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Journal of Archaeological Science 34 (2007) 675–680

Journal of Archaeological SCIENCE

http://www.elsevier.com/locate/jas

Ancient DNA and the genetic signature of ancient Greek manuscripts

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Received 4 January 2006; received in revised form 19 June 2006; accepted 20 June 2006

Abstract

Determination of the species origin of historic objects is one of the common tasks of ancient DNA (aDNA) analysis. DNA recovered from archaeological and palaeontological remains allows going back in time and revealing the genetic signature of several human tools. Comparisons of this signature with DNA sequence from recent animals (wild and domestic goats) from several Mediterranean regions are expected to allow us to identify a geographical origin for the biological material used to produce the Greek parchment manuscripts. Here, we have realised an experiment based on which it is possible to recover DNA from ancient parchment fragments (three Greek parchment manuscripts of relatively recent age: 13th to 16th century AD). The analysis of the three Greek manuscripts has shown that most signature documents have goat-related sequences (*Capra* spp.). As demonstrated, DNA of animals whose skins furnished the parchment pages of ancient and medieval books may survive in that parchment, enabling not only to determine the species of the animal from which the skin had been taken, but moreover, it might even be possible to reconstitute the history of the herds from which they originated.

Keywords: Ancient DNA; Deamination; Greek manuscripts; Evolution

1. Introduction

Animal skin parchment is one of the oldest substances used by human. Like rocks, sticks or other vegetable matter, people had access to animals and tried to put every piece of an animal to full use. Skins have been used for clothing, tools, and weapons. In historical times, another form of animal skin, parchment (pergamena in Latin), was an essential ''vehicle'' of cultural activity in Eurasia [2]. It is known that skins were used as writing surfaces as early as 2700 BC in the IV dynasty in Egypt. In an effort to stifle any possible challenge to the amassing of knowledge that was taking place in the famed libraries of Alexandria, the Egyptians banned the export of papyrus, which was the most common writing surface of the time.

Parchment is a material for the pages of the codex, the manuscript book, made from fine sheepskin, goatskin or calfskin. According to the historians, it was invented about the beginning of the 2nd century (Before Christ, BC), in Pergamon, as a substitute for papyrus. Pergamon or Pergamum (modern day Bergama in Turkey) was a Greek city, in northwestern Anatolia, 16 miles from the Aegean Sea, located on a promontory on the north side of the river Caicus (modern day Bakir), that became an important kingdom during the Hellenistic period, under the Attalid dynasty, 282–129 BC. Pergamon had a great library that rivaled the famous Library of Alexandria. As prices rose for papyrus, while the reed was overharvested towards local extinction in the Nile delta, Pergamon ''adapted'' by increasing use of parchment. Though

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doi:10.1016/j.jas.2006.06.013

the Assyrians and the Babylonians impressed their cuneiform on clay tablets, they also wrote on parchment from the 6th century BC onward [36]. The use of parchment for making manuscript books expanded throughout Mediterranean, Asian and African countries. Parchment was also the main material of making manuscripts books until the 16th century AC. It was used as well by the chancelleries of the ancient and medieval states and the ecclesiastical authorities until 19th century.

The ability to recover biomolecules, most importantly DNA, from ancient remains has opened new research that has many implications [13,24,34 and references therein]. Access to ancient DNA (aDNA) provides the opportunity to study the genetic material of past organisms and identify individual and population histories. There are numerous studies in which the retrieving of ancient DNA from post-mortem material provides scientists with powerful molecular evidence to examine the genetic changes and phylogenetic relationships between extant and extinct taxa, even when morphological and anatomical information is limited.

The parchment on which most medieval manuscripts were written was made from animal skin by removal of hair and flesh, followed by drying and scraping [36]. When an organism dies, its DNA normally becomes degraded by endogenous nucleases. Under fortunate circumstances, such as rapid desiccation, low temperatures or high salt concentrations, nucleases can themselves become destroyed or inactivated before all nucleic acids are reduced to mononucleotides [13]. The treatments used in this process were fairly mild, and there is a good chance that some of the DNA in the skin would have been left intact. But will it have survived through time? Past investigations have shown that ancient DNA can be detected not only in various palaeontological, archaeological and museum materials [13,24,34 and references therein], including parchments [36,3,30]. The post-mortem instability of nucleic acids is central to the methodological problems inherent in aDNA research. Early research showed that post-mortem DNA decay was characterized by strand breaks, baseless sites, miscoding lesions and crosslinks, and that these caused both sequencing artefacts and the preferential amplification of undamaged contaminant DNA [15,22,23,24,34]. However, the relative rates of various types of DNA damage and their mode of accumulation remain poorly characterized [6,7].

