Genetic Analyses of Bovid Remains and the Origin of Early European Cattle

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Dissertation presented at Uppsala University to be publicly examined in Lindhalsalen, EBC, Norbyvägen 14, Uppsala, Friday, November 10, 2006 at 10:00 for the degree of Doctor of Philosophy. The examination will be conducted in English.

Abstract

The aurochs Bos primigenius, extinct since 1627, was the wild progenitor of cattle. It is believed that all European cattle originate from one domestication event in the Near East 10,000 years ago. However, it is evident from the archaeological record that the aurochs survived into historic time and spent many years existing alongside domestic cattle. Thus, a question posed is whether aurochs were locally domesticated or incorporated into early domestic cattle stock.

In this thesis, genetic techniques are applied to ancient and modern DNA from bovids in order to study questions relating to the origin of early European cattle. DNA from ancient specimens is fragmented and in greatly reduced quantity. Therefore mitochondrial DNA, present in many copies in the living cell, has long been dominating the ancient DNA research field. Analyses of ancient DNA presented in this work are based on both mitochondrial DNA and nuclear DNA, through the study of Single Nuclear Polymorphism (SNPs). A method for typing ancient SNPs was developed and applied to ancient cattle bones.

Mitochondrial DNA of cattle is structured into five geographically distributed lineages, the dominant lineage in Europe is also found in the Near East where additional lineages are found. This pattern has been attributed to the proposed domestication event in the Near East from where cattle carrying the single lineage were brought to Europe. However, the results presented here show that cattle domestication was more complicated than previously suggested. SNP data from extant cattle and bones from cattle and aurochs point towards a hybridisation event. European cattle appear indeed to have been domesticated in the Near East and brought in to the European continent from there. However, once in Europe, hybridisation with local aurochs took place. It appears therefore that today’s cattle descend both from both Anatolian and European aurochs.

Keywords: Bos taurus, cattle, domestication, aDNA, mtDNA, SNPs

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ISSN 1651-6214
ISBN 91-554-6688-5
urn:nbn:se:uu:diva-7201 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-7201)
List of papers

The thesis is based on the following papers, referred to by their roman capitals throughout the text.


IV Anderung C, Hellborg L, Seddon J, Hanotte O, Götherström A Identification of X-and Y- specific single nucleotide polymorphisms (SNP) and insertion/deletions diagnostic of taurine *Bos taurus* and indicine *Bos indicus* cattle. Manuscript


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Art work on front page by Thérése Anderung
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
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<tr>
<td>aDNA</td>
<td>Ancient DNA</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>HG</td>
<td>Haplogroup</td>
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<td>HT</td>
<td>Haplotype</td>
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<tr>
<td>bp</td>
<td>Base pairs</td>
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<tr>
<td>BC</td>
<td>Before Christ</td>
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<tr>
<td>BP</td>
<td>Before Present (ref. date 1950 AD)</td>
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<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<td>U</td>
<td>Unit</td>
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<tr>
<td>UNG</td>
<td>uracil N-glycosylase</td>
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<tr>
<td>$\pi$</td>
<td>Average pairwise difference</td>
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Introduction

Humans have spent a comparatively short period herding animals and cultivating plants. We lived quite successfully for many years as hunters and gatherers, collecting wild plants and hunting animals. A classical view is that about 20 000 to 10 000 years ago, around the time of the last ice age when the climate got warmer and dryer, humans started to colonise new previously uninhabited areas (Clutton-Brock, 1999). At the same time there is evidence for the extinction of many species of large mammals in these areas, although climate change may also lie behind this event (Martin and Klein, 1984; Shapiro et al., 2004). However, if one accepts the view that human hunting activity played a role in the extinction, this may indicate a generally increasing pressure on a growing human population to find adequate food by hunting and gathering which could have provided the impetus to find new ways of acquiring food.

It was also during the end of the last ice age that wild dogs started to be associated with humans. The scavenging behaviour of wild dogs probably meant that they were drawn to Mesolithic hunter camps, and occasionally a puppy would have a placid enough behaviour and stay with the humans as if they were its pack (Bökönyi, 1974; Clutton-Brock, 1999; Zuner, 1963). The habit of keeping pets is believed by some to have played an important role in the domestication of animals in general (Bökönyi, 1974; Clutton-Brock, 1999). The initial process of domestication of animals has been described as being like a willing partnership (Bökönyi, 1974; Clutton-Brock, 1999). The extreme result is the reproduction in isolation from the wild. Thus, one way to define domesticated animals can be, once-wild animals that have had their behaviour, life cycle or physiology altered as a direct result of their breeding and living conditions being controlled by humans (Clutton-Brock, 1999). The species that were to become domesticated needed to have certain physiological and behavioural qualities making them suitable. One desirable trait is a natural social behaviour based on a dominance hierarchy within a herd or pack, leading more easily to the acceptance of a human as a leader (Clutton-Brock, 1999; Diamond, 2002; Jensen, 2002).

The domestication of wild animals and plants in Europe was a process that started around 10 000 years ago. It began in the Fertile Crescent, the region that today is Turkey, and it was to dramatically affect the evolution of societies (Childe, 1925; Diamond, 2002; Smith, 1995; Zeder, 2006b). For more than 100 years, researchers have been trying to answer questions relat-
ing to this process. Archaeologists and anthropologists have addressed these questions through the study of remains from ancient settlements. More recently, the development of new analytical tools based on biomolecular techniques have shown the promise to give substantial additional insights into the domestication process.

The classical way to study the emergence of agricultural practices is through the archaeological record. The archaeological excavations of settlements in different regions provide the possibility to directly study the cultural context of the domestication process. With the help of different dating techniques it is possible to pinpoint the origin in time and follow its pace as it spreads across Europe (Smith, 1995). The domestication process and later the emergence of agriculture did not only affect the biology of the animals and plants involved; it also brought about great social and environmental changes. It sparked an economic revolution leading to a basic change in human way of life: the development of agricultural economies (Bökényi, 1974; Clutton-Brock, 1999; Diamond, 1997; Zeder, 2006b).

Over the period of 1000 years, humans changed their subsistence economy from hunter-gatherer to one based on more reliable food sources, domesticated animals and plants, which in turn allowed for the expansion of societal organisation and hierarchies (Childe, 1925; Smith, 1995). Although the question of whether this changed way of life brought on the subsequent population expansion, or if the population expansion itself triggered the change is still debatable. However, once underway, this change certainly allowed human populations to grow further. By tracing the spread of attributes associated with the settled communities it was possible to observe in the archaeological record how these new ways expand into adjacent areas.

The first European livestock to be domesticated were goats and sheep, followed by cattle, pigs and horses. Domestic animals tend to be smaller than their wild progenitor (Bökényi, 1974; Reed, 1984). Although it is not known how fast this morphological change took place in each species it is sometimes used to identify and separate wild fauna from domestic forms in the archaeological record. The use of this criterion gets even more difficult when the archaeological remains are fragmented. Recent research has shown that the reduction of body size might not be so reliable in the study of the early stages of animal domestication (Zeder, 2006a; Zeder and Hesse, 2000). For example initial stages of goat domestication were detected through demographic profiling of goat bones from the Zagros mountains of the Near East. These signs of herd management predate the morphological changes by 500-1000 years (Zeder and Hesse, 2000).

Biologists and geneticists have been engaged in the study of domestication process through analysis of DNA from both modern species and from ancient animal bone remains. Technical advances have increased the number of studies concentrating on livestock domestication. Genetic analysis of extant livestock have confirmed and refined archaeological theories concerning the
origin and spread of domestic animals and the number of domestication event (Bruford et al., 2003; Luikart et al., 2001) as well as how livestock, once domesticated, are affected by more modern breeding strategies (Freeman et al., 2006a). However, studies on modern DNA can be skewed by thousands of years of artificial selection. Ancient DNA on the other hand allows direct studies on the genetic composition of past and extinct animals, and has been used in studies of cattle (Troy et al., 2001) horses (Vilà et al., 2001) dogs (Leonard et al., 2002) goats (Fernández et al., 2002) and pigs (Watanobe et al., 2002).

The questions of which animals were domesticated and when and where the domestication took place have been approached and understood reasonably well. On the other hand, the questions of who domesticated these animals, for which reasons and in what manner are still largely unsettled. The domestication process is intrinsically linked to human cultural change: much debate has gone on about whether the spread of domestication is linked to colonisation or indigenous adaptation, migration or acculturation (Ammerman and Cavalli-Sforza, 1971; Childe, 1925; Pinhasi et al., 2005; Price, 2000; Zvelebil and Lillie, 2000). Given the complex nature of human behaviour and interaction, it seems likely that different combinations of these theories may have applied on different occasions. The answering of such questions is a long-term goal for those working in this field, of which it is hoped that the work presented here will provide some contribution and direction.

The research described in this thesis focuses on the domestication of European cattle, *Bos taurus*, based on genetic analyses. I shall begin with an overview of the techniques used in the genetic analysis of ancient DNA and the associated challenges. I shall then provide a brief summary of what is known of the history of the livestock species commonly found in Europe: goats and sheep, pigs, horses and, of course cattle. Since this thesis focuses on European domesticates, I shall not discuss Asian or New World species in any length.

**Analysing ancient DNA**

DNA sequences from ancient remains give us a unique opportunity to directly assess genetic change over time, as opposed to DNA from extant organisms where it is necessary to make an inference and reconstruct the changes that have taken place (Willerslev and Cooper, 2005). Apart from domestication studies, two examples of areas to which ancient DNA has contributed with information are phylogenetic relationships among extinct species (Krings et al., 1997; Loreille et al., 2001; Orlando et al., 2003; Orlando et al., 2006; Sorenson et al., 1999) and population changes during the
last glacial maximum (Barnes et al., 2002; Leonard et al., 2000; Shapiro et al., 2004).

One of the first ancient DNA studies, that identified the extinct quagga being a member of the horse family, used cloning as method to isolate DNA (Higuchi et al., 1984). However, when the polymerase chain reaction was developed in the late 1980s (Mullis, 1990; Saiki et al., 1988) this method, requiring smaller sample quantities, superseded the cloning method (Pääbo, 1988a). Today, the PCR is the dominant method in research involving ancient DNA. The advantages with the PCR are that it is highly sensitive: just a few starting molecules are needed. It is also specific to a particular sequence of target DNA, so one can repeat the amplification and in that way confirm the results. On the other hand, the PCR can cause artefacts due to nucleotide misincorporation that occurs for a number of different reasons (Hofreiter et al., 2001a).

Degradation

It is well known that ancient DNA is degraded, and that degradation can result in altered bases (Lindahl, 1993). The breakdown process starts as soon as an organism dies. Therefore ancient DNA is often of low molecular weight and has been subject to oxidative and hydrolytic damage (Pääbo, 1989). Oxidation affects the nitrous bases and the sugar-phosphate backbone of the DNA and alters it (Höss et al., 1996). If the DNA strand is broken, then the elongation step in the PCR will be blocked; however, depending on its nature the damage can also result in the insertion of altered bases rather than blocking the DNA polymerase. Depending on the proportion of DNA molecules that are amplified correctly to those that contain errors, sequences derived from the PCR product may or may not contain errors.

It was realised early on that the most commonly observed change was deamination of cytosine to uracil (Lindahl, 1993; Hofreiter et al., 2001a; Stiller et al., 2006) which results in substitutions, changing C to T and G to A. This is especially problematic with a low number of starting molecules and when the substitutions happen in the first cycles of the PCR. “Jumping PCR” or template switching (Pääbo et al., 1990) can also occur due to damaged DNA in the PCR, and this also has a tendency to cause nucleotide misincorporation and create chimaeric DNA strands.

