

Cytochrome *b* sequences of ancient cattle and wild ox support phylogenetic complexity in the ancient and modern bovine populations

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Summary

Mitochondrial DNA has been the traditional marker for the study of animal domestication, as its high mutation rate allows for the accumulation of molecular diversity within the time frame of domestic history. Additionally, it is exclusively maternally inherited and haplotypes become part of the domestic gene pool via actual capture of a female animal rather than by interbreeding with wild populations. Initial studies of British aurochs identified a haplogroup, designated P, which was found to be highly divergent from all known domestic haplotypes over the most variable portion of the D-loop. Additional analysis of a large and geographically representative sample of aurochs from northern and central Europe found an additional, separate aurochs haplotype, E. Until recently, the European aurochs appeared to have no matrilineal descendants among the publicly available modern cattle control regions sequenced; if aurochs mtDNA was incorporated into the domestic population, aurochs either formed a very small proportion of modern diversity or had been subsequently lost. However, a haplogroup P sequence has recently been found in a modern sample, along with a new divergent haplogroup called Q. Here we confirm the outlying status of the novel Q and E haplogroups and the modern P haplogroup sequence as a descendent of European aurochs, by retrieval and analysis of *cytochrome b* sequence data from twenty ancient wild and domesticated cattle archaeological samples.

Keywords ancient DNA, aurochs, cattle, *cytochrome b* gene, domestication, mitochondrial haplotypes.

Introduction

All modern cattle were domesticated from the extinct progenitor, the aurochs (*Bos primigenius*). After the last glacial maximum 15 000 years ago, *B. primigenius* was found throughout almost the whole of Eurasia and North Africa. While zooarchaeological data points towards the Near East and the Indus Valley as domestication and diffusion centres of modern cattle (Helmer *et al.* 2005), a debate regarding possible contribution of local aurochs populations, for example in Europe, continues (Götherström *et al.* 2005; Beja-Pereira *et al.* 2006; Achilli *et al.* 2008).

The previous analyses of sequence variation in the mitochondrial chromosome control region have shown a strong phylogenetic structuring of bovine mtDNA haplotypes within Eurasia and Africa. The primary feature of this variation is the existence of two families of sequences (alternately of *Bos taurus* and *Bos indicus* origin), each of which clusters in a tight phylogeny but is separated from the other family by a long internal branch. This pattern of distinct, divergent clusters is a recurring feature in domesticated *Bovini* and likely represents limited episodes of capture from a more phylogenetically complex wild population (MacHugh & Bradley 2001; Finlay *et al.* 2007).

All *B. taurus* individuals belong to the T haplogroup, which can be further divided into haplotypes designated as T, T1, T2, T3 and T4, all defined by polymorphisms within 240 bp of the mtDNA D-loop (Loftus *et al.* 1994; Bradley *et al.* 1996; Mannen *et al.* 1998; Cymbron *et al.* 1999; Troy *et al.* 2001; Magee *et al.* 2002). These haplotypes are geographically distributed: T1 is almost exclusively African

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(Troy *et al.* 2001); T, T1, T2 and T3 are all found in the Near East, with T3 predominating in Europe; and haplogroup T4 has been thus far only been detected in Japanese cattle (Mannen *et al.* 1998). Achilli *et al.* (2008) also suggest a further taurine haplotype, T5, which is distinguished by variants elsewhere in the mtDNA genome. *Bos indicus* mtDNA sequences are highly divergent from *Bos taurus*, and cluster into two haplogroups, I1 and I2 (Baig *et al.* 2005; Lei *et al.* 2006; Magee *et al.* 2007).

There is an absence in modern samples of intermediate haplotypes, and the predominant phylogenetic branch separating *B. indicus* and *B. taurus* is unpopulated. An interesting question is whether the missing phylogenetic complexity of the wild population may be discoverable by either ancient DNA analysis of wild ox or by more extensive sampling of modern cattle. The first study on archaeological aurochs samples was published by Bailey *et al.* (1996). The authors succeeded in sequencing a 220-bp fragment of the mitochondrial control region from two British *B. primigenius* individuals. They demonstrated that these individuals, which cluster closely together and were designated as haplogroup P, were divergent from modern cattle and yet positioned more closely to extant taurine than indicine individuals. Haplogroup P is distinguished from modern cattle haplotypes by eight unique control region mutations (Bailey *et al.* 1996; Troy *et al.* 2001). From an extensive analysis of control region variation in archaeological skeletal samples ranging from Great Britain to Hungary, it is clearly identifiable as the *B. primigenius* haplotype of Central, Northern and Western Europe (Edwards *et al.* 2007). Interestingly, this study also found an additional divergent aurochs haplotype, E. This was discovered in one individual found at the Early Neolithic German site of Eilsleben. This novel D-loop haplotype shares two of the eight mutations that characterize the P haplogroup, but also contains two private mutations and two that it shares with *B. indicus* and *Bison bison* individuals. Additionally, it exhibits five mutations that are shared with only very few taurine individuals (Edwards *et al.* 2007).