Numerous parchments (more than 3000 manuscripts) are available in several Greek libraries (Mount Athos, Athens, Patmos, Jerusalem, Alexandria, Sinai, etc.) about the origins of which we have limited knowledge. A number of questions concerning the origin and production of the Greek parchments may be addressed using DNA analysis. Based on radiocarbon and other analyses, these manuscripts date between the 8th and 16th century and were for the most part written on what is thought to be goat or sheepskin parchment. Here, we tried in collaboration with the Cultural Foundation of National Bank of Greece (CF-NBG) that has the opportunity to have access to these manuscripts, to reveal the genetic signature of the Greek manuscript.

2. Materials and methods

2.1. DNA extraction and amplification

Standard ancient DNA handling conditions and dedicated equipment were used for parchment collection, cleaning and DNA extractions [17,21]. Equipment and reagents were dedicated solely for ancient DNA work and extractions, and amplifications were carried out in a laboratory where no mammalian DNA except human had been previously used. Disposable equipment was used whenever possible, and reusable equipment was soaked in 0.5% sodium hypochlorite and then exposed to UV light for 1 h prior to use.

Genomic DNA was extracted from six parchments (Hellenic manuscripts), the age of which ranged between 13th and 16th century AD, using silica-based and phenol–chloroform extraction methods, but the phenol–chloroform method was more. The following protocol was used for phenol–chloroform DNA isolation. DNA was extracted from small pieces (approximately 0.5 cm^2) of parchment in a solution containing 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 10–25 mM EDTA, 2% SDS, and at least 200 μg/ml proteinase K for 16–24 h at 56 °C, followed by phenol–chloroform extraction. DNA purification was completed by precipitation with isopropanol or by concentrating with Centricon YM centrifugal filter devices (with Amicon filter).

Independent extracts from the same sample or samples from different parchments were used as template for PCR amplification. PCR was performed using AmpliTaq Gold (Perkin–Elmer, USA) with a $MgCl_2$ concentration of 3 mM, following the supplier's instructions, except for the addition of bovine serum albumin to a final concentration of 0.25 mg/ml. Two pairs of primer were used to assemble a contiguous sequence of up to 320 bp from the cytochrome *b* gene (cyt *b*). Primers were designed to target overlapping DNA fragments of 150–200 bp (Fig. 1A). Amplifications were done in a M.J. Thermocycler with a 5 min activation step at 94 °C, followed by 60 cycles of 94 °C for 30 s, 42 °C for 60 s and 72 °C for 30 s. PCR products were isolated from 3% agarose gels and melted in 50 μl ddH_2O, of which 5 μl were used for reamplification for 30 cycles under the PCR conditions described, except that the activation step was prolonged to 7 min and the annealing temperature was 45 °C.

At least two sterile negative controls were used for each reaction to detect contamination throughout the extraction and amplification reaction. For each sample studied the entire analysis was undertaken twice to confirm the sequences obtained.

2.2. Cloning and sequencing

Reamplification products product were then directly ligated overnight at 4 °C into the pGEM (Promega) plasmid, followed by transformation in *Escherichia coli* JM109 competent cells (Promega). Positive colonies were checked for correctly sized inserts using a PCR assay to amplify the region between the vector's SP6 and T7 promoter sites. PCR products of the

