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Origin of European rabbit (*Oryctolagus cuniculus*) in a Mediterranean island: Zooarchaeology and ancient DNA examination

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Abstract

Mammalian species presently living on Mediterranean islands have been brought in by man. The question of their geographical origin and of the time of their introduction is often a matter of debate. We studied this problem using a population of rabbits (European rabbit: *Oryctolagus cuniculus*) living in Zembra, an island off Tunisia. Archaeological surveys show that rabbit has been introduced to the island by Bronze Age or Roman people, between the IIIrd Millenium B.C. and the IIIrd century A.D. Part of the 16S-rRNA gene of mitochondrial DNAs from fossil bones of different ages (dated back to 130–390 A.D.) was characterized and compared to that of present day rabbits of differing geographical origin. The data suggest that animals present on Zembra in late Roman times belonged to the same maternal lineage as present populations from Northern Spain and Southern France.

Introduction

Paleontological, archaeological and historical data have shown that, during early historical times, man played a fundamental part in migrations of animals in various places around the world. This role was crucial for restricted areas such as islands. The genus *Oryctolagus*, which originated in Southern Spain 6–6.5 Myr ago (Lopez-Martinez, 1977), is now represented by only one species: *Oryctolagus cuniculus* (European rabbit). The most ancient known fossil of this species has been found in

Andalusia in middle Pleistocene layers (Lopez-Martinez, 1977); remains reported in Southern France are post Mindel glaciation (only 200 000 years ago). Its present distribution all over Western Europe, in Morocco and Western Algeria and a few surrounding Mediterranean and Atlantic islands, is the consequence of transportations by man. The origin of the animals transported, the time of their transport throughout the Western Mediterranean Basin and the date of their earliest introduction are a matter of controversy (Bijou-Duval et al., 1991; Bijou-Duval, 1992). Phoenicians were the first to report the presence of rabbits in Spain (Bodson, 1978), however this does not prove they actively transported these animals to the Mediterranean islands. We had the opportunity ot examine the question of such man-made migrations through the study of the present and past rabbits of Zembra, a small island (about 340 ha) in Tunis Bay.

Rabbits presently living on Zembra are well differentiated from domestic stocks by nuclear genetic markers (Benammar and Cazenave, 1982). Their mitochondrial DNA (mtDNA) shows very low polymorphism and is very close (1 restriction site difference over the 100 sampled) to the type carried by domestic stocks. They both belong to the same maternal lineage referred to herein as B) which also includes mtDNAs from populations sampled from Northern Spain to Central France, whereas populations from Southern Spain belong to a rather divergent (4%) maternal lineage (Biju-Duval et al., 1991), hereafter referred as A.

In order to reconstruct past rabbit migrations a zooarchaeological survey has been conducted at Zembra since 1986. Rabbit remains have been collected in several layers (Vigne, 1988). More recent excavations lead to the proposal of an introduction between about 1000 B.C. and 100 A.D.

A few years ago, several groups developed techniques for the analysis of DNA from ancient bones: Hagelberg et al. (1989), Horai et al (1989), Hänni et al. (1990), Williams et al. (1990).

With a view to discovering the origin of rabbits on Zembra island we characterized mtDNA from bones of different ages. The data suggest that the rabbits living on Zembra at late Roman times were of the same maternal lineage as those of Northern Spain or Southern France.

Materials and methods

Origin of the bones

Three kinds of bone were used: present bones from Las Lomas (LLR), recent bones from Zembra (ZV) and fossil bones from Zembra (Z7).

Present bones come from two individuals (LLR1 female and LLR5 male) caught alive at Las Lomas, near Cadix (Andalusia, Southern Spain). They were cleaned by boiling and papain hydrolysis of soft tissues in 1987.

Recent bones from Zembra were collected in 1986 from the ground after natural decomposition of animals which died a few months or years before. These bones were cleaned with fresh water.

Table I. Description of the stratigraphic succession of the layers in the "abri du casino" shelter of Zembra.

Stratigraphic unit	Depth under ground level	NISP Invertebrates	NISP Vertebrates	NISP Rabbit	No. fragments of pottery + glass
S.U. 0	0/−37 cm	19	141	19	86 + 9
S.U. 1	−35/−58 cm	22	267	24	72 + 5
S.U. 2	−35/−58 cm	13	55	10	132 + 4
S.U. 3	−58/−65 cm	0	16	2	123 + 1
Fire place on flagstone pavement					
S.U.4	−65/−72 cm	0	1	0	20 + 0
Substratum	more than −72 cm	0	0	0	0 + 0

S.U.: Stratigraphic Unit; NISP: Number of identified specimens.

