieval sites, but in spite of this, they are not considered to have been a part of the natural fauna on Gotland in prehistory (Noréhn 1958 D1:43p, Lindqvist & Possnert 1997:69).
- **Wild boar**, (*Sus scrofa*). The Question whether this animal has been present on the island or not has been widely debated due to its similarity to domesticated wild boars and has never conclusively been answered (Rowley-Conwy & Storå 1997:124p, Lindqvist & Possnert 1997:64p). Bones are abundant from Neolithic sites on the island and have also been found at Stora Förvar, dated to 3350 cal BC, which is the earliest dating for this species (Lindqvist & Possnert 1997:64p).

### 2.5.3 The mountain hare

The mountain hare (Fig 4) is a circumpolar species, existing primarily in countries of Northern latitudes, from the British islands eastwards to Japan (Kurtén 1968:230p, Angerbjörn & Flux 1995:3). It is divided into several subspecies based on geographical distribution and distinct morphology with two subspecies in Sweden: (*Lepus timidus timidus*) in the north above 59°N and (*Lepus timidus sylvaticus*) south of this limit, with an intervening hybrid zone (Bergengren 1969:427&444p, Angerbjörn & Flux 1995:1pp). Fossil records from archaeological sites in Europe confirm that mountain hares were present from the Pyrenees to Hungary during the Pleistocene (Kurtén 1968:230p), and relict populations still exist in the Alps (*Lepus t. varronis*), Scotland (*Lepus t. scoticus*), and Ireland (*Lepus t. hibernicus*) (Bergengren 1969:449p, Angerbjörn & Flux 1995:1pp). The populations in Ireland and Scotland are genetically divergent and different routes of colonization have been proposed as underlying mechanisms (Hamill *et al.* 2006:363). Skeletal remains from Ireland has been dated to 24000-20000 BP, showing that the mountain hare was present there during the glacial period but if the population survived until today is not known (Hamill *et al.* 2006:356 & 363). Compared with the Irish and Scottish populations, the mountain hare population in Fennoscandia have a high genetic diversity (Thulin *et al.* 2003:49p, Hamill *et al.* 2006:363) and the reason for this has been proposed to be due to bidirectional colonization (Thulin *et al.* 2003:49p). Another explanation of the high genetic diversity is that the colonization progressed slowly, without leading edge colonization (Hamill *et al.* 2006:363).

The mountain hare is primarily active at night and live in mixed forests but also in habitats such as tundra. Preferred foodstuff is somewhat determined by season and availability and include twigs, grasses, leaves, moss, bark and lichens (Kurtén 1968: 230, Angerbjörn & Flux 1995:5). The mountain hare itself is preyed upon by species such as the red fox, wolf, wolverine (*Gulo gulo*), lynx (*lynx lynx*) mink and several birds of prey (Angerbjörn & Flux 1995:6). The home range for mountain hare vary depending on geographical location but

mean home ranges of 116 hectares for females and 280 hectares for males have been recorded (Angerbjörn & Flux 1995:6). They generally stay within their home range, although dispersal, primarily by males, has been recorded during mating season (Dahl & Willebrand 2005:313p). Dispersal up to 200 km have been recorded (Angerbjörn & Flux 1995:6), while other studies indicate that they generally disperse no longer than 5 km (Dahl & Willebrand 2005:314p).

Today, the distribution of mountain hares in Sweden has retracted northwards, possibly due to interspecific interaction with the brown hare (Thulin & Tegelström 2002:302p, Thulin 2003:34pp), which is considerably larger than the mountain hare (Kurtén 1968:230, Angerbjörn & Flux 1995:1, Thulin 2003:32pp). Interspecific introgression has been observed between the two species of hares, and the hybridization is unidirectional i.e. that mountain hare mtDNA is present in the brown hare, but not the other way around (Thulin & Tegelström 2002:302p, Thulin 2003:34pp). The mountain hare male is outcompeted by the brown hare male during mating causing the mountain hare female to mate with the brown hare males, producing fertile hybrids (ibid).

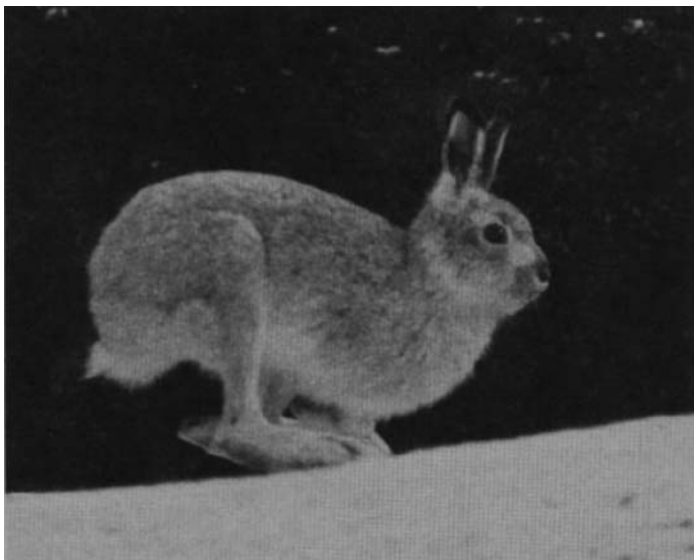


Figure 4. The mountain hare. Photograph by A. Angerbjörn (Angerbjörn & Flux 1995:1)

### **The mountain hare and the island of Gotland**

Both the mountain hare and the brown hare live on Gotland today. The latter was introduced to the island in the beginning of the 20<sup>th</sup> century and it is now the numerous of the two, a fact that can be seen on the game bags of hares shot on Gotland during the last 5 years (Virgin, Pers comm., E-source 4). The mountain hare is, beside humans, the earliest terrestrial mammal present on archaeological sites on Gotland from the Mesolithic and predating the second terrestrial mammal to appear on the island, the red fox, by almost 2000 years (Lindqvist & Possnert 1997:43p). An interesting fact is that the mountain hare and the squirrel are the only animals on Gotland of which hunting restrictions are stated in Gotalagen, dated to the Medieval Period (Wessén & Holmbäck 1943:102p). This might illustrate their importance as well as indicate the lack of other hunt worthy species on the island during the time. Remains from the mountain hare are less frequently found in archaeological contexts after

5900 cal BC, i.e. 400 years after the earliest radiocarbon dating of the red fox 5500 cal BC (Lindqvist & Possnert 1997:40).

Outbreaks of worm infestations in liver and lungs heavily reduced the population of mountain hares during the beginning of the 20<sup>th</sup> century (Noréhn 1958:103pp). Despite translocations from the mainland, the population has not recovered since then, possibly due to interspecific competition with the larger brown hare (Noréhn 1958:103pp). Because of the small mountain hare population on Gotland and because of extensive translocation, the samples in this study will not be compared to the present population of mountain hares.



Figure 5 a, b. Hare-shaped fibula found in Bjärs, Hejnum parish on Gotland, dated to the Roman Iron Age (Hildebrand 1903:151, E-source 13).

Bones, primary hind feet, from mountain hare are frequently found in graves from the middle Neolithic on Gotland (Ahlström 2009:91pp), three samples in this study derive from a Neolithic double grave, Västerbjers (67:2) (Gejvall 1974:154, Ahlström 2009:91pp). The hare foot has a symbolic meaning; it was used as a lucky charm in Anglo-Saxon folklore (ibid). This is a modern meaning and should not be applied to the Prehistoric. Although the symbolic meaning of hare feet in graves cannot be elucidated, it can show that the hare has had some particular importance. An example of the meaning of the hare can also be exemplified by a hare shaped fibula found on Gotland fig 5 a, b.

#### 2.5.4 How the terrestrial mammalian fauna reached the island

As mentioned above, the terrestrial mammalian fauna on Gotland is scarce and the reason for this is because of its short history and because Gotland is an island. This topic has been discussed in several studies prior to this, and theories on how animals reached the island are as follows:

##### **1. They walked on ice, swam or float ashore on a log or an ice floe**

This theory has been proposed for several terrestrial mammals on the island, except rats and voles that might have been brought by humans by accident, and hedgehogs that hibernate and thus can not use the ice (Noréhn 1958 D:2:578p, Lindqvist & Possnert 1997:40). There are recent finds of animal carcasses that have floated ashore on Gotland, both from roe deer and

elk. They have been interpreted as animals that have drowned while crossing thin ice (Noréhn 1958 D1:46&79, Noréhn 1958 D2:579).

## **2 They walked on a connecting land bridge or archipelago from the mainland**

This suggestion is based on the theory that Gotland has been connected to the mainland (Österholm 1989:25). This would have been during the Ancylus Lake, prior to the first human expansion to the island (Noréhn 1958 D2:579). The theory of a land bridge is based on the fact that there is an underwater plateau south of Gotland that connects to the mainland south of Öland, the current dept is 20-40 meters (Noréhn 1958 D2:579).

