

ANCIENT DNA EXTRACTION AND AMPLIFICATION OF HUMAN BONE SAMPLES FROM THE AREA OF DELPHI: A PILOT CASE STUDY

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Abstract: The present work is a preliminary effort, an experiment on the extraction of ancient DNA from human remains from a proto-Byzantine context in the area of Delphi. The first results are encouraging; however, the interpretation of such analyses needs to be very careful. DNA and other scientific methods have to take into consideration all historical and socio-economic characteristics of a past society before the proposal, for example, of the existence or migration of specific ethnic groups in an area. The theoretical and methodological thinking of Archaeology in the last decades suggest that all scientific analyses have to evaluate the specific context and the complex nature of human existence before the application of any general-based model.

Περίληψη: Η παρούσα εργασία αποτελεί μια προκαταρκτική, πειραματική προσπάθεια για την εξαγωγή αρχαίου DNA από ανθρώπινα κατάλοιπα που βρέθηκαν σε ταφές της πρωτο-Βυζαντινής περιόδου στους Δελφούς. Τα πρώτα αποτελέσματα είναι ενθαρρυντικά, όμως η ερμηνεία τους χρειάζεται ιδιαίτερη προσοχή. Πρέπει να ληφθούν υπόψη στη συγκεκριμένη περίπτωση αλλά και γενικότερα, το σύνολο των ιστορικών και κοινωνικο-οικονομικών χαρακτηριστικών κάθε κοινωνίας, πριν από τη διατύπωση οποιουδήποτε συμπεράσματος για θέματα που σχετίζονται για παράδειγμα με την ύπαρξη ή τη μετακίνηση ομάδων σε μια περιοχή. Οι θεωρητικές και μεθοδολογικές αναζητήσεις της Αρχαιολογίας τις τελευταίες δεκαετίες οδηγούνται στο συμπέρασμα ότι η σύνθετη φύση της ανθρώπινης συμπεριφοράς και ο χαρακτήρας του συγκεκριμένου κοινωνικού context είναι ιδιαίτερης σημασίας και πρέπει να συνεκτιμηθούν πριν τη διατύπωση γενικευμένων μοντέλων.

Keywords: dna, early byzantine delphi, molecular archaeology, scientific methods, archaeological theory

Introduction

DNA extraction and its successful amplification with the PCR (Polymerase Chain Reaction) process from ancient samples or for forensic medical purposes is the most significant application in diagnostics over the last years. Particularly in reference to archaeological finds it offers the potential for phylogenetic studies and the analysis of anthropological evolution and migrations. The possibility to detect genetic diseases and observe their progress is another potential of this specific method. On these grounds, a preliminary analysis was undertaken on skeletal material samples (580-620 AD) from burials found within the limits of an Early Byzantine Villa (South Eastern Villa) in Delphi.

Archaeological research showed that the material under study belonged to two successive burials: a) of a man, and b) of a woman and a child.

The theoretical context: a brief account

As the specific attempt for analysis and amplification of archaeological DNA is undertaken in terms of a pilot project, a brief account of theoretical and practical issues involved is necessary. The discussion about theories and practices in Archaeology, and more specifically about the

contribution of exact sciences to Archaeology is a matter not yet dealt with effectively. The contribution, limits, and interpretation of the results of various processes and techniques applied by exact sciences to Archaeology must be defined and more widely discussed within the speculation framework of Aegean Archaeology.

The revolution in biological sciences over the last decades led to the development of a distinct research branch called Molecular Biology. It was not long after that its application was put into practice also in studies of the past for the analysis of various remains, which resulted in one more field of archaeological research referred to as Molecular Archaeology. The advance of this specific branch set a new basis for the study of certain historical and archaeological problems.

The significance and application of scientific methods in Archaeology, which usually derive from other scientific branches, is not a novelty for research. However, their meaning and the application process of their results in the interpretation of past societies was re-assigned within the wider speculation context of archaeological methodology and practice. The discussion and criticism of the post-processual Archaeology, among other goals, aimed at proving that the interpretation of past phenomena is promoted by the study of the ideological

and symbolic parameters of these societies, and not that of their economy and environment that can be studied using general rules and mathematical models, borrowed from other scientific fields. The application of Molecular Biology in Archaeology coincided with the criticism exerted on “scientific Archaeology” in being, according to its exponents, the only objective one due to the potential for a verification of its results.

