Ancient DNA: genomic amplification of Roman and medieval bovine bones.

L. Pariset[1], F. Gabbianelli[1], D. De Bernardis[2], M.T. Fortunato [2], F. Alhaique [2], E. De Minicis [2], A. Valentini[1].

[1] Dipartimento di Produzioni Animali; Facoltà di Agraria, Università degli Studi della Tuscia, Viterbo, Italy [2] Dipartimento di Scienze del Mondo Antico, Facoltà di Conservazione dei Beni Culturali, Università degli Studi della Tuscia, Viterbo, Italy

Corresponding Author: Lorraine Pariset, Dipartimento di Produzioni Animali, Università della Tuscia, via S. Camillo de Lellis, 01100 Viterbo, Italy. Tel. +39 0761 357447, Email: pariset@unitus.it

ABSTRACT

Cattle remains (bones and teeth) of both roman and medieval age were collected in the archaeological site of Ferento (Viterbo, Italy) with the aim of extracting and characterising nucleic acids. Procedures to minimize contamination with modern DNA and to help ancient DNA (aDNA) preservation of the archaeological remains were adopted. Different techniques to extract aDNA (like Phenol/chloroform extraction) from bovine bones were tested to identify the method that applies to the peculiar characteristics of the study site. Currently, aDNA investigation is mainly based on mtDNA, due to the ease of amplification of the small and high-copied genome and to its usefulness in evolutionary studies. Preliminary amplification of both mitochondrial and nuclear aDNA fragments from samples of Roman and medieval animals were performed and partial specific sequences of mitochondrial D-loop as well as of nuclear genes were obtained. The innovative amplification of nuclear aDNA could enable the analysis of genes involved in specific animal traits, giving insights of ancient economic and cultural uses, as well as providing information on the origin of modern livestock population.

Introduction - DNA molecules are characterised by extremely stable biochemical conformation. Amplification of even damaged and scarcely represented DNA, like aDNA (ancient DNA) from fossil remains, is feasible employing Polymerase Chain Reaction. The present study analyzes roman and medieval bovine bones collected from Ferento, an archaeological site near Viterbo, central Italy. Biometric analyses of ancient remains can give information expecially about dimension, whereas molecular biology techniques can help in authenticating data from archaeological sites or give further information on the specific characteristics of the animals. Mitochondrial DNA is the nucleic acid most frequently used in aDNA research and has proven to be very useful for inferring the origins and phylogenetic hystory of many species. Informations gained from nuclear DNA can be used to answer questions about phenotypic characteristics (Bunce et al. 2003) unachievable by biometric analyses of remains. However, nuclear DNA is much more difficult to amplify than mtDNA from the same aDNA extracts, mainly because nuclear genes are typically 5,000–10,000 times less abundant per cell than those of mitochondrial origin (Poinar et al., 2003; Binladen et al, 2005). However, recent studies reported successful nuclear DNA sequencing (Greenwood et al., 1999, 2001; Edwards et al, 2003; Cymbron et al., 2005; Noonan et al., 2005).

The aim of this study was to obtain sequences from mitochondrial and, possibly, nuclear DNA in order to reveal specific traits of animal populations breed in historical times. Among the several possible nuclear genes, two particular genes were chosen: one easily selected by man as it determines coat colour, the melanocortin receptor 1 gene (*MC1R* or melanocyte stimulating hormone receptor, *MSHR*) and the Y-encoded, testis-specific protein gene (*TSPY*), present only in males, to determine the sex of the remains. Among the main genes affecting coat colour in cattle, MC1R is reported as the one showing most variations between breeds (Maudet and Taberlet 2002). Klungland et al. (1995) have shown that MC1R encoded by the extension locus (E-locus) is an important gene in the regulation of the synthesis of two pigments within melanocytes.