## **3. They were brought there by people**

Most of the current terrestrial mammalian fauna on the island have been introduced during the last two centuries (Noréhn 1958 D2:569pp). This way of transportation could have been deliberate, like the introduction of the animals during recent time or with the domesticated animals (Noréhn 1958 D:579, Lindqvist 1997:71). The transportation might also be done without the awareness of the people in the boat as is the case with the brown rat.

In a similar study, the origin and introduction of the mammals to Ireland were analysed using mitochondrial DNA from modern samples (Edwards & Bradley 2009). Ireland has a similar situation to Gotland regarding how animals are believed to have reached the island, with the exception that Ireland probably was a refugia during the last glacial period (Edwards & Bradley 2009:215).

### **2.5.5 Prehistoric introductions of wild animals**

It is safe to say that people have globally affected the current dispersal of wild animals through introductions. Somewhat more controversial is the proposal that these kinds of introductions were also carried out in Prehistory. There are several examples where wild animals are believed to have been introduced to remote locations that can not have been colonized by the animal itself. Cyprus has not been connected to the mainland for 5 million years (Marra 2005:10). The island has housed populations of dwarf elephants (*Elephas cypriotes*), and dwarf hippos (*Phanourios minutus*), now extinct (Croft 2002:172, Marra 2005:10, Blondel 2008:511). The reason for this extinction has been proposed to be due to humans (Croft 2002:172). These people are not believed to have settled on the island until about 10 000 years BP (Croft 2002:172). This time they brought animals, sheep (*Ovis orientalis ophion*), pig (*Sus*), goats (*Capra aegagrus*) and also the Persian fallow deer (*Dama mesopotamica*), the latter is found in archaeological contexts covering a period of 6000 years (Guilaine *et al.* 2000:76, Croft 2002:174pp). Since fallow deer are not believed to have been domesticated, it was probably introduced to Cyprus as wild game (Croft 2002:174pp). Another example is the introduction of the brown hare on the British Isles (Thulin 2003:33). It has been proposed to have been introduced by the Romans during their colonization, but skeletal remains have shown that the brown hare was present on the island almost 2000 years prior to their arrival (ibid).

### 3. Skeletal remains

The source material for this study consists of bones from mountain hares from a number of prehistoric sites on the island and it was chosen because of the spatial and temporal diversity that it represents.

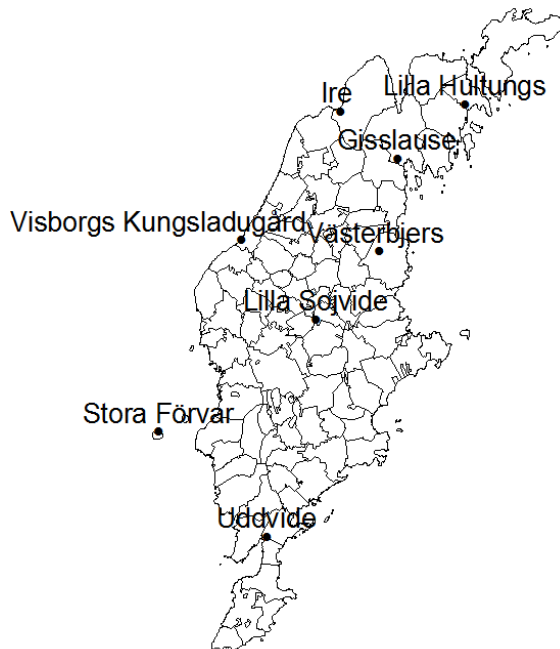


Figure 6. Gotland and the archaeological sites where the prehistoric samples derive from. © Lantmäteriet Gävle 2011. Medgivande I 2011/0094

The greater part of the bone material in the study has been stored at the Museum of National Antiquities since the excavation took place, parts of the material for as long as 120 years. Nowadays, bone materials are stored at facilities with regulations on the indoor climate with reference to humidity and temperature, although this has not always been the case. This means that most of the bones used for this study have been stored in what would today not be considered a suitable environment. The implications for DNA preservation in bones during long term storage is not entirely elucidated and further research is needed to clarify whether DNA can survive truly long term storage. However, two recently excavated materials from Gotland have also been included in the study. One specimen originates from an archaeological site in Lilla Hultungs, Bunge parish on northern Gotland – excavated during the summer of 2009 by Dan Carlsson. The other specimen is from Gisslause, in Lärbro parish, was excavated by Jan Apel during the summer of 2010.

#### 3.1 Excavation sites

##### **Stora Förvar**

The Stora Förvar cave is situated on Stora Karlsö, an island 6 km west of the coast of Gotland. The material used for this thesis originates from excavations performed during 1888-1893 (Schnittger & Rydh 1940:5, Lindqvist & Possnert 1999:65). The culture layer had a varying dept, up to 4.5 meters, excavated in layers of 3 dm and comprised of material from the Mesolithic to recent time. The cave is sloping upwards making the chronology of the

layers difficult and not directly comparative to each other (Schnittger & Rydh 1940:61). Furthermore, it is not known how the cave was used. Was it filled from the inner and outwards or was the whole cave used continuously? There also seems to be disturbance in the layer composition and possibly even some mix up of the finds that have been placed in the wrong context (Schnittger & Rydh 1940:61pp, Lindqvist & Possnert 1997:70). The complexity of the layer composition emphasizes the importance of radiocarbon dating the samples and this will be done on a later occasion. From this site, seven bones were sampled of which six derive from a Mesolithic context and one from an Iron Age context (Lindqvist & Possnert 1997:67). Two samples were selected using MNI (see 4.1) and the other derives from different layers and parcels.

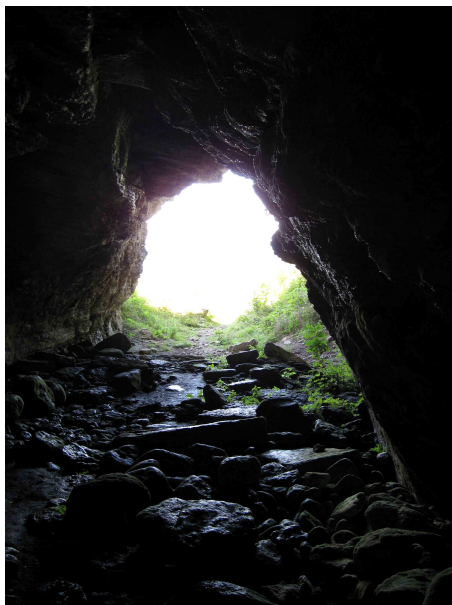


Figure 7. View from the inside of Stora Förvar Cave looking out. Photograph by H. Ahlgren

### **Visborgs Kungsladugård**

This is one of the Mesolithic sites on Gotland, Visby parish, excavated in 1907. A considerably large amount of bones from hare were found on this site, 103 bones are mentioned in the excavation report and six samples were selected using MNI (SHM13326 E-source 14).

### **Gisslause**

This archaeological site in Lärbro parish was excavated the first time in 1929. The cultural layer was located between 0.8-1.5 metres below a layer of gravel from the Littorina transgression and constituted of sand, ash and charcoal (Hansson & Munthe 1930). Radiocarbon dating from charcoal from a hearth on the site gave the value  $7265 \pm 145$  BP (Österholm 1989:55), and that gives a calibrated date between 6433-5881 calBC (OxCal 4,1,7, IntCal 09, 2011-05-11). There is also a date from an unspecified animal bone,  $7865 \pm 100$  BP Apel & Vala 2011, calibrated to 7043-6557 calBC (OxCal 4,1,7, IntCal 09, 2011-05-11). The layers were excavated in dept of 5 cm and every parcel of 1 square meter was divided in 4 parts 50\*50 cm and named a-d. From the 1929 excavation one sample was included. The rest derive from an excavation performed by senior lecturer Jan Apel in 2010.

This excavation was adapted to prepare the bone materials for further DNA-analysis, mainly due to the fact that human remains had previously been found on this site. Gloves were used at all time, and in case any human remains would be discovered; a full suit and facemask were available. Water sieves were used so small bones could be retrieved. To pour water on recently excavated bones is not ideal for the long time preservation of bone and especially not if they are intended for DNA analysis, but they would not have been found otherwise. From this excavation, ten bones and teeth from different squares and layers are included.

### **Västerbjers**

This Neolithic site, located in Gothem parish on the eastern side of Gotland has been excavated on several occasions during the last century. The material used in this study was excavated in the mid 1930s (Janzon 1974:7 SHM 21234). The bones from this site, three using MNI, are of special interest since they are all part of a collection of hind feet bones that had been placed in a grave (67:2) (Gejvall 1974:154). In the grave, a man and a boy were buried and collagen extracted from one of the humans was dated to 4250  $\pm$ 50 BP (Gejvall 1974:154, Eriksson 2004:150).