In reference to archaeological methodology and practice, the limits and processes in which techniques and analytical methods from other sciences can contribute to archaeological research must be defined. These paths of approach should certainly not be considered as nostrum for the solution of certain problems. Moreover, questions worded in each occasion as well as the interpretation of results must be set forward with caution. The results of various methods of exact sciences that are applied in archaeological research must be incorporated effectively in the interpretive process of cultural phenomena. A complete evaluation of all parameters and their attentive analysis constitutes the most serious proposal for an objective approximation to the historical truth, since the limits of knowledge cannot be absolutely defined, and a new discussion of this issue is necessary (see for instance Shanks, Hodder 1995; Evison 1996; Brown and Pluciennik 2001).

More specifically in reference to matters of Genetics and Biology and their application in Archaeology, criticism focuses on the impression that these approaches degrade the history and diversification of groups and larger population masses, as they connect certain characteristics of linguistics, cultural identity, material culture, and economy with biological facts. An unlimited connection of biological and genetic facts with cultural elements and characteristics creates specific national identities, and features groups and populations with specific “labels” of characterisation. In this way, languages and material culture are not comprehensible as a whole but can be perceived each time within a specific framework that does not permit the study and comprehension of their complexity. The analysis of cultural phenomena at a level of individualism, the gender /age roles, the specific social context in each case etc. must be taken into consideration during the application process of results provided by exact sciences for the interpretation of the past. General conclusions and research models referring to the migration of groups, which still define thinking in Archaeology, cannot be generally effective but must be assigned within each context separately and further studied. The relation between biological and cultural data is not always immediate; consequently, this relation is not to be taken for granted but must be subjected to research and clarification. Characteristic is the point of Ammerman and Cavalli-Sforza (1984), who believe that the spreading of the Neolithic culture is evident in the frequency and diffusion of evolved genes. However, there is also another approach according to which the reconstruction of the past

based on genetic and linguistic facts does not lead to a correct interpretation of historical processes (Pluciennik 1996, 14). Genes do not define cultural identities, and for this reason interpretations based exclusively on the frequency of genes must be dealt with special attention. Social activities and ideology, relations, languages, material culture, even the concepts of landscape and space of an environment vary; thus, genetic facts should be related with different elements in each case (Evison 1996).

The problem of the diffusion of the agricultural economy in Europe makes a most characteristic example for the connection between genetic facts and specific historical processes. According to the Ammerman and Cavalli-Sforza model (1984) (which has been supported, modified, as well as refused by many researchers), the diffusion of the first farmers affected the genetic composition of the population in Europe (for different models and views see for instance, Runnels and van Andel 1988; Halstead 1996; Kotsakis 2000). As mentioned above, the role and diversity of local societies are not being thoroughly examined. However, this model and its later modifications do combine certain facts of culture, linguistics, and genetics (Sims-Williams 1998). In other cases, genetic and linguistic characteristics analysed at a global level discover relations between populations. The case of how, when, and from where the Indo-European language was diffused, gives a common example of connection between genetics and linguistics.

This brief discussion on the problems of interpreting and applying the results provided by research in biological sciences for the interpretation of the past aims at showing that these methods’ deductions of an indisputable significance must be set within the framework of research, necessities, and limits of Archaeology.

In general terms, there are two distinct phases of research and analysis of human group’s genetic characteristics: The first relates to the study of classical genetic characteristics, which is gradually replaced by the mitochondrial DNA and the Y chromosome analysis (Renfrew 1999). To this first phase belongs the so-called principal component analysis, the spreading of which was related with that of the first farmers; while, the analysis of a second detected component was difficult to interpret. Nevertheless, it was proved that the largest part of demographic processes took place during the Upper Palaeolithic.

The relatively new methods of ancient DNA analysis have already developed and are still so, since many problems of methodology have not been successfully dealt with yet.