Deamination products of cytosine, in summary, cause incorrect bases to be inserted during the PCR (Pääbo, 1989). Although other mechanisms may cause the general pattern of nucleotide misincorporation, the fact that the changes of C-to-T and G-to-A bases are mainly caused by deamination of cytosine residues makes it possible to overcome them through the use of uracil N-glycosylase (UNG) (Hofreiter et al., 2001a). This is an enzyme that removes uracil from DNA (Lindahl, 1993). The enzyme is added to the PCR
reaction and only requires one extra initial temperature step at the beginning of the PCR.

Contamination

The contamination problem is an aspect that one cannot ignore when working with DNA from ancient remains due to the extreme sensitivity of the PCR reaction and the small number of ancient DNA molecules. However, whether or not contamination poses a serious problem depends on the organism being investigated (Gilbert et al., 2005). For example, it is more of a problem when working with ancient human remains compared to working with remains from an extinct animal. Although not realised by all, a remarkable find of dinosaur DNA (Woodward et al., 1994), was later found out to be a human mitochondrial gene insertion in the nucleus (Zischler et al., 1995). Those who were active in this field early on and claimed the first successes with DNA extracted from ancient tissue (Higuchi et al., 1984; Pääbo, 1985) subsequently realised the problems involved, and that these early results also were almost certainly artefacts from contamination. Some of these pioneers have since become vocal proponents of rigorous protocols for authentication (Cooper and Poinar, 2000; Handt et al., 1996).

Modern DNA can be present in the specimens before sample removal (Bouwman et al., 2006; Malmström et al., 2005; Richards et al., 1995; Sampietro et al., 2006), or introduced at any stage during the extraction procedure; in chemical reagents, on laboratory disposables, or through intake of air at any stage of sample processing (Handt et al., 1994; Schmidt et al., 1995). The PCR can amplify minute traces of nucleic acids, and will amplify modern as well as ancient traces of DNA. It is clear that working with DNA from animals avoids the problem of direct contamination from the human DNA of those handling the samples. However, when performing the analyses described here care was still needed in order to avoid contamination from modern bovid DNA such as could be found from e.g. food, leather articles and reagents derived from animal tissues.

Standard basic precautions used by ancient DNA laboratories are cleaning work and floor surfaces with harsh chemicals such as bleach and HCl, wearing protective sterile clothing and separate pre- and post-PCR working areas. Chemicals are treated with UV light in order to crosslink any possible contamination. The outer layer is removed, the specimen UV treated and sometimes the bone is even treated with bleach (Kemp and Smith, 2005).

Authentication

The importance of authentication of results cannot be overstressed in ancient DNA studies, and more and more research is investigating this aspect of the field. Different suggestions for how to deal with the problem have been pro-
posed (Binladen et al., 2006; Bunce et al., 2003; Gilbert et al., 2005; Handt et al., 1994a; Pruvost et al., 2005). However, even following the general guidelines suggested (Cooper and Poinar, 2000) is no guarantee for reaching authentic results (Malmström et al., 2005). Since some of the work presented in this thesis is based on DNA extracted from ancient bovid remains, it was necessary to take a number of precautions. Firstly, it was verified that sequences retrieved made phylogenetic sense. Other precautions used include the use of separate work areas for pre- and post-PCR steps, the use of negative extractions and PCR blanks, repeated PCR results from multiple extractions, and independent replication of a proportion of the results in an independent laboratory. The likelihood of authenticity is affected by the history of the sample: how has it been handled? Also, the likelihood of an authentic result is linked to the likelihood that the bone samples contained DNA in the first place. Likely DNA survival can be evaluated firstly through the inspection of gross morphological preservation. However, a proper analysis of biochemical preservation is always to be recommended.

Extracting ancient DNA

There are several factors that need to be taken into account for when attempting to extract ancient DNA (aDNA). Preferably, the material from which the DNA is to be extracted should be well preserved, protected from contaminating DNA, and of sufficient quantity to last for independent replications. All these constraints have placed limits on the scope of ancient DNA studies.

That ancient DNA is fragmented and only present in minute amounts is an established fact. In a study of the relative likelihood of aDNA survival (Smith et al., 2003) it was shown that shorter DNA fragments have a greater chance of surviving compared to longer fragments. However, very few studies take into account these findings when setting out on the quest to retrieve ancient DNA.

The main problems when working with ancient DNA are the low amount of starting molecules and the presence of PCR inhibitors (Hagelberg and Clegg, 1991b; Tuross, 1994). There are thus two primary aims when extracting ancient DNA from bones to maximise the chance of a successful PCR. Firstly, as much of the target DNA as possible should be extracted. Secondly, the extract finally used in the PCR should be as pure as possible: unwanted biomolecules (inhibitors) co-extracted with the target DNA and other contamination such as other DNA (microbial or from humans handling the samples) can all have a negative impact on the chance of a successful PCR.

Early strategies to overcome these problems were to increase the amount of the enzyme Taq polymerase used in the amplification step, sometimes up to 10U per PCR reaction (Hänni et al., 1995), or to dilute the DNA extract, thereby diluting the co-extracted PCR inhibitors (Kaestle, 2000). However,
most of the efforts were focused on improving the extraction and purification procedures. Many different extraction techniques are in use: although some methods have been more influential than others, none has yet achieved general acceptance as being clearly superior to the others. Thus, there are almost as many ancient DNA extraction techniques as there are ancient DNA laboratories.

Of the many ancient DNA extraction protocols suggested over the years, a few have approached the problem in an unconventional manner, such as the use of the dye Dextran Blue as an inhibitor carrier (Kalmár et al., 2000), the use of pure water (Petrishchev et al., 1993) and the use of the somewhat unusual reagent Coca Cola (Scholz and Pusch, 1998).

The extraction techniques that have had most impact on general ancient DNA work have focused on purifying extracted DNA with silica binding (Boom et al., 1990; Höss and Pääbo, 1993; Yang et al., 1998), and decalcifying bone with EDTA (Hagelberg and Clegg, 1991b; Hänni et al., 1995; Yang et al., 1998).

Bone apatite as a DNA adsorber has been recognised in several studies (Götherström et al., 2002; Hagelberg and Clegg, 1991b; Salamon et al., 2005; Tuross, 1994). Taking the role played by bone apatite into consideration, most of the suggested extraction protocols fall within one of three categories: those attempting to release DNA by degrading the hydroxyapatite (Fisher, 1993; Hagelberg and Clegg, 1991b; Hänni et al., 1995; Krings et al., 1997; Yang et al., 1998), those attempting to release the DNA from the bone apatite by adding competing ions (Götherström and Lidén, 1996; Persson, 1992), and those that do not consider the bone apatite (Faerman et al., 1995; Höss and Pääbo, 1993; Kalmár et al., 2000; Meijer et al., 1992).

Another way of categorising the methods would be via the purification strategy employed. Most popular are methods based on phenol-chloroform extraction and alcohol precipitation (Hagelberg and Clegg, 1991b; Hänni et al., 1995), and silica binding (Höss and Pääbo, 1993; Yang et al., 1998). However other methods have also been suggested, like using chelex (Faerman et al., 1995), centricon filters (Anzai et al., 1999) and Dextran Blue (Kalmár et al., 2000). A commonly used method today is based on a combination of EDTA decalcification and silica purification (Krings et al., 1997; Yang et al., 1998).

The main method used in the studies presented in this thesis has been developed based upon a method called “target hooking” (Tofanelli et al., 1999), in which the DNA is extracted using biotinylated probes and magnetic separation. The DNA is released from the bone through ProteinaseK digestion, followed by a phosphate buffer extraction (Persson, 1992). The extract is cleaned through centrifugal filters at the same time the phosphate buffer is exchanged for a binding and washing buffer with a pH and salt concentration suitable for the next step: binding the DNA fragment to biotin-tagged probes. A short DNA molecule complementary to the specific target
DNA sequence is added to the extract (Figure 1a), and the sample heated up to denature the DNA molecules (Figure 1b). On cooling down, the double helix attempts to reform, and the biotinylated oligos hybridise with the target DNA (Figure 1c). Thereafter, streptavidin coated magnetic beads (Dynabead® M-280) are added. The biotin tags of the primer bonds strongly to the beads, and magnetic separation is used to retain the magnetic beads with the DNA (Figure 1d) and allow it to be washed.

The motivation for this technique is to allow a sample of valuable ancient material to be reused for amplification of a number of different specific targeted sequences, by simply hybridising the extract again with a new set of biotinylated probes. The technique also allows for effective cleaning of the extract in order to remove inhibitors, and perhaps most importantly; to concentrate as much DNA as possible in the PCR.

Figure 1. Extraction method mainly used for ancient DNA extraction in this thesis. (a) The biotinylated probe is added to the extract. (b) The DNA is denatured. (c) The probe is attached to target DNA strand. (d) The probe binds to the magnetic bead. (Figure assembled and kindly provided by Per Persson.)
Pyrosequencing

Once the DNA has been extracted and amplified, the next step is to determine the actual sequence. Standard chain termination DNA sequencing is the most commonly used technique, sometimes after cloning. However another method does exist, that is particularly suitable for short DNA fragments. The Pyrosequencing® technology is based on detection of flashes of light generated by pyrophosphate (PPi), which is released as a result of nucleotide incorporation in a real-time sequencing-by-synthesis reaction (Ronaghi, 2003; Ronaghi et al., 1998). This means that you can determine the sequence nucleotide by nucleotide as the reaction proceeds.

This technique involves sequencing of single stranded PCR products. To extract the required strand, one of the primers in the PCR reaction is biotinylated. The biotinylated fragments are immobilised onto sepharose beads and the double-stranded DNA is separated by NaOH denaturing, washed, and neutralised. Finally, the appropriate sequencing primer is annealed to the immobilised fragment.

In the pyrosequencing process, each nucleotide is added one at a time to the sequencing mixture (Figure 2). If the nucleotide being added is complementary to the nucleotide in the sequence being analysed, the polymerase will incorporate it and release pyrophosphate. The pyrophosphate is converted to ATP, which is then consumed by firefly luciferase to produce a flash of light. The amount of light produced is proportional to the number of nucleotides incorporated, so that e.g. if there are two identical nucleotides in the target sequence, double the amount of light will be produced. Enzymes in the reaction consume excess dispensed nucleotide so that successive dispensations can be clearly distinguished.

Pyrosequencing is ideal for ancient DNA applications, since short DNA fragments can be sequenced while directly screening for foreign DNA, and the reading of the sequence starts from the first base after the sequencing primer. The pyrosequencing principle has also been implemented in the very latest of sequencing techniques (Margulies et al., 2005). This technique has been applied to ancient DNA studies of a whole woolly mammoth (Mammuthus primigenius) genome (Poinar et al., 2006) and on a large-scale ancient DNA degradation investigation (Stiller et al., 2006).
Genetic markers used in domestication studies

Genetic markers applied in livestock domestication studies have been able to identify the wild progenitors of domesticates, as well as locations and timing of domestication events (Troy et al., 2001; Vilà et al., 2001). Here is a short description of the genetic characteristics used in such studies.
Mitochondrial DNA

The haploid mitochondrial DNA (mtDNA) has a maternal mode of inheritance, a fast rate of sequence evolution, and does not recombine (Pakendorf and Stoneking, 2005). It has therefore been chosen as a marker to study many domestic species. Animal mtDNA is a closed circular molecule, 15-20 kilobases (kb) long; it is fairly small compared to nuclear DNA. However, each cell contains up to 10,000 copies of mtDNA as opposed to one or two copies as for nuclear DNA (Robin and Wong, 1988). When studying material that has already lost much of its DNA due to degradation, this makes it an ideal target for genetic analysis (Bruford et al., 2003; Pakendorf and Stoneking, 2005; Savolainen, 1999).

Both the Cytochrome b region and the control region (also called the displacement-loop or D-loop) are used in livestock studies. The D-loop is a non-coding region with the highest rate of divergence. The high mutation rate means that there is a high degree of variation between species and even within species. In livestock studies, mtDNA is used to detect differentiation between domestic lineages and track down number and location of domestications, as has been done with cattle (Troy et al., 2001) and pigs (Larson et al., 2005). This high mutation rate (compared to nuclear DNA) also makes it possible to investigate if a population has undergone recent demographic expansion (Luikart et al., 2001).

