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Ancestral DNA — an incontestable source of data for Archaeology*

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Abstract. *The DNA is present in every cell of a person's body, not only in the cell's nucleus but also in its cytoplasm, in mitochondria. Of great importance is the fact that, except for the rare occurrence of a mutation, the DNA in every cell of the person's body is identical. As a result, DNA can be taken from saliva, sweat, blood, hair, skin or bone cells for individual identification. The many opportunities to obtain DNA evidence can be seen, for example, in the number of places where saliva has been identified: a bite mark, an area licked, bed linens, a mask worn, paper tissue, a washcloth, a cigarette butt, a toothpick, the rim of a bottle or glass, but all of those sources are available just for present DNA. In the case of old DNA, also called ancient DNA (aDNA), the things are different and the possibilities to analyse the substrate of genetic information are limited to bone fragments or teeth. Even in these conditions, the DNA analysis is a very accurate and powerful tool for getting useful information in Archaeology.*

Rezumat. *Fiecare celulă dintr-un organism conține ADN (Acid dezoxiribonucleic) care este prezent atât în nucleu, cât și în mitocondriile din citoplasmă. Cu excepția unor mutații genetice, ADN-ul este identic în toate celulele unui organism. Din acest motiv există foarte multe posibilități de obținere a ADN-ului cum ar fi: analiza salivei, a transpirației, a sângelui, a părului, a pielii sau a oaselor. Multiplele posibilități de obținere a ADN-ului pot fi exemplificate foarte bine prin numeroasele locuri din care poate fi recuperată saliva unui individ: o mușcătură, o zonă linsă, lenjeria de pat, o mască purtată, șervețel, prosop, țigară, scobitoare, sticlă sau pahar. Dar toate acestea sunt valabile doar pentru ADN-ului actual. În cazul probelor vechi, obținerea informației genetice se poate face, în general, doar prin analiza oaselor și a dinților. Chiar și în aceste condiții, analizele ADN reprezintă o importantă sursă de informații pentru arheologie.*

Keywords: ancient DNA, nuclear DNA, mitochondrial DNA, haplogroups, archaeological analysis.

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Introduction

DNA analysis can describe the latest developments in different scientific areas, with a clear and accessible discussion of the results of mitochondrial DNA and Y-chromosome analysis and of their integration with the archaeological[5] and geo-climatic records[6].

If we are interested in peoples of a specific region[7], like countries[8] or even continents, how long ago the peoples move into this specific area[9], how many peoples moved, how many times they moved, we can get the genetic data (mainly the mitochondrial DNA sequences) that will show us the patterns of variations in population and we can compare this data with patterns of variation from different populations[10], but we need calibration points from archaeological records[11], to know when we see evidences of past societies and how they are related with other communities[12]. Generally, it is a comparative process involving genetics, archaeology and sometimes linguistics or even fossil records if we are going back deep in time[13], which requires a multidisciplinary analysis, because we need to create a complete image of that times. Even if genetics is a powerful tool to look into the history, it couldn't tell us anything by itself, it has to be integrated in a comparative framework[14].

The analysis of ancient DNA (aDNA) is a relative new research tool in a wide variety of fields, from history and anthropology through genetics and emerging diseases to forensic medicine[15]. The human evolution and population history was often investigated using aDNA study[16]. During the last three decades since the first genetic data supporting the recently *out of Africa* hypothesis were produced[17], hundreds of analyses of mtDNA, Y chromosome, and nuclear markers have largely continued to develop the model. The degree in which the molecular studies have included, in general, other anthropological information[18], and the degree in which all this data where included in the archaeological models, is still highly variable[19]. However, in the introduction to a volume dedicated to reviews of human

[5] BADRO *et al.* 2013, 1–11.

[6] van ANDEL 2003, 31–39.

[7] de-la-RUA *et al.* 2015, 306–311.

[8] CHILVERS *et al.* 2008, 2707–2713.

[9] IZAGIRRE, de la RÚA 1999, 199–205.

[10] REICH *et al.* 2009, 489–494; MILLER *et al.* 2011, 12348–12353; NOVEMBRE *et al.* 2008, 98–101; YANG *et al.* 2012, 725–731.

[11] HELED and DRUMMOND 2012, 138–149; DORNBURG *et al.* 2011, 519–527; WILKINSON *et al.* 2011, 16–31; NOWAK *et al.* 2013, e66245.