### **Ire**

This Neolithic site is located in Hangvar parish in northwestern Gotland. The two bones used in this study derive from different excavations, S28 was excavated in the 1950s and S31 was excavated in 1976. Their spatial distance on the site was c. 35 meters (Janzon 1974:263, SHM 31118, SHM 32442).

### **Lilla Sojvide**

The bones from this Iron Age site (n=2) using MNI, were found while excavating a grave from the large cemetery Lilla Sojvide in Sjunhem parish. It was a cremation grave in a cist, covered by a grave mound built of stone and soil. The animal bones were not cremated. The grave was excavated in 1932 (SHM 20147, E-source 1).

### **Uddvide**

The bones from this site (n=2 using MNI) derive from an excavation at the Iron Age cemetery Uddvide 1:20 in Grötlingbo parish, excavated in 1983 (SHM 34667, E-source 15).

### **Lilla Hultungs**

The samples from this site in Bunge parish, northern Gotland, were found in 2009 during an excavation of a house deriving from the Early Medieval Period. Five bones were selected using MNI, the dating are somewhat unclear but range within Iron Age and the Early Medieval Period (Hongslo Vala Pers. Comm. 2010-09-30)



Table 1. Complete list of samples; S. stands for sample, invnr. = inventory number. Aprox. Dat. = approximate dating, I.A. = Iron Age, M.P. is Medieval period, Mes. = Mesolithic period, Neo. = Neolithic period. L. mm = length in millimetre, W. mg = weight in milligram. Samples 1-15 do not have inventory numbers. For s1-s5, l = layer, s = shaft, the numbers are X and Y coordinates. For s6-s15, s = layer (stick), the letter means area in the shaft, and the numbers are X and Y coordinates.

S.	Invnr.	ID	Parish, Site	Aprox. Dat.	Type, Part, Side	L. mm	W. mg	Powder, mg
S1		l2s5 16 23	Bunge, Lilla Hultungs	I.A./M.P.	radius, prox, dex	70,8	1842	<100
S2		l3s5 19 20	Bunge, Lilla Hultungs	I.A./M.P.	radius, prox, dex	40,3	856,37	<100
S3		l2s3 17 17	Bunge, Lilla Hultungs	I.A./M.P.	radius, prox, dex	34,7	1034	<100
S4		l2s2 17 27	Bunge, Lilla Hultungs	I.A./M.P.	radius, prox, dex	14,3	177,5	<100
S5		l2s5 16 22	Bunge, Lilla Hultungs	I.A./M.P.	radius, prox, dex	35	921,55	<100
S6		s1a103 199	Lärbro, Gisslause	Mes.	pm/m, maxilla	15,4	214,07	<100
S7		s1a101 199	Lärbro, Gisslause	Mes.	mt 3, dex	25,1	285,31	<100
S8		s2c103 199	Lärbro, Gisslause	Mes.	mt, dist	22	200,54	<100
S9		s3a100 200	Lärbro, Gisslause	Mes.	phalang 1	21,9	274,97	<100
S10		s4b100 201	Lärbro, Gisslause	Mes.	mt, prox, sin	19,8	208,77	<100
S11		s5c103 199	Lärbro, Gisslause	Mes.	incisiv, mandibula	24,6	290,68	126,2
S12		s6c103 199	Lärbro, Gisslause	Mes.	pm/m, maxilla	9,7	130,56	124,6
S13		s6c100 200	Lärbro, Gisslause	Mes.	pm/m, mandibula	12,7	179,07	130,9
S14		s7b103 199	Lärbro, Gisslause	Mes.	incisiv, maxilla	17,4	151,37	130,9
S15		s7c100 200	Lärbro, Gisslause	Mes.	phalang 2	11	82,13	<100
S16	19178	524584	Lärbro, Gisslause	Mes.	tibia, dist, dex	23,6	694,96	115,7
S17	34667	581011	Grötlingbo Uddvide	I.A.	tibia, dex	41,4	1318,19	120,4
S18	34667	581011	Grötlingbo Uddvide	I.A.	tibia, dex	45,5	1623,96	113,5
S19	20147	218662	Sjonhem Lilla Sojvide	I.A.	calcaneus, dex	35	1670,92	119
S20	20147	218662	Sjonhem Lilla Sojvide	I.A.	calcaneus, dex	27	629,06	113,7
S21	13326	155765	Visby, Visborgs Kungsladugård	Mes.	humerus, dist, dex	53,6	1234,12	141,5
S22	13326	155765	Visby, Visborgs Kungsladugård	Mes.	humerus, dist, dex	82,8	2232,24	144,9
S23	13326	155765	Visby, Visborgs Kungsladugård	Mes.	humerus, dist, dex	69,3	1625,85	128,2
S24	13326	155763	Visby, Visborgs Kungsladugård	Mes.	humerus, dist, dex	25,2	620,21	140,4
S25	13326	155763	Visby, Visborgs Kungsladugård	Mes.	humerus, dist, dex	42,5	1069,31	143,8
S26	13326	155763	Visby, Visborgs Kungsladugård	Mes.	humerus, dist, dex	75,2	1790,59	133,9
S27	21234	878369	Gothem Västerbjers	Neo.	mt 5, sin	51,2	834,45	103,3
S28	32442	848614	Hangvar Ire	Neo.	phalang 1	21,5	290,09	124,5
S29	21234	878368	Gothem Västerbjers	Neo.	mt 5, sin	49,8	746,51	108,3
S30	21234	878368	Gothem Västerbjers	Neo.	mt 5, sin	54,2	1044,1	107,7
S31	31118	477332	Hangvar Ire	Neo.	radius, dist, sin	45,4	1085,16	117,2
S32	8983	150966	Eksta Stora Karlsö Stora Förvar	Mes.	radius, prox, dex	68	1826,25	121,8
S33	8983	151300	Eksta Stora Karlsö Stora Förvar	I.A./M.P.	humerus, prox, sin	82,5	3583,95	115,9
S34	8983	151374	Eksta Stora Karlsö Stora Förvar	Mes.	coxae	6,5	3444,54	133,2
S35	8983	151374	Eksta Stora Karlsö Stora Förvar	Mes.	coxae	6,6	3804,2	114,6
S36	14344	157643	Eksta Stora Karlsö Stora Förvar	Mes.	mandibula, sin	57,3	2634,32	100,6
S37	14344	157658	Eksta Stora Karlsö Stora Förvar	Mes.	femur, dist, sin	70,5	4575,08	112,9
S38	14344	151901	Eksta Stora Karlsö Stora Förvar	Mes.	scapula, sin	54,2	2079,38	115,7

## 4. Method

### 4.1 Sampling and preparation

The source material used in this study is bone and teeth, selected to include a maximum number of individuals. This was primarily done using the MNI method described in (Marshall & Pilgrim 1993, During 1996). MNI means that the minimum number of individuals in a context is calculated, using bone type, number and size as parameters (Marshall & Pilgrim 1993:261pp, During 1996:106). This method is known to show a much lower number of individuals than the one present in the material (*ibid*), and it was not possible to solely use MNI for this study. Sampling was also based on spatial distance, meaning that the remains originated from different layers and areas so that the bones are more likely to not originate from the same individual. A third method to distinguish individuals is radiocarbon dating. Unfortunately, it was not possible to radiocarbon date the samples until the finishing stages of this thesis.

Since DNA extraction is a destructive process, the bones were extensively analysed and documented prior- and post analysis, according to species, length, weight and type of bone. All bones used in this study were also documented using a digital camera. All work surfaces are washed with bleach or Hydrochloric acid HCl 0.01 M, which break the DNA-strands (Hummel 2003:135p). To remove outer contamination, the bones were irradiated at 1 joule of UV-light and the outer and inner surfaces were removed using an electric drill prior to grinding. Some researchers prefer to soak the bones in bleach, which with its destructive properties destroys the contaminant DNA by breaking its strands (Kemp & Smith 2005:54). This procedure is somewhat risky since it has been shown that bleach might enter bone and teeth and destroy endogenous DNA (Dissing *et al.* 2008:1448). Due to the risk that bleach destroys the endogenous DNA and since this study treats faunal remains, the material was not soaked in bleach. The bones and teeth were ground to a powder using an electric drill or an agate mortar. Prior to use, the mortar was washed with detergent and ethanol 95% and then placed in a glass beaker with ionized water that was then placed in sonicator for 4 minutes, washed once more in ionized water and sterilised using ethanol 95% and a burner. The electric drill was cleaned with HCl 0.01 M and ethanol 95% and the drill bit was changed between every sample. Protective clothes are worn all the time, lab coats and disposable gloves in the bone lab and full suit cover, face mask and double pair of disposable gloves in the ancient-DNA lab. The mortar was advantageous to minimize the loss of bone powder and to avoid accidents with the electric drill, when the bone size was small. The drill was favoured if the bones were well preserved. In some cases the bone was first chopped into smaller pieces using a saw drill bit and then ground in the mortar. This proved to be the best method to avoid loss of bone powder. The drill also generates heat at high speeds and this has shown to affect the recovery of DNA from bones negatively (Adler *et al.* 2011:960p). Between 100-150 mg powder was extracted per sample and the powder was collected in 20 ml tubes and placed in a -20° C freezer, pending further analysis.