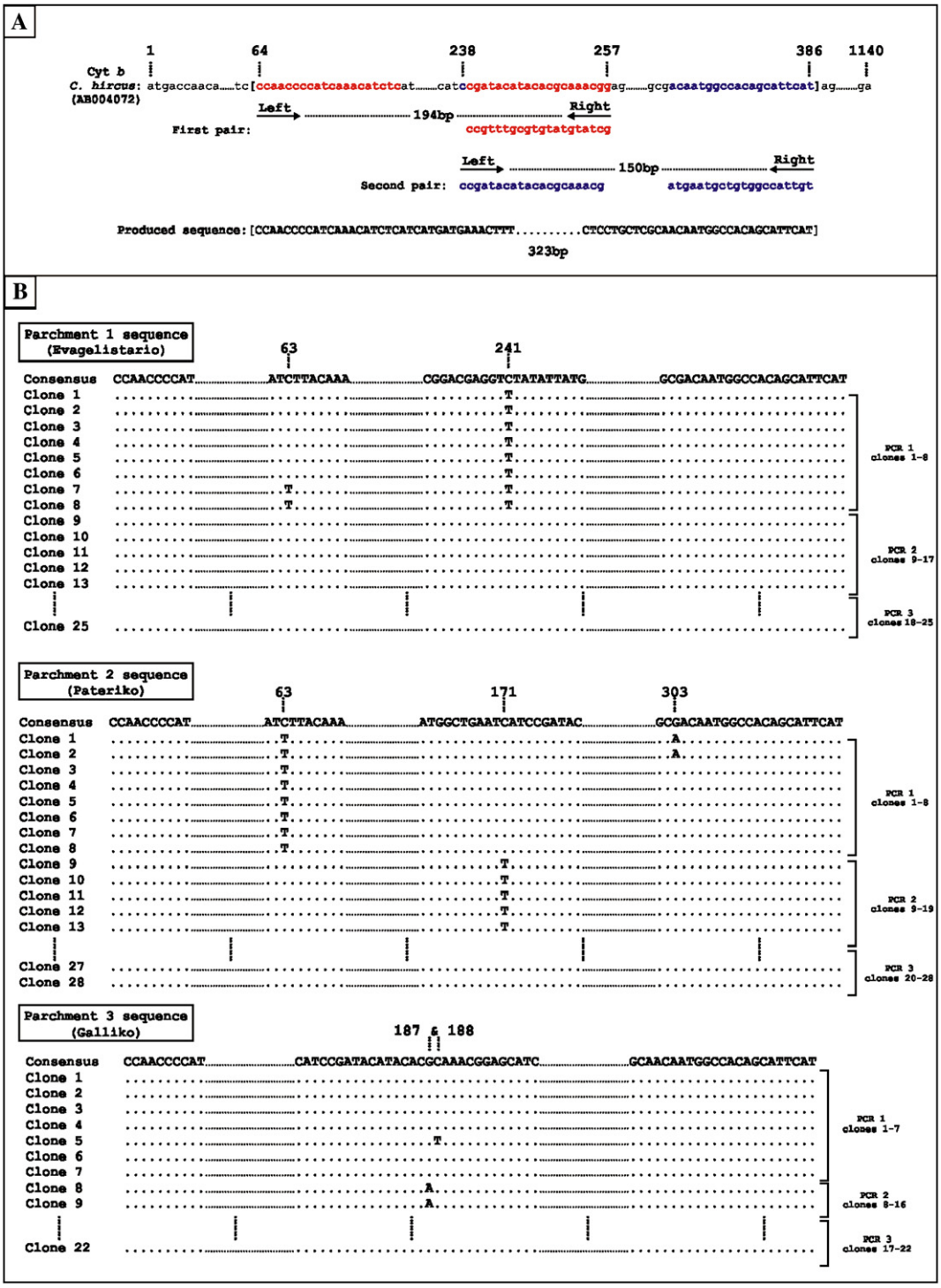

Fig. 1. (A) Position of the targeted fragments of mitochondrial cyt *b* gene, the primers and the produced sequence: schematic map. The length of each fragment is indicated in base pairs. The nucleotide numbering is based on the sequence of *Capra hircus* (GenBank AB004072). (B) Alignment of the mitochondrial DNA clones sequenced from three amplifications products for each one the three ''successful'' parchments used in this study. The consensus sequence was generated by Consensus Confidence Program (v.1.12). The high percentage of C/G→TA changes is probably due to deamination of C residues in the ancient DNA templates. Numbers above the sequences indicates consistent, singleton and other substitutions among DNA sequences.

correct size from this assay were used as templates for automated cycle sequencing on an ABI 377 (Applied Biosystems), according to manufacturer's instructions. GenBank accession numbers for the sequences obtained are DQ778621–DQ778623.

