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Ancient DNA from Bronze Age bones of European rabbit (*Oryctolagus cuniculus*)

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Abstract. The European rabbit (*Oryctolagus cuniculus*) is now widely distributed throughout the world as a result of transportation by man. The original populations, however, were confined to southern France and Spain. In order to investigate the role of human intervention in determining the genetic diversity of rabbit populations, we are studying the origin of rabbits introduced onto a small Mediterranean island (Zembra) near Tunis over 1400 years ago, by examining ancient DNA extracted from rabbit bones found both on Zembra and on the European mainland. Ancient DNA was successfully extracted from rabbit bones found at two archaeological sites dated to at least the Early Bronze Age (more than 3500 years ago) in south-central France, and compared to that found in modern mainland and island populations using a small variable region of the cytochrome *b* gene. The results confirm that the Zembra Island population is descended from that present over 1400 years ago. The technical aspects of DNA extraction from bones and the implications of this type of research for determining the origin of introduced rabbit populations are discussed.
Key words. Ancient DNA; mitochondrial DNA; *Oryctolagus cuniculus*; cytochrome *b* gene.

Introduction

The European rabbit (*Oryctolagus cuniculus*) originated in the Iberian Peninsula, and the oldest known fossils of this genus, found in southern Spain, date from 6.5 million years (Myr) ago[12]. Prior to their active transportation by man, rabbits were confined to southern France and Spain[3,6,17]. Within this range, modern rabbits show considerable diversity in morphology, mitochondrial DNA (mtDNA) polymorphisms and other polymorphic markers[2,5,7,18,21]. Two main maternal lineages termed A and B have been described from mainland Europe which show around 5% nucleotide divergence in the mitochondrial DNA. They are thought to have separated about 2 Myr ago. This estimate is based on a mtDNA divergence rate of around 2% difference developing between two molecules every million years[5]. Type A is found only in southern Spain and type B is present in northern Spain and France. The distribution of the two lineages may have been influenced by glaciation events in Europe between 2 Myr and 12000 years BP (before present), and more recently, man has played an important part in the dissemination of rabbits both by domestication and by release for hunting.

The discovery of rabbits of maternal lineage type B from Zembra Island near Tunis[5] is at variance with the proposed division of rabbits into two subspecies[9], since although the Zembra island rabbits are morphologically more related to those from southern Spain[22], their mtDNA clearly belongs to that of rabbits from the north of the range[4]. The presence of rabbits on this island is probably the result of introduction by man in the Bronze Age or in Phoenician, Greek or Roman times[10].

We are interested in determining the role of man in maintaining or altering the genetic diversity of rabbits introduced into Mediterranean islands as well as in modern mainland populations. To approach this problem, we are using zooarcheology and ancient DNA analysis to characterize rabbit populations present in historic and prehistoric times, before man became involved in rabbit transportation.

An initial study conducted on Zembra Island[10] demonstrated the feasibility of ancient DNA extraction from rabbit bones, and that the rabbits on this island are descended from those originally introduced more than 1400 years ago. In this study, we report the extraction of ancient DNA from rabbit bones dated to at least the early Bronze Age (more than 3500 years ago) from archaeological sites in southern France, and confirm the data for Zembra Island. DNA sequences derived from a highly variable region of the mtDNA cytochrome *b* gene from both modern and ancient samples were determined, which can be used to give information about rabbit populations in ancient times.

Methods

Rabbits

Modern rabbits used in this study are shown in table 1. The recent bone (Las Lomas R5) comes from the individual LL 17 caught at Las Lomas in southern Spain and cleaned by boiling and papain hydrolysis in 1987[10]. Nomenclature of mtDNA subtypes is as described[14].

Table 1. Rabbit DNA Sources[a].