#### 4.1.1 Steps to avoid and detect contamination

A set of authentication criteria has been set to minimize the risk of getting false results due to contamination (Cooper & Poinar 2000:1139). These criteria cannot be used as a definite confirmation that a result is authentic but only support them.

The aDNA lab is separated from other facilities and no modern source material has been brought there. Only personnel with reasons to be in the DNA lab are permitted. PCR-product carry over can be avoided by separating the pre-PCR from the PCR-and post-PCR lab and it is not allowed to enter the ancient-DNA lab after working in the PCR-area the same day (Götherström & Lidén 1998:56, Hummel 2003:133p). The ancient DNA-lab is irradiated with UV-light for a minimum of 2 hours prior to entering, although the effect of UV-irradiating surfaces in open spaces is poorly elucidated (Hummel 2003:135p). Contamination in the chemicals can be detected by using negative blanks in the extraction step, i.e. extracts that is treated the same way as the samples, without DNA (Götherström & Lidén 1998:56, Hummel 2003:134). Additional blanks are introduced at the PCR step. To see if there is contamination in the chemicals, it can be a good idea to run a few blank samples through the whole extraction and amplification process.

#### 4.2 Extraction

The method for extraction and purification used in this study followed a modified version of Yang *et al.* protocol C (Yang *et al.* 1998:541). To be able to extract DNA from bones the cells first need to be broken down and this is done by a mix of, EDTA that breaks down the hydroxyapatite, Triton X that lyses the bone cells and Proteinase K, an enzyme that digest proteins in the bone powder. A mastermix is prepared containing per sample; 2900 µl EDTA, 30 µl Triton X and 100 µl Proteinase K. This 3030 µl solution is mixed with the bone powder and placed in an incubator at 37° C, for 3 days. To detect contamination, one negative control is added for every fifth sample. A negative control is a sample with all chemicals, but with no bone powder, which is treated like the other extract from the extraction step to the gel electrophoresis.

#### 4.3 Purification

Next step is purification of the samples in order to remove all substances except the DNA. A modified version of the PCR Purification protocol provided by the manufacturer (Qiagen) was used.

After the incubation, the samples were centrifuged at 4000 g for 5 minutes and then transferred to Eppendorf tubes and centrifuged once more, 5000 g for 6 minutes. These steps make the greater part of the bone powder and other particles to remain in the bottom of the tubes. After centrifugation, the DNA-extract is transferred to Amicon Ultra-4 Centrifugal filter devices and centrifuged at 5000 g for 20 min. This step separates solutes of low molecular weight <30 kDa from the DNA. The remaining extract was transferred to 2 ml Eppendorf tubes and mixed with 1 ml PB-buffer QIA per sample. The extracts were then transferred to silica columns (the PB-buffer makes the DNA to bind to the columns) and

centrifuged at 17900 g for 1 min. The columns were then transferred to new 2 ml Eppendorf tubes and 650 µl PE-buffer was added and centrifuged at 17900 g for 1 min, this step is performed to wash the DNA and was repeated twice, with new Eppendorf tubes every time. The columns were then transferred to new Eppendorf tubes and centrifuged at 17900 g for 1 minute to dry. Then the columns were transferred to 1.5 ml Eppendorf tubes and 70 µl EB-buffer was added and centrifuged at 17900 g for 1 min, to elute the washed DNA. As a last step, the purified samples were placed in a freezer at -20° C pending further analysis.

#### 4.4 PCR

The polymerase chain reaction (PCR) is used to amplify DNA segment to manageable amounts by imitating the way the cell transcribes DNA. The first step in the PCR cycle is denaturation where a rise in temperature to 94°C, makes the hydrogen bounds in the double stranded DNA to release and the segment to open. The next step is annealing, where the temperature is lowered to 50-65°C, depending of the primer, which makes the primers bind to their designated area. The next and last step is elongation, where the temperature is raised to 72°C which makes the DNA-polymerase start building the nucleotides on the primer (Hummel 2003:81pp, Freeland 2005:16pp). The desired segment is now doubled and the cycle starts from the beginning. The number of cycles depend on the quality of the sample but usually range within 35-50 cycles with low quality samples such as with aDNA (Hummel 2003:81pp).

For primer design, sequences from the mitochondrial D-loop from mountain hares from different places in Northern Europe (Thulin *et al.* 1997), found on the National Center for Biotechnology Information (NCBI) accession nr: Y15300-Y15314 were used for alignment. By using the software Multalin (Corpet 1988, E-source 2), areas with consensus among the different individuals were chosen to place the primers. A 25 base pair primer pair was picked using Primer-BLAST, forward primer 5' CTAATAACAAATCCAAGTACCTTGT 3' and reverse primer 5'AATGGTCTAATGTTGATTATGAAAT 3', resulting in a 130 base pair sequence. The primers were run in nucleotide BLAST to verify that they were species specific and then tested on two samples from modern mountain hares at the Department of Zoology at Stockholm University.

Next step was to run a PCR with the ancient samples. This is a process of trial and error since there is no answer on how to combine the PCR-mix, and what cycling conditions the PCR machine should be set at, but only guidelines. PCR amplifications were conducted in a MJ Research PTC-200 Thermo Cycler. Amplification was performed following the HotStarTaq Plus DNA Polymerase protocol (Qiagen) with a 5 min denaturation step at 94°C, to activate the Tag polymerase plus. After that, PCR was run for 40 cycles at 94°C for 20 s for the denaturing step, at 53°C for 30 s for the annealing step, and for 72°C for 15 s for the extension step. The PCR was completed with a final extension step for 7 minutes at 72°C. The 25 µl reaction mix contained; 2 µl DNA extract, 0,4 µl Taq polymerase plus, 1 µl dNTP, 1,5 µl MgCl, 1 µl BSA, 2,5 µl Buffer, 1,25 µl primer 1, 1,25 µl primer 2 and 14,1 µl H<sub>2</sub>O.

There were some initial problems with contamination in the PCR-blank. The contaminated sample was sequenced and run in nucleotide BLAST (E-source 5) and was not similar to any hare species. The contamination disappeared when the cycle condition was optimized, the chemicals used for the PCR-mix were replaced with new and the working area in the lab was extensively cleaned.

## 4.5 Post-PCR

After the PCR, the samples were transported to the Department of zoology at Stockholm University, for further analysis.

### 4.5.1 Gel electrophoresis

Prior to sequencing, the DNA molecules are controlled using agarose gel electrophoresis, which can be described as a sieve that separates DNA molecules. The DNA extracts are mixed with a loading dye and placed in small wells in the gel. The gel slab is then covered with TAE-buffer. The loading dye does not simply colour the extracts but gives it a higher density than the TAE-buffer, making it stay in the wells. The gel contains a mix of TAE-buffer, agarose and ethidium bromide and when connected to an electric current; the negatively charged nucleic acids travel towards the positive pole (Hummel 2003:113pp, Campbell 2008 405p). Smaller molecules travel faster than the larger ones and are hence separated, a high concentration of agarose makes the molecules move slower and are therefore suited for smaller segments. A 2% solution was mixed for this study (Hummel 2003:113pp). The ethidium bromide makes it possible to visualize the DNA-extracts in UV illumination (Hummel 2003:113pp, Campbell 2008 405p). If nothing was visible in UV-light, new samples could be run through PCR with different PCR mix and/or cycle preconditions.

### 4.5.2 Sequencing

In order to determine the nucleotide sequence, the DNA extracts are sent to sequencing. By using the principle of DNA replication, a mix of the DNA template, a primer, the four deoxyribonucleotides (dNTP) and the four dideoxyribonucleotides (ddNTP) was prepared. The ddNTP are similar to dNTPs but lack an OH-group, causing the synthesis to stop when it binds to the DNA-template (Freeland 2005:23p, Campbell 2008:408p). The ddNTP binds at random so that fragments of different lengths are created and since the ddNTP are dyed, the sequencing machine can recognize which nucleotide are located at a certain position of the sequence (Freeland 2005:23p, Campbell 2008:408p). The sequences were analysed using both the forward and reverse primers.