Achilli *et al.* (2008), in an investigation of whole mtDNA genome diversity in modern Near Eastern and European cattle, discovered a third divergent haplotype, designated Q, although this divergence is not marked within the control region alone.

In several surveys of modern mtDNA control region diversity, featuring in excess of 3000 samples from throughout the range of *B. taurus*, no European aurochs haplotypes had been encountered (e.g. Loftus *et al.* 1994; Bradley *et al.* 1996; Mannen *et al.* 1998; Cymbron *et al.* 1999; Troy *et al.* 2001; Magee *et al.* 2002; Mannen *et al.* 2004; Hiendleder *et al.* 2008). However, in 2005, as part of a GenBank deposition of whole mtDNA genome sequences from animals of East Asian and European ancestry, Shin & Kim included a sample, DQ124389, which displays a control region sequence that clearly clusters with the ancient

aurochs P haplogroup. The remainder of the mitochondrial genome is also unlike typical modern variation and this deposition may represent a single recorded example of matrilineal introgression of typical European aurochs into the domestic gene pool (Achilli *et al.* 2008). Shortly before the submission of the whole mitochondrial genome DQ124389 (Shin H.D & Kim L.H., unpublished data) in 2004, Kim S.J., Suh J.Y., Kim K.C. & Suh D.S. (unpublished data) found AY337527, a mitochondrial control region that exhibits the P haplotype. In 2006, Zeng Y.T., Yan J.B. & Huang S.Z. (unpublished data) found sequence data from the mitochondrial control region of a Chinese Holstein (AY998840), appearing to be another living member of the P haplogroup.

Thus, in addition to the two major extant clades of T and I, some sequence information from three other divergent aurochs haplogroups (P, E and Q) are known. Here, we extend the mtDNA sequence haplotypes of 20 ancient cattle samples, including individuals belonging to the control region T, P and E haplogroups. We aligned the *cytochrome b* gene sequences of 169 published complete mitochondrial genomes and screened for possible clade-specific sites (data not shown). This alignment suggested that DQ124389 (putative modern haplogroup P) differed in two positions from all other *B. taurus* sequences. At position 14873, DQ124389 exhibited an A whereas all *B. taurus*, and the majority of *B. indicus*, individuals showed a G allele. At position 15134, DQ124389 showed a T allele, which it shares with *B. indicus* individuals. We sequenced 227 bp of *cytochrome b*, including these informative sites, and used these data to confirm ancient sequence affinity with the single extant P chromosome, and also illustrate the phylogenetic outlying character of the German aurochs E haplotype.

Materials and methods

Samples

A total of 20 Mesolithic and Neolithic aurochs and domestic cattle from five sites in the United Kingdom, Germany and Slovakia were sequenced. All samples had been previously differentiated as domestic or wild, on the basis of size or date, by the researchers who carried out archaeozoological studies of the various sites. All samples included in this study had been genetically assessed and replicated in the previous studies and had been proven to contain sufficient mitochondrial DNA (Bollongino *et al.* 2006; Edwards *et al.* 2007). Thirteen of the 20 were classified as *B. primigenius*; 12 of these (CPC03, CPC05, CPC06, CPC07, CPC08, CPC11, CPC12, CPC13, CPC14, CPC98, EIL6 and NORF) exhibited the ancestral P haplotype of the European aurochs, while one (EIL4) exhibited the novel ancestral E haplotype. Of the remaining seven, five individuals could not clearly be identified as either *B. primigenius* or *B. taurus* in the morphometric assessment. Four (CPC04, CPC10,

227 bp comprised two fragments from the centre of the *cytochrome b* gene, ranging from positions 14811 to 14900 and 15031 to 15167 (numbering according to Anderson Reference Sequence; Anderson *et al.* 1982). One specimen (WH06) could not be unambiguously typed due to low preservation quality and was removed from further analyses. The sequences reported in this study have been deposited in the GenBank database under accession numbers FJ392894–FJ392913.