Rabbit	Origin	mtDNA type
Dom (pOcc)[b]	Domestic, France	B1
Ba16	Badajoz, Spain	B3 (B3sa)[c]
Ce2	Cerisay, France	B3 (B3sb)[c]
Ax1	Arjuzanx, France	B3 (B3sc)[c]
Tv9	La-Tour-du-Valat, France	B4
Z18	Zembra Is., Tunisia	B6
Z10	Zembra Is., Tunisia	B7
Se1	Sesma, Spain	B8
Tu1	Tudela, Spain	B9
Tu3	Tudela, Spain	B10
Cn2	Castejon, Spain	B11
LL27	Las Lomas, Spain	A2
LL85	Las Lomas, Spain	A4
LL13	Las Lomas, Spain	A5
LL30	Las Lomas, Spain	A6
LL17 (R5)[d]	Las Lomas, Spain	A7
Ba6	Badajoz, Spain	A10

[a]Nomenclature of rabbits and mtDNA subtypes is as described[14].
[b]Entire mtDNA from a Domestic rabbit (Fauve de Bourgogne) cloned into Bluescript plasmid[13].
[c]New type B3 nomenclature based on sequence data.
[d]Bones from this rabbit were kept as museum specimens.

Archaeological sites

Rabbit bones used for these experiments came from 4 different archaeological sites: the 'abri du Casino' on Zembra Island, Tunisia[10,22], and three sites in south-central France – Montou Cave[19], an open site at Laouret[8], and Abeurador Cave[20]. The sites at Montou[19] and Zembra[10,22] are the most accurately dated. Bones were taken from Montou from three well preserved and radio-carbon dated Bronze Age layers (Ly): MIV: Ly 5907: 2850 ± 45 BP, calibrated: [1245–835] BC; MVII: Ly 5909: 3070 ± 45 BP, calibrated: [1540–1120] BC and MX: Ly 3857: 3640 ± 100 BP, calibrated: [2320–1755] BC (F. Treinen-Claustre, pers. commun.) and from a site dated to late Roman times (1400 BP) on Zembra[10]. The bones from Abeurador came from layers attributed to the Mesolithic (around 6000–8000 BP) with less precise carbon dating[20]. The site at Laouret is assigned to the Late Bronze Age (IIIA) (950–850 BC) on the basis of pottery and ceramic remains[8].

DNA extraction

Total DNA was extracted from soft tissue and bones as previously described[10]. Powdered bone (approximately 1 g) was washed at least three times in 20 ml 0.5 M EDTA over 2 days at 37 °C until no further coloured matter was present in the supernatant. Following these washes, DNA was extracted overnight at 37 °C in 10 ml of 0.5 M EDTA pH 8.5, 0.5% N-lauryl sarcosine, 100 µg/ml proteinase K. The supernatant was then extracted 2–3 times with phenol:chloroform until the interface was clear of protein. Following this, the supernatant was concentrated by Centricon dialysis and washed with TE buffer (10 mM Tris-HCL, 1 mM EDTA pH 8), and the DNA was typically recovered in 100–200 µl TE buffer. The presence of DNA was determined by examining aliquots of 5–10 µl on a 2% agarose gel.

PCR

PCR was performed on total DNA extracts in the buffer supplied by Promega (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100), using 150 µM NTPs and 100 pmol of each primer. In addition 160 µg BSA was added to PCR reactions containing ancient DNA. PCR was performed in a Kontron Cycler in 100 µl over 30 cycles for modern DNA and 40–50 cycles for ancient DNA. Each cycle consisted of 93 °C, 30 sec; 55 °C, 60 sec; 72 °C, 30 sec. Following the last cycle, the PCR was incubated a further 3 min at 72 °C. Primers (5′–3′) used were:

– cytb 4: ACTGGTTGGCCTCCGATTCA (15774–15755)
– cytb 6: GCTCTTGTCTTATCTATCCTTG (15623–15644)
– cytb 11: ATGTCTAAACAACGTAGCATG (15671–15691)
– cytb 12: CTTGCGAGGGGTATAAGAATA (15774–15755)
– cytb 13: GCTACTTGTCCAATGGTGATG (15807–15787)
– th3: CTCCATTCCTGGCTTACAAGAC (15937–15915)

The numbers in brackets after the sequences indicate the nucleotide positions of the 5′ and 3′ nucleotides respectively according to the human mitochondrial genome[1].