The lack of recombination means that the mtDNA is inherited in an entity following a maternal mode of inheritance without being mixed; this means that each individual has a single haplotype. A mitochondrial haplotype is defined as a particular combination of genetic characteristics, while a haplogroup is defined as a monophyletic group of haplotypes sharing particular characteristics. Mitochondrial data within a species or a breed are difficult to display using standard rooted phylogenetic trees because of parallel mutation and recurrent mutations. Instead, a network diagram is often used where each haplotype is displayed as a node, with its genetic distance to its nearest neighbour represented by the length of the branch between them (Bandelt et al., 1999; Bandelt et al., 1995).

While the characteristics of mtDNA are useful for studying divergence times between wild and domestic forms under a relative short timescale, suitable for the periods of time involved in domestication, it does not say anything about paternal inheritance. Although less variable, nuclear DNA (nDNA) has shown itself to be very useful.

Nuclear DNA

The Y-chromosome, like mtDNA, is ideal for phylogenetic studies. Although less variable, this sex chromosome is paternally inherited and it does not undergo homologous recombination at meiosis. Analyses based on the
Y-chromosome in combination with mtDNA can reveal different patterns: while mtDNA will tell you something about the female lineage, the Y-chromosome provides information about male mediated gene flow. In livestock animals this can be very important, as has been shown in the study of male *Bos indicus* introgression in African cattle: African cattle mtDNA display a *Bos taurus* type (Hanotte et al., 2000; Loftus et al., 1994b). Although present in fewer copies compared to mtDNA, it has been shown that it is possible to analyse chromosomal markers in ancient DNA studies (Bunce et al., 2003; Huynen et al., 2003) and it is possible to target Single Nucleotide Polymorphisms (SNPs) (Paper III).

The other main class of nuclear markers used in domestication studies is the diploid microsatellites or Short Tandem Repeats (STRs) located on the autosomal chromosomes. They mutate fast compared to SNPs. STRs are short repetitive elements, and the number of repeats vary between alleles; they are also co-dominantly inherited (Ellegren, 2004). STRs have proved to be very useful in detecting diversity at the population and breed level, and in detecting admixture among livestock populations (Kumar et al., 2003; Loftus et al., 1994). They are also able to detect recent population bottlenecks and selection (Luikart et al., 1998). The number of bases that need to be analysed for microsatellites and their instability makes them difficult to apply to ancient DNA; however in one study of medieval cattle remains from Dublin it was possible to analyse three short STRs (Edwards et al., 2003) and thereby draw the conclusion that the animals were local rather than imported.

As the availability of polymorphic nuclear markers is increasing, an alternative approach to microsatellite analysis would be to target SNPs located on the autosomes, thereby obtaining information from both neutral and selected markers relating to selection.

**Domestication of common livestock animals**

When wild animals are domesticated, a limited number of individuals are removed from their wild population and become substantially isolated from the larger wild gene pool. This leads to a bottleneck effect, which in itself causes a reduction in genetic diversity. Furthermore, the breeding of the domesticated population can then be controlled through artificial selection of desirable and particular traits such as behaviour, milk yield, meat and coat quality or size. When artificial selection for a particular trait is being practiced, other tightly linked loci can also be selected: this leads to a further reduction of diversity and changes in allele frequencies through a selective sweep. Here, I describe some of what is known about the domestication of common European livestock, and how genetic analyses have contributed to this knowledge.
Caprines: sheep and goats

Caprines (sheep and goats) are versatile and hardy ruminants, and were the first livestock to have their physical appearance and genetic makeup altered by humans some 10 000 years ago. They belong to the order Artiodactyla, family Bovidae and tribe Caprini. They have a remarkable talent for surviving in the harshest climates, and could probably inhabit most of the mountainous regions in Europe and Asia if they were allowed to roam freely. Their success can probably be ascribed to their ability to adapt to different environmental conditions (Clutton-Brock, 1999). Archaeological studies show that sheep and goats were initially domesticated in the fertile crescent region of the Near East (Smith, 1995; Zeder and Hesse, 2000) and in the eastern margin of the Middle East, i.e. today’s Afghanistan, eastern Iran and Pakistan (Meadow, 1993).

The taxonomy and nomenclature of sheep and goats are complicated: early on, sheep were classified according to their diploid chromosome number. Goats are commonly classified according to the shape and curvature of their horns (Clutton-Brock, 1999). Recently, a number of molecular analyses of modern DNA from both goats and sheep have been published. To date, both species generally display one widely spread geographical mitochondrial lineage and several minor lineages.

Sheep

The home ranges of wild sheep today are the mountain regions of central Asia, extending west into Europe and east into America (Clutton-Brock, 1999; Ryder, 1984). Based on the karyotypes (Ryder, 1984) it was concluded that modern domestic sheep were domesticated from the Asiatic mouflon (Ovis orientalis), as they had the same chromosome number, but mouflon is now considered to be a relic of the first domestic sheep that were brought to Europe by early farmers around the 7000 BC (Clutton-Brock, 1999).

Initially two mitochondrial DNA haplogroups named A and B were identified in modern sheep from New Zealand (Wood and Phua, 1996); these main haplogroups were later also found in European breeds. The B haplogroup was widespread and the A haplogroup was mainly found in Asia (Hiendleder et al., 2002; Hiendleder et al., 1998a; Hiendleder et al., 1998b). More recent studies identified a third haplogroup denoted C, in the Middle east (Pedrosa et al., 2005) and in Asia (Guo et al., 2005). A fourth haplogroup D was found in Caucasus (Tapio et al., 2006). Haplogroups A to C have also been found in sheep from China (Chen et al., 2006). The estimated divergence time between these maternal lineages predates domestication which suggests multiple domestication events (Pedrosa et al., 2005).

Studies of these modern sheep mtDNA lineages (Pereira et al., 2006; Tapio et al., 2006) have shown the possibility of tracing trading routes and
the movement of animals (and perhaps people) during ancient times. Analysis of Iberian sheep has identified a Mediterranean trading route, and analysis of sheep from Caucasus, where the D haplogroup was found, has identified a route linking the Near East with North Europe.

In a study of indigenous sheep breeds from Portugal (Pereira et al., 2006), the C haplogroup was found, which had previously only been found in the Near East and Asia (Hiendleder et al., 1998b). The investigation of Iberian sheep revealed an unusually high genetic diversity, and since modern breeds of sheep were avoided in the analysis, it was concluded that this was a signature of ancient introduction, rather than modern introduction of Asiatic sheep. The interesting question is how the C haplogroup arrived on the Iberian Peninsula. As there are so far, no records of this “Asiatic” lineage on main continental Europe, it is most probable that the trading route went via the Mediterranean Sea, either over water or along the coast (Pereira et al., 2006). It would be interesting to study the genetics of African sheep breeds, since the Straits of Gibraltar can act as an alternative trading route linking Asia via Africa with Europe. The study of ancient DNA would add a temporal as well as a geographic framework to the origin of European sheep.

Recent analysis of Y-chromosomal haplotypes in domestic and wild sheep identified two paternal lineages present in modern domestic sheep. That the mouflon is a feral relict from the early domestic sheep was confirmed by the finding that the most common haplotype observed was fixed in Mouflon (Meadows et al., 2006).

**Goats**

The wild forms of goats inhabit the mountain ranges of Europe, Africa and Asia. The domestic goat (*Capra hircus*) has, with the help of humans, spread all over the world (Clutton-Brock, 1999; Pidancier et al., 2006). Traditionally, the classification of all living goats is done based on horn morphology; and wild goats are thereby divided into four groups: bezoars (*C. aegagrus*), turs (*C. cylindricornis*), markhor (*C. falconeri*) and ibex (*C. ibex*) (Clutton-Brock, 1999; Mason, 1984). Two wild species have been suggested to be the ancestor of domestic goats: the bezoar and the markhor (Clutton-Brock, 1999). This theory has been strengthened by genetic analysis (Manceau et al., 1999; Mannen et al., 2001; Takada et al., 1997). The markhor is found on mountains from east Kashmir to the Hindu Kush and south to Quetta in Baluchistan; and the bezoar is found in the mountain ranges of western Asia. Their geographical distribution correlates with the area where it is suggested that goats were first domesticated.

Studies of modern domestic goat mitochondrial DNA have so far identified six mtDNA lineages (Joshi et al., 2004; Luikart et al., 2001; Sardina, 2006; Sultana et al., 2003). Initially lineages A, B, and C were identified in 88 goat breeds from the old world (Luikart et al., 2001). Mitochondrial lineage A predominates, and is believed to derive from the initial domestication
event. Lineage B was found in India, Malaysia, Mongolia and Pakistan; and lineage C was found in Slovenia, Switzerland and Mongolia. Later investigations identified lineage C in goats from Pakistan (Sultana et al., 2003) and two additional lineages, D and E, were identified in Indian goats (Joshi et al., 2004). These lineages (D and E) might correspond to the suggested centre of domestication in eastern Pakistan (Meadow, 1993). Lineages A, B, C and D have also been found in Chinese goats (Chen et al., 2005).

Recently an investigation of goat breeds from Sicily identified three domestic goat breed haplotypes that clustered with the wild Bezoar (C. aegagrus). This could either be a new mtDNA lineage, or be the result of historical introgression from wild goats (Sardina, 2006).

The several mitochondrial haplogroups detected in domestic goats in different locations and the star-like pattern observed in the network diagram constructed is in accordance with goats being domesticated on more than one occasion (Luikart et al., 2001). The most recent common ancestor of these domestic goat lineages was dated to between 200 000 – 300 000 years ago which implies that the existence of the different lineages in domesticated goats comes from domestication of different wild populations. The relatively weak phylogeographic structure found among goats is probably a sign of the high mobility of the species, and is probably induced by human movements in historic times (Luikart et al., 2001).

A more detailed picture of goat origins (evolution of the genus Capra) was reached through combining mtDNA and Y-chromosomal DNA from domestic breeds and wild species (Pidancier et al., 2006). Typing of Y-chromosomal data revealed two well defined clades. One clade consisted of domestic goats (C. hircus), bezoar (C. aegagrus) and the markhor (C. falconeri): this supports the view that domestic goats originate from one or both of these wild species. All other wild species belonged to the other clade. It is interesting to note that horn morphology mostly agreed with the Y-chromosome results. Two clades were also found when analysing mitochondrial DNA, but the species grouping to the mtDNA clades were different from those grouped in the Y-chromomose investigation. All capra species but one belonged to the same mtDNA clade. The discrepancy between the mtDNA lineages and the Y-chromosomal lineages are explained by the existence of two isolated ancestral populations and hybridization between these two taxa.

Pigs

Domestic pigs belong to the order Artiodactyla and family Suidae; they descend from the wild boar Sus scrofa (Clutton-Brock, 1999; Epstein and Bichard, 1984). Between 16-25 subspecies of Sus scrofa have been described (Clutton-Brock, 1999; Epstein and Bichard, 1984; Fang et al., 2006). Wild boar are widely distributed throughout the Old world, overlapping with
their domestic form; hence, detecting pig domestication based on geographical location is difficult (Albarella et al., 2006).

Pigs are quite different in their behaviour and biological features from the other members of the even-toed ungulates such as caprines and cattle. A wild adult boar can be very dangerous, but the piglets can easily be tamed. They are also omnivorous and eat virtually anything that humans do. Pigs build nests and have large litters of piglets and, unlike other livestock, are born physically weak and thus remain around their nest for several weeks after birth. It is also easy to adapt pigs to certain eating and sleeping regimes (Clutton-Brock, 1999). Thus, pigs are very adaptable and it is easy to see how human could have initiated a closer and more controlled relationship with wild boars, since like dogs they probably were drawn to human settlements. Both in modern and ancient times, pigs have been kept under fairly loose control, foraging freely in forests (Albarella et al., 2006; Clutton-Brock, 1999) which means that hybridisation and introgression between the wild and domestic forms further complicates the detection of prehistoric pig domestication.