Fossil bones from Zembra came from a small cave (Z7 = "abri du casino"; Vigne, 1988) with four archaeological alluvial clay layers described in Table I. The clearest division is that between S.U. 3/2, which is a continuous hardened surface. The fossil bones of rabbits used for DNA examination, came from S.U. 2 (fragment Z7-C2) and S.U. 1 (fragment Z7-C1).

Origin of DNAs

Preliminary experiments were conducted on mtDNA extracted from liver of present day wild animals caught in Las Lomas (LL27, LL85), near Badajoz (Ba16, Ba6), both localities in Southern Spain, and on Zembra (Z10). MtDNAs of these animals had been previously characterized (Biju-Duval et al., 1991; Biju-Duval, 1992) and the phylogenetic relationships of the types established (Fig. 1). These

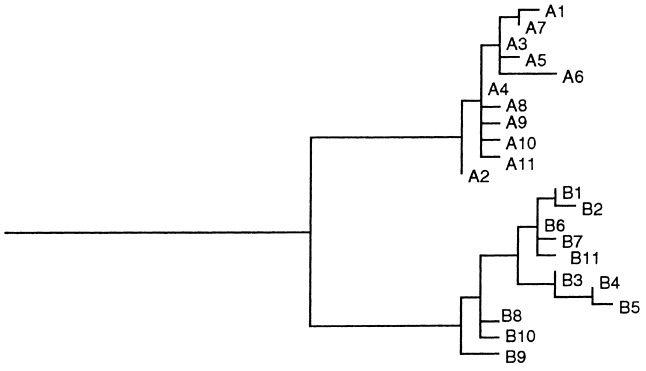

Fig. 1. Phylogenetic relationships between mtDNA molecules. The molecules studied so far have been organized into two maternal lineages A and B (Biju-Duval, 1992). Within each lineage the numbers refer to the different types as measured by RFLP analysis (Biju-Duval et al., 1991, Biju-Duval, 1992).

Table II.–Characterization of mtDNA from different sources

DNA Source	ENZYME		mtDNA TYPE†
	*Hin*fI†	*Sty*I†	
a: *Modern (soft tissues)*			
*LL27	xx	x	A1
*LL85	xx	x	A4
*Ba6	xx	x	A10
*Z10	x	xx	B6
*Ba16	x	xx	B3
pOCCmtDNA	x	xx	B1
b: *Present Day Bones*			
LLR1 Femur	xx	x	A
LLR5 Femur	xx	x	A
c: *Recent Bones*			
Z V1 Femur	x	xx	B
Z V2 Scapula	x	xx	B
c: *Ancient Bones*			
Z7-C1 Femur (IVth–XXth c. A.D.)	x	xx	B
Z7-C2 Femur (Vth–VIth c. A.D.)	x	xx	B

The presence of sites for two restriction enzymes, *Hin*fI and *Sty*I, in either the 16S-rRNA gene amplified with primers 16sar-16sbr (Simon et al., 1991) or 16S3-16S5 (present study), was determined for mtDNA of different sources and different ages. mtDNA has been extracted from liver (*) or from bones (ancient and modern); the plasmid pOCCmt containing an entire rabbit mtDNA molecule from liver of domestic rabbit, is used as a control. Depending on the mtDNA type (A or B, Biju-Duval, 1991, 1992), the enzymes cut once (x) or twice (xx). †: The numbers accompanying the letters indicate mtDNA subtypes according to Figure 1.

types are: A1 for LL27, A4 for LL85, A10 for Ba6, B3 for Ba16, B6 for Z10, Figure 1 and Table II. This first approach involved a plasmid containing an entire mitochondrial DNA molecule, of mtDNA type B1 (Fig. 1), coming from the liver of a domestic rabbit (pOCCmt, Mignotte et al., 1990).

The bones of animals described above were used for other experiments.

DNA extraction, amplification and sequencing

Present and ancient bones were ground and mtDNA were extracted following the technique described by Hagelberg and Clegg (1991). All bone extractions were performed singly using sterile techniques in a separate laboratory to those used for extracting modern DNA in order to minimise contamination both between samples and by modern DNA.