The extraction and analysis of ancient DNA confronts specific problems and limitations. DNA “degrades” with an organism’s death. It is relatively hard to be extracted in the laboratory, since it is usually detected in small quantities; thus, its amplification process with other practices is

demanding, which creates more possibilities for its mixture with modern DNA.

The difficulty of analysing small DNA quantities, either degraded or chemically modified, was overcome with the application of a new technique [Polymerase chain reaction (PCR)], with a necessity of merely small DNA quantities (Sykes, Renfrew 1999). It enabled a quick application of this method for a variety of human, animal, as well as palaeontological and plant remains, preserved in different environments and conditions. Important studies have been undertaken, e.g. on mummies from Egypt and Peru that were preserved in dry conditions, but also in conditions of frost, as in the case of Greenland, or generally alkaline or acidity percentages in humid environments where greatly affect preservation (Brown 1992, 11-12). The DNA extraction and analysis from Neanderthal skeletal remains (Renfrew 1999) is particularly encouraging for future efforts.

In Greece, several groups of specialists have begun working on human remains, as in the case of the successful DNA extraction from the very early skeletal material (Upper Palaeolithic – Mesolithic) recovered from the Theopetra Cave in Thessaly (Evison *et al.* 2000) or that of the skeletal remains analysis from the cemeteries at Mycenae (Bradley *et al.* 1999). The analysis of plants, mainly wheat in the case of Northern Greece, from which domesticated categories of this species could derive, offers new perspectives of research (Kotsakis 2000, p.175, with relevant bibliography).

As already mentioned, DNA does not in any circumstance respond to all of the questions about Biology and the Past or the origins of archaeological remains. Although it is possible to deduct certain data about the migration and spreading of population groups, the limits of interpreting these results are not always clear; while, no databases for the comparison of ancient DNA series exist. These cases of research require systematic studies of modern population groups in a large scale. Nevertheless, limits always occur, since these populations' DNA relates solely to those family lines that have survived (Pääbo 1999; Renfrew 1998). The definition of national identities and groups must be examined with special attention. The way that national, or better, social identities are expressed in prehistoric times is an especially complex matter, and requires the estimation of multiple factors together prior to drawing any conclusion (e.g. the use and significance of material culture, ideology, symbolism, social organisation etc.) (see for instance Jones 1997).

Certainly, it is possible under specific conditions to approach the subject of family relationships among individuals. It is possible for some biologic characteristics to arise, as for example the definition of sex, while this is not effective for other characteristics of disparity (e.g. mental capacities). Clinical researches of modern DNA

with the PCR process can detect certain diseases such as hepatitis or other parasitical ones such as malaria.

DNA analysis has also been applied on animal bones from several archaeological sites, and yielded important information on the animal populations reproduced. It is particularly possible to determine the wild ancestors of domesticated species.

Plants offer different research potentials in reference of their geographic and genetic features (Allaby 1999). If a specific cultivation shows special genetic characteristics, it may present a series of types that would allow the supervision of its spreading in space as well as the geographical movement of each variety through time (Brown 1992). Apart from the domestication of wheat, the analysis of the domesticated cattle's origins, for example, has been similarly successful (Bradley *et al.* 1996), as well as the origins definition of the Cretan agrimi-goat (Bar-Gal *et al.* 2002). However, the finds show that a parallel domestication in different areas is possible, though this process would not refer to one and only case of evolution, as supported by traditional views on domestication evolution within a nuclear zone and its diffusion from a single centre to the remainder of the regions (Bradley 1999).

Bio-Molecular Archaeology is not absolutely identical to Archaeo-Genetics, as it can offer data that are not exclusively genetic. For example, the study of lipids in ancient food provides data for ancient dietary habits, as in the case of analysis from vases' contents.

In reference to the preservation and contamination of samples, DNA is affected by various environmental factors, such as bacteria, the heat, and several chemical factors, the study of which has not been completed yet. There are four major DNA contamination sources to be outlined: 1) between the moment of death and that of burial, when the dead body is being touched by several individuals, according to their burial customs; 2) between the moment of burial and that of excavation: little is known about the passage of DNA to the area of the burial and the additional contamination of the samples, especially in the case of multiple burials; 3) special attention must be paid during the excavation of the material, the selection of the samples, and the following analytical procedures in the laboratory. Particularly important is moreover the storage process of the samples with the avoidance of any bacteria adaptation. As about the selection of samples, although long bones are commonly used, there is no general rule for a selection of the part most appropriate for analysis.