Within the NCBI database, there are 304 *B. taurus* and *B. indicus* complete *cytochrome b* sequences available. Additionally, two *Bison bison* individuals were employed as an outgroup. The publicly available sequences included a dataset of 136 sequences sampled from Chinese endogenous cattle breeds (Cai *et al.* 2007; downloaded May and August 2008). This was the largest contiguous data set and, because of unusual variability, was considered separately. Several other anomalous aspects of these data were apparent. Firstly, this collection of sequences seemed to have a high number of haplotypes (23 in 136 samples) compared with the rest of the bovine sample (12 haplotypes observed in 168 individuals). Secondly, the dataset displayed a significantly higher number of transversions relative to transitions (tv:ti); that is, 8:18 compared with 0:17 in the wider data set (Fisher Exact Test, $P = 0.011$). Note that mtDNA is known to display an especially high transition–transversion ratio and published samples of other *Bovini cytochrome b* data (banteng, mithun and yak) also show zero or very few transversions despite appreciable transition variation (Anderson *et al.* 1982). Thirdly, a comparison with the other cattle and *Bovini* data sets shows the Chinese samples display a much higher non-synonymous to synonymous (Pn/Ps) ratio, with 10:16 compared with 3:14 in other cattle (Fisher Exact Test, $P = 0.13$). Because of these outlying statistics, we decided to exclude this Chinese dataset from further analyses.

The remaining 165 partial *cytochrome b* sequences, the 19 ancient sequences and the two *Bison bison* outgroup sequences were aligned (Table 1). The 227-bp fragment displayed 27 polymorphic sites, 10 of which were exclusive to the *Bison* outgroup. Within the remaining 17 polymorphic sites were eight singleton transitions (14847, 15047, 15068, 15082, 15092, 15102, 15155 and 15157), and six transitions which appear to indicate a subspecies-specific split between *B. indicus* and *B. taurus* (14825, 14858, 14897, 15105, 15134 and 15146).

Two of the polymorphic sites, 14873 and 15134, are of particular interest for this study because they seem to differentiate between the *B. taurus* and the *B. primigenius* clades. At site 15134, the data suggest that the ancestral allele is a T and the vast majority of domesticated cattle display a C. The only exceptions are the Korean modern P sequence (DQ124389) and the divergent Q haplotype (EU177866 and EU177867). The ancient specimen EIL4 and *B. indicus* individuals possess the ancestral allele. The

sequences gained from the Neolithic samples fall in both groups. The individuals EIL2, CPC04, CPC10, SVO1 and WH10 show a C whereas all other sequences exhibit a T at this site. These also possess a T3 D-loop haplotype. Of the remaining 14 ancient samples, 13 (CPC03, CPC05, CPC06, CPC07, CPC08, CPC11, CPC12, CPC13, CPC14, CPC98, EIL6, NORF and SVO3) possess D-loop sequences belonging to the aurochs haplogroup P. The individual EIL4 typed with a divergent D-loop haplotype, designated E (Edwards *et al.* 2007), showed the T allele, supporting its placement as an outlying haplotype. The potentially ancestral allele at site 14873-A is exhibited by 10 of the 14 members of the P haplogroup, including DQ124389. Only two *B. indicus* and *Bison bison* individuals share this allele. CPC04 and CPC10 are interesting samples because these bovine bones were robust and fell within a size range typical of aurochs rather than domesticates. However, they gave T3 D-loop haplotypes (Edwards *et al.* 2007). Here, they also possess the alleles at sites 15134 and 14873 typical of domesticates.

To place the ancient *cytochrome b* variation in a richer phylogenetic context, we constructed a median network (Bandelt *et al.* 1995) of the 19 ancient samples analysed here, plus 140 modern cattle samples for which alignable positions in both the *cytochrome b* gene and control region were available (Fig. 1). Mutations in the D-loop fragment were down weighted in the network from 10 to 1 because of the known presence of hypermutable sites.

The network shows clearly the genetically distinguishable bovine sub-species, *B. taurus* and *B. indicus*, separated by six *cytochrome b* substitutions. All *B. indicus* haplotypes cluster closely together, as do all *B. taurus* sequences. All *B. primigenius* individuals typed with a P haplotype sit closer to the *B. taurus* cluster than to the *B. indicus* cluster. Within this P grouping is the modern sample, DQ124389, distinguished from other modern *B. taurus* at both the 14873 and 15134 sites. At site 15134, the derived state is possessed by each of the T haplogroups, and among modern *B. taurus* is absent only in the single modern P and the two individuals reported with the divergent Q haplotype; these are separated by four mutations from the T3 cluster.