Part of the 16S-rRNA gene (homologous to nucleotides 2147–2413 in rat mtDNA) of mtDNAs previously extracted from liver of present day animals (Biju-Duval et al., 1991) was amplified using two primers 16sar and 16sbr (Simon

et al., 1991) specific to this gene and well conserved in Metazoa. Amplification was accomplished through 30–40 cycles: 1 min at 95° C, 1 min at 50° C, 1 min at 70° C. The PCR conditions were: 67 mM Tris HCl pH 8.8, 6.7 mm $MgSO_4$, 16.7 mM NH_4SO_4, 10 mM β-mercaptoethanol, 0.1% Triton X100, 250 μM dNTPs and 1 μM primers. After PCR the products were purified, end-filled to produce blunt-ended fragments and cloned into Bluescript vectors (Stratagene) for sequencing single strand DNA (Sambrook et al., 1989).

Amplification of mtDNA from bones was performed using 16sar and 16sbr primers (Simon et al., 1991) and/or two new primers, 16S3 and 16S5, specific for rabbit mtDNA. These two new primers are 224 nucleotides apart within the region amplified with the use of 16sar and 16sbr (see Fig. 2). Amplification was conducted

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   16sar---------------->
 1 cgcctgttta tcaaaaacat CACCTCTAGC ATTACTAGTA TTAGAGGCAC

                    RsaI
51 TGCCTGCCCA GTGACATACG TTCAACGGCC GCGGTATCCT GACCGTGCAA
                   G

101 AGGTAGCATA ATCACTTGTT CCTTAATTGG GGACTAGCAT GAATGGCAAC


151 ACGAGGGTTA AACTGTCTCT TTCTTCCAAT CAGTGAAATT GACCTCCCCG

                                          16S3--------------
201 TGAAGAGGCG GGGATAAAAT AATAAGACGA GAAGACCCTA TGGAGCTTTA

   ---->                                                  StyI
251 ATTATTTAAC CCAACACTTC CTTTATTCTA CTCTACAACG AGCCTAACTC
                                               T           -

301 AAGGAAATCC CTGGGTTAAA AATTTTGGTT GGGGTGACCT CGGAGTATAA

                                  HinfI
351 ATCAACCTCC GAATGATTTT AGCCTAGACT CAACAAGTCA AAGCAATTAT
                                    C
                                                       StyI
401 AATCATAAAT TGACCCAAAA AATTTGATCA ACGGAACAAG TTACCCTAGG
                          T
                             HinfI <-----------------16S5
451 GATAACAGCG CAATCCTATT TTAGAGTCCC TATCGACAAT AGGGTTTACG

                                       BpvI
501 ACCTCGATGT TGGATCAGGA CATCCTAATG GTGCAGCCGC TATTAAAGGT
                                     C         T
                                    <-------------------16sbr
551 TCGTTTGTTC AACGATTAAA GTCCtacatga tctgagttcag accgg
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Fig. 2. Sequence of part of the 16S-rRNA gene in rabbit mtDNA. Sequence of the 16SrRNA gene of Type A European rabbit mtDNA between the universal primers 16sar and 16sbr (Simon et al., 1991) is given on the main line. The seven nucleotide differences presented by Type B mtDNA are indicated below the line. Numbering of the nucleotides begins with the first nucleotide of primer 16sar. The location of rabbit specific primers 16S3 and 16S5 used to amplify DNA from bone extracts are indicated above the sequence. Diagnostic restriciton endonuclease sites between the two types are also indicated above the sequence.

using identical conditions for modern DNA except that 160 μg/ml Bovine Serum Albumin was included in the PCR reaction.

Separate digestion (according to suppliers' instructions) of the products with *Hin*fI and *Sty*I allowed us to assign them to a maternal lineage according to the presence/absence of the sites tested (see Results).

Results

Dating fossil rabbit bones from the "abri du Casino"

The carbon samples of S.U. 3 have been dated Ly-4382: 1760 $\pm$ 90 b.p. (i.e. calibrated: 130–390 A.D.) and all the determinable potteries of the same layer indicate the end of the IIIrd into the IVth centuries A.D. The archaeological assemblages of these layers is very homogeneous, no perturbation has been observed during excavation and this part of the archaeological sequence is sealed by the hardened surface. We can thus conclude that: (i) rabbits were present at Zembra during the IIIrd–IVth c. A.D., i.e. during the Late Roman periods, and (ii) the rabbit bone Z7-C2 dates from Late Classical Antiquity (Vth–VIth c. A.D.).