1 **Material and methods -** Stringent standards for the authentication of ancient DNA were applied
2 (Cooper & Poinar, 2000; Hofreiter et al. 2001): (*i*) for all samples aDNA was extracted in a
3 dedicated laboratory room and at least two independent DNA extractions were performed; one
4 single sample a day was extracted with a relative extraction control; (*ii*) all aDNA amplifications
5 were performed in two separated laboratory; modern DNA was manipulated in a third one; in each
6 amplification both extraction and PCR controls produced negative results; (*iii*) amplification of long
7 DNA fragments, unusual in ancient DNA analyses, was not observed; (*iv*) at least two independent
8 amplifications of the same fragment in each sample were performed to validate results. An
9 independent set of extraction, amplification, and sequencing by using the uracil-*N*-glycosylase
10 treatment is in progress, to ascertain the presence of sequence artefacts due to postmortem damages.
11 DNA was extracted from remains recovered from two different find areas within Ferento site,
12 starting from 2004 and dated on the basis of the potteries finds. Each bone, after a pre-tratment with
13 sand paper and UV irradiation to clear surface contamination, was grinded using a drill in a sterile
14 flow-hood in a laboratory dedicated to aDNA extraction. A blank control was performed in each
15 extraction. We tried both a phenol/clorophorm and a silica-based spin column extraction (Yang et al
16 1998) to identify the method giving best results in term of yield and purity. aDNA was obtained
17 from a total of seven bovine bones (2 romans and 5 medieval), each belonging to a different
18 individual, by using standard commercial kits (tissue and blood kits; Qiagen, Chatsworth, CA).
19 DNA quantification was performed using a DTX Multimode Detector 8800 (Beckman). A mean of
20 30 ng DNA per g of bone powder was observed, even if this value is possibly affected by sample
21 condition.
22 Primers were designed from the AF547663 (MC1R) and AY539852 (TSPY) sequences to allow
23 amplification of fragmented aDNA, targeting to fragments inferior to 200bp in lenght (table 1).
24 For MC1R, a first PCR amplification was performed using 20 ng of DNA template, 0.5 pmoles of
25 each primer (MWG biotech), 1X PCR buffer containing 1.5 mM $MgCl_2$, 0.2mM each dNTPs, 1 U
26 of a mix of *Pfu* DNA polymerase and Go*Taq polymerase* (Promega) 1:5 in a 20μl final volume,
27 using primer pairs 1-2 or 1-3. A nested PCR amplification was performed using the same condition
28 in a final volume of 40 μl, using primer pairs 4-2 or 1-5.
29 To amplify TSPY gene the same PCR conditions were used (using primer pairs 6-7), while
30 employing a touchdown method by starting 7°C above the Tm of the primer set in the first cycle
31 and reducing the annealing temperature by 0.5°C per cycle over 15 cycles. A further 15 cycles were
32 then carried out using the calculated annealing temperature of 55°C.

33 Table 1. Table of primers designed and/or used in the present work.

sequence 5'-3'
1- CAAGAACCGCAACCTGCACT*
2- GCCTGGGTGGCCAGGACA*
3- AGATGAGCACGTCGATGACA
4- TTTATCTGCTGCCTGGCTGT
5- CGTCGATGACATTGTCCAG
6- TCCTGTGCTTTCAGTGATGG
7- GGATCAAGCTAAGCCAACCA

34 *Primers described by Klungland et al (1995)
35
36 All sequences were obtained for both DNA strands on a CEQ8800 (Beckman coulter) by using the
37 DCTS quick start sequencing kit (Beckman coulter) following manufacturers instructions. Raw
38 sequences were edited and aligned by using Bioedit software (Hall, 1999). Sequencing of
39 amplification aliquots was also outsourced to MWG-biotech, to confirm sequencing results.
40
41 **Results and conclusions -** Mitochondrial DNA was successfully and consistently amplified and
42 sequenced. Genomic DNA was also amplified. The amount of DNA obtained in the nested PCR, as

quantified by Picogreen, was in the range normally used for gel evaluation or subsequent sequencing reaction. In fact clear sequences of the considered genes (*MC1R* and *TSPY*) were obtained, mostly with a quality comparable of that of modern DNA.