Prior to sequencing, the samples were purified from remaining PCR-products by performing a similar purification step as described under 4.3, using the protocol provided by the manufacturer (QIAGEN). Sequencing was performed at KIGene using ABI 3730 and 3130XL PRISM® DNA Analyzers (E-source 6).

### 4.5.3 Phylogenetic analysis

Two methods were used to analyse the genealogies of the samples. The sequences were aligned using the software Multalin. Pairwise comparison of sequence similarities is given a

score and sequences are grouped using hierarchical clustering, used to build a bifurcating phylogenetic tree (Corpet 1988:10881pp, E-source 3). The phylogenetic tree gives information of the evolutionary relationship between genetic lineages by arranging the taxa in different branches, depending on how genetically similar they are (Avice *et al.* 1987:493pp, Corpet 1988:10881pp, Freeland 2005:155pp). The place where the branch bifurcates, the node, represents a common ancestor and genetically similar taxa are hence close in the tree (ibid).

A statistical parsimony network was created using the software TCS v1.21 (Clement *et al.* 2000:1657, Freeland 2005:155pp). A network sort haplotypes by their evolutionary relationship based on pair wise differences due to mutations (ibid). In a comparison with a phylogenetic tree, a network can be multifurcating i.e. that one haplotype can be ancestor to several genetic lineages, it includes missing haplotypes and can be used despite little variation (ibid). The haplotype presumed to be the oldest can be recognized by a few criteria, they are supposed to be in the centre of the network and to have several connections, they are also presumed to be the most abundant and geographically widespread (Freeland 2005:167pp).

The samples were compared to 67 known mountain hare haplotypes from different places in Europe; Austria, Ireland, Scotland, France, Norway, Sweden, Finland, Russia, Ural, Italy and Switzerland (fig. 8) (Melo-Ferreira *et al.* 2007). Because the sequence was short, 65 bp, several haplotypes were grouped that would probably otherwise be separated.

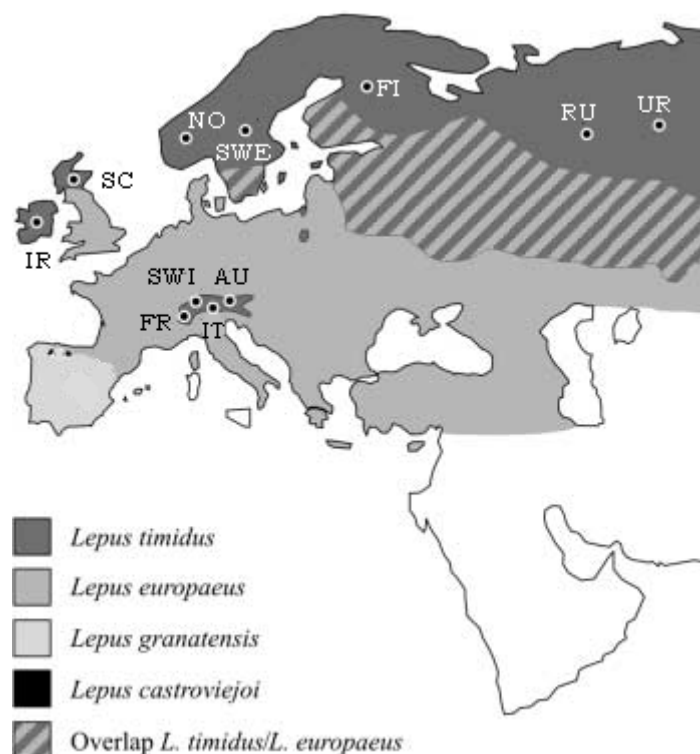


Figure 8. Areas sampled for modern DNA, revised from Melo-Ferreira *et al.* 2007

## 5. Results

Of the 38 bones that were sampled, 20 yielded DNA, 53%. The success of the analysis was somewhat correlated with the site, samples from Visborgs Kungsladugård (n=6), Uddvide, (n=2) and Ire (n=2) did not yield DNA. Regarding the damage on DNA caused by long time storing of bones, both bones from Lilla Sojvide, excavated in 1932, and Stora Förvar excavated between 1888-1893 yielded DNA, and this result do not convincingly show that long time storing is harmful for the DNA.

Table 2. Samples that yielded DNA marked (Y) and the samples that did not yield DNA (N).

Sample	site	Approximate date	Skeletal element	Length (mm)	Weight (mg)	DNA
S1	Lilla Hultungs	Medieval Period	radius	70,8	1842	Y
S2	Lilla Hultungs	Medieval Period	radius	40,3	856,37	Y
S3	Lilla Hultungs	Medieval Period	radius	34,7	1034	Y
S4	Lilla Hultungs	Medieval Period	radius	14,3	177,5	Y
S5	Lilla Hultungs	Medieval Period	radius	35	921,55	Y
S6	Gisslause	Mesolithic Period	pm/m	15,4	214,07	Y
S7	Gisslause	Mesolithic Period	meta tarsal 3	25,1	285,31	Y
S8	Gisslause	Mesolithic Period	meta tarsal	22	200,54	N
S9	Gisslause	Mesolithic Period	phalang 1	21,9	274,97	N
S10	Gisslause	Mesolithic Period	meta tarsal	19,8	208,77	N
S11	Gisslause	Mesolithic Period	incisiv	24,6	290,68	Y
S12	Gisslause	Mesolithic Period	pm/m	9,7	130,56	N
S13	Gisslause	Mesolithic Period	pm/m	12,7	179,07	Y
S14	Gisslause	Mesolithic Period	incisiv	17,4	151,37	Y
S15	Gisslause	Mesolithic Period	phalang 2	11	82,13	N
S16	Gisslause	Mesolithic Period	tibia	23,6	694,96	N
S17	Uddvide	iron Age	tibia	41,4	1318,19	N
S18	Uddvide	iron Age	tibia	45,5	1623,96	N
S19	Lilla Sojvide	Iron Age	calcaneus	35	1670,92	Y
S20	Lilla Sojvide	Iron Age	calcaneus	27	629,06	Y
S21	Visborgs Kungsladugård	Mesolithic Period	humerus	53,6	1234,12	N
S22	Visborgs Kungsladugård	Mesolithic Period	humerus	82,8	2232,24	N
S23	Visborgs Kungsladugård	Mesolithic Period	humerus	69,3	1625,85	N
S24	Visborgs Kungsladugård	Mesolithic Period	humerus	25,2	620,21	N
S25	Visborgs Kungsladugård	Mesolithic Period	humerus	42,5	1069,31	N
S26	Visborgs Kungsladugård	Mesolithic Period	humerus	75,2	1790,59	N
S27	Västerbjers	Neolithic Age	meta tarsal 5	51,2	834,45	Y
S28	Ire	Neolithic Age	phalang 1	21,5	290,09	N

S29	Västerbjers	Neolithic Age	meta tarsal 5	49,8	746,51	Y
S30	Västerbjers	Neolithic Age	meta tarsal 5	54,2	1044,1	Y
S31	Ire	Neolithic Age	radius	45,4	1085,16	N
S32	Stora Förvar	Mesolithic Period	radius	68	1826,25	N
S33	Stora Förvar	Medieval Period	humerus	82,5	3583,95	Y
S34	Stora Förvar	Mesolithic Period	coxae	6,5	3444,54	Y
S35	Stora Förvar	Mesolithic Period	coxae	6,6	3804,2	Y
S36	Stora Förvar	Mesolithic Period	mandibula	57,3	2634,32	Y
S37	Stora Förvar	Mesolithic Period	femur	70,5	4575,08	N
S38	Stora Förvar	Mesolithic Period	scapula	54,2	2079,38	Y

In a network created in TCS, samples from this study were compared to 67 haplotypes, deriving from modern hares from Europe (Melo-Ferreira 2007:607). The haplotypes in the network were sorted into two groups (Table 3).

Table 3. The two main groups in which the haplotypes where sorted in the network.