A major consideration was the avoidance of contamination in the PCR reactions. All pre-PCR reactions were set up under sterile conditions in a Biohazard Hood, in a room separate from that used for the PCR reactions, and were run with both a blank and a control DNA extract from a pig or *Caprini.* (sheep/goat) bone to detect any 'carrier effect'[15]. In addition, the primer pairs were chosen to be specific for rabbit mtDNA to avoid amplifying non-rabbit DNA. PCR primers cytb6, cytb11, cytb12 and cytb13 were designed in such a way that the 3′ sequence of each primer was unique for rabbit relative to other known cytochrome *b* sequences[11]. Primer th3 is in the threonine tRNA gene.

In order to avoid the problem of sequence errors associated with cloning[16], asymmetric PCR (primer ratios between 50:1 and 100:1) was conducted over 40 cycles under the same PCR conditions, except that BSA and the final 3 min at 72 °C were omitted, using 1 µl of the first reaction mixture directly, or following purification of the PCR products from an agarose gel using QIAX (Qiagen, Germany). Prior to sequencing, the single strand PCR products were phenol extracted, ammonium acetate was added to 2.5 M and precipitated away

from the primers in 60% ethanol for 15 min at room temperature, and this was followed by centrifugation at 13000 rpm for 15 mins. Each PCR and ssDNA preparation was done at least twice independently.

DNA sequencing

Sequencing of single strand PCR products was conducted using dideoxy nucleotides with the T7 DNA Polymerase Kit supplied by Pharmacia.

Results

Origin of ancient rabbit bones

The origin and dates of ancient bones used in these experiments are summarized in table 2. Examples of bones used for DNA extraction from each site are shown in figure 1.

Ancient DNA extraction

Each extraction of rabbit bones was accompanied by parallel extractions both without a bone (blank) and with a bone from another animal (pig or sheep/goat) found in the same level of the site as a control against possible contamination originating from the archaeological site itself. Differences can be noted in the quality of preservation of the bones depending on the site of origin. In particular, bones from Laouret were considerably less well presesrved than those from the caves or the Zembra Island rock shelter. This was reflected by the relative ease with which the bones from Laouret could be crushed. The wash supernatants of bones from Laouret were also considerably more coloured than those from the better-preserved bones from the other sites. In addition, coloured material was always present in greater quantity in bone fragments than in extracts from complete bones. Following incubation in extraction buffer the supernatants again appeared yellowish to reddish. Most of this colouration, however, was removed during the Centricon dialysis steps.

In all extracts, large MW DNA (>20 kbp) could be easily detected (fig. 2). In extracts from recent bones, this may include large fragments of rabbit nuclear DNA, but in ancient bones it derives almost certainly from contaminating microorganisms, as evidenced by the PCR data described below. By contrast, relatively little low molecular weight DNA is evident in ancient bone extracts, although large quantities are present in extracts from a 6 year old bone (R5) preserved as a museum specimen (fig. 2). DNA was then used directly for PCR.

PCR

PCR products for the primer pairs cytb 4/cytb 6 and cytb 11/cytb 12 were usually detected after 40 cycles. Ancient DNA extracts were tested for modern DNA contamination by using a primer pair cytb 6/th 3 (amplifying a 311 bp product) to test for the presence of larger DNA molecules (fig. 3). No PCR products of 311 bp could be detected in any of the ancient DNA samples. PCR products of 152 bp (primer pair cytb 4/cytb 6) and 185 bp (primer pair cytb 11/cytb 12) were

Table 2. Bone samples used for ancient DNA extraction.