[12] DODGE 2012, 22–30.

[13] GREEN, SHAPIRO, 2013, 286–288.

[14] FU *et al.* 2013, 553–559.

[15] ELANGO 2007, 28–36; KUMAR, RAO 2007, 36–77.

[16] DASKALAKI 2014, 14–15; KIRSANOW, BURGER 2012, 121–129.

[17] CANN *et al.* 1987, 31–36.

[18] GREALY *et al.* 2015, 37–47.

[19] MATISOO-SMITH, HORSBURGH 2012, 112.

dispersals, C. Renfrew identified the *out of Africa* hypothesis as an archaeo-genetic very inspiring story which take into considerations the genetic, archaeological, and linguistic data in the pursuit of more accurate and nuanced reconstructions of early prehistory[20].

The aDNA analysis contributed to the understanding of human evolution, studying various hominids and their relation to modern humans[21]. The Denisovans and Neanderthals complete mitochondrial genome[22] and the draft nuclear genome for both species[23] has led to the evaluation of their contribution to present-day ancestry and their geographic ancestor area[24]. Later studies demonstrated the origin of Denisovans and Neanderthals in Africa that migrated to Eurasia, much earlier than the modern humans[25].

Also, the picture of the Neolithic process in Europe was mostly revealed by mtDNA analysis of human remains from Central Europe, Northern Europe and Iberia[26]. The aDNA was an important tool in understanding the process of plants and animals' domestication. Recent studies show that dogs are derived from wolves of Europe[27], contradicting the theories that suggest dogs were domesticated either in the Middle East or in East Asia[28]. All these results were possible because the researchers working on human genetics and the one doing archaeology have moved to a more balanced understanding of the potential and limits within both disciplines[29]

Recently, K. Kristiansen considers that the next generation of sequencing of ancient DNA which is now able to produce genomic data represents an important part of the so-called *Third Science Revolution*[30]. The mtDNA provided interesting information about major changes in the genetic composition of Europeans during the Neolithic[31]. The increasing data generated new haplogroups, some with possible origins in the east[32], others in the Iberian Peninsula[33]. All these changes where almost completed by the Bronze Age[34].

[20] RENFREW 2010, 162–165.

[21] DASKALAKI 2014, 14–15.

[22] REICH *et al.* 2010, 1053–1060; GREEN *et al.* 2008, 416–426.

[23] MEYER *et al.* 2012, 222–226; GREEN *et al.* 2010, 710–722.

[24] NOONAN *et al.* 2006, 1113–1118.

[25] KRAUSE *et al.* 2010, 231–236; LALUEZA-FOX, GILBERT 2011, 1002–1009; LOWERY *et al.* 2013, 83–94; REICH *et al.* 2010, 1053–1060;

[26] BRAMANTI *et al.* 2009, 137–140; HERVELLA *et al.* 2012, e34417; LEE *et al.* 2012, 571–579; MALMSTRÖM *et al.* 2009, 1758–1762.

[27] THALMANN *et al.* 2013, 871–874.

[28] DASKALAKI 2014, 14–15.

[29] BROWN, PLUCIENNINK 2001, 101–104.

[30] KRISTIANSEN 2014, 13.

[31] HAAK *et al.* 2015, 207–211.

[32] HAAK *et al.* 2010, 1–16.

[33] OLALDE *et al.* 2015, 1–11.

[34] ALLENTOFT *et al.* 2015, 167–172.

Within the *Genetic Evolution: New Evidences for the Study of Interconnected Structures. A Biomolecular Journey around the Carpathians from Ancient to Medieval Times* (GENESIS) project we intend to create a database which will give us the possibility to connect our genetic approach and results with other European research programs which are targeting South-eastern Europe[35]. We are focusing on understanding at a regional scale the genetic characteristics, the appearance of different haplogroups and the dynamic of some communities from prehistory to the Middle Ages. A timespan that yielded major genetic changes at a European scale and represents a central theme of our investigations is Early Bronze Age. One can say that some of the *moving communities* which can be traced archaeologically from the Steppes towards Central European regions originate in the Steppes Pit Grave culture[36].

Despite its huge potential, the archaeological genetics has a variety of challenges, from DNA preservation and contamination to the sample access[37]. These challenges, have led this field to become stricter and more highly method oriented, in order to overcome this issues and to recover all genetic information that can be obtained from a specimen.