The unusual haplotype E sequence, EIL4 from a German *B. primigenius* sample, is distinguished by several *cytochrome b* mutations, confirming it as an outlier to both modern cattle and other aurochs. However, a neighbour-joining tree of the cattle sequences and a reasonable sample of *Bovini* outgroups places it within the same clade as *B. taurus* (Fig. 2). Despite its unusual divergence from modern samples, there is adequate bootstrap support for excluding the possibility that the E bone sample was a different species that was misidentified as aurochs.

Discussion

The discovery of the P haplogroup in North and Central European wild oxen supports an important archaeological

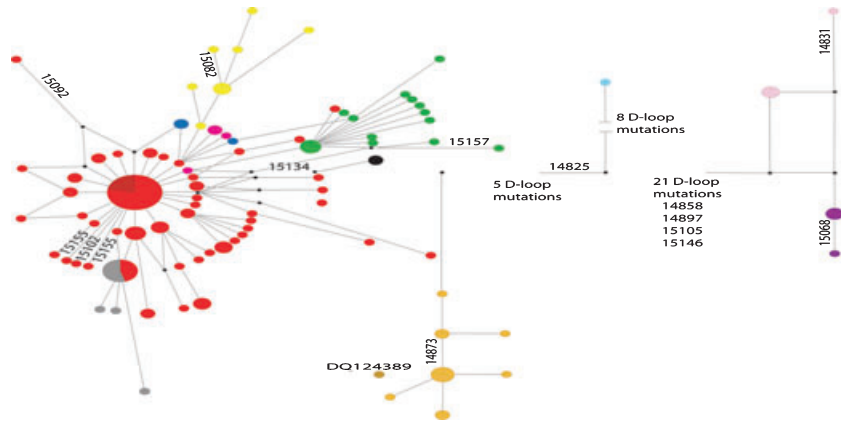


Figure 1 A median phylogenetic network including 159 individuals and a total of 429 bp from both the *cytochrome b* gene and the D-loop. Colours as follows: T: dark blue; T1(a): yellow; T2: bright green; T3(a): red; T4: grey; T5: pink; Q: black; P: light brown; E: light blue; I1: dark purple; I2: light purple. All *cytochrome b* mutations are clearly indicated. The size of a circle in the network is proportional to the number of individuals, and the length of the branches is proportional to the number of mutations. An exception to this rule is indicated in the branches leading to haplogroups E, I1 and I2.

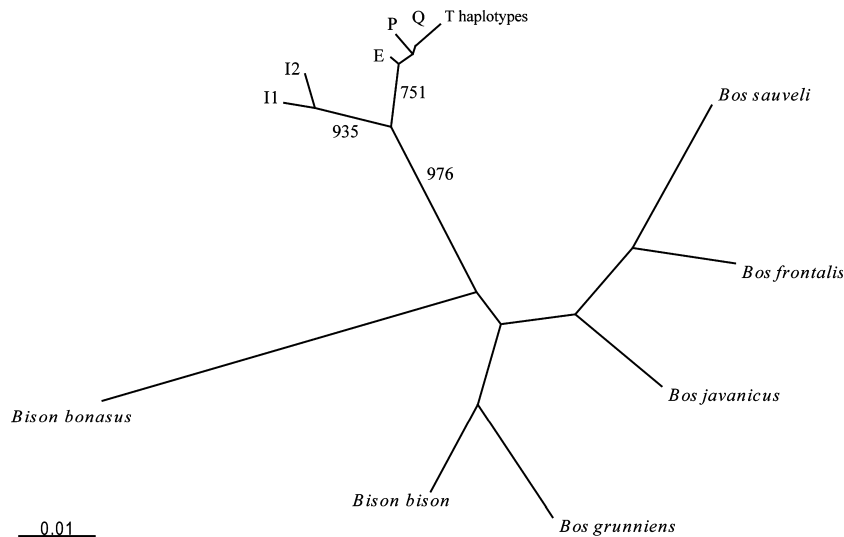


Figure 2 Neighbour-joining tree. Bootstrap values were calculated from 1000 pseudoreplicates of the data. The three highest bootstrap values are given, confirming E as an outlier to the taurine cluster, and thus excluding a misidentification with a member of one of the other *Bovini* outgroups.