Thus, it is difficult to identify wild and domestic forms of pigs in the archaeological record. It is also likely that pig domestication was a very gradual process, involving intermediate stages where genotypic and phenotypic changes were minimal. It becomes clear that a number of techniques and approaches are needed when defining and detecting pig domestication (Albarella et al., 2006). The osteological material is fragmented, and since pig domestication probably involved different types of human management it is difficult to pinpoint when and if the process started at a specific site. A prehistoric site that has provided a long enough temporal context to study the domestication of pigs is Çayönü Tepesi in south eastern Turkey (Ervynck et al., 2001). Although this site is one of the earliest where pig domestication has been detected, it cannot be ruled out that other independent domestication events happened as well.

Although local domestication has been suggested for pigs, the classic view was that the domestication of pigs was geographically limited (Childe, 1925). However, new archaeological and genetic evidence (Giuffra et al., 2000; Larson et al., 2005) supports the suggestion that pigs could have come under the influence of human control in more than once place and during different times in prehistory (Zvelebil, 1995).

European pig domestication has been investigated through analysis of modern wild boars, modern pigs and recent historical remains from around the globe. The results support the multiple domestication hypothesis and that, during historic times, European pig breeds have been crossbred with Asian pigs (Giuffra et al., 2000; Kijas and Andersson, 2001; Larson et al., 2005). Two European (D1 and D4) and one Asian (D2) mitochondrial clades were found when European and Asian wild boar and domestic pig breeds were analysed. European domestic pigs and wild boars from Europe and
Israel cluster in the D1 lineage. The D2 lineage was made up of Japanese wild boar, a Chinese domestic breed and some of the European domestic breeds. The D4 lineage was detected in Italian wild boar (Giuffra et al., 2000; Kijas and Andersson, 2001; Larson et al., 2005). A study of mtDNA diversity in European and Asian pigs suggests a population expansion prior to domestication (perhaps beginning after the last glaciation period), and also showed introgression of Asian pigs into European pig mtDNA: a Y-chromosome analysis is suggested to allow male-mediated gene flow to be assessed (Fang and Andersson, 2006).

Ancient DNA from prehistoric pig remains could provide a geographic location and a temporal framework for the apparently complicated pig domestication process. However, the only ancient DNA investigations to date have been done on Asian pig remains (Watanobe et al., 2002).

Horses
Domestic horses belong to the order Perissodactyla and the family Equidae. In spite of the great morphological variation displayed among different breeds, types or groups of domestic horses, they all belong to a single species; Equus caballus. The Equid family can be divided into two groups: stenoids (zebroid, i.e. zebras and asses) and caballoid (true horses). These are believed to have diverged some time between 1-2 and 4 million years ago (Forstén, 1992; Jansen et al., 2002; Oakenfull, 2000). This implies that domestic horses have their origin in a caballoid form.

The determination of the wild progenitor of the domestic horse has occupied researchers for many years. The only extant wild horse is the Equus ferus przewalskii, the Prezwalski wild horse of the Mongolian steppe (Clutton-Brock, 1999). The last individuals were captured in the middle of the 20th century, and were subsequently reintroduced following their extinction in the wild. It has been suggested that this is the ancestor of domestic horses. However, both mtDNA and Y-chromosomal data suggest that this might not be the case (Ishida et al., 1995; Lindgren et al., 2004; Oakenfull, 2000; Wallner et al., 2003). Prezwalski horses are closely related to domestic horses but appear not to be their direct progenitor; this appears instead to be some other extinct Equus ferus, yet to be identified.

As with the other four main domestic animals sheep, goat, pig and cattle, the domestication of horses has been investigated with the help of mtDNA variation (Jansen et al., 2002; Lister, 1998; Vilà et al., 2001). The most striking difference between horses and other domesticates is the high mitochondrial divergence and lineage divergence found in horses (Jansen et al., 2002; Vilà et al., 2001): this has been found even in small horse populations (Wang et al., 1994). The diverse state of wild horses is perhaps not surprising, considering the extensive paleontological record of wild horses: they must have been domesticated on a number of occasions, and individuals from a wide
geographic region must have been incorporated into the early stock of domestic horses (Lister, 1998). Ancient DNA from Alaskan wild horses dated to 12 000 – 28 000 BP confirms this statement (Vilà et al., 2001). The ancient wild horses displayed a high diversity: none of the samples had the same haplotype and they were all novel, although one of the two clades found clustered with domestic horses. Therefore it appears that modern horses do not have a single origin; they descend from multiple maternal lineages and it is likely that different populations were involved. However, this high mitochondrial diversity is in striking contrast to the very low diversity observed in the Y chromosome (Lindgren et al., 2004). This suggests that the number of breeding males contributing to the modern horse population must have been low in contrast to the number of females, which fits with the way that horses are bred in captivity but would also agree with the way that horses live in the wild where a single stallion controls a harem with many females (Jensen, 2002).

The domestic cow

The wild progenitor of European cattle Bos taurus was the now-extinct wild ox Bos primigenius (aurochs). This large mammal roamed a wide geographic area of Europe, northern and western Asia and North Africa. The last herd of aurochs was kept in a Polish game reserve: the written sources tell a sad story of how the number of animals decreased until 1627 when the last aurochs, a cow, was shot (Bökönyi, 1974; Ekström, 1993; Zeuner, 1963). It is suggested that a mammal with such a large range should be made up of a number of populations creating a cline across its distribution area. However, it is difficult to make any such conclusions based on the fragmentary osteological record. Grigson (Grigson, 1974; Grigson, 1978) presents a summary of the early osteological analyses.

During the 19th century, two forms of Bos primigenius distinguished by size were recognized in Europe. Ancient domestic cattle remains were also divided into two forms based on size and referred to as brachycere and primigene. Based on this evidence it was assumed that two separate wild ancestors must have been domesticated. However, it was later concluded that the difference between the two forms was not large enough to exclude a monophyletic origin with the size difference being due to sexual dimorphism. This explanation was supported by the fact that the differences in the two wild forms were very similar to the differences seen between the sexes in domestic cattle. The small form of Bos primigenius was simply the female of the species, and this is today the accepted view.

The earliest documentation of the aurochs is the Palaeolithic cave paintings from continental Europe. One of the most skilfully painted caves is Lascaux in south west France (Zeuner, 1963). Although one cannot be cer-
tain how representative the pictures are, these paintings have been used to gain an impression of the appearance, colouring and stature of the Palaeolithic aurochs. The bulls were black with a white stripe along the back, they had light coloured curly hair between their horns, and their muzzle was grey or white (Zeuner, 1963). The cows were mostly reddish in colour, but some had the colour of the bulls with a saddle of lighter brown colour (Ekström, 1993; Zeuner, 1963). However, the paintings also show a good deal of variation in colouration and horn shapes.

In the archaeological record, remains of the aurochs dominate in central and eastern Europe during the Neolithic period, where 60-70% of identified fragments belongs to this animal (Zeuner, 1963). Although the osteological remains are fragmented and few large scale surveys exist, the aurochs appears to have been a morphologically diverse animal. The dimensions of the bones were said to show signs of crossbreeding and it appears that the aurochs was still hunted when domestic cattle were also being kept (Bökőnyi, 1974; Zeuner, 1963). By early Bronze Age (1800 BC), the finds of aurochs bones decline substantially (Bökőnyi, 1974; Zeuner, 1963).

The archaeological evidence available indicates that cattle were domesticated in the Eastern Mediterranean and Near Eastern region, about 10 000 years ago. There are early finds of domesticated bovid bones at the archaeological site Çatalhöyük in the Middle East dating to around 7000 BC (Russell and Martin, 2000). The assessment of the remains from Çatalhöyük as domesticated or wild is based on size, and on the gradual reduction in size of bovid remains at this site (according to discussion with Louise Martin, 2006). The strongest direct evidence for the presence of domestic cattle comes from a site on Cyprus (Shillourokambo) dated to 8200-7500 BC (Vigne et al., 2000). Aurochs are not considered to be part of the contemporary indigenous fauna, which means that bovid remains found here must have been domesticated cattle brought to the island by humans. Compared to other domestic animals, cattle require considerable attention and care: physically they are very sensitive, and need to maintain their cycle of eating and ruminating (Clutton-Brock, 1999). Their sheer size must have been a great problem in the beginning, and supply of fodder in permanent settlements must have caused logistical difficulties. Therefore it is likely that the relation between cattle and humans began with humans attracting them through clearing pastures and possibly supplying them with salt (Clutton-Brock, 1999). Humans keeping herds of cattle in this manner would no doubt also attract wild aurochsen which would take advantage of the arrangement.

A number of routes have been suggested for the introduction of domestic cattle into Europe from the Near East. A Mediterranean route (Zilhao, 2001), which may also have included Africa (Cymbron et al., 1999) extended to the Iberian peninsula. Another route is proposed following the Danube into central Europe. The appearance of domestic cattle on the European continent
coincides with Europe’s neolithisation and the proposed human migration associated with it (Renfrew 1987); and by 6000 BC cattle had become of great economic importance in the Linearbandkeramik (LBK) culture (Benecke, 1994). From that time domestic cattle and aurochs can be found together at archaeological LBK sites in Central Europe: this was taken as evidence for local domestication of aurochs in Europe (Bökönyi, 1974; Zeuner, 1963), although this is at odds with the common view that European cattle descend from animals imported from the south-east (Benecke, 1994).

One might wonder what the initial motivation was to attempt to domesticate such a large animal as the aurochs, which is much harder to care for than the other domesticated animals such as pigs and goats. The answer probably lies in the versatility of the cow: it can provide meat and milk, the dung may be used to fertilise crops, fuel and as a building material, and the hide can be used to make leather articles. At a later stage, the cow also became a beast of burden.

Domestic cattle were initially divided into two groups based on the absence or presence of a hump, respectively *Bos taurus* and *Bos indicus*. It was originally believed that these two types of cattle derived from a single domestication event some 10 000 years ago. However, mitochondrial DNA analyses of the two taxa indicated a divergence time 200 000 years ago, and it was therefore concluded that these two types of cattle were domesticated on separate occasions, probably from separate subspecies of *Bos primigenius* (Bradley et al., 1996; Loftus et al., 1994).

![Figure 3. Geographical distribution of taurine mtDNA haplogroups T, T1, T2, T3, T4 (displayed as networks), and Y chromosomal haplotypes Y1, Y2. Figure kindly provided by Dr. D.G. Bradley.](image-url)
Mitochondrial DNA analysis of modern *Bos taurus* has identified five geographically distributed haplogroups designated T, T1, T2, T3 and T4 (Loftus et al., 1994; Mannen et al., 1998; Troy et al., 2001) (Figure 3). The dominant haplotype cluster in Europe is T3; this cluster is also found in the Near East where additionally clusters of T, T1 and T2 are found. The dominant clusters in Africa and the Far East are T1 and T4 respectively; it is suggested that these originate from wild oxen domesticated separately in those areas.

The pattern on the European mainland, with T3 dominating, and the higher diversity in the Near East, consisting of T3, T, T1 and T2, has been attributed to the proposed domestication event in the Near East from where cattle with the T3 haplotype were brought to Europe. When DNA from British bone finds of *Bos primigenius* were compared to the modern sequences it was found to be genetically different and it was concluded that they could not have been an ancestor of European domesticates (Bailey et al., 1996; Troy et al., 2001), implying that the maternal lineage of domestic cattle in Europe originates from the domestication event in the Near East rather than local domestication of wild European aurochsen. It is suggested that the mtDNA diversity seen in today’s cattle has accumulated since the Neolithic times (6000 years ago (Bollongino et al., 2006) when domestic cattle was introduced.