In this study we will describe technical aspects associated with successful, authentic and reliable DNA recovery from old samples. We hope to give an interesting and comprehensive overview of the process of analyzing DNA obtained from archaeological remains and of their possible use in the archaeological analysis.

The basic DNA structure

DNA, or deoxyribonucleic acid, is the hereditary material in almost all living organisms, a small percent being RNA based organism. Every human cell has the same genetic information and the same DNA structure. Most DNA is located in the cell nucleus (referred as nuclear DNA) and a small amount of DNA can also be found in the mitochondria (referred as mitochondrial DNA or mtDNA).

DNA consists of two parallel spiral strands that form a double-helix[38]. Each strand is actually a linked chain in which the links consist of a very large number of units called nucleotides, representing the genetic information stored as a code made up of four chemical bases. Every nucleotide is made up of three smaller chemical compounds: a phosphate, a sugar, and a base[39]. There are four different bases, which are referred to by using the first letter of their names: A (adenine), T (thymine), G (guanine), and C (cytosine). A and G are double-ringed nitrogen-containing compounds, called purines; T and C are single-ringed

[35] Excellence Cluster Topoi, Research Project A-2-1. *Pastoralism on the Eurasian Steppe*. www.topoi.org/project/a-2-1/.

[36] BOLOHAN *et al*., 2014; BOLOHAN *et al*., 2015.

[37] DASKALAKI 2014, 15.

[38] LODISH *et al*. 2004, 102–108.

[39] ANTHONY-CAHILL *et al*. 2012.

nitrogen-containing compounds, called pyrimidines[40]. The base is the important identifying part of a nucleotide[41]. Each phosphate group is linked to a sugar molecule, which, in turn, is attached to one of the four nitrogen-containing bases. The phosphate group of each nucleotide is also chemically bonded to the sugar molecule of the adjacent nucleotide, forming the polynucleotide chain[42].

Nuclear and mitochondrial DNA

The offspring of sexually reproducing organisms inherit approximately half of their DNA from each parent. In a diploid, sexually reproducing organism for example, this means that within the nuclear genome one allele at each locus came from the mother and the other allele came from the father[43]. This is known as biparental inheritance. However, even in sexually reproducing species, not all DNA is inherited from both parents. Two important exceptions are the uniparentally inherited organelle genomes of mitochondria (mtDNA) and plastids, with the latter including chloroplasts (cpDNA)[44]. These are both located outside the cell nucleus. Mitochondria are found in both plants and animals, whereas plastids are found only in plants. Organelle DNA typically occurs in the form of supercoiled circles of double-stranded DNA, and these genomes are much smaller than the nuclear genome. For example, at between 15000 and 17000bp the mammalian mitochondrial genome is approximately 1/10000 the size of the smallest animal nuclear genome[45], but what they lack in size they partially make up for in number – a single human cell normally contains anywhere from 1000 to 10000 mitochondria. Molecular markers from organelle genomes, particularly animal mtDNA, have been exceedingly popular in ecological studies[46] because, as we shall see below, they have a number of useful attributes that are not found in nuclear genomes. Nuclear and mitochondrial chromosomes consist of two types of nucleotides: (1) those that make up the genes, called coding sequences, and (2) those whose function is largely unknown, referred to as noncoding regions[47]. The nucleotides in coding and noncoding portions of a chromosome are exactly alike in chemical composition and bonding characteristics; they differ solely in whether or not they contribute to one or more of the individual's traits (phenotype).

[40] ALBERTS *et al.* 2002.

[41] MAO 2004, 2036–2038.

[42] ANTHONY-CAHILL *et al.* 2012.

[43] RIDLEY 2006.

[44] BUTLER 2005, 123–145.

[45] LEWIN 2004; GREGORY *et al.* 2007.

[46] HARRISON 1989, 6–11.

[47] WATERS 2013.

Finally, the nuclear chromosomes and cytoplasmic mitochondria are transferred from one generation to the next along different paths, which greatly affects their applications in Archaeology.