2.3. Phylogenetic analysis

Sequences were aligned using the ClustalX program package [27]. The MEGA computer package (v.2) [29] was used to determine the number and type of nucleotide substitutions in

pair-wise comparisons of sequences, to measure the degree of divergence between sequences and to identify the unique sequences for phylogenetic analysis. For phylogenetic analysis, a data set of 24 cyt *b* sequences was used (including the three parchment sequences of this study). Bayesian inference [10,35] analysis and Maximum Likelihood (ML) [4] were performed with the MrBayes (v3.1B) [10] and PAUP*4.0b10 [8], respectively, using a model of substitution [HKY + *G* model [11] with *Ti*/*Tv* = 11.8563, $a = 0.091$ and $A = 23.1$, $C = 24.7$, $G = 18$, $T = 34.2$] that was chosen by performing hierarchical likelihood-ratio tests [14] in Modeltest (v.3.06) [1]. For BI, the analysis was run with four chains for 10^7 generations and the current tree was saved to file every 100 generations. This generated an output of 10^5 trees. The $-ln$L stabilized after approximately 10^4 generations and the first 10^3 trees (10% "burn-in" in Bayesian terms, chain had not become stationary) were discarded as a conservative measure to avoid the possibility of including random, sub-optimal trees. The percentage of samples recovering any particular clade in a BI analysis represents that clade's posterior probability [35]. We used one of the methods of [19] to assure that our analyses were not trapped on local optima. In particular, the posterior probabilities for individual clades obtained from separate analyses (four runs) were compared for congruence [28], given the possibility that the analyses could appear to converge on the same *ln*-likelihood value while actually supporting incongruent phylogenetic trees.

3. Results and discussion

When an organism dies, its DNA normally becomes degraded to small average size, generally between 100 and 500 bp [13,23] by both enzymatic and nonenzymatic (hydrolytic and oxidotic) processes [9,22]. Nevertheless, DNA has been recovered from archaeological and palaeontological remains [13,24,34], as long as several fortunate environmental conditions, such as rapid desiccation, low temperatures or high salt concentrations, may prolong DNA survival [13,24,34,22,31,33].

However, the field of ancient DNA is fraught with technical pitfalls and needs stringent criteria to ensure the reliability of results. Quite a few review articles reported the basic authenticity criteria for the determination of ancient DNA sequences [13,24,34,20,18,26]. Critical steps such as extraction and PCR controls, inverse correlation between amplicon length and amplification efficiency, amplification from a second extract, cloning, phylogenetic sense are widely accepted. In addition when an unexpected result is obtained, the reproduction in a second laboratory is necessary [13].

Given that authentic ancient DNA is typically highly degraded, shorter target DNA fragments should be sought. For this reason, we designed two pairs of primers (Fig. 1A) based on previously published caprinae sequences that amplify short, nonhuman, mtDNA fragments (194 and 150 bp, respectively).

For each extraction (30 in total, five for each specimen), amplifications were performed using these specific pairs of primer. It is known that if low amounts of DNA are preserved in a specimen, some extracts will fail to contain DNA molecules by chance [13]. Consequently, it was an expected result the fact that endogenous DNA was amplified from three out of six specimens of ancient Greek parchment remains used in this study (Table 1).

While most post-mortem DNA damage events fragment the molecule and prevent it from being amplified, a small proportion merely generates miscoding lesions [34]. These are manifested as base modifications in the amplified sequence, changing the appearance of aDNA template. The few detailed studies of miscoding lesions concur with earlier hypotheses [e.g., see 23,22,15] that the majority of changes arise from the deamination of C to uracil (U), an analogue of *T*, or the deamination of *A* to hypoxanthine (HX), an analogue of *G* [6,7,26,5,32]. Ancient DNA template is a mixture of molecules comprising varying amounts of the correct endogenous sequence, damaged endogenous sequence, contaminant sequence and damaged contaminant sequence. Therefore, PCR products from aDNA template are also likely to be a mixture of misamplified damaged template and contaminant template.