Six out of the seven individuals examined show a E^+/ E^+ genotype, found in many European breeds like Chianina, Normande, Belgian Blue, Rendena (Maudet e Taberlet, 2002), associated with a white, light brown, roan and grey coat colour. Only one sample (medieval Q4) shows an E^D/ E^D genotype, which determines a white and black coat coloration (fig. 1).

As for the sexing, a simple electrophoretic analysis on agarose gel revealed that six individuals, but the medieval sample Q5, were males (fig. 2). When identical or very similar sequences are found in modern samples the authentication of aDNA sequences with absolute certainty is virtually impossible (Beja-Pereira et al., 2006). However, we are confident that the sequences we present are endogenous, because before their arrival in the molecular laboratory the remains were manipulated by archaezoologists following established procedures to minimize contamination particularly with human DNA (use of latex gloves, extraction of deep samples) and to preserve aDNA (samples quickly set under vacuum and later set aside at –20°C). During molecular analysis ancient and modern DNA were extracted and amplified in different isolated rooms and stringent criteria and protocols for DNA validation were applied (Willerslev and Cooper 2005).

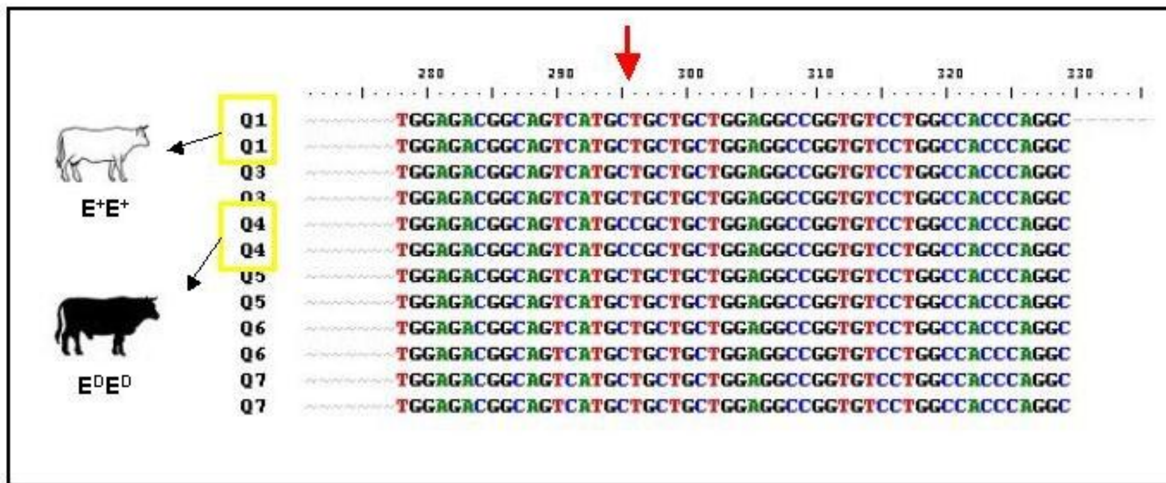

Fig 1. Sequencing results of *MC1R* gene.

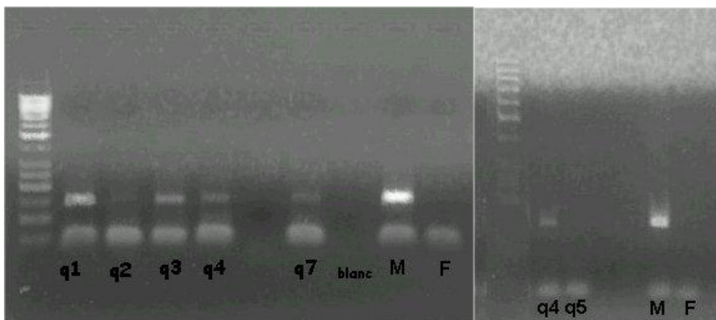

Fig 2. *TSPY* amplification of the samples. M= male control, F= female control.