These findings, however, only give part of the picture. Male-mediated gene flow is not captured by analyses of mitochondrial DNA. When the Y-chromosome was investigated (Paper III) polymorphisms divided cattle from Europe and Anatolia in two haplotypes denoted Y1 and Y2, distributed in a north-south gradient (Figure 3). The southern haplotype Y2 is shared between Near Eastern cattle and European cattle; the northern Y1 haplotype seems not to be shared and suggests aurochs introgression into domestic stock. Typing of ancient specimens for the polymorphism diagnostic for distinguishing between the two haplotypes supports this scenario.

European cattle was domesticated in the Near East and brought in to the European continent from there. In Europe hybridisation with local aurochsen took place, thus it appears that today’s cattle descend from Anatolian as well as European aurochsen.
Research aims

1. To develop methods and markers suitable for analyses of ancient cattle remains.
2. To investigate whether European early domestic cattle did hybridise with local aurochsen, and whether there is a genetic continuity between modern and ancient cattle.
3. To evaluate the theory that cattle were domesticated only once in the Near East by searching for signs of local domestication on the European mainland.
4. To measure genetic diversity in ancient cattle populations in order to trace routes of introduction of domestic cattle from the Near East.
Investigations

Paper I: Prehistoric contacts over the Straits of Gibraltar indicated by genetic analysis of Iberian Bronze Age cattle

Genetic investigation of modern cattle mitochondrial DNA from Europe and Africa has revealed certain genetic patterns. These differences have been described and grouped into haplogroups denoted T, T1, T2, and T3. Genetic analyses of ancient (pre-domestic) bone specimens from England have furthermore identified a *primigenius* type (Bailey et al., 1996; Troy et al., 2001). The most common haplogroup in Europe is T3, this haplogroup together with T and T2 is common in cattle from the Near East. Haplogroup T1 is common in Africa, where it may also have originated (Bradley et al., 1996; Troy et al., 2001). This African haplogroup has also been observed in extant cattle from Iberia and Latin America (Beja-Pereira et al., 2003; Cymbron et al., 1999; Magee et al., 2002; Miretti et al., 2002). It has been presumed that the bulk of cattle on the Iberian peninsula, like those in the rest of Europe, have their origin in the Near East. The African feature/contribution in Iberia is believed to have partly been introduced in AD 710 (Cymbron et al., 1999) with the Muslim expansion and also in more recent time during the 1960s and 1970s (Beja-Pereira et al., 2003; Beja-Pereira et al., 2002).

In this paper we investigate a possible earlier contact between Iberia and Africa through the analysis of ancient DNA from Iberian cattle remains dated to the Bronze Age.

Material and methods

Forty-seven domestic cattle teeth and bones were sampled from Europe: 14 Iberian Bronze Age specimens were collected from the Portalón cave in northern Spain, 16 additional specimens of various ages from other sites in Spain were also collected, and finally 17 Early to Late Neolithic Central European (German) samples were also included in the analysis. General guidelines to avoid contamination and reach authentic results were followed. DNA was extracted from pulverised bone and teeth using a method where the DNA is released from the bone apatite complex by using a phosphate...
buffer. The cattle control region was amplified in three overlapping fragments and sequenced using Pyrosequencing® and standard chain termination DNA sequencing.

Results and discussion

Of the 47 samples analysed, 30 gave reproducible sequence results and were included in the phylogenetic analysis. Eight of the German samples and 19 of the Iberian samples were identified as belonging to haplogroup T3. One Iberian sample belonged to haplogroup T. These findings are in agreement with the Near Eastern origin of European cattle. However, two specimens from Iberia, radiocarbon dated to the Bronze Age, displayed deviating haplotypes. One sample grouped with previously published aurochs sequences, and one grouped with the African haplogroup T1.

Sample MAD15, a permanent upper left premolar radiocarbon dated to 1740 cal B.C (Ua-22027), was of the \textit{primigenius} haplogroup. Sample MAD16, a permanent lower right molar radiocarbon dated to 1800 cal B.C, belonged to the African T1 haplogroup.

The unexpected identification of an aurochs haplotype in the Bronze Age material assemblage suggests a tempting speculative scenario. Since the material was classified among 1000 bovid remains as being of domestic \textit{Bos taurus} (Clark, 1979), it could be suggested that this is a sign of local domestication or backcrossing occurring between domesticates and aurochs females. However, it should be appreciated that, with the material from the Bronze Age section being fragmented, many diagnostic morphological features normally used to distinguish between wild and domestic forms are not obvious. Sample MAD15 could instead represent a hunted wild animal: in favour of this interpretation are the low-frequency finds of hunted red deer and wild boar at the Portalón site.

More interesting is the identification of T1; this is to our knowledge the earliest identification of this haplogroup on the Iberian Peninsula. If this haplogroup is indeed of African origin (Troy et al., 2001), it would mean that there is a contact between this part of Europe and Africa during the Bronze Age or earlier. Another possibility is that the T1 haplogroup was present among the aurochs population from which these cattle were domesticated. However, the European aurochs remains so far analysed only display the distinct \textit{primigenius} haplogroup (Bailey et al., 1996; Troy et al., 2001). To resolve this would involve analysing morphologically classified aurochs remains and at present it is not possible to either support or dismiss the possibility of local aurochs domestication or introgression on the Iberian Peninsula. Although a subject beyond the scope of this thesis, another explanation that could be proposed would be that the haplotype is a result of recurring mutations. However, if the African origin of the T1 haplogroup is accepted, as supported by microsatellite analysis of African cattle (Hanotte et al.,
2000), this investigation shows that by analysing ancient cattle remains it was possible to detect a signal of an interchange between Africa and Europe predating AD 710.

Paper II: Tracing genetic change over time with nuclear SNPs in ancient and modern cattle

Ever since the early days of DNA analysis of ancient material, maternally inherited mitochondrial DNA (mtDNA) (Pääbo, 1988a) has been the target molecule. This is due to a number of practical and technical reasons; for instance it is maternally inherited and therefore does not recombine. It is also present in a very high copy number per cell compared to nuclear DNA, and evolves quickly. The mode of inheritance and evolutionary rate are useful in evolutionary and population genetic studies, especially when it comes to studies of animal domestication (Loftus et al., 1994; Troy et al., 2001) and where genetic change is to be followed over time (Hadly et al., 2004; Shapiro et al., 2004).

However, there are drawbacks to using mtDNA. One such drawback is that it only traces the maternal line, and does not detect male mediated gene flow or selection of traits for breeding purposes. In order to assess the feasibility of working with chromosomal DNA, and at the same time contribute to a more detailed picture of cattle keeping and cattle breeding in prehistory we developed a method of analysing ancient nuclear DNA.

Nuclear DNA is present in ancient specimens (Bunce et al., 2003; Huynen et al., 2003), and microsatellites have been applied on cattle bones dated to the 12th century (Edwards et al., 2003). However, microsatellite analysis has some difficulties, (stutter band and jumping PCR) when applied to highly degraded ancient DNA. Our approach involved targeting short fragments of informative DNA in the form of Single Nucleotide Polymorphisms (SNPs) linked to phenotypic traits likely to have been desirable. The SNPs were typed using pyrosequencing.

Material and methods

The initial analysis was conducted using a single SNP, on material from the major medieval town Lądöse in south-west Sweden. A coding locus was targeted: the Melanocortin receptor 1 (MC1R) that influences coat colour contains a SNP, T/C, which results in dominant black coat colour (Klungland et al., 1995). PCR primers were designed close to the SNP resulting in fragment lengths of 40bp including primers. The first results demonstrated the viability of this approach and the analysis was expanded with more material and two additional markers.
In the end, 123 archaeological cattle bones were selected from ten Swedish, one Lithuanian and one Hungarian site. Modern samples were also analysed for comparison purposes, with 93 genomic DNA samples from 19 breeds originating from Sweden, Lithuania and the European continent. The additional markers were the Leptin \((LEP)\ C/T\) polymorphism associated with body fat deposition in beef cattle and with milk and milk protein yield (Buchanan et al., 2002; Buchanan et al., 2003), and the Toll-like receptor 4 \((TLR4)\) with the SNP C/T, a candidate gene for disease resistance (White et al., 2003).

DNA from the ancient samples was extracted at least two times and for every four samples extracted two blank extractions were included. The nucleotide dispensation order for a particular SNP analysis was automatically calculated by the PSQ™ 96MA SNP software (Biotage AB, Uppsala). The SNPs were analysed using the PSQ™ 96MA, and the SNPs were scored automatically by the PSQ™ 96MA SNP software and checked by eye.

Archaeological material producing homozygous results was amplified and typed a minimum of four times in order to detect possible allelic dropout. A sample was accepted as being a heterozygote if the heterozygous state was detected at least twice or if the two different homozygous alleles were detected at least twice each.

Results and discussion

Twelve of the 123 samples did not yield any DNA, or did not reach the criterion of two successful PCRs from each extraction in any of the SNPs.

A logistic regression of the three coding loci displays a decrease of individual heterozygosity over time especially since Medieval time; this is something one can expect as cattle breeding becomes more intensive and organised (Myrdal, 1994).

This work was interesting in context of this thesis since it involves the development of a method for ancient SNP typing. The amplification success was high: the Hungarian material came from an open air site almost 4000 years old, and the fact that all three markers were successfully typed in this material indicates the robustness of the strategy we applied. The successful outcome of the analysis is due to the short target sequence, and the efficiency of the pyrosequencing typing method. The results show that SNP assays can successfully be applied to ancient material and that it should be possible to expand the study to even older material. Thus, effective SNP analyses should enable investigations of ancient remains covering a long time frame, so that detailed questions may be addressed such as breeding for specific traits, interbreeding of domestic and wild stock, or transport of livestock throughout history and prehistory.
Paper III: Cattle domestication in the Near East was followed by hybridization with aurochs bulls in Europe

The wild progenitor of European cattle *Bos primigenius*, or aurochs once roamed a wide geographic area of Europe, northern and western Asia and North Africa. The last record of the aurochs dates to 1627 (Bökönyi, 1974; Ekström, 1993). Because domestic cattle and aurochs evidently did exist side by side, both local domestication and interbreeding between the two bovids have been suggested (Bökönyi, 1974; Zeuner, 1963). Genetic analysis of mitochondrial DNA from both modern and ancient specimens (Troy et al., 2001) and archaeological evidence points towards a Near Eastern origin of European modern cattle dated to about 10,000 years ago (Clutton-Brock, 1999). Thus, looking at the maternal lineage of domestic cattle in Europe no local introgression from wild European aurochs took place.

Returning to the information from the archaeological record; bones morphologically classified as those of aurochs and domestic cattle have been found together at a number of prehistoric sites in different locations (Bökönyi, 1974). The aurochs did survive long into historic times and it is possible that, either by accident or by human design, they did hybridise with domestic stock. The nature of early cattle keeping probably involved free roaming herds and it seems possible that wild aurochs bulls might have interbred with domestic cows. The mitochondrial DNA surveys done so far would not be able to detect such male-mediated gene flow. Thus, the paternally inherited Y chromosome could bring additional information and reveal patterns that mitochondrial DNA cannot detect. This may in turn enable a wider understanding of how cattle were once introduced to Europe. We therefore developed Y-chromosomal markers on modern material and subsequently applied them to both ancient and modern material. Y chromosome sequences were screened for Single Nucleotide Polymorphisms (SNP) and the markers detected in the modern material were then analysed in ancient material. SNP analysis was chosen since it allows for short strands of DNA to be targeted, maximising the chance of success in highly fragmented ancient DNA.