In order to determine the nature of the DNA sequences amplified, the two overlapping cyt *b* fragments (194 and 150 bp, Fig. 1A) were cloned and the inserts of 22–28 clones were sequenced (Fig. 1B) for the three "successful" specimens. To obtain the true aDNA sequence, it is not sufficient to generate a single direct sequence of the mixture, even where the authentic aDNA is the most abundant in the component mixture. Only bacterial cloning can elucidate if the sequence is real or not. Bower et al. [12] calculated that the whole number

Table 1
List of the specimens of Bovidae used in molecular analyses (species name, number of specimens, samples age, and GenBank accession numbers of sequence data)

Specimens	Samples	Age	GenBank accession No.
Parchment 1 (Evagelistario)	1	13th century AD	DQ778621
Parchment 2 (Pateriko)	1	13th century AD	DQ778622
Parchment 3 (Galliko)	1	16th century AD	DQ778623
Capra hircus	6	Recent	AF217254, X56289, AB004072, D84201, AB004075, AJ231402
Capra ibex	1	Recent	AF034735
Capra pyrenaica	2	Recent	AJ010048, AJ213406
Capra falconeri	1	Recent	AF034736
Capra caucasica	1	Recent	AF034738
Capra cylindricornis	1	Recent	AF034737
Capra aegagrus	1	Recent	AF034739, AJ231408
Capra nubiana	1	Recent	AF034740
Capra sibirica	1	Recent	AF034734
Ovis aries	1	Recent	AF034730
Ovis ammon	1	Recent	AF242350
Bos Taurus	1	Recent	NC001567
Suc scrofa	1	Recent	AJ314558
Homo sapiens	1	Recent	AY255180

of clones that need to be sampled in order to be 95% confident of identifying the most abundant sequence present at 70% in the ancient sample are 20.

We generated the consensus sequence using the free-access web-based program (Consensus Confidence Program, v.1.12, http://www.mcdonald.cam.ac.uk), which constructs the most reliable consensus sequence from the user's input clone sequences and analyses the confidence limits for each nucleotide position and for the whole consensus sequence.

In total, 75 parchment clones for each fragment (194 and 150 bp) from nine initial PCR reactions (three for each "successful" parchment) were analyzed. When we scrutinized the nucleotide substitutions observed among the clones (Fig. 1B), we found that for two amplification products (PCR 1 in position 241 of parchment 1 and PCR 1 in position 63 of parchment 2, Fig. 1B) all clones that had been sequenced carried substitutions that distinguished them from all the clones of other independent amplifications from the same DNA extract. Hofreiter et al. [32] stated that substitutions (consistent substitutions) that occurred consistently among clones within one amplification, but were not reproducible in other amplifications are likely to be the result of nucleotide misincorporations that occurred during the first cycle of the PCR in cases where the amplification started from a single DNA molecule and they represent errors induced during replication of the ancient DNA molecules [20,32,16]. Moreover, one singleton substitution was observed in a single clone (PCR 1, clone 5, position 188 in the sequence of parchment 3, Fig. 1B), which probably is due to *Thermus aquaticus* (*Taq*) DNA polymerase misincorporation in later cycles of the PCR [32] and is rather an error that occurs during replication of newly synthesised molecules than a modification present in the original DNA molecules extracted from the parchment. Finally, for the rest PCR amplifications (four PCRs and 59 clones), where no consistent or singleton substitutions were observed, four other substitutions that we found in a few clones (Fig. 1B), could be explained either by DNA damage present in the ancient DNA template or by subsequent PCR errors during amplification [32].

It is noteworthy that among all substitutions (two consistent, one singleton substitution, and for other substitutions), five were C→T substitutions and two were G→A substitutions, showing an extreme bias (G/C→A/T changes). This phenomenon, which is quite common in the DNA extracted from archaeological and palaeontological samples, could be explained by cytosine deamination and/or by the tendency of *Taq* polymerase to add deoxyadenosine residues when it reaches the ends of templates [6,7,26,32].

To ensure the authenticity of the ancient DNA sequences [24,34] we repeated the extractions, the amplifications, the cloning and the sequencing of the amplifiers (see Section 2).