Site	Bone	Code	Age (years BP)	Weight	
				Total	Extracted
Zembra	Rabbit femur[a]	Z7 C1	< 1400	0.89	0.89
	Rabbit femur[a]	Z7 C2	1400[b]	1.14	1.14
	Pig ulna[a]	Z7-86 C2	1400[b]	0.90	0.90
Montour	Rabbit femur	MT89-R9-210 (MIV)	2850[b]	1.92	1.22
	Rabbit femur	Mt83-H7-506 (MVII)	3070[b]	1.62	1.31
	Rabbit femur	MT83-I6-391 (MX)	3640[b]	2.62	2.08
	Capra/Olvis Phalange[a]	MT91-I9-R12 (MIVC)	2850[b]	1.14	0.54
	Capra/Ovis Tibia[a]	MT83-J7.6.2485-505 (MXC)	3640[b]	6.93	1.43
Laouret	Rabbit femur[a]	LA2-I13-180	2800	0.90	0.90
	Rabbit femur[a]	LA-P15-150	2800	0.87	0.87
	Capra/Ovis. Tibia[a]	LA2-D13-510	2800	1.05	1.05
Abeurador	Rabbit humerus	BA-H4-F64-155	6-8000[b]	1.65	1.22
	Rabbit ulna	H4-F6b-460 (AbU)	6-8000[b]	1.03	1.03
	Wild Boar exoccipitale[a]	BA-I3-F6-103	6-8000[b]	3.35	2.10

[a]Fragment of bone only.
[b]Uncalibrated radio-carbon datings.

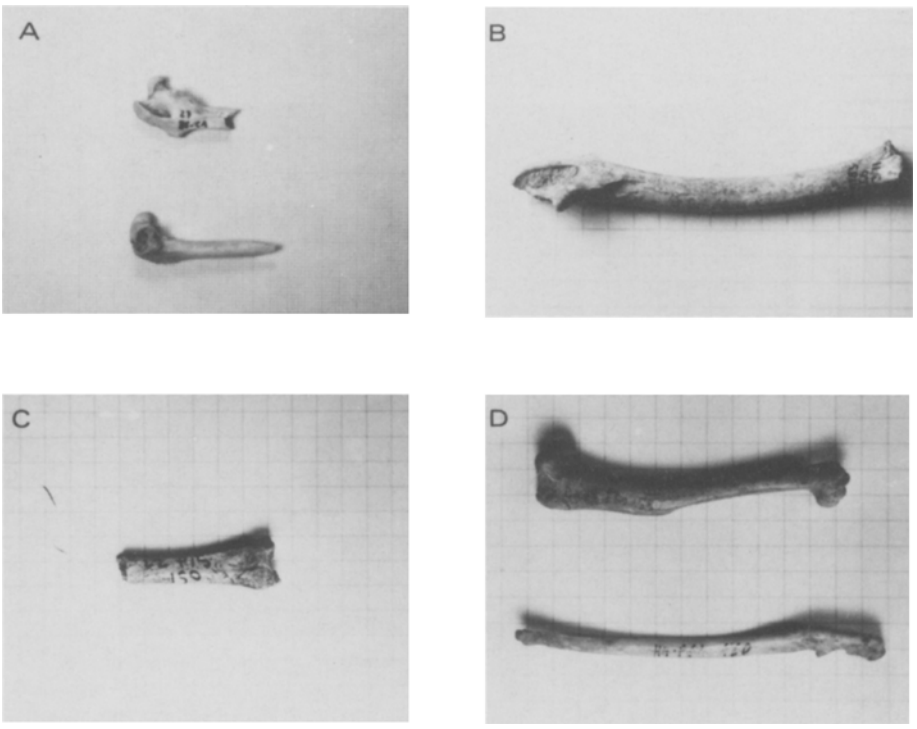

Figure 1. Examples of bone remains used for DNA extraction (see table 2). *A* Zembra femur fragment Z7C2; *B* Montou femur MT83-I6-391 (MX); *C* Laouret tibia fragment (LA-P15-150); and *D* Abeurador humerus BA-H4-F64-155 (AbU) and ulna H4-F6b-460.