Archaeological evidence

Early Byzantine Delphi

The bone assemblage that became the object of laboratory analysis as presented below derived from systematic

excavations undertaken by the French School of Archaeology at two points of the archaeological site in Delphi, i.e. the Roman Forum and the South Eastern Villa*. At the Roman Forum, one of the magazine/workshops was excavated, the deposits of which yielded very rich material of all categories of finds. This material dates from the second half of the 4th century AD, a period when the excavated room ceased to function after its northern wall had collapsed. It was then that its door was sealed, and it was filled with soil up to a height of 5 m. A possible first function seems to be related to that of a workshop of glassware manufacture.

The South Eastern Villa constitutes the largest to date excavated architectural complex in Delphi, and employs triclinia, storerooms, and small but elaborate in construction private therme. Its function as a residence dates from the 5th to the 6th century AD. About 580 AD, the Villa is abandoned, while maximum a decade later the workshops of pottery, metallurgy, and tannery or dye-works are installed inside its rooms. The final abandonment of the building is dated around 620 AD (Fig. 1).

Within the limits of the South Eastern Villa, the occurrence of burials, both adults and infants, has also been confirmed. Burials were located:

1. either inside a roof tile placed directly into the earth, as in the case of infant burial T342 (TS 97 34), in room C15,
2. or inside a roughly formed grave, with the arrangement of horizontal and vertical slabs and roof tiles, as in the case of adult burial T324 (TS 97 11), recovered within the upper levels of a pottery pit (C30),
3. or, finally, inside a well-attended, built pit possible

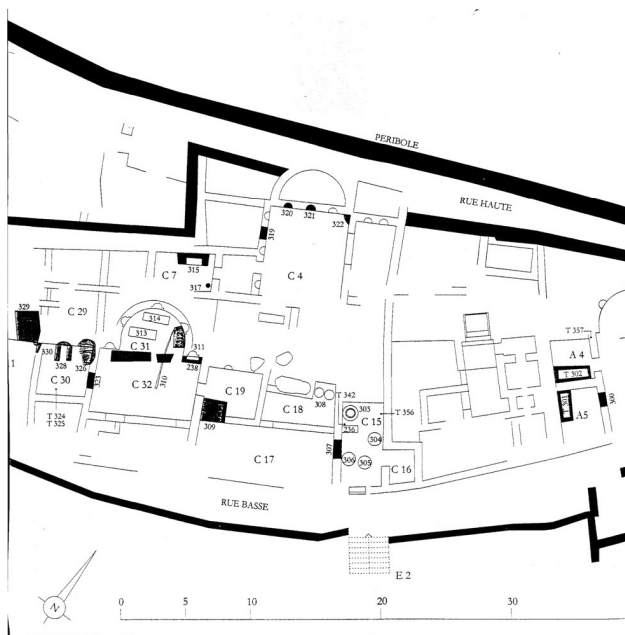


Figure 1. View of the South Eastern Villa, the area where Burials T301, T342, T356, T324, T325 were located.

used as a grave in a secondary phase, and covered with the part of an inscribed stele also in secondary use, as in the case of two adults' and a child's burial (T301) recovered in room A5, next to the eastern triclinium (TS 91 38). These belong to burials mentioned in page 1. Along them, a pair of silver earrings and some small copper coins were found accompanying the dead.

Materials and methods

The study of the anthropological material and the selection of the samples took place in the Museum of the Department of History and Archaeology, University of Athens, while the samples' analysis at the Laboratory of Molecular Immunopathology / Histocompatibility of the Onassis Cardiac Surgery Center.

The methodology applied was that by Kalmar and co-operators (Kalmar *et al.* 2000), modified. The extraction method of the archaeological DNA comprises the following processes:

1. Prevention of secondary contamination

In order to prevent the osteological material from any possible contamination incidents, all process stages were realised under sterile conditions (i.e. the use of gloves and mask). All tubes, bowls etc to be used, as well as the process area had been cleaned and UV-irradiated at least for 30 minutes.