Material and methods

Initially 3.5 kb of intronic Y chromosome sequences from 20 modern European bulls were screened using the method as described in Hellborg and Ellegren (2003). Polymorphic sites were identified from sequence alignments. Two haplogroups were identified, denoted Y1 and Y2, and the analysis was expanded further to include 180 bulls from 48 breeds in total, 45 European breeds and three Anatolian breeds.

Thirty-nine ancient *Bos* samples from Italy, Austria, Germany and Sweden dated to between 5,000 - 10,000 BP were then analysed for the SNP
marker that distinguishes between haplogroups Y1 and Y2. Pyrosequencing was used for SNP typing.

Results and discussion

Two co-segregating sites were identified in the modern material, an A/C SNP in UTY intron 19 and a 2bp insertion/deletion polymorphism in ZFY intron 5. The markers formed two haplotypes denoted Y1 and Y2, which were distributed with a clear geographic structure amongst the samples studied. Y1 dominated in northern European breeds, Y2 was found with a high frequency in Iberian, French, and Swiss breeds and was fixed in Italian and Anatolian breeds, while Central European breeds showed intermediate haplotype frequencies. Analysis of *Bos indicus*, bison and gaur revealed that Y1 is the derived form.

There are several possible hypotheses to explain the north-south gradient found in the distribution of the haplotypes. Clines in allele frequencies can be created by adaptive genetic differences along latitudinal gradients or between geographic areas as a response to selection (Beja-Pereira et al., 2004). This could potentially manifest itself in Y chromosome haplotype frequencies. However, since most genes on the mammalian Y chromosome are involved in male reproduction, this does not appear to be a very likely explanation as it is not clear how artificial selection during domestication would involve different selection regimes related to such traits across a geographical cline. Another explanation is that the two haplotypes represent two different migrations of stock from the Near East, one northern route and one southern Mediterranean route. Sampling and bottleneck effects could have affected the different occurrences of Y1 and Y2 along these routes; however, the absence of Y1 in modern Anatolian breeds is not explained by this scenario since it implies that both Y1 and Y2 were part of the original population in Anatolia. It should be noted however that only 15 Anatolian animals were analysed.

We suggest a plausible scenario in which the first cattle brought from the Near East carried Y2. Spreading north through Europe, these animals hybridised with local aurochs bulls carrying Y1, leading to subsequent introgression of this haplotype into the breeding population. If this was the case we expect to see the northern Y1 haplotype at high frequency among European aurochs.

The introgression hypothesis was tested through the analysis of ancient bone samples of European *Bos* from Germany, Sweden, Italy and Austria. The material dates from Late Pleistocene to Iron Age (from older than 9500 BC to 100 BC). The UTY SNP distinguishing between Y1 and Y2 was successfully amplified in 21 samples; all but one sample showed the SNP diagnostic for the Y1 allele. Eleven samples were classified as being aurochs based on morphology or due to a pre-domestication date.
This analysis shows that a SNP diagnostic for Y1 was present at a high frequency among aurochs and early domestic cattle. However, we have no knowledge of what the remaining 3500 bp look like in aurochs and ancient cattle. Thus, we cannot be certain whether the observed Y1 type in the ancient specimens is the same type seen in the modern breeds, or a new Y type.

Although the Y1 haplotype is the derived form, the observation of Y1 in the ancient specimens rejects an origin in a post-domestic mutation from the Y2 haplotype becoming fixed in a domestic population migrating northward. The presence of Y1 in central European modern cattle is not a recent effect of cattle breed structure or artificial selection, since it is present in early domestic cattle from Germany. Looking at the Y chromosomal data, it appears that modern domestic cattle from northern Europe have a closer affinity with local aurochs than with south European and Anatolian cattle.

Our results therefore point towards a more complex scenario of cattle domestication in Europe. This scenario entails local hybridisation (in areas other than the suggested centre for domestication) with wild ancestors.

Paper IV: Identification of X- and Y-specific single nucleotide polymorphisms (SNPs) and insertion/deletions diagnostic of taurine Bos taurus and indicine Bos indicus cattle

There are two types of domestic cattle, Bos taurus (taurine) and Bos indicus (indicine). Bos taurus are the European cattle once domesticated in the Near East but also present in Africa (Loftus et al., 1994; Troy et al., 2001). The most frequently observed mitochondrial haplogroup in African taurine cattle is T1, and it may have originated on this continent (Bradley et al., 1996; Troy et al., 2001). The indicine cattle were domesticated on the Indian subcontinent (Bradley et al., 1996; Hanotte et al., 2002; Loftus et al., 1994). Admixture between taurine and indicine cattle occurs both on the African continent and in the Near East (Freeman et al., 2006b; MacHugh et al., 1997). In studies of mitochondrial DNA, African cattle are assigned to the taurine group. However, it is known that African cattle have different levels of indicine input.

Newly developed X-chromosomal markers and previously published Y-chromosomal markers (Paper III) discriminating between taurine and indicine cattle were analysed in order to evaluate the admixture between the two types of cattle.
Material and methods

Exon and intron organisation, different exonic regions and sequence variation for two X-chromosomal fragments (KZFX3 and KZFX5) were identified by aligning bovine ZFX and ZFY cDNA with human genomic sequences of the same region. Primers were designed to have the 3’ ultimate base at the identified markers. Other X-chromosomal primers were developed according to published method (Hellborg and Ellegren, 2003; Lyons et al., 1997). Only females were used for the X chromosome amplification to avoid unwanted amplification of the Y chromosome, since the primers were not entirely X chromosome specific.

Twenty European taurine cows and three indicine cows were screened. Nine X chromosomal fragments were targeted (DBX4, DBX5, DBX9, DBX12, SMCX2, SMCX5, SMCX14, KZFX3 and KZFX5) as well as 5 Y chromosomal markers (Paper III) previously shown to separate between indicine and taurine cattle.

The sites variable between the taurine and indicine cattle were investigated in 57 African bulls and 33 African cows. The African samples were made up of six breeds: Raya Azebo (10 bulls, 5 cows), Nguni (9 bulls, 5 cows), Caprivi (8 bulls, 7 cows), Danakil (9 bulls, 6 cows), Kilimanjaro zebu (10 bulls, 5 cows) and South Kavirondo zebu (10 bulls, 5 cows).

Results and discussion

About 3.5 kb X chromosome sequences were amplified for both *Bos taurus* and *Bos indicus*, and differences between the two taxa in the form of three SNPs and three indels were identified. Of these, two substitutions were A/G transitions and one an A/T transversion. Two indels in the KZFX3 were insertions of 4bp and 6bp in the taurine lineage, while one indel in the KZFX5 fragment is a 1bp deletion in the taurine lineage compared to the indicine lineage. The polymorphic sites separating indicine and taurine types were present in the African samples, in pure types as well as in recombined types. All the African breeds analysed had a bias towards indicine types in the Y chromosome rather than in the X chromosome. The east African breeds had higher levels of indicine admixture compared to southern breeds.

Three African breeds (Raya-Azebo, Danakil and South Kavirondo Zebu) had males with exclusively indicine origin, while one breed (Nguni) had males with exclusively taurine origin. Two breeds, Caprivi and Kilimanjaro Zebu had mixed male origin.

The Kilimanjaro Zebu animals investigated exhibited no pure taurine type in the X chromosome. All other breeds had X haplogroups of mixed taurine and indicine origin or taurine origin only. The Nguni breed seemed to have exclusively taurine origin for both males and females; however one sample displayed a recombinant X chromosome.
Indicine introduction in African cattle appears to have taken place largely through male transmission, the bias toward indicine markers being observed on the Y rather than on the X chromosome clearly visible in our results. Although the indicine genetic influence of African cattle is ancient and extensive, it is interesting to note that it has not completely exhausted the taurine alleles. This can probably be attributed to different breeding strategies: African taurine cattle are known to be trypanotolerant while indicine cattle are more adapted to cope with heat and drought (Hanotte et al., 2000).

A parallel can be drawn between the situation in Africa, where the taurine cattle received genetic input from indicine cattle, and the hypothesised situation in post-domestication Europe where domestic cattle receive genetic input from wild aurochs bulls. In neither case is the hybridisation visible the mitochondrial. Therefore, techniques that are capable of tracing indicine genetic input in African *Bos taurus* may potentially be used in ancient DNA studies to trace post-domestication aurochs introgression in domesticated cattle. This would however be a challenging project involving large scale sequencing of aurochs remains to establish suitable genetic markers. The techniques for these kind of ancient genome analyses are now becoming available (Noonan et al., 2005; Poinar et al., 2006). Markers in the form of SNPs are ideal since analyses of short fragments are suitable for fragmented ancient DNA.

Paper V: Mitochondrial DNA variation in ancient European cattle: tracing the introduction routes of domestic cattle

The domestication of animals, and especially cattle, is one of the most important processes in Eurasian prehistory (Childe, 1925; Diamond, 2002). Data from mitochondrial DNA analyses suggest that cattle domestication was a process mainly taking place in the Near East (Troy et al., 2001), where after domesticated cattle entered Europe via different routes (Troy et al., 2001): the Mediterranean route (Zilhao, 2001) that might or might not have include Africa (Cymbron et al., 1999) (and see paper I), and another mainland route along the Danube (Bökönyi, 1974; Childe, 1925). An alternative hypothesis, popular in the 70s and 80s is that European aurochsen were used in the domestication. This idea of local European domestication had gained some support lately due to typing of Italian aurochs mtDNA (Beja-Pereira et al., 2006), and also by studies of the MHC nuclear genes (Vilà et al., 2005) and cattle Y chromosomes (Paper III). If cattle were locally domesticated in Europe we would expect to find similar amount of diversity in all European regions inhabited by aurochsen, while Neolithic cattle import would result in higher diversity in the areas where the domestic cattle en-
tered Europe. By estimating the average pairwise difference ($\pi$) over an entire sequence data set, we tested the most plausible of the two scenarios.

Material and methods

Ancient DNA was extracted from bone and teeth specimens using the method outlined in paper I, with the exception that the digestion step was performed using 100µg ProteinaseK in the presence of 2M Urea. The samples originated from Sweden, Spain, Germany, Greece, Ukraine and Lithuania. The PCR was performed as in paper I with the addition of 0.2 U of UNG. One hundred and nineteen sequences covering nucleotide positions 16042-16158 and 16164-16262 according to the cattle reference sequence (Anderson et al., 1982) were generated. Thirty-one sequences previously published (see paper I) were also included in the analysis. Differences in nucleotide diversity ($\pi$) between populations were assessed by non-parametric bootstrapping, treating the Iberian, Ukrainian, German and Greek sample sets. Modern sequences from GenBank representing central Europe were also analysed. In order to get a graphical representation of the sequences, a reduced median network (Bandelt et al., 1995) (version 4.1.1.2) was constructed, using DNA Alignment (version 1.1.3.0) with reduction threshold set to 1.

Results and discussion

One hundred and thirty ancient sequences were included in the network; 41 haplotypes were identified. The T3, T2, T1 and T haplogroups were present in the prehistoric samples, in patterns closely similar to those in modern European cattle.

The average pairwise difference ($\pi$) for the modern samples was significantly greater than for all the ancient samples analysed together. This could be expected, since the high rate of mutation in combination with global gene flow due to industrialised breeding would cause modern cattle to diverge. Comparing the ancient samples from different regions with one another, the samples from central Europe could be seen to have lower average pairwise difference than the samples from Greece, Iberia and Ukraine.

Higher values of $\pi$ were measured in the ancient samples from the areas where early domestic cattle may have entered Europe compared to the ancient cattle from central Europe. This supports an external origin of European cattle and that the European cattle stock, once dispersed, consisted of a number of small populations with limited gene flow. Once cattle had been introduced into Europe, intentional or unintentional hybridization with wild aurochs bulls may well have occurred (see paper III), but this would not affect the mitochondrial DNA.
Thus, through the analysis of ancient DNA we could trace entrance routes via south-eastern Europe and over Gibraltar by a signature in the total genetic diversity as measured by average pairwise difference.