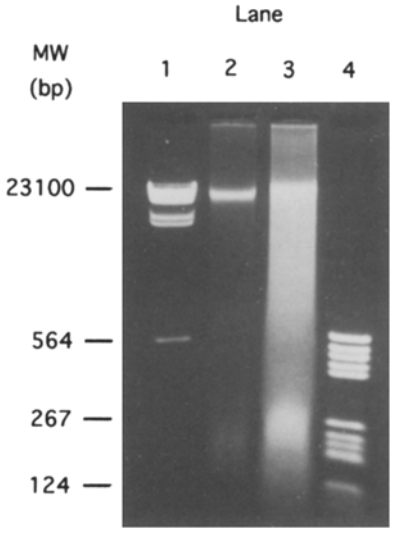

Figure 2. Examples of DNA extracts from rabbit bones.
2% agarose gel stained with ethidium bromide.
Lanes 1 = λ *Hind*DIII; 2 = Montou femur MT83-I6-391 (MX), 3640 BP; 3 = Las Lomas R5, 6 BP; and 4 = pBR322 *Hae*III. The amount of DNA corresponds to that extracted from approx. 0.3 g bone.

obtained for all 3 rabbit bones from Montou (MX, MVII and MIV), one bone from Abeurador (AbU) and the 2 bones from Zembra (Z7C1 and Z7C2). In addition, a product of 233 bp using the primer pair cytb 6/cytb 12 were obtained from the oldest bone from Montou (MX). No PCR products could be obtained using the 2 rabbit bone fragments from Laouret, even when primer pairs cytb 11/cytb 13 (137 bp) and 50 cycles were used.

mtDNA sequence analysis

Preliminary sequence analysis using a region of the mtDNA 16S rRNA gene[10], although useful for distinguishing between the two main rabbit lineages A and B, did not show sufficient variability to determine the various RFLP subtypes within each lineage. However, the 190 bp region between primers cytb 6 and cytb 12 in the 3′ region of the cytochrome *b* gene (fig. 4) is sufficiently variable to allow distinctions to be made among individuals, both between and within the A and B lineages. The two lineages are clearly different and nine different subtypes (4A and 5B) can be distinguished in a total of 17 individual rabbit DNA extracts (table 1). The results for modern DNA show an interesting feature. In the B lineage, although no distinction can be made between the mtDNA sequences from six individuals; Ba16, Tv9, Se1, Tu1, Tu3 and Cn2 (types B3, B4, B8, B9, B10 and B11 respectively), two other type B3 individuals from southwest France (Ax1 and Ce2) show unique sequences compared to the type B3 individual Ba16. These new B3 subtypes based on sequence data have been designated B3sa (Ba16), B3sb (Ce2) and B3sc (Ax1). The individuals Z18 (type B6) and Z10 (type B7), representing the two type B lineages currently found on Zembra Island, show an identical sequence,