In the last decade, advances in genomic sequencing are starting to provide insight into the complex narrative of human ancestry embedded in human DNA, particularly through genetic variation. Genetic variants are sequences of DNA base pairs that differ from more common ancestral sequences and can be traced to specific human populations, either present or past by ancient DNA. The genetic analysis of human ancestry is a search for variants in an individual's genetic code and determine how related that person is to specific ethnic and geographic populations. For males, if the samples are very good preserved, the Y chromosome is sequenced since this allele is always passed down from father to son; for females, mitochondrial DNA is sequenced, since a daughter inherits the DNA from her mother. Genetic variants found in these sequencing data can be used to construct a paternal or maternal evolutionary tree, showing how certain human populations connect to each other.

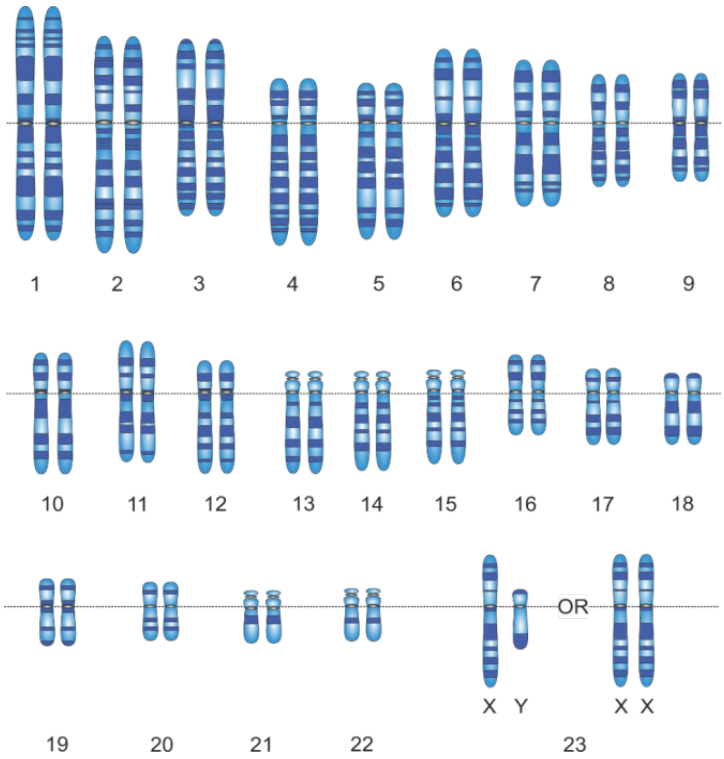

Figure 1. Graphical representation of the normal human karyotype showing the organization of the genome into chromosomes. The karyotype indicates the number and morphology of chromosomes in a eukaryotic cell undergoing mitosis (44 somatic autosomes and 2 sex chromosomes XX for females or XY for males).

Nuclear DNA

Although DNA in the nuclear chromosome and the cytoplasmic mitochondria of a cell are composed of complementary poly-nucleotide chains, their numbers, sizes, and geometric arrangements are quite different. A normal human somatic cell has 46 or, 23 pairs of chromosomes (Figure 1), having received one of every pair of homologous chromosomes from each parent. The number varies slightly depending on whether the set of chromosomes being considered includes the X, resulting in more base pairs, or the Y chromosome.

The total human genome, or 99.9% of it, was originally decoded and published in 2001 and updated in 2002 and 2004[48]. This was the culmination of a joint multinational effort and nearly 10 years of work. The total size of the human genome was found to be approximately 3.2 billion bp.

In nuclear chromosomes, the coding and noncoding sequences are distributed intermittently along the length of each DNA double helix (Figure 2).