**Paper VI: Medieval remains from Lithuania indicate loss of a mitochondrial haplotype in *Bison bonasus***

*Bison bonasus*, the wild bison or wisent of Europe, was once widespread throughout the deciduous woodland in Europe and western Asia. Since the early Pleistocene it existed alongside the aurochs (*Zeuner, 1963*). Today it is only present in some forests in Poland and Russia (*Clutton-Brock, 1999*). The wild population of wisent actually became extinct in the 20th century and has since been reintroduced from zoological gardens. Since the reintroduced wild population is founded from few individuals, the genetic diversity is very low. In this paper we screen medieval wisent specimens from Lithuania for mitochondrial DNA variation and compare the results with modern sequences from GenBank.

While not a domestic bovid, the European wisent *Bison bonasus* is interesting to compare with the extinct aurochs *Bos primigenius*. They were both present in Europe long into recent historical times. However, in contrast to the aurochs, the wisent is still alive, albeit from re-introduced stock.

**Material and methods**

Four phalanges dated to the end of the 13th-14th century from Lithuania (*Baubliene et al., 2004*) were collected. DNA was extracted twice from each sample using a silica-based spin column method (*Bouwman and Brown, 2002*). The mtDNA control region was amplified in three overlapping fragments and sequenced using standard chain termination DNA sequencing. The modern sequences were aligned together with modern reference sequences.

**Results and discussion**

The modern sequences were all of the same haplotype, while the four ancient sequences yielded two haplotypes: three yielded a novel haplotype and one was identical to the modern sequences.

Not surprisingly, the finding of an extinct mitochondrial haplotype in a small medieval sample set is in concordance with a loss of genetic diversity probably due to the near extinction of this species in the wild and the reintroduction of animals from zoological gardens.
By the time the aurochs became extinct, in the 17th century, the wisent had also disappeared in west and central Europe (Zeuner, 1963). Two subspecies of wisent, the lowland and mountain wisent were once distinguished, but the mountain wisent no longer exists (Wolinski, 1984). In southern Sweden the wisent existed in the Pre-boreal period, while the aurochs existed from the end of the Pleistocene epoch into the Atlantic period (Ekström, 1993). Although never domesticated, the European wisent were kept in royal zoological gardens together with aurochsen in the 16th century and they were systematically fed (Wolinski, 1984). Although today confined to certain geographical areas and displaying low mtDNA diversity the wisent does still exist in a somewhat wild form. The aurochs started to become rare by the 6th – 10th century and became extinct in 1627.

However, it may be misleading to consider the modern wisent population as a survivor from ancient times in a way that the aurochs is not: one can draw a parallel between the modern wisent stock and domestic cattle, in that their existence today is purely due to human intervention.
Concluding remarks

In this thesis it is demonstrated that cattle domestication in Europe was a more complex process than previous investigations suggest. Through genetic analyses it was possible to detect local aurochs introgression in prehistory. It appears that today’s European cattle (*Bos taurus*) are the descendants of both Anatolian and (male) European aurochs; whether this aurochs introgression took place by accident or by human design must remain the subject of speculation. Also, the accepted view of the origin of the T1 haplogroup in Iberia could be challenged.

Applying modern genetic patterns to draw conclusions about processes taking place in antiquity will not always give satisfactory answers. The combination of modern and ancient DNA, however, becomes a powerful tool in tracing male and female demographic histories and selection in past and present times. In this thesis, the utility of both traditional mitochondrial DNA analyses and the more challenging nuclear DNA analyses is demonstrated.

Genetic analyses of both ancient and modern DNA confirm that domestication was a long process, happening at different times and in different locations depending on the animal in question. Knowing when and where the domestication process took place and what was domesticated are first steps along the road towards being able to answer questions of why humans decided to settle down after thousands of years living as successful hunters and gatherers. Agriculture, the keeping of livestock and artificial selection can be regarded as changes that have brought progress to humanity. However, as artificial selection for desirable traits in farm animals becomes more intense, unwanted side-effects may increase. Understanding of what has happened in the past could give us a vital perspective about this process.
Svensk sammanfattning

Bakgrund

Många europeiska husdjur domesticerades vid slutet av den senaste istiden för cirka 10 000 år sedan, i det område som kallas den bördiga halvmånen i nuvarande Turkiet. Forskare har under många år ställt frågorna varför man började odla växter och avla djur, och varför man blev bofast. Människan hade under många årtusenden levtt som framgångsrika jägare och samlare, och varit beroende av vilda djur och växter. Men något hände i förhistorien som fick människan att ändra sin livsstil på ett dramatiskt sätt. Man spred sig till tidigare obebodda områden, och i samband med den förändringen försvann många stora däggdjur. Försvinnandet av däggdjur anses av vissa bero på klimatförändringar, men en annan teori är att jakten på vilda djur ökade i takt med att populationen av människor ökade. Till slut fanns det så få djur kvar att man var tvungen att hitta nya sätt att försörja sig på, man var tvungen att börja odla grödor och hålla sig med domesticerade djur. Vad det än var som startade processen så kom människan att ändra sitt förhållande till djur, från att jaga dem till att hålla dem levande vid sin sida.

Denna process som alltså initierades för 10 000 år sedan satt igång en utveckling som resulterade i bildandet av små samhällen vilka senare utvecklades till byar och städer. Husdjur som får, getter, hästar och kor, som alla en gång i tiden har varit vilda, har utgjort en stor och viktig ekonomisk del i många samhällen idag såväl som i förhistorien.

I denna avhandling behandlas frågor som rör domesticeringen av europeisk boskap *Bos taurus* med hjälp av DNA-analyser från moderna och förhistoriska bovider. Kor domesticerades i Mellanöstern och deras anfädare var den numera utdöda uroxen *Bos primigenius*. Uroxen fanns spridd över hela Europa, norra och västra Asien och norra Afrika, men blev utrotad i samband med att den sista individen, en ko, sköts i Polen 1627.

En klassisk fråga som många forskare ställt sig är huruvida den europeiska uroxen domesticerades lokalt i Europa eller om tamkor och uroxar hybridiserade under förhistorien. Ben av tamkor och uroxar från arkeologiska platser i centrala Europa vittnar om att båda djuren existerade samtidigt. Eftersom uroxar förekom ända fram till 1600-talet så verkar en hybridisering mycket trolig.

Mitokondriellt DNA, som är avs på mödernet, har analyserats i nu levande kor och utifrån dessa resultat har lokal hybridisering inte kunnat påvisas.

Undersökningarna i den här avhandlingen baseras i huvudsak på DNA från förhistorisk vävnad, så kallat gammalt DNA. Med den typen av analyser får man en direkt bild av vad som hände i förhistorien speciellt när dateringar av vävnads materialet föranckrar resultaten i tid. När man inkludera resultat från moderner DNA får man information över en lång tidsaxel.

Mitokondriellt DNA är en användbar markör för flera frågor rörande domesticering, medan andra markörer lämpar sig bättre för att studera hybridisering. Eftersom det finns fler kopior av mitokondriellt DNA i cellerna, jämfört med kärn-DNA (celler har oftast bara en kärna), lämpar sig mitokondriellt DNA även för analyser av gammalt DNA som är nedbrutet och finns i mycket få kopior. Nya tekniker och mer känsliga metoder har utvecklats med vars hjälp man kan studera nukleära markörer i gammalt DNA. Det innebär att även större studier av gammalt DNA från arkeologiska ben och tänder numera är möjliga. Den senaste tiden har analyser av gammalt DNA ökat och med en bättre förståelse av problemen associerade med förhistoriska DNA såsom kontamination och degradering är det bara de arkeologiska frågeställningarna som sätter gränserna.

Artikel I: Genetiska analyser av boskap från Spanien indikerar kontakter över Gibraltarsundet redan under Bronsåldern

Mitokondriellt DNA från nutida europeisk och afrikansk boskap grupperar sig i kluster, så kallade haplogrupper, vilka har namngivits T, T1, T2 och T3. Mitokondriellt DNA från engelska förhistoriska uroxben har en helt annorlunda typ, en uroxtyp. I Europa är T3 den vanligaste förekommande haplogruppen, medan kor från mellanöstern tillhör T3, T och T2. I Afrika är T1 vanligast förekommande, och den har möjligtvis sitt ursprung där. Den afrikanska haplogruppen har även identifierats i nu levande spanska kor, och antas ha införts från Afrika till den Iberiska halvön på 700-talet i samband med den muslimska expansionen över Gibraltar.

I denna studie undersöker vi om det har funnits en tidigare kontakt med den afrikanska kontinenten genom att analysera DNA från iiberiska och europeiska kollen. Totalt analyserades 47 ben och tänder från Spanien och Tyskland. Av dem var 14 daterade till bronsåldern (C14 daterade till mellan
1635 till 1800 f Kr) och kom från en grotta (Portalón) i norra Spanien, 16 var av varierande ålder från olika platser i Spanien och 17 var Neolitiska ben (3100-4900 f Kr) från Tyskland.

Av de 47 proverna som analyserades gav 30 reproducerbara DNA resultat. Åtta tyska prover och 19 spanska prover visade sig tillhöra den vanliga haplogruppen T3 medan ett spanskt prov tillhörde T-haplogruppen. Ett prov från Portalóngrottan, daterat till 1740 f Kr, visade sig tillhöra den i England identifierade uroxtypen, och ett annat prov från samma plats, daterat till 1800 f Kr, tillhörde den afrikanska T1-haplogruppen.


Upptäckten av den afrikanska haplogruppen T1 i ett ben som daterats till bronsåldern är den tidigaste indikationen på en afrikansk signatur på den Iberiska halvön. En alternativ tolkning kan dock vara att den här haplogruppen inte är specifikt afrikansk utan även har funnits i förhistoriska uroxar på andra platser i Europa och därmed kom att ingå i lokalt domesticerade kor. Detta är dock inte troligt eftersom alla hittills genetiskt analyserade och morfologiskt identifierade uroxar tillhör uroxtypen. Att avgöra denna fråga skulle dock kräva en bredare undersökning av den spanska uroxens DNA.

Vi har i denna studie kunnat påvisa att T1-haplogruppen, vilken anses vara en afrikansk mitokondriell markör, fanns i Spanien tidigare än den antagna introduktionen i samband med den muslimska invasionen på 700-talet, och vi tolkar det som bevis på tidiga transporter av boskap över Gibraltar.

Artikel II: Genetisk förändring över tid analyserad med nukleära markörer i modern och förhistorisk boskap

Mitokondriellt DNA (mtDNA) har varit den genetiska markören som dominaterar analyser av förhistoriskt material. Detta av tekniska och praktiska orsaker: den är i rakt nedstigande led på mödernet och den rekombinerar därför inte, det finns många kopior av den i cellerna, vilket sällan är fallet med nukleärt DNA, och den har en hög mutationshastighet som innebär att korta sekvenser ger mycket information. Dessa egenskaper gör att den används flitigt inom studiet av domesticerade djur eftersom det är lätt att tolka det genetiska mönstret skapat över tid och det är relativt lätt att extrahera mtDNA från förhistoriskt material som innehåller nedbrutet DNA. Det finns
dock begränsningar med mtDNA. Man får inte ut någon information om vad som händer på fädernet. Man kan heller inte studera om eller på vilket sätt man selekterat specifika egenskaper, något som man antagligen gjort med boskap under många år. För att undersöka möjligheterna att analysera kromosomalt DNA och också undersöka hur man kan ha avlat boskap under medeltid har vi utvecklat en metod för analyser av nukleär gammalt DNA. Gammalt DNA är nedbrutet och fragmenterat, men genom att vi inriktat oss på korta informativa DNA-fragment kommer vi förbi problemet. Enbaspolymorfer eller punktmutationer (SNPs) är variabla baser som skiljer sig åt mellan individer och kromosomer i en individ. En individ kan alltså vara homozygot eller heterozygot. Genom att analysera SNPs länkade till tänkbara fenotypiska egenskaper och genom att typera dessa med hjälp av pyrosekvensering har vi lyckats analysera ett stort antal historiska prover. Moderna boskapsprover har också analyserats i jämförande syfte. Tre SNPs vardera i en av tre gener valdes ut, \textit{MC1R} (Melanocortin receptor 1) som kodar för pigmentering och påverkar pälsfärg har en SNP (T/C) som resulterar i svart pälsfärg; \textit{LEP} (Leptin) som har en SNP C/T vilken är associerad med kroppsfett i köttboskap och med mjölk och proteinmängd; samt \textit{TLR4} (Toll-like receptor 4) som är en kandidatgen för sjukdomsresistens och har SNP:n C/T.