Group 1			
Sample site	Number of samples	Haplotype	Aproximate Date
Stora Förvar	4		Mesolithic
Gisslaue	5		Mesolithic
Scotland	6	t31, t32, t33, t34, t35, t36	Modern
Finland	5	t24, t25, t26, t27, t28	Modern
Sweden	4	t1, t3, t9, t1	Modern
Italy	3	t47, t51, t58	Modern
Urals	2	t64, t66	Modern
Russia	1	t67	Modern
Group 2			
Sample site	Number of samples	Haplotype	Aproximate Date
Västerbjers	3		Neolithic
Lilla Sojvide	2		Iron Age
Lilla Hultungs	5		Iron Age/Medieval period
Stora Förvar	1		Iron Age/Medieval period
Sweden	16	t2, t4, t5, t6, t7, t8, t10, t12, t13, t14, t15, t16, t17, t18, t19, t20	Modern
Italy	15	t46, t48, t49, t50, t52, t53, t54, t55, t56, t57, t59, t60, t61, t62, t63	Modern
Ireland	3	t37, t38, t39	Modern
France	3	t40, t41, t42	Modern
Norway	3	t21, t22, t23	Modern
Switzerland	2	t43, t44	Modern
Finland	1	t29	Modern
Scotland	1	t30	Modern
Austria	1	t45	Modern
Urals	1	t65	Modern



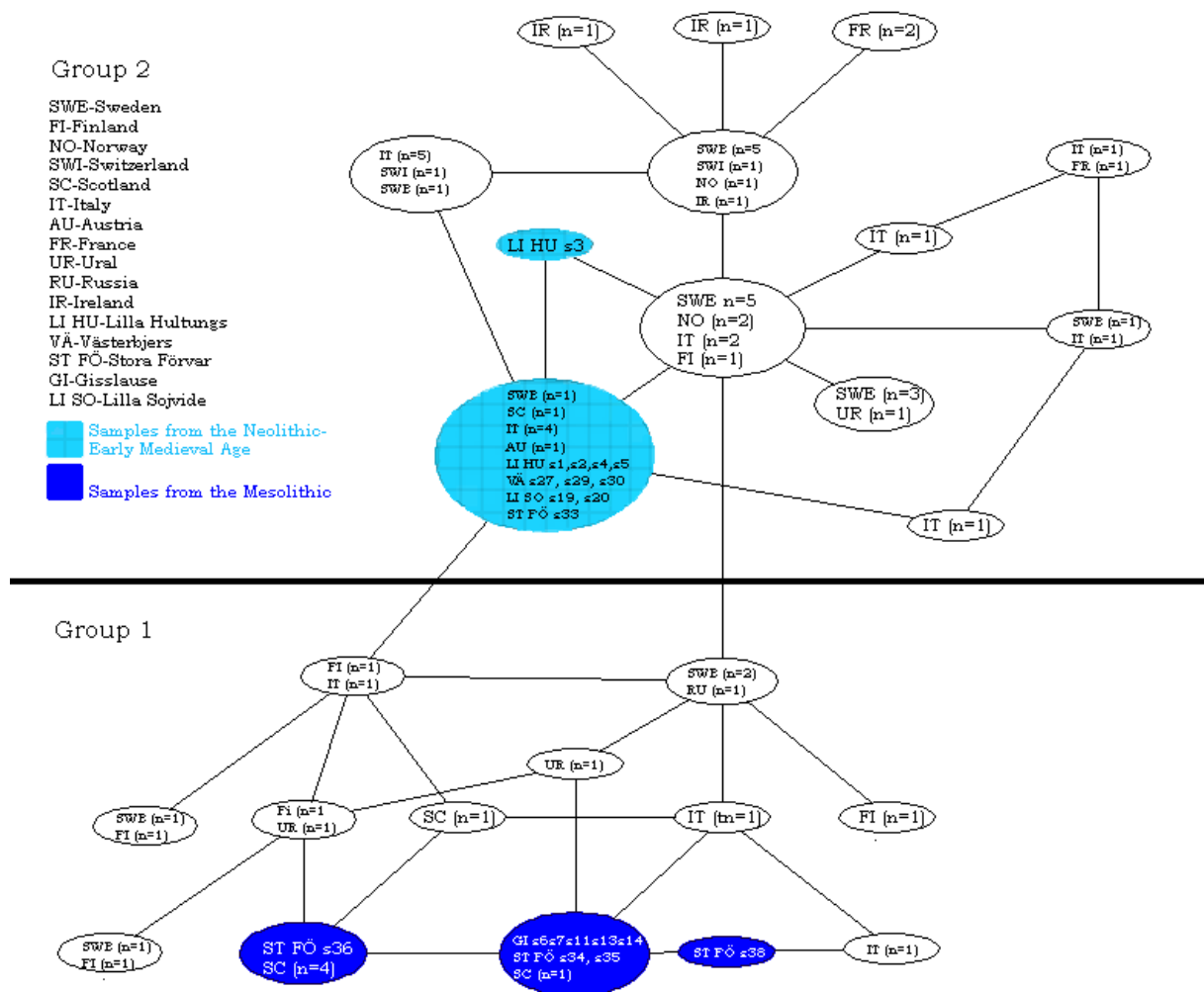


Figure 9. Statistical parsimony network based on a 65 base pair sequence for 67 European haplotypes (Melo-Ferreira *et al.* 2007), compared to the prehistoric samples, and based on Clement *et al.* 2000.

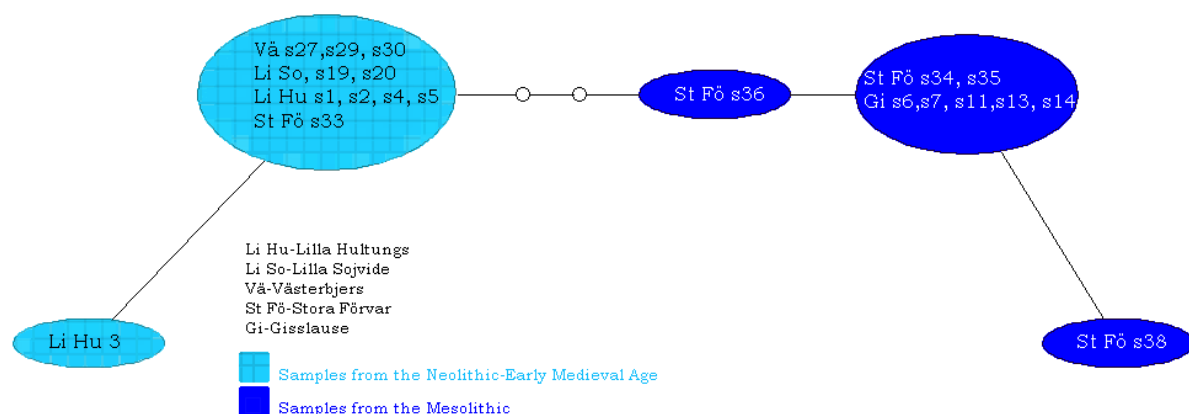


Figure 10. Statistical parsimony network based on the prehistoric samples, based on Clement *et al.* 2000.

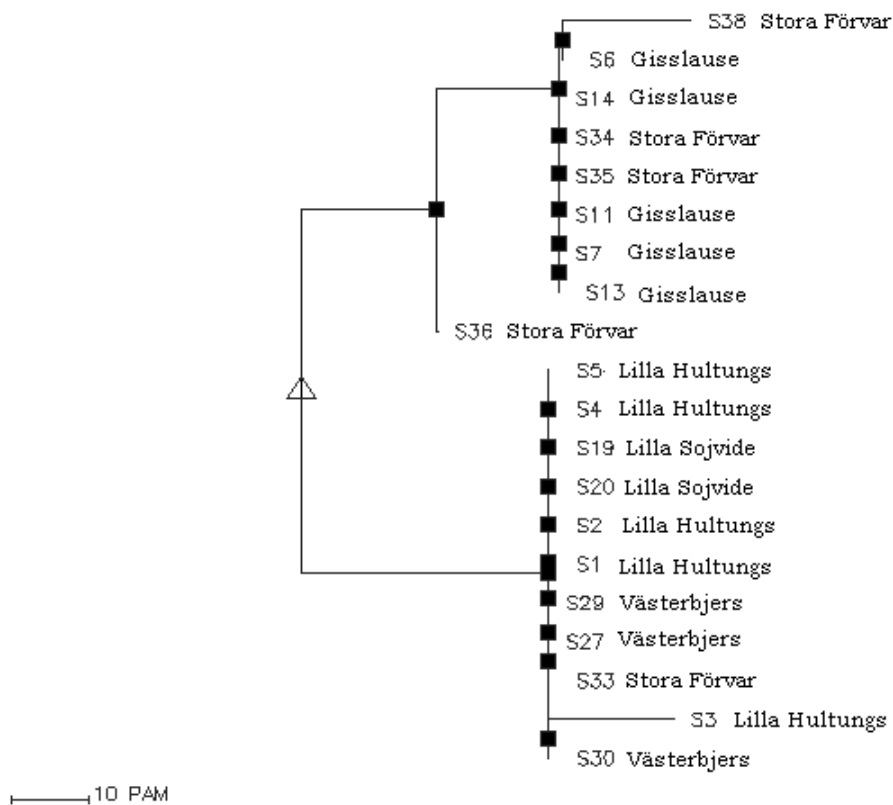


Figure 11. Phylogenetic tree of all samples in the study based on Corpet 1988.

In the phylogenetic tree, the samples form two genetically distinct clades (fig11). The samples from Stora Förvar which have their relative dating to the Mesolithic, and the samples from Gisslausa, that were found under the Littorina transgression wall with a dating to prior 6200 cal. BC form one clade (Lindqvist & Possnert 1999:66). The samples from Västerbjers  $4250 \pm 50$  BP and younger, as well as the sample from layer f2 in Stora Förvar form one clade.