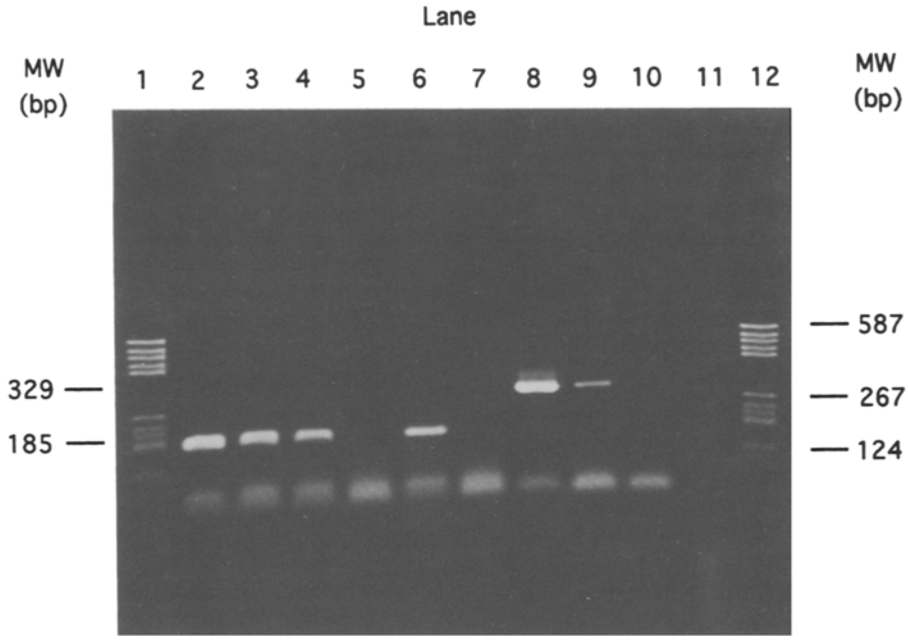

Figure 3. PCR of DNA extracts from rabbit bones. 2% agarose gel stained with ethidium bromide.
Lanes 1, 12; DNA marker: pBR322 *Hae*III
Lanes 2–7; primers cytb11, cytb12
Lanes 8–11; primers cytb6, th3
DNA sources are described in tables 1 and 2:
1, pBR322 *Hae*III; 2, pOcc; 3, Las Lomas R5; 4, Montou rabbit (MX); 5, Montou *Caprini.* (MXC); 6, MX + MXC; 7, Blank (No DNA); 8, pOcc; 9, Las Lomas R5; 10, Montou rabbit (MX); 11, Blank (No DNA); 12, pBR322 *Hae*III. 40 cycles: 93 °C, 30″; 55 °C, 60″; 72 °C, 30″.

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                                              *     *  *                                  *               * *
                                             20                  40                  60                  80                 100
MODERN     Dom    (B1)      TTCTAGCCTTCATCCCATTCCTTCATATGTCTAAACAACGTAGCATGATATTCCGACCCATTAGTCAAGTCCTATTCTGAGTTCTCGTCGCAGATCTTCT
           Ba16   (B3sa)    ....................................................................................................
           Ce2    (B3sb)    ....................................................................................................
           Ax1    (B3sc)    ....................................................................................................
           Z18    (B6)      ...............................C....................................................................
           Z10    (B7)      ...............................C....................................................................
           Other  (B)       ....................................................................................................

           LL27   (A2)      ......................C.....A...................................C...............A.C.................
           LL85   (A4)      ............................A...................................C...............A.C.................
           LL13   (A5)      ............................A...................................C...............A.C.................
           LL30   (A6)      ............................A...................................C...............A.C.................
           LL17   (A7)      ............................A...................................C...............A.C.................
           Ba6    (A10)     ............................A...................................C...............A.C.................

ANCIENT    Z7C1             ...............................C....................................................................
           Z7C2             ...............................C....................................................................
           MIV, MVII, MX    ....................................................................................................
           AbU              ....................................................................................................

                            *     *           *  *  *              *     *        *           *           *     *
                                               120                 140                 160                 180
MODERN     Dom    (B1)      CACACTCACATGAATCGGAGGCCAACCAGTAGAACACCCGTTCATCACCATTGGACAAGTAGCATCTGTCCTCTACTTCACCACCATCCT
           Ba16   (B3sa)    ..................G.....G..............A..................................................
           Ce2    (B3sb)    ........................G..............A............................................T.....
           Ax1    (B3sc)    ........................G..............A..................................................
           Z18    (B6)      ..................G.....G..............A..................................................
           Z10    (B7)      ..................G.....G..............A..................................................
           Other  (B)       ..................G.....G..............A..................................................

           LL27   (A2)      G.....G...........G..T..G..............A.....T....................C...........T.....A.....
           LL85   (A4)      A.....G...........G..T..G..............A.....T........G...........C...........T.....A.....
           LL13   (A5)      G.....G...........G.....G..............A.....T....................C...........T.....A.....
           LL30   (A6)      G.....G...........G.....G..............A.....T....................C...........T.....A.....
           LL17   (A7)      G.....G...........G.....G.....................T....................C...........T.....A.....
           Ba6    (A10)     G.....G...........G.....G..............A.....T....................C...........T.....A.....