Av de 123 proverna var det 12 som inte gav något resultat. Antingen amplifierades inget DNA eller så uppfylldes inte kriterierna för korrekt typning. En logistisk regressionsanalyse av resultaten från alla tre lokus som typats visade en nedgång i genetisk variation över tid, speciellt mellan medeltid (1300-1400-talet) och historisk tid (1700-talet). Något som sannolikt sammanfaller med en mer organisad och riktad avel.

Mitt intresse i denna studie var först och främst att testa och utvärdera möjligheterna att analysera förhistoriskt kärnDNA med hjälp av pyrosekvensering. Resultaten visar tydligt att den här metoden mycket väl lämpar sig för degraderat material. Genom att i framtiden analysera flera markörer borde man kunna få en ganska god bild av hur boskap har avlats i historisk och förhistorisk tid.
Artikel III: Domesticering av boskap i Mellanöstern följes av hybridisering med europeiska uroxtjurar


Men om man återgår till de arkeologiska fynden och kontexterna där både uroxben och tamkoben har hittats samt det faktumet att uroxar och tamkor existerade sida vid sida under en ganska lång tid, så förefaller hybridisering fullt möjlig. Ett troligt scenario skulle kunna vara att uroxtjur hybridiserade med tamkor, men eftersom mtDNA endast säger något om vad som händer på möddernet skulle sådana analyser som baserar sig på mtDNA inte kunna påvisa den här typen av genflöde (från uroxtjur till tamko). Därför har vi utvecklat Y-kromosomala SNP-markörer (enbaspolymorfier) på modernt material och applicerat dessa markörer på både modernt och förhistoriskt material.


En tredje och mer sannolik förklaring är att de först införda domesticerade korna från Mellanöstern förde in Y2, men på sin väg norrut genom Europa

Resultaten från den här studien påvisar att domesticeringen av boskap var mer komplicerad än vad mtDNA avslöjar. Scenariot verkar innehålla lokal hybridisering i andra områden än i Mellanöstern.

Artikel IV: Identifiering av X- och Y-specifika punktmutationer och insertioner/deletioner som skiljer Bos taurus från Bos indicus

Boskap delas in i två grupper, Bos taurus som domesticerades i Mellanöstern och är den boskap som nu finns i Europa och Bos indicus, som domesticerades i Indien. Afrikansk boskap klassificeras som Bos taurus om man tittar på mitokondriellt DNA, men det är känt att olika grader av Bos indicus har avlats in i afrikanska kor. I den här studien analyserar vi tidigare publicerade Y-kromosomala markörer (Artikel III) och utvecklar nya X-kromosomala markörer som skiljer de två huvudgrupperna Bos taurus och Bos indicus åt. Markörerna applicerades sedan på 6 afrikanska boskapsraser, Raya Azebo (10 tjurar, 5 kor), Nguni (9 tjurar, 5 kor), Caprivi (8 tjurar, 7 kor), Danakil (9 tjurar, 6 kor), Kilimanjaro zebu (10 tjurar, 5 kor) och South Kavirondo zebu (10 tjurar, 5 kor).

boskap är tolerant mot trypanosomer som orsakar bovid sömnsjuka och sprids med tsetseflugan, medans *Bos indicus* boskap tål betta och torka.

Intressant i det här sammanhanget är att kontrastera det afrikanska förhållandet med förhållandet mellan domesticerad boskap och uroxar i förhistoriska Europa, där uroxar sannolikt hybridiserade med tamkor. I båda fallen visar mtDNA i första hand på *Bos taurus*. En liknande undersökning för att utröna graden av uroxinblandning skulle involvera en storskalig analys av ett uroxgenom, något som numera inte är omöjligt eftersom tekniken för den typen av analysen finns och har tillämpats på andra utdöda djurarter såsom mammutar och grottbjörnar.

**Artikel V: Variation i mitokondriellt DNA i förhistoriska europeiska kor: kartläggning av introduktionsrutten av den domesticerade boskapen**

Domesticeringen av boskap var en viktig händelse i förhistorien som möjliggjorde utvecklingen av bofasta samhällen. Kor domesticerades för ca 10 000 år sedan i det område som kallas den bördiga halvmånen i Mellanöstern. Domesticerad boskap infördes därifrån till Europa via olika vägar. En föreslagen introduktionsrutt gick västerut mot Iberiska halvön längs medelhavskusten och inkluderade eventuellt även den afrikanska kusten. En annan föreslagen väg gick från Mellanöstern norrut upp genom Europa. Kor som fördes in till det europeiska inlandet från domesticeringsområdet kan förvandas bilda små geografiskt isolerade populationer med begränsat genflöde medan områden som ligger på introduktionsrutten skulle uppvisa högre diversitet. Det finns dock en alternativ hypotes vilken var populär under 70 och 80 tal, och aktualiserats i och med studier av italienskt urox DNA, MHC i moderna djur, och Y-kromosomer på moderna och förhistoriska djur. Lokala europeiska uroxar kan ha använts i större utsträckning än vad som antas, och en stor del av den variation som vi ser i moderna europeiska kor kan ha sitt ursprung i den vilda europeiska faunan.

Här analyserar vi gammalt DNA (150 mtDNA sekvenser) från boskap härrörande från de olika områdena längs de hypotetiska införselsvägarna (Spanien, Tyskland, Ukraina och Grekland). Istället för att endast titta på de få nukleotiderna som definierar de europeiska haplogrupporna T, T1, T2 och T3, tar vi hänsyn till den totala diversiteten genom att uppskatta den genomsnittliga parvisa nukleotiddiversiteten ($\pi$) i stickproven. För att ge en grafisk bild över variationen i sekvenserna gjorde vi även nätverk.

DNA extraherades och amplifierades och sekvenserna grupperades efter geografiskt ursprung. Även moderna centraleuropeiska sekvenser från andra publicerade arbeten analyserades i jämförande syfte.
Det visade sig att diversiteten i de moderna centraleuropeiska kosekvenserna var högre jämfört med alla förhistoriska sekvenser tillsammans. En jämförelse av diversiteten i de olika områdena Spanien, Tyskland, Grekland och Ukraina visade däremot att de tyska proverna hade lägst diversitet. Resultaten kan tolkas som en founder-effekt och stödjer hypotesen att de europeiska tamboskapen kan söka sitt ursprung utanför Europa, i alla fall på mödernet.

Artikel VI: Analyser av mitokondriellt DNA från medeltida litauiska visenter påvisar en förlorad haplotyp

Den europeiska visenten *Bison bonasus* var liksom uroxen en gång spridd över ett stort område i Europa och även i västra Asien. Den existerade också under lång tid parallellt med uroxen. Liksom uroxen hölls den i kungliga inhågnader under medeltiden. Skillnaden mellan de båda djuren är att visenten, trots att den utrotats i det vilda, finns återintroducerad i polska och ryska skogar. Återintroduktionen av visenter skedde med hjälp av få individer från zoologiska parker vilket betyder att den genetiska diversiteten är låg bland de befintliga djuren.

I den här studien analyserar vi mitokondriellt DNA från medeltida visentben från Litauen med avsikten att jämföra resultaten med moderna visentsekvenser. Även om visenten aldrig domesticerades så finns det intressanta paralleller med den helt utdöda uroxen eftersom båda existerade i Europa som populära jaktvillebråd under en lång tid och in i historisk tid.

Vi extraherade och amplifierade mitokondriellt DNA från fyra ben som daterats till medeltiden (1400-1500-tal). Sekvenserna som genererades jämfördes med sekvenser från nu levande visenter. Föga förvånande så tillhörde alla de moderna sekvenserna samma haplotyp i det fragment vi undersökte. De fyra medeltida sekvenserna uppvisade två haplotyper, varav en var identisk med de moderna sekvenserna medan tre medeltida sekvenser hade en avvikande haplotyp. Resultatet stämmer med att visenterna antagligen tappade sin diversitet i samband med utrotningen i det vilda och återintroduktionen från zoologiska parker.

När uroxen dog ut hade visenten utrotats från västra och centrala Europa, men finns nu återigen i vilt tillstånd, i begränsade områden i Polen och Ryssland. Men det är troligtvis missvisande att påstå att visenten är en överlevare från förhistorien och inte uroxen. Man kan likna de idag existerande visenterna med tamkor, vars existens är helt beroende av människor.
Acknowledgements

A lot of people should be thanked for making sure this thesis was eventually finished!

I would first like to thank my supervisor Anders Götherström who is always full of good ideas, always had time for me and always managed to find solutions to problems.

I would also like to thank Professor Hans Ellegren for accepting me as a PhD student and providing a stimulating research environment.

Lots of thanks go to the other members of my small but dynamic research group, Helena Malmström and Emma Svensson. Thank you for being such good friends, for your support, and for cheering me up when I needed it, and good luck in the future: don’t hesitate to ask for help.

Thank you to the various funding bodies, without whose support many of the investigations in the thesis would not have been possible: The Göransson–Sandviken fund, the Sven and Lilly Lawski’s fund for scientific research, Rosa and Valter Tengborgs fund, the friends of the Athens institute fund, the Swedish Archaeology Society fund, the German Academic Exchange Service, and Birgit and Gad Rausing’s foundation for humanities research.

Thanks to my pun-tastic officemates, Erik, Frank and Mikael, indeed what shall we do with the collected money? Also thank you Mikael and Frank for help with computer problems and pulling me up by my bootstraps.

A special mention to Nilla, thank you for help with the sequencing so I could spend more time in the ancient DNA lab wearing my attractive and comfy lab overalls.

Thanks to Per Persson for your nightly networks, and for being the one who suggested the subject being researched in this thesis in the first place.

Also a big thank you to my collaborators in Spain, to Professor Juan Luis for providing lab space and material, and to José and Ana in Burgos for help with osteological analyses and for finding the material. To Cris for being
such a good and helpful friend and to Love for providing alternative interpretation of star-like patterns.

To Rengert Elburg, thank you for helping me dig through the hundreds of boxes of bones, and to navigate my way through the Landesamt in Dresden; and thanks to Karen Privat for finding Ukrainian bones for me. Thanks to Colin Smith, Matthew Collins and Mike Buckley for checking if there might have been any DNA in the first place…

And thanks to Henrik Sundberg for giving me this crazy idea that an archaeologist could do genetics and get away with it (almost)…

Thank you Kjel and Helena Knutsson for teaching me about prehistory, and making me take the step over from archaeology to the world of archaeogenetics, and to Terry Brown for teaching me everything worth knowing about the DNA molecule. And while we’re in the UK, thanks to Abi for swiftly replicating my results.

Thanks to AJ for stopping me disappearing altogether into academia, and pulling me (sometimes kicking and screaming) into the more glamorous world of fashion, premieres and cocktails. And Lotte, för det tydliga sven-skat.

And, a big thank you goes to all the rest of my colleagues past and present at the department, who brightened my days at EBC and maintained the cheerful atmosphere.

And last but not least, Mike for washing and cooking and correcting and telling me to stop writing and go to bed!

If you’ve managed to get this far: thanks for your attention, and I hope to learn from your criticism.
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Acta Universitatis Upsaliensis

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