## 6. Discussion

### 6.1 On the origin of the mountain hare on Gotland

The process of post-glacial colonization for different species from refugias south of the ice rim is complex and not fully understood. What is known today derive from skeletal remains and the current distribution of modern organisms. This does not necessarily represent the dispersal of species in the past which can have some implications when interpreting the results. Hares have been moved around by people to increase population size through time, e.g. we know that the contemporary hare population on Gotland has been reintroduced with hares from the mainland and is not covered in this study. The same goes for the population on the mainland with hares from Russia (Thulin *et al.* 1997:471). Translocations can explain why one haplotype from Scotland is found in group 2 (fig 9), because the specimens with that haplotype were sampled on the isle of Mull, where we know Irish hares had been reintroduced (Melo-Ferreira *et al.* 2007).

The results show that there is a discontinuity between the Mesolithic and the Neolithic periods in the mountain hare populations on Gotland, thus it seems likely that hares on the island originate from multiple source populations. The haplotypes in group 1 (fig 9, table 3) mainly consists of hares from Scotland, Finland, Sweden and Italy but also from the Urals and Russia. The earliest samples are genetically most similar to the subspecies *Lepus t. scoticus*, now present in Scotland. This subspecies is believed to be a remnant population from the last glacial period that colonized the British Isles by a land bridge and was later isolated by the English Channel (Thulin 2003:31). Although it could be an explanation, this does not imply that the earliest hare population on Gotland came from Scotland. As the ice cover retreated at the end of the last glacial period, the flora and fauna that had dwelled in refugia south of the ice moved northwards. The haplotypes in group 1 seems to have more of an eastern origin, and hares with the same origin of descent as the hares in Scotland may have travelled northwards from a common refugia. Eventually they reached the shore of the Baltic Sea and from there these hares reached the island of Gotland. Sweden was connected to the continent by a land bridge during the Yoldia Sea stage and hares, as well as other animals, could colonize this area using this passage.

The later populations of hares, Group 2 (fig 9, table 3) derive from samples from the Middle Neolithic to the Medieval Period. The haplotypes from modern samples in group 2 mainly consist of hares from Sweden, Italy, and all sampled hares from Norway, Ireland, Austria, and France. It also includes some haplotypes from Switzerland, one from Scotland, one from Ural and one from Finland. This possibly gives an indication of what refugia these mountain hares dwelled in during the last glacial period. This suggests that the latter hare population came to the island from the west, given that the contemporary population of hares in Sweden is representative for the hares that lived in Sweden in prehistoric time. Therefore, to get a fuller understanding of post-glacial colonization history of the north, DNA analyzes of skeletal remains of hares from different archaeological sites are necessary. There is also a need to use a longer sequence, to get a higher resolution of the origin of the mountain hare. However, this study show how ancient DNA analysis can contribute to the field of phylogeography since it can reveal haplotypes that have been present historically, which can reveal different events of colonization.

## 6.2 The genetic relationship between the mountain hares in the study

The samples in the study form two main genetically distinct groups, shown in the statistical parsimony network (figs 9 & 10, table 3), and the phylogenetic tree support this result (fig 11). Group 1 consists of samples from Gisslause (n=5), that were found beneath the Littorina transgression wall and radiocarbon dating from the site range between 6433-5881 calBC and 7043-6557 calBC. Group 1 also include the samples from Stora Förvar, layer G8 and F10-12 (n=4), all found in Mesolithic layers (Lindqvist & Possnert 1999:67). Group 2 represent the Neolithic samples from Västerbjers (n=3), the Iron Age samples from Lilla Sojvide (n=2) and the Iron Age/Medieval Period samples from Lilla Hultungs (n=5) and Stora Förvar layer F2 (n=1).

The discrepancy between the samples can be caused by several factors. The haplotypes common in group 2 may have been present on the island during the Mesolithic period, although not sampled for this study. Still, the haplotypes present in group 1 are not represented in the later material either. There is though, a possibility that the haplotypes in group 1 have been lost due to genetic drift.

So, if the result actually represents the haplotypes present on the island at the different occasions. The discrepancy seems to indicate that the female lineage of mountain hares disappeared between the Mesolithic and the late Iron Age, and that the samples from Västernbys show that this break can have occurred as early as  $4250 \pm 50$  BP. This result is also strengthened by the fact that skeletal remains from mountain hare become rare after the Mesolithic 5900 BC. Multiple scenarios could have been the cause for this decrease or extinction. It could be due to stochastic factors such as diseases, climatic oscillations or the introduction of a new species. As seen in figure 12, there are about 1500 years between the two groups, and several events that could have contributed to the decline of the mountain hare population occurred during this time. The climate changed from the warm Atlantic period to the cold Sub-boreal period. At the same time, the Littorina transgression caused habitat loss as the area of Gotland decreased, from the current 3100 square km, to 1900 square km (Lindqvist & Possnert 1997:52). The red fox is present in the archaeological material from 5500 BP and this new predator could have had a major effect on the island population. A small isolated population is already vulnerable and if a climate change caused the population to decrease, its vulnerability would increase. Extinction could also be caused by deterministic factors such as overhunting, or a combination of stochastic and deterministic factors, creating an extinction vortex that caused the population to go extinct.

The result also indicates that a second introduction of hares could have occurred on Gotland. The samples from the intermediate period Västernbys, show that the haplotype that is abundant in later periods were present on the island as early as  $4250 \pm 50$  BP. The Gotland samples in group 2 represents only two haplotypes in a time period covering as much as 3500 years. As seen in figure 9, haplotypes tend to cluster when they are cut and additional haplotypes may appear if a larger sequence is analyzed. Few haplotypes may also be the result of a bottleneck, a founder effect caused by a colonization of the island by a small number of mountain hares. This would indicate that there have been no, or limited gene flow after the second colonization. There is a possibility that the population was stable after the second colonization and that there was no need to move new hares to the island. There is also the possibility that the mountain hare lost its role as a terrestrial food source when domesticated animals were introduced to the island during the shift from the Mesolithic to the Neolithic. This could have made the need to move hares to Gotland less necessary. However, if the second introduction was done by people, hares obviously were important enough to be reintroduced. This importance could of course also have been symbolic, as shown by the hare feet in graves from the Neolithic. For further research in this area, a longer sequence should be analyzed to see if more haplotypes are revealed. A comparison should also be made with nuclear DNA that will show the paternal lineage, to see if the result corresponds. A larger

sample size and more samples from the intermediate period 7000-4000 BP would also be eligible.