REFERENCES – **Beja-Pereira**, A., Caramelli, D., Lalueza-Fox, C., Vernesi, C., Ferranda, N., Casoli, A., Goyache, F., Royo, L. J., Conti, S., Lari, M., Martini, A., Ouragh, L., Magid, A., Atash, A., Zsolnai, A., Boscato, P., Triantaphylidis, C., Ploumi, K., Sineo, L., Mallegni, F., Taberlet, P., Erhardt, G., Sampietro, L., Bertranpetit, J., Barbujani, G., Luikart, G., Bertorelle, G., 2006. The origin of European cattle: Evidence from modern and ancient DNA. PNAS. 103:8113–8118.

1 **Binladen**, J., Wiuf, C., Thomas, M., Gilbert, P., Bunce, M., Barnett, R., Larson, G., Greenwood, A.
2 D., Haile, J., Ho, S. Y. W., Hansen, A. J., Willerslev, E., 2006. Assessing the Fidelity of Ancient
3 DNA Sequences Amplified From Nuclear Genes. Genetics. 172:733–741. **Cooper**, A., Poinar, H.
4 N., 2000. Science 289:1139. **Cymbron**, T. Freeman, A.R., Malheiro, M. I., Vigne, J. D., Bradley,
5 D. G., 2005. Microsatellite diversity suggests different histories for Mediterranean and Northern
6 European cattle populations. Proc. R. Soc. B. 272:1837-1843. **Edwards**, C. J., Connellan, J.,
7 Fallace, P. F., Park, S. D. E., McCormick, F. M., Olsaker, I., Eythòrsdòttir, E., MacHugh, D. E.,
8 Bailey, J. F., Bradley, D. G., 2003. Feasibility and utility of microsatellite markers in archaeological
9 cattle remains from a Viking Age settlementin in Dublin. Animal Genetics,:34:410-416.
10 **Greenwood**, A. D., Capelli, C., Possnert, G., Pääbo, S., 1999. Nuclear DNA sequence from late
11 Pleistocene megafauna. Mol. Biol. Evol. 16:1466-1473. **Greenwood**, A. D., Lee, F., Capelli, C.,
12 DeSalle, R., Tikhonov, A., 2001. Evolution of endogenous retrovirus-like elements of the woolly
13 mammoth (Mammuthus primigenius) and its relatives. Mol. Biol. Evol. 18:840–847. **Hall**, T. A.,
14 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for
15 Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98. **Hofreiter**, M., Serre, D., Poinar, H. N.,
16 Kuch, M., Pääbo, S., 2001. Nat. Rev. Genet. 2:353–359. **Klungland**, H., Vage, D. I., Gomez-Raya,
17 L., Adalsteinsson, S., Lien, S., 1995. The role of melanocyte-stimulating hormone (MSH) receptor
18 in bovine coat color determination. Mamm. Genome. 6:636–639. **Maudet**, C., Taberlet, P., 2002.
19 Holstein's Milk Detection in Cheeses Inferred from Melanocortin Receptor 1 (MC1R). Gene
20 Polymorphism. J. Dairy Sci. 85:707–715. **Noonan**, J. P., Hofreiter, M., Smith, D. Priest, J. R.,
21 Rohland, N., 2005. Genomic sequencing of Pleistocene cave bears. Science. 309:597–599. **Poinar**,
22 H., Kuch, M., McDonald, G., Martin, P., Pääbo, S., 2003. Nuclear gene sequences from a late
23 Pleistocene sloth coprolite. Curr. Biol. 13:1150-2. **Willerslev**, E., Cooper, A., 2005. Ancient DNA.
24 Prooceedings of the Royal Society B. 272:3-16. **Yang**, D. Y., Eng, B., Waye, J. S., Dudar, J. C.,
25 Saunders, S. R., 1998. Technical Note: Improved DNA Extraction From Ancient Bones Using
26 Silica-Based Spin Columns. Am. J. Phys. Anthropol. 105:539-543.