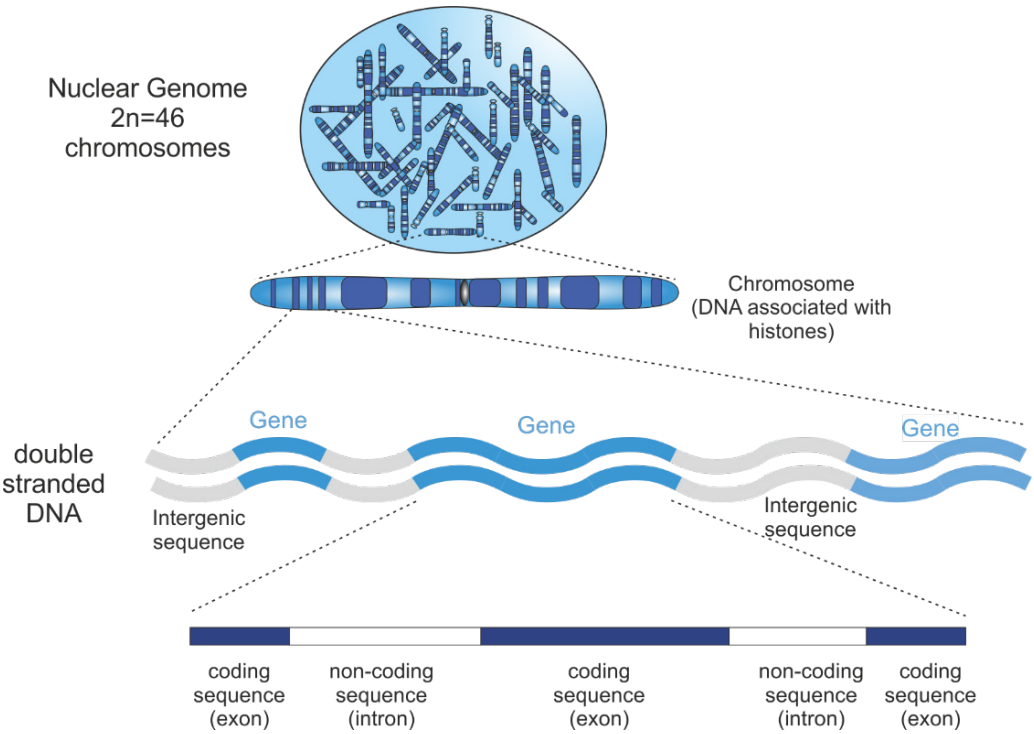

Figure 2. Chromosome internal structure.

[48] LANDER *et al.* 2001, 860–921; VENTER *et al.* 2001, 1304–1351; COLLINS *et al.*, 2004, 931–945.

The ability to designate whether a sample originated from a male or female contributor is extremely valuable in case of incomplete human remains, bone fragments, if sex identification is required. The most popular method for sex typing is the short tandem repeat (STR) analysis. Recently, Y chromosome-STR analysis has become available and has provided identification where STR analysis was not definitive. Several genetic markers have been identified on the Y chromosome that are distinct from autosomes markers, being useful for human (male) identification. The Y-STR markers are found on the noncoding region located on both arms of the Y chromosome. The Y-STR markers produce a haplotype profile when amplified from male DNA and are extremely valuable in the analysis of lineage and the reconstruction of family relationships. Because these markers are only paternally inherited, they are useful in paternity-related matters. In order to determine the sex of the individuals studied a dimorphism in the amelogenin gene can be analysed. This gene encodes a protein involved in the formation of dental enamel; it is a single copy gene located at position Yp11.2 of Y chromosome with its counterpart in the region Xp22.3–p.22.1 the X chromosome. In the X chromosome a 6bp deletion is present. This marker is used in forensic studies and it is of great importance in archaeology, allowing sexing individuals even when not all the skeletal parts are available to establish it by their morphological characteristics. In this case, a small region where the dimorphism is located can be amplified and the sizes of the fragments obtained can be compared in 2.5% agarose electrophoresis.

In addition, Y-STR markers' use and effectiveness in lineage studies can extend to answering questions of common ancestral geographical origin. Y-STR markers, together with mitochondrial DNA (mtDNA) markers will complement each other in these ancestral analyses.

Mitochondrial DNA

The mitochondria (singular, mitochondrion) are cytoplasmic structures (organelles) involved in cells' energy production[49]. Although mitochondria contain their own DNA genomes, mitochondrial genes are inherited in a different manner from nuclear genes because the zygote's mitochondria come only from the mother's egg, because the father's sperm contributes only nuclear DNA to the new embryo. For this reason, all sons and daughters have the same mitochondrial DNA (mtDNA) as their mothers, and mtDNA is passed on, virtually unchanged, from one generation to the next through the maternal line of a family. No meiosis is involved in mtDNA replication, and therefore no segregation of alleles or independent assortment takes place. Since little to no genetic recombination occurs on the mitochondrial chromosome[50], all genes are inherited as if they were a single unit. Because only maternal DNA is present, mtDNA can be considered haploid for mitochondrial genes.

[49] HENZE, MARTIN 2003, 127–128.