The acetic ammonium NH₄-acetate extraction buffer (with no PROTEINASE K), the Dextran Blue solution, and the ionized, distilled-sterilised water are irradiated for 30 minutes before their use. All stages (cutting of bones, cleaning of surface, pulverisation, extraction, and amplification) are executed on an isolated surface. In all procedures sterile filter tips are used.

2. DNA extraction

The sample is rinsed with a solution of chlorine and distilled water. The part of a bone measuring ca. 2 X 5 cm is cleaned at its surface and at a depth of 2-3 mm with the assistance of a sand disk, in order to remove any modern mixture of genomic material. Next, the bone undergoes UV-irradiation for 30 minutes, and the process of mechanical pulverisation follows with a sterile porcelain mortar and pestle.

The powder (750 mg) is dissolved in 1,6 ml of the extraction buffer solution (0,1 M EDTA, 0,5% N-LAUTYLSARCOSINE-NA SALT, 100 µg/ml PROTEINASE K), then stirred (vortex), and incubated over night at 37^o C under continuous vertical rotation.

The sample is centrifuged at room temperature at 12.000 rpm for 10 minutes; 250 µl of supernatant is transported

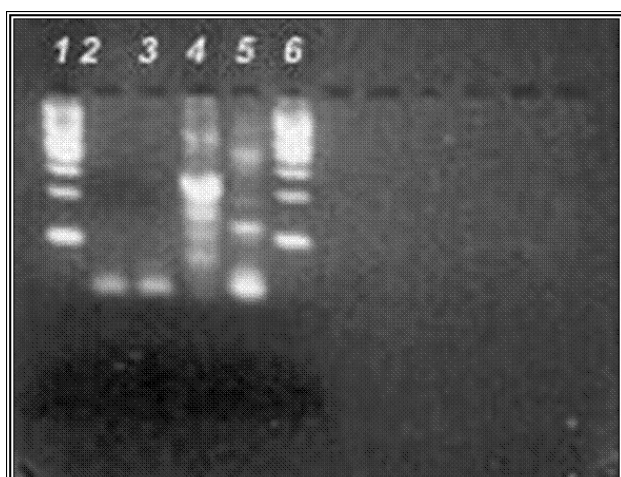


Figure 2. Agarose DNA electrophoresis (2%) of PCR reaction products for *b*-globin gene, [lanes 2-4: lane 2, blank, lane 3, sample, lane 4, control DNA, lanes 1, 6: molecular marker ϕ X-174 HaeIII] as well as undiluted DNA (lane 5).

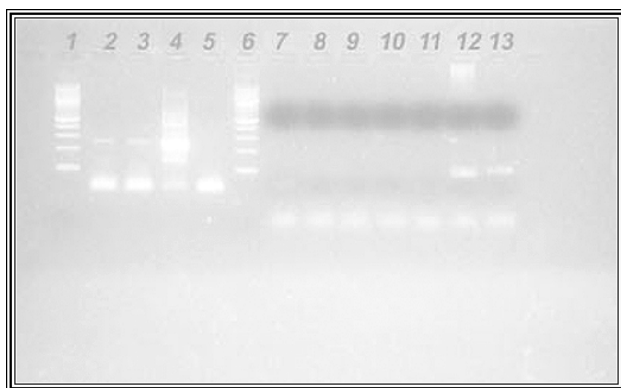


Figure 3. Agarose DNA electrophoresis (2%) of PCR reaction products for sex identification, [lanes 7-13: lane 7, blank, lane 8-11, samples, lane 12, female control DNA, lane 13, male control DNA, lanes 1, 6: molecular marker ϕ X-174 HaeIII].

to a 1,5 ml Eppendorf tube; 3,5 μ l 1 μ g/ μ l Dextran Blue, 250 μ l 4 M NH_4 -Acetate, and 500 μ l 96% EtOH are added and vortexed.

It should be noted that Dextran Blue inhibits the PCR reaction in a dose-dependent manner and only in a concentration > 125 μ g/ml.