Most of the potteries of the whole upper part of the deposit (S.U. 1/0) date from Late Classical Antiquity, but some of them, and the main part of the glass fragments, are modern (end of the XXth century). These deposits probably accumulated in the cave during the construction and utilisation of the nearby casino, from 1960. It is thus very difficult to date the Z7-C1 rabbit bones more precisely than between the IVth and the XXth century A.D.

Setting up a method for easy analysis of ancient mtDNA

Before attempting to extract DNA from ancient bones, sequence data for the two types of rabbit mtDNA were required in order to estimate the nucleotide variability within and between the two maternal lineages (A and B) previously identified (Biju-Duval et al., 1991; Biju-Duval, 1992). It was decided to use PCR as a rapid method for obtaining large quantities of DNA for cloning and sequencing. Amplification of mtDNA was performed for wild individual mtDNAs chosen from 4 subtypes: Types A4 (LL85), A10 (Ba6), B6 (Z10) and B3 (Ba16) and the plasmid pOCCmt (Type B1) as a control (Tab. II and Fig. 2).

The sequence data revealed no differences between members of the same lineage (A or B) and the presence of 7 nucleotide changes in 554 bp (1.3%) between the two primers 16sar and 16sbr (Simon et al., 1991): 5 transitions, 1 transversion, and 1 insertion in Type A (Fig. 2). Four of the seven differences lead to polymorphisms (RFLPs) for four restriction enzymes: *Bpv*I, *Hin*fI, *Rsa*I and *Sty*I (Figs 2 and 3).

Compared to data published on other mammals (Man: Anderson et al., 1981; Mouse: Bibb et al., 1981; Bovine: Anderson et al., 1982; Rat: Gadaleta et al., 1989) these sequences allowed us to select a new primer pair (16S3, 16S5) specific for

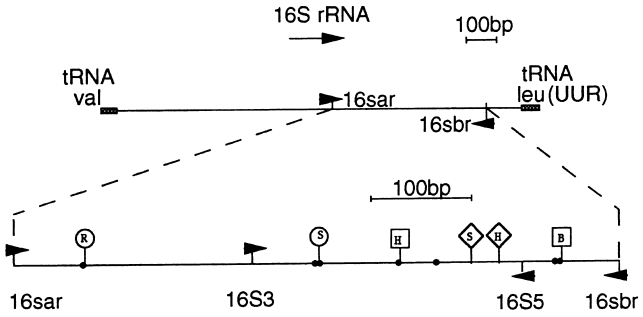

Fig. 3. Diagnostic restriction sites in the rabbit 16S-rRNA gene. On the upper line are indicated the relative positions of the val-tRNA, 16SrRNA and leu-tRNA genes and that of the universal primers: 16sar and 16sbr (Simon et al., 1991). The lower line represents and enlargement of the domain amplified with the use of these primers. The restriction sites including the polymorphic sites are indicated as well as the location of the primers specific for rabbit mtDNA (16S3 and 16S5). The abbreviations and the signs are as follows: B = *Bpv*I, H = *Hinf*I, R = *Rsa*I, S = *Sty*I, ▶ = oligoprimers, ● = mutation site, □ = restriction sites only present in type A mtDNA, ○ = restriction site only present in type B mtDNA, ◇ = restriction sites present in both A and B mtDNAs.

rabbit and delimiting a shorter domain (224 nucleotides). The primers were chosen on the basis of four positive criteria. Firstly, the two primers are separated by only 224 nucleotides (a size ideal for amplification of ancient DNA less than 1000 years old, Pääbo, 1989) which includes 4 of the 7 nucleotide changes, 2 of them leading to RFLPs (*Hin*fI, *Sty*I). Secondly, there is an addtional site for each enzyme between the primers which serve as internal controls. Thirdly, the final 3′ nucleotides of the primers differ from those present in the sequences of some of the most likely sources of external DNA contamination in the bone samples (human, rat, mouse, etc.) preventing amplification of non-rabbit DNA. Finally, the *Hin*fI site is specific for Type A, the *Sty*I site is specific for Type B (Tab. IIa).

DNA extraction from bones

Whenever possible, entire femurs or fragments of this bone were used since they are often the largest and best preserved bones. Extractions were attempted on between 0.4 and 2.8 g of powdered individual bone. Overall, six bone samples were used in the preliminary extractions. Two present-day bone samples from Type A rabbits (LLR1—femur, 2.79 g, and LLR5—femur, 2.15 g), two recent bones from Zembra (ZV1—femur, 2.70 g and ZV2—scapula, 0.46 g) and two ancient bones (Z7-C1—femur fragment, 0.89 g, IVth–XXth c. A.D., and Z7-C2—femur fragment, 0.46 g, IIIrd–IVth c. A.D.). DNA was isolated in each case, although the oldest sample (Z7-C2) showed considerably less nucleic material than the rest. In all cases, the final DNA solutions showed the presence of yellow or blue-grey large molecular weight contaminants. Much of this, but not all, could be removed by

repeated phenol extraction of the samples. The presence of DNA was confirmed by DNAse digestion of one sample which removed the ethidium bromide-stained material.