The consensus sequences of the three parchments used for a preliminary phylogenetic analysis with several Bovidae species (*Capra* spp., *Ovis* spp., and *Bos Taurus*, see Table 1). Phylogenetic analysis is perhaps the final criterion for the authenticity of ancient DNA sequence [24,34]. We suspected the origin of the DNA extracted from the three legal Greek manuscripts (parchments), since for their construction sheep, goat, or calfskin were used. However, the morphological species determination was impossible. The sequences of parchments are 323 bp and correspond to positions 64–386 of *Capra hircus* sequence (GenBank AB004072, Fig. 1A). During the phylogenetic analyses (Maximum likelihood and Bayesian Inference, see Section 2) identical topologies were recovered (Fig. 2). The parchment sequences were significantly different from the human sequence, demonstrating that the parchment material was not contaminated by human DNA either in the handling of the parchment during collection or during the laboratory manipulations and were most closely related to that of goats (*C. hircus*), giving us the opportunity to establish the genetic signature unique for each parchment. To further test all the possible competing monophyly hypotheses, we used the Shimodaira–Hasegawa test [25] to compare the ML tree topology with the alternative ones (forcing the parchment sequences within other Bovidae species). The SH test in all cases was in favor ($p < 0.0001$) of the topology presented in Fig. 2.

As demonstrated, DNA of animals whose skins furnished the parchment pages of ancient and medieval books may survive in that parchment. It might be possible not only to determine the species of the animal from which the skin had been taken, but moreover, it might even be possible to reconstitute the history of the herds from which they originated. In a broader perspective, aDNA obtained from the parchment fragments may help answer some interesting questions as: what is the origin of the manuscripts in the Greek libraries collections? Which items can be grouped together as originating

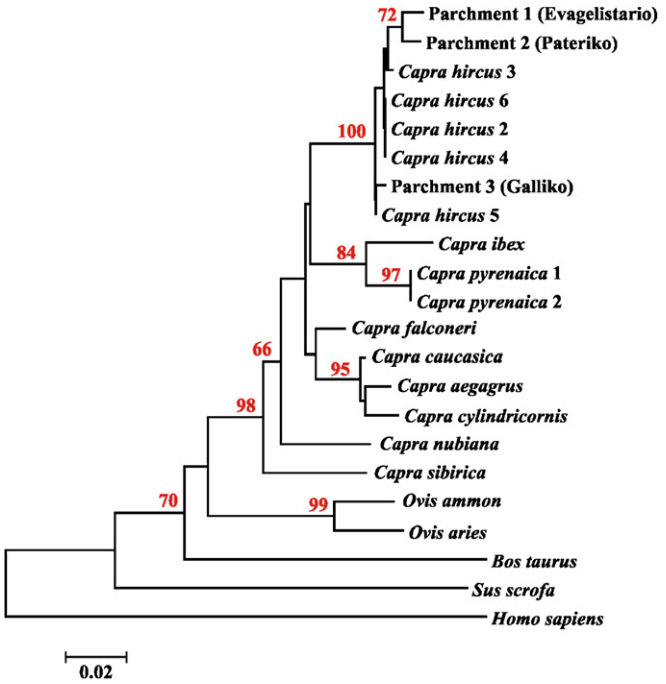

Fig. 2. Cyt *b* phylogeny of several Bovidae sequences, including the produced sequences of the three parchments of this study. Phylogenetic analyses of maximum likelihood (ML) and Bayesian inference (BI) produced trees with the same topology with regard to the major lineages. Only the BI tree is presented. Numbers indicate posterior probabilities values of BI.

from the same or closely related parchments, craftsmen and/or localities? Did different scribes use parchment originating from various sources for their manuscripts, or did some have specific providers? Does the collection contain manuscripts from a single locality, or is it a collection representing contributions from a wide region? What is the temporal element of these questions? From another point of view, what are the phylogeographic relationships of contemporary domestic animals with the stocks bred several hundreds years ago?

This study is the beginning of a hopefully fruitful project that will continue over the next few years. We hope that the analysis of DNA from parchment fragments will add a new level of critical analysis to scroll knowledge, given the availability of more than 3000 manuscripts from several libraries of Greece (Mount Athos, Athens, Patmos, Jerusalem, Alexandria, Sinai).

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