[50] EYRE-WALKER *et al.* 1999, 477–483; AWADALLA *et al.* 1999, 2524–2525.

The number of mitochondria varies greatly with the type of cell and stage of its development, ranging usually between 200 and 1000; the number of nucleotides in a mitochondrial DNA molecule is fixed at 16569 base pairs[51] (Figure 3). Each mitochondrion, however, typically contains two or three DNA molecules.

In a mitochondrial ring chromosome, the coding and noncoding areas are entirely separate, with the noncoding portion of the chromosome being located in a region referred to as the control region (also called the displacement loop or D-loop). The control region contains about 1,100 base pairs and is divided into 2 distinct sections, hypervariable 1 (HV1) and hypervariable 2 (HV2). The various base sequences of the control region nucleotides are the most useful in identifying a, human genetic variation and population genetics, but also the human populations migration.

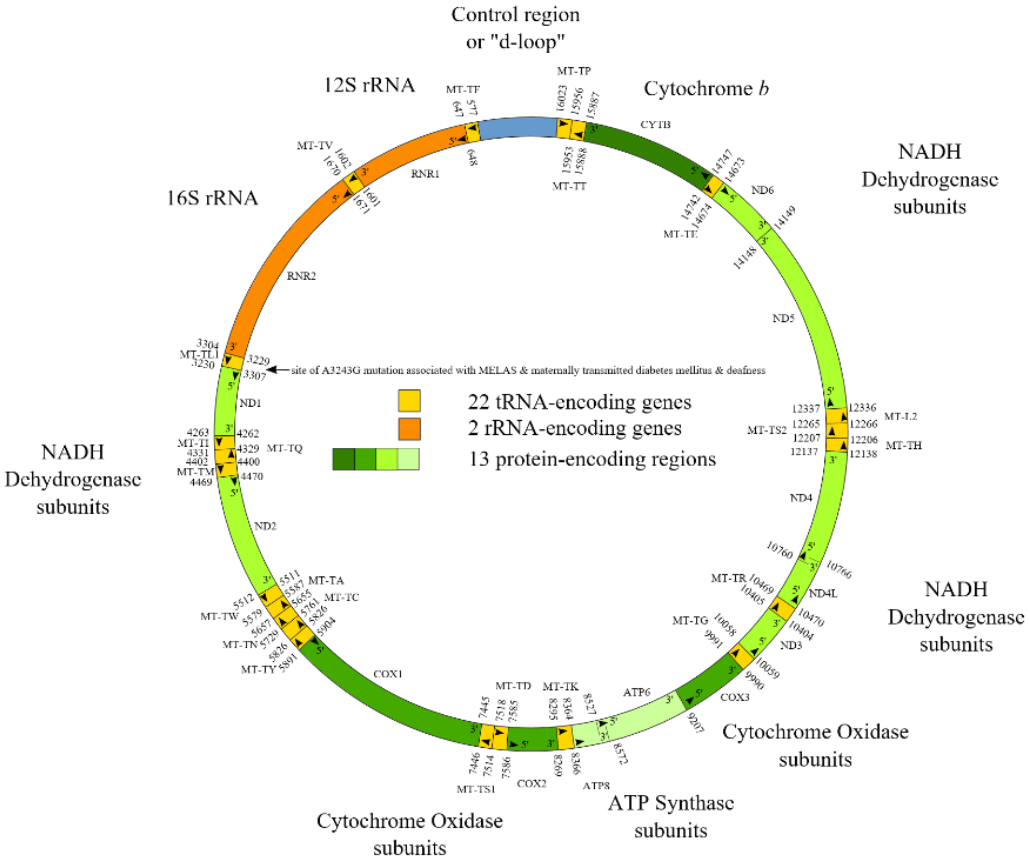

Figure 3. Human mitochondrial DNA.

[51] ANDERSON *et al.* 1981, 457–465.

Furthermore, the ends of each mitochondrial DNA molecule are bonded together, forming a total of two or three circular DNA rings per mitochondrion. By common agreement, the ring chromosome is viewed as the face of a clock with the base pairs numbered from 1 at the 12 o'clock position and proceeding clockwise to 16569.