Characterization of mtDNA extracted from bones

PCR products were obtained with primers 16S3 and 16S5 for samples LLR1, LLR5, and ZV1. PCR was similarly successful with the same primers for ZV2, Z7-C1 and Z7-C2, but only after using 10-fold less DNA in the reaction (diluting the putative inhibitor of the *Taq* polymerase). Each PCR product was digested with *Sty*I and *Hin*fI. Restriction profiles showed that mtDNA from LLR1 and LLR5 are characteristic for Type A, whereas ZV1, ZV2, Z7-C1 and Z7-C2 resembled Type B (Tab. 2b, c and d). PCR products larger than 270 bp (up to 600 bp using the other primer combination) could only be obtained with DNA from LLR1 and LLR5 bones.

Discussion

Paleontological data show that there were no rabbits in Morocco and Western Algeria during the Middle and Upper Pleistocene (Jaeger, 1975; Kowalski and Rzebik-Kowalska, 1991). A rich faunal assemblage from an (Early?) Upper Pleistocene deposit confirms this result for Zembra (Vigne, unpublished data). At the start of the Holocene the island had recently isolated from the mainland; a Late Neolithic layer studied at the "abri du Scorpion" (Z21, Vigne, unpublished data) has been carbon-dated from Ly-5570: 3465 ± 155 b.p. (i.e. calibrated: 2244–1453 B.C.) but is probably slightly older as judged from the pottery assemblages (IIIrd millenia B.C.?). This layer provided no rabbit bones. The oldest rabbit bones at Zembra are a scapula and a rib from S.U. 3 of the "abri du casino" firmly dated from the IIIrd–IVth c. A.D. by its stratigraphic position and the pottery dating. Preliminary surveys show that the island was occupied by Punic people from the VIth to the IInd c. B.C. (Chelbi and Ghalia, unpublished data) but the corresponding layers have not yet been excavated.

These data indicate that rabbits were introduced to Zembra between the Late Neolithic and the IIIrd c. A.D., i.e. by Bronze Age, Punic or Roman people. This is consistent with the fact that Phoenicians were the first to mention the existence of rabbits in Spain and the Romans reported their presence on some Mediterranean islands (Bodson, 1978).

The characterization of restriction sites in a part of the 16S-rRNA gene of mtDNA extracted from recent (ZV1 and ZV2), Late Classical Antiquity (Z7-C2 from the Vth–VIth c. A.D.) and intermediate (Z7-C1, IVth–XXth c. A.D.) bones from Zembra has clearly demonstrated that they are all related to the same maternal lineage: B.

If we assume that the few bones analyzed are representative of the entire populations living at any time, this shows that either Man has brought rabbits

several times to the island from populations belonging to the same original maternal lineage, or that the present rabbits of Zembra are descended from a unique introduction earlier than the IIIrd c. A.D. The few measurements of the fossil rabbit bones of Zembra, which do not seem significantly different from present bones from the island (Vigne, 1988 and unpublished data), might indicate that the second possibliity is more probable.

The fact that all bones studied exhibit a mtDNA type related to the maternal lineage B gives an indication as to the geographical origin of the rabbit ancestors. Based on mtDNA analysis, present rabbit populations exhibit a well defined geographical structure with the mtDNA type A only present in Southern Spain and mtDNA type B in Northern Spain and Southern France (Biju-Duval, 1992). Whether this distribution has remained stable over the two last millenia remains to be seen. If so, rabbits introduced to Zembra originated from Northern Spain or Southern France.

We intend to collect new fossil bones in layers between the IIIrd c. A.D. and the VIth c. B.C. in order to specify which peoples brought rabbits to the island and to identify the mtDNA type of the oldest animals. Definitive accuracy is expected in the future by radiocarbon dating (accelerator) of the bone from which mtDNA is extracted. A more precise molecular approach is being developed to detect the precise origin of rabbits introduced to Zembra among the various possibilities of populations carrying the mtDNA type B from Spain or Southern France.

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