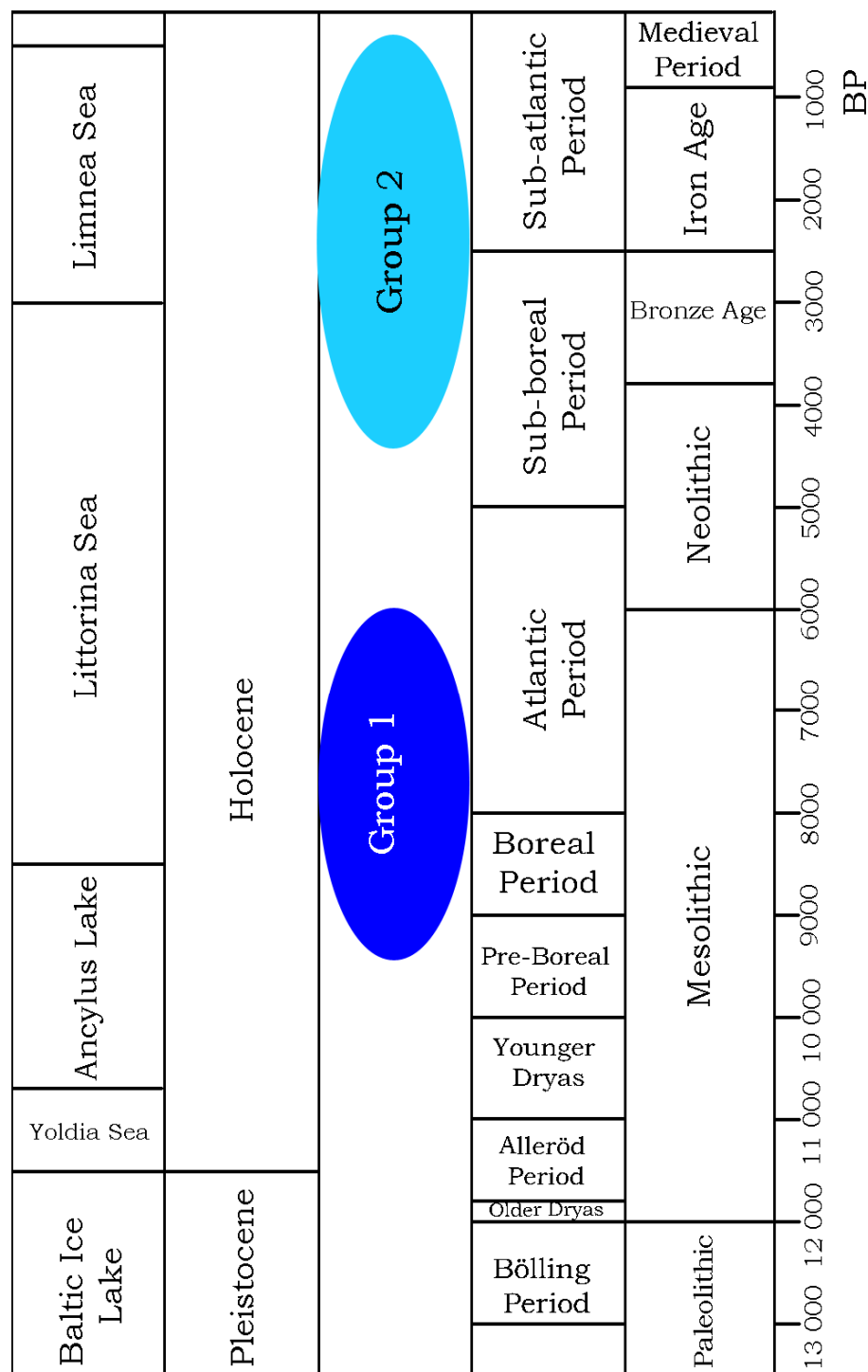


Figure 12. A rough chronology; Baltic Sea stages, geological epochs, the genetic groups, climatic phases, archaeological periods.

### 6.3 On how the mountain hare reached Gotland and what this can say about the early people on the island

Prehistoric skeletal remains are most often found in cultural layers on archaeological sites, deposited there by people. There is a possibility that hares were present on Gotland before the first humans but that will not show in the archaeological material. Hence, it is not possible to deduce who first set their feet on the island. Skeletal remains from the mountain hare are found on the earliest archaeological sites from several geographical locations on Gotland, the earliest from Stora Förvar, dated to 7420 years cal BC.

Although the sequence used for this study was relatively short, only 130 BP, and the sample size representing the early phase on the island was limited, (n=9), three haplotypes were found. Additional haplotypes may be found with a larger sample size and if complementing sequences are analyzed. Multiple lineages of mtDNA indicate that the earliest population was founded by several individuals. This result could be expected if a land bridge or archipelago connected the island to the mainland, a land bridge that current research does not support (Liljegren & Lagerås 1993:11, Björck 1995:27p). The lack of a land bridge is also supported by the low number of species on Gotland today, and because the wild terrestrial mammalian fauna has been brought to the island during the last centuries except for the red fox and some rodents. A comparison with the prehistoric fauna on the Island of Bornholm, which has been connected to the continent by a land bridge, show that this island housed several large terrestrial mammals such as, roe deer, red deer, wild boar, reindeer (*Rangifer tarandus*), beaver (*Castor fiber*) and elk (Casati & Sørensen 2009:248p). The last seems to have gone extinct as Bornholm became an island (ibid). These animals were all absent on Gotland during the Mesolithic.

Several haplotypes would also be expected if a number of hares were introduced to the island by people, at the same time or at several occasions. This way, a number of haplotypes may be brought there by chance. There is also the possibility that a number of hares were present on the island prior to human colonization through dispersal over the ice, and that this population was refilled by translocations. The haplotype variation among the earliest hares could also be the result of mutations. Long dispersals by mountain hares have been recorded (Angerbjörn & Flux 1995:6), and it would physically manage to disperse to Gotland. However, the number of hares that have dispersed or travelled over sea from the mainland to Gotland should most likely be limited, causing a low genetic variation. Since the result show several haplotypes, this may not be the case, but a longer sequence and a larger sample size from the earliest phase is required to conclude this. The lack of wild terrestrial mammals also shows the difficulties in founding an island population. Except the red fox, no larger animal than the mountain hare seem to have succeeded this during the prehistory of Gotland.

Translocations of wild animals to remote locations have been observed before the introduction of farming and the hare is easy to catch alive and transport. The brown hare has been introduced to the British Isles, possibly as early as 4000 years BP. There is also the transportation of fallow deer to Cyprus as early as 10 000 years BP. The number of species on

Gotland was low when the first people settled and it is possible that hares were brought to the island to complement the present fauna on the island. It does not necessarily mean that nourishment was the main reason, but symbolic or practical uses of hare remains might also have been the cause, as shown by the hare feet in Neolithic graves. Whatever reason, this would be an early example of human niche construction, where the environment is adapted for the benefit of the people who change it (Riede 2011:793pp).

#### 6.4 Some concluding remarks

First, the MNI method has its shortcomings (During 1996:106) and could not conclusively be used as a sampling method on the sites Ire, Stora Förvar and Gisslause. On the Ire site, the bones were found 35 meters from each other and probably represent two individuals. These did not yield DNA. On the Stora Förvar site, the bones were found in different layers and parcells and were believed to derive from different individuals. Four haplotypes were found among the five samples that yielded DNA from Stora Förvar, and the samples with the same haplotypes, s34 and s35 could be distinguished using MNI. The samples from Gisslause that yielded DNA were found in different parcells and/or layers but only one individual could be distinguished using the MNI method. Only one haplotype was found on the site and it was not possible to distinguish between individuals based on genetic differences, although this could change when a larger sequence is used. The samples that yielded DNA were found in different layers except s6 and s7 that were in the same layer but two meters apart, and it is not possible to conclusively conclude that they derive from different individuals. There are 35 cm between the top and bottom layers where the samples were found and these will be radiocarbon dated. If the other three samples derive from different individuals cannot fully be elucidated prior to the radiocarbon dating and the complementing DNA analysis. Second, the genetic marker used in this study is mitochondrial DNA, which is undoubtedly the best choice for this kind of study because of the characteristics of mtDNA. The fact that only the female lineage is represented has some implications on the interpretation for these questions, and studies on nuclear DNA are needed to conclude if an extinction has occurred. Third, the sequences in this study were picked because they were highly variable and were tested to be able to identify haplotypes that represented different geographical regions. However, when working with aDNA, there are limitations on sequence size, with a higher success rate when using shorter sequences. This is not necessarily a greater problem other than that the resolution is low, the sequences will be extended. Last, the sample size for this study is limited and it is possible that not all haplotypes present on the island during different periods have been covered.

## 7. Conclusion

The results indicate that there were two prehistoric populations of hares on Gotland, and that they may be of different origin. The first population on Gotland, group 1 consists of the Mesolithic samples from Stora Förvar and Gisslause. They group together in the network and are genetically most similar to modern mountain hares from Scotland, a population believed to be a remnant from the last glacial period. The hares on Gotland may therefore originate from the same refugia as the hares on Scotland. The haplotypes in group 1 seem to have more of an eastern origin. The second population of hares on the island, group 2, dating from  $4250 \pm 50$  BP and onwards consist of the Neolithic samples from Västerbjers, the Iron Age samples from Lilla Sojvide the Iron Age/Medieval Period samples from Lilla Hultungs and Stora Förvar. They are genetically most similar to hares from Italy and the Swedish mainland, and seem to have more of a western origin.

The discrepancy between the samples indicates that the female lineage of the hare population on Gotland got extinct prior to the Iron Age, and possibly as early as the Neolithic period,  $4250 \pm 50$  BP where the haplotype common in group 2 first appear. This result is consistent with the archaeological finds where the mountain hare becomes rare after the Mesolithic period. The population decline also coincides with the Sub-boreal period which is characterized by a considerably colder climate, the Littorina transgression and the appearance of the red fox. These factors as well as overhunting may have caused an extinction. The results indicate that different events of colonization of hares to the island have occurred. Only two haplotypes were found in Group 2 which shows that a population lived on the island for 3000 years, without being refilled by translocation from the mainland. This can possibly show the decreased role of wild animals on the island in the Neolithic.

Archaeological evidence suggest that translocation of wild animals to isolated locations have occurred in prehistoric time, e.g. on Cyprus and the British Isles. The same thing could have happened on the island of Gotland which probably did not house any terrestrial mammals during the time when humans first arrived, and have not been connected by a land bridge. In that case, this would be an early example of human niche construction in Scandinavia, where the surroundings are adapted to become more suitable to live in. If the mountain hare reached the island by walking on ice or floating there on an ice flow, a low number of haplotypes would be expected. The result shows three haplotypes in group 1 and this indicate that the earliest population was founded by several individuals, although mutations may be an explanation.



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#### Personal comments

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