In addition, mtDNA contains no STRs (Single Tandem Repeats) and is analysed, instead, for the sequence of bases in its DNA. STR DNA typing does not work for all biological samples. MtDNA analysis can, however, frequently be used to obtain some DNA typing information when samples contain DNA that is highly degraded or insufficient for nuclear DNA STR analysis. Older biological samples that contain very little nucleated cellular material (for example, hair, bones, and teeth) cannot be analysed for STRs, but such samples can frequently be analysed for mtDNA[52]. Although nuclear DNA contains much more information than mtDNA, it is present in only two copies per cell; a cell contains hundreds to thousands of copies of mtDNA. For archaeological purposes, mtDNA is considered to be inherited solely from one's mother. Because a mother passes her mtDNA to all of her children, all siblings and maternal relatives have the same mtDNA sequence, and unlike nuclear DNA, mtDNA is not unique to an individual. This pattern of maternal inheritance is helpful in identifying the genetic structure of human populations at different moments in time and the evolution of these populations from an ancient time to present, also the estimation of the admixture degree between populations[53].

MtDNA is analysed by sequencing, a process that determines the order (sequence) of the DNA nucleotides in a DNA segment. The particular regions of the mtDNA genome sequenced are those that are the most variable among individuals, that is, the hypervariable control regions HV1 and HV2[54]. Methods for sequencing DNA are usually performed with the same CE instruments that are used for STR analysis; different PCR and CE analysis strategies, however, are used for this type of DNA analysis.

For mtDNA sequencing, the DNA of each hypervariable region is first amplified. The amplified PCR product for each particular region is then individually used in another PCR reaction, in which, in addition to the usual dNTP building blocks, special types of nucleotides that stop DNA replication (dideoxyribonucleotide triphosphates: ddNTPs) are also present. Each of the four ddNTPs is labelled with a fluorescent dye of a different colour. When a ddNTP is added to a growing segment of DNA instead of a dNTP, DNA extension stops immediately, and no new nucleotides are added. Because both types of NTPs are present, different PCR products will be terminated at different points on the DNA template, and a mixture containing a series of DNA fragments, each differing by one base pair in length, is formed. CE then separates these fragments, and because each has the label of the last base (ddNTP)

[52] RENFREW 2001, 4830–4832.

[53] ELHAIK *et al.* 2014.

[54] STONEKING 2000, 1029–1032.

added, the entire sequence of bases in the DNA region examined can be obtained. After the sequence is generated, it is compared to a reference sequence for mtDNA, and differences are noted. MtDNA coming from the same person or from a person with the same maternal lineage is expected to have the same DNA sequence and therefore the same differences from the reference sequence.

The analysis of mtDNA

When sample size is limited, as is the case when only a small segment of bone or a tooth is found, mitochondrial DNA sequencing is the method of choice to determine the origin of such samples[55]. Mitochondrial DNA sequencing is also useful when an evidentiary biological specimen is degraded by environmental factors or aging, and nuclear DNA testing fails. Unlike nuclear DNA, mtDNA is present in high copy number, with hundreds of mitochondria present in most cells.

The mitochondrial genome is a closed circle of DNA that consists of 16569 base pairs. The two strands of the molecule are referred to as the heavy (H) and light (L) strands. The former strand has the largest number of guanine nucleotides. These bases have the largest molecular weight of all four DNA building blocks. As a result, the H strand can easily be separated from the L strand by centrifugation. The genome contains regions that code for 36 gene products, including specific proteins and ribonucleic acids that are involved in the structure and function of the mitochondrion as well as a control region, whose purpose is to regulate mitochondrial DNA replication. The control region contains two segments of DNA that are highly polymorphic and described as hypervariable (HV). Thus, the researcher is primarily interested in regions HV1 and HV2. The first, HV1, has a sequence of 342 bp (16,024–16,365) and the second, HV2, has a sequence of 268 bp (73–340)[56]. All of these bases (610 bp combined) are sequenced in ancient mtDNA analysis.

It would be very difficult to totally sequence exemplars (known reference samples) and evidentiary items and then report this total sequence information from beginning to end. To avoid any confusion in the comparison of two specimens, the researcher compares each specimen's mtDNA sequence to a reference sequence, and then describes differences found at specific sites. These differences are used for identifying the haplotypes.