ANCIENT    Z7C1             ..................G.....G..............A..................................................
           Z7C2             ..................G.....G..............A..................................................
           MIV, MVII, MX    ........................G..............A..................................................
           AbU              ..................G.....G..............A..................................................
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Figure 4. Comparison between modern and ancient DNA sequences for the 190 bp between primers cytb6 and cytb12 at the 3′ end of the mtDNA cytochrome *b* gene from various rabbit A and B mtDNAs: Other (B) = rabbits Tv9 (B4), Sel (B8), Tul (B9), Tu3 (B10) and Cn2 (B11). Subtypes are indicated in brackets. Positions 1 and 190 of the sequences correspond to positions 15645 and 15834 respectively of the human mtDNA sequence (1). The 17 variable nucleotide positions are designated by asterisks above the sequences. Abbreviations for rabbits and bones are described in tables 1 and 2.

distinguished from those of all the other rabbits by a T→C nucleotide change at position 32 (position 15676 relative to the human mitochondrial genome) (fig. 4). In lineage A, four sequences can be distinguished from the six lineage RFLP subtypes examined (A2, A4, A7 and A5, 6, 10).

The sequences for DNA extracted from the ancient bones all have the pattern corresponding to a B lineage (fig. 4). The DNAs for the two bones from Zembra Island are identical to types B6 and B7 of individuals Z18 and Z10 respectively, and all three sequences from Montou are identical to that present in the type B3sc in the rabbit Ax1 from Arjuzanx. The bone from Abeurador has the same sequence as is seen in the 6 other non-type B1 rabbits (Ba16, Tv9, Se1, Tu1, Tu3 and Cn2).

Discussion

The major problem encountered with ancient DNA extracts is their apparent dependence on the quality of preservation of the bones. The vast majority of bones are present as fragments, and whole unbroken ones are relatively rare. The results presented here indicate that small or broken bones do not always harbor ancient DNA of sufficient quality or quantity to conduct DNA analyses. However, under certain circumstances, such as preservation in protected caves like Montour[19] and Abeurador[20] and on Zembra Island[22], ancient DNA is present in sufficient quality and quantity even after considerable periods of time to permit the recovery of fragments of around 200 bp using PCR. However, further comparisons between the quality of bone preservation, age, and the presence of ancient DNA will be needed to clarify the relationship between these characteristics.

Divergence between mtDNA in lineages A and B (around 5%), seen in RFLP analyses of whole mtDNA from rabbits[4], is not uniformly shown at the DNA sequence level. For instance, only 7 nucleotide differences were found in a 555 bp region of the 16SrRNA gene[10], whereas 19 nucleotide changes at 17 sites are present in the 190 bp sequence between the primers cytb 6 and cytb 12 near the 3′ end of the cytochrome *b* gene (fig. 4). Indeed, the cytochrome *b* gene and other regions of the mtDNA contain sections variable enough to distinguish between individual rabbits which were not separated by RFLP of whole mtDNA. Although not all rabbit mtDNA RFLP subtypes could be distinguished in this cytochrome *b* gene region, the sequence data show that type B6 or B7 was present 1400 years ago on Zembra Island. Similarly, the sequence currently found in Arjuzanx (type B3sc) was present more than 3500 years ago at Montou. By contrast, it is not possible to determine which of the other non-type B1 rabbits in lineage B were present at Abeurador around 6000 years ago using this region of the mtDNA. Two mtDNA varieties (B1 and B3) are currently distributed throughout southern France, and the absence of type B1 in the four bone samples from France where mtDNA was sucessfully extracted could be due to the absence of the type B1, or to insufficient sample size.

The ability to extract and successfully amplify DNA from rabbit bones dated between the Early Bronze Age and the Mesolithic (over 3500 years ago) has two important implications. It permits the analysis both of the origin of introduced populations and of their diversity in historic times. Examination of the level of diversity present in ancient populations should be possible since rabbit remains are particularly abundant in archaeological sites from the Late Pleistocene and Holocene.

The results presented here demonstrate the approach we have taken to determine the origin of the Zembra Island stock and reinforce the previous conclusion[10] that the rabbits from Zembra are descended directly from those introduced over 1400 years ago. We can probably conclude that the animals taken for introduction on Zembra Island did not originate from the regions in northern Spain and southern France examined here (including Abeurador and Montou). We aim to sample both modern and ancient DNA from more individuals and from more archaeological sites, using diagnostic sequences to determine the origins of this and other rabbit populations known to have been manipulated by man, such as present-day domestic and feral animals.

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