premise; that European cattle matriline have a domestic origin in the Near East rather than from local European strains (Troy *et al.* 2001). Edwards *et al.* (2007) examined 59 bone samples from Mesolithic, Neolithic and Bronze Age European contexts that had been designated as aurochs either by measurement or dating evidence. They found a mixture of P and T haplotypes; a distinction which we confirm here in the same samples by further sequencing of informative regions of the *cytochrome b* gene. The presence of the P haplogroup in Central and Northern European samples confirmed that this aurochs variant is the typical mainland European type; it had previously only been encountered in Britain (Bailey *et al.* 1996; Troy *et al.* 2001). The bones giving T haplotypes were either of intermediate

size between wild and domestic or were fragmentary and so could not be measured effectively and so domestic origin could not be excluded for these animals. Each amplifiable archaeological specimen unambiguously identified as aurochs, either by measurement or time depth, gave a P haplotype.

In over 10 years of studies of bovine control region sequence variation in modern cattle from disparate geographical origins, only three of over 3000 submitted haplotypes in GenBank show a P sequence: AY337527, AY998840 and DQ124389. DQ124389 is a complete mitochondrial genome and has been shown to be distinct from all domesticates (Achilli *et al.* 2008). This surprising finding required confirmation, and thus we here present

cytochrome b sequence from 13 aurochs P individuals and compare them with DQ124389. Our data confirm that this published modern P individual matches the consensus ancient Northern European aurochs sequence at two diagnostic polymorphic sites, strongly suggesting that this individual is indeed the product of a rare, and seemingly unlikely, matrilineal introgression from the wild into the European domestic gene pool. It is also conceivable that the P lineage originates from the as yet unexplored ancient Asian population. Whereas AY998840 and DQ124389 are sampled from individuals of the Holstein breed, AY337527 is an individual of the indigenous Korean Youngju Yellow breed. Introgression of local Asian aurochs populations has also been suggested by Mannen *et al.* (1998, 2004) who reported a new taurine haplotype, T4, which has been so far only detected in Asian cattle populations. However, the phylogenetic relationship of T4 to other T haplotypes (most especially the fact that it roots through T; Achilli *et al.* 2008) does not rule out a Near Eastern/Anatolian origin of all T haplotypes.

The results here confirm some assertions of the survey by Edwards *et al.* (2007). Importantly, the divergent D-loop sequence of a single aurochs from Eisleben (EIL4; haplogroup E) is confirmed as an outlier by *cytochrome b* sequence data. Edwards *et al.* (2007) report this as an unusually robust archaeological bone and their data place it phylogenetically within cattle sequences (thus excluding an identity as a Bison or more distant bovid); the results here confirm it as a more divergent relative to modern cattle than haplogroup P. The *cytochrome b* mutation at site 14873 also suggests the existence of a third rare aurochs sequence type, Q, distinct from modern cattle sequence types, which has already been identified as a outlying sequence by Achilli *et al.* (2008). Q may be a Southern European or Near Eastern variant, but its provenance is impossible to ascertain as it has been encountered only once, in a double sample from a single Italian breed.

Thus it seems that the missing history in the standard modern cattle phylogenetic structure is populated by rare divergent mitochondrial types. These appear to represent matrilineal captures from the wild, or, alternatively, might point to a higher mitochondrial variability of domestic cattle than previously thought. The genetic variation of *B. primigenius* was geographically structured – P seems to be the typical European variant, with predominance across Europe, from Britain to the Hungarian plain. Outliers appear rarely, for example E as a single, and morphometrically robust, sample. There is an inadequate sample of aurochs from the Near East with which to build an estimate of phylogenetic complexity, but one might surmise that T-like variation was typical, given its predominance in modern *B. taurus*. It is very unlikely that the divergent modern P haplotype was domesticated in the Fertile Crescent; its extreme rarity and the provenance of the wild haplotype

suggests it to have resulted from a secondary, possibly European, introgression from the wild. Thus, in the course of European domestic history, it does appear that at least one wild-born female cow was captured and bred as a domesticate, an event which these data suggest to be of vanishing rarity.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Table of archaeological bone samples studied, with associated codes and skeletal element used.

Table S2 Primer pair sequences, given in 5' → 3' direction, and their positions in the mitochondrial genome numbered according to Anderson Reference Sequence V00654 (Anderson *et al.* 1982).

Table S3 PCR cycling conditions for (a) Dublin and (b) Mainz primer pairs.

Table S4 Sequence information.

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