DNA precipitates at -70°C for 7 minutes, and centrifuged at 14.000 rpm and 4°C for 15 minutes. The pellet is dissolved in 20-30 μ l of ionised-distilled water. At this stage, it can be stored at -20°C .

3. Amplification

Typical PCR amplification occurs in 2-7 μ l of extract with 1U Taq DNA Polymerase, 160 μ g/ml BSA, 200 μ M out of each dNTP, 20 pmol out of each primer for human *b*-globin gene, or DR gene of the HLA system, or X and Y-chromosomes identification genes in a PCR bugger

of 25 μ l final volume. Many attempts of increasing archaeological DNA concentration took place with Quick spin columns (Qiagen).

Denaturation occurs at 93°C for 5 minutes, followed by 35 circles of denaturation at 93°C for 1 minute, annealing temperature at 58°C for 1 minute, and extension at 72°C for 1 minute. The last cycle is followed by additional extension at 72°C for 5 minutes (primer extension).

As molecular markers, recent human DNA as well as a 50-1000 bp DNA ladder have been used.

Results

The first experiments proved the human origin of the archaeological sample (Fig2, position 5). After repeated denaturation processes, the imprint of the archaeological DNA was confirmed to vary between 250 and 350 bp.

The use of the Quick spin column did not offer the expected condensation, and for this reason the identification of sex genes or the HLA-DR genes was not possible for the present (Fig.3). Efforts for an improvement of the results are already being attempted with the use of special supplementary condensation columns, as the type Micropure E2-Enzyme (Millipore).

Discussion

The still limited references in the international bibliography (Halgelberg *et al.* 1989; Hanni *et al.* 1995) show that the potential of extraction and amplification of archaeological material depends on the age of the samples, due to the DNA deterioration fact. Thus, for finds up to approximately 5000 years old it seems that the genomic DNA isolation is possible, while for earlier samples one has to resort to mitochondrial DNA isolation from bone remains. The latter is an exceptionally tedious and expensive method. On the contrary, the potential of genomic DNA amplification is more feasible, though difficulties and expenses also in this case are not insignificant. The results are encouraging, and sex identification has reached already a satisfactory level.

The ancient DNA extraction and amplification from burials discovered in the South Eastern Villa in Delphi is a primary effort realised with the combination of technical and alternative ways of processing already known methods. Attempts on Greek samples are still limited, similarly perhaps to the knowledge of the potentials offered by these scientific methods to the excavator, for a deeper knowledge of the materials brought to light. This experimental attempt by a Greek laboratory and its prospective, systematic occupation with ancient DNA extraction, apart from its modern and multiple applications for other purposes, are expected to give a significant impetus to this subject and new important data for the research of the past, parallel

to the occupation of other scientists from different institutions.

The application of these methods on a larger scale in Aegean Archaeology is expected with particular interest. The progress of research with the systematisation of laboratory procedures and methodology by specialists but also the application of specific questions by archaeologists, or better a series of hypothesis that can be tested, form the next stage of the research process. The continuation of research of prehistoric cemeteries of a specific date in the Aegean would be the next step in an attempt of understanding burial customs, family relationships, and sex diversification, in a long term prospective followed by the approach of matters related with population movements.

This paper also aims to connect theory with science that both are very important in archaeological research. Despite the fact that these approaches contradict most of the times with each other in terms of methodology and ideological background, they can be linked systematically for providing better understanding of ancient societies. The Science versus anti-Science (Thomas 1991) concept is no longer useful in Archaeology and both approaches' results and research potentials should be evaluated as serving a single objective.

Taking into consideration the limited purpose of this pilot programme and its primary encouraging results, the successful issue of analogous attempts in the future can be foreseen. The continuous progress in Molecular Biology and the application and adaptation of new and improved techniques will possibly assist for an analysis of "more laborious" samples, even with a small percentage of degraded or contaminated DNA.

Notes

Under the direction of V. Dèroche and Pl. Petridis, a large group of collaborators consisted of archaeologists and students of Archaeology from Greece, France, Belgium, and Switzerland, architects and conservators from Greece and France, as well as topographers, one numismatologist, and several specialists of clay analysis and environmental studies, specifically shells and bones.

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