A haplotype is a group of genes within an organism that was inherited together from a single parent. The word "haplotype" is derived from the word "haploid", which describes cells with only one set of chromosomes, and from the word "genotype", which refers to the genetic makeup of an organism. A haplotype can describe a pair of genes inherited together from one parent on one chromosome, or it can describe all of the genes on a chromosome

[55] LUTZ *et al*. 1996, 205–209.

[56] STONEKING 2000, 1029–1032.

that was inherited together from a single parent. This group of genes was inherited together because of genetic linkage, or the phenomenon by which genes that are close to each other on the same chromosome are often inherited together. In addition, the term "haplotype" can also refer to the inheritance of a cluster of single nucleotide polymorphisms (SNPs), which are variations at single positions in the DNA sequence among individuals[57].

In human genetics, a human mitochondrial DNA haplogroup is a group of similar haplotypes that share a common ancestor having the same single nucleotide polymorphism (SNP) mutation in all haplotypes[58]. Because a haplogroup consists of similar haplotypes, it is possible to predict a haplogroup from haplotypes. An SNP test confirms a haplogroup. Haplogroups are assigned letters of the alphabet, and refinements consist of additional number and letter combinations. Haplogroups are used to represent the major branch points on the mitochondrial phylogenetic tree[59]. Understanding the evolutionary path of the female lineage has helped population geneticists trace the matrilineal inheritance of modern humans back to human origins in Africa and the subsequent spread around the globe[60].

The letter names of the haplogroups (not just mitochondrial DNA haplogroups) run from A to Z. As haplogroups were named in the order of their discovery, they (meaning the accidental dictionary ordering of the letters) do not reflect the actual genetic relationships.

The hypothetical woman at the root of all these groups (meaning just the mitochondrial DNA haplogroups) is the matrilineal most recent common ancestor (MRCA) for all currently living humans. She is commonly called Mitochondrial Eve[61].

aDNA recovery

Initially, the development of the polymerase chain reaction (PCR) induced an early trend of DNA recovery reports from various source like plant fossils[62], dinosaurs[63] and insects trapped in amber[64]. Throughout time, more of these early reports are regarded with caution and considered to be products of contamination or artefacts[65]. However, despite its controversial past, the field of archaeogenetics is a reliable research area due to recent methodological improvements[66]. Due to the recent PCR development, that allows

[57] The International HapMap Consortium 2003, 789–796.

[58] SHARMA *et al.* 2005, 497–506.

[59] Van OVEN, KAYSER 2009, E386–E394.

[60] TOLK *et al.* 2001, 717–723; BEKADA *et al.* 2013, e56775.

[61] SHARMA *et al.* 2005, 497–506.

[62] GOLENBERG *et al.* 1990, 656–658; SOLTIS *et al.* 1992, 449–451.

[63] WOODWARD *et al.* 1994, 1229–1232.

[64] CANO *et al.* 1993, 536–538; DeSALLE *et al.* 1992, 1933–1936.

[65] AUSTIN *et al.* 1997, 467–474; PÄÄBO *et al.* 2004, 645–679; HEBSGAARD *et al.* 2005, 212–220; PENNEY *et al.* 2013, e73150.

[66] RIZZI *et al.* 2012, 21–29.

amplification of aDNA even if a small number or just a single copy is available, a rapid increase and diversification of ancient DNA research[67] was observed. However, the aDNA recovery is still a challenge, mainly determined by the aDNA damage and the contamination with modern DNA[68].The archaeological remains presents, in general, a small amount of endogenous genetic material, ancient DNA, able to be amplified by PCR. Mainly two types of damage are likely to affect DNA in archaeological deposits. The DNA macromolecule possess a limited chemical stability without a repair mechanisms[69]. After cell death, the DNA is cut into fragments by nucleases[70] or is digested by micro-organisms[71]. Long-term degradation of the DNA is done by the hydrolysis of amino groups, resulting the loss of purine or pyrimidine residues[72], or by oxidative damage mediated by free radicals[73].

Another major issues in aDNA recovery is the contamination risk, especially when dealing with human ancient DNA than with animal or plant ancient DNA[74]. Several studies reported that modern human DNA contamination is the top priority in the amplification process from aDNA template[75]. Also, it is impossible to completely remove modern human DNA from ancient bones even with extensive UV or bleach treatments[76]. An alternative in order to avoid contamination with modern DNA is to use teeth when are well preserved and directly removed from the jaw or maxilla. They present a lower risk of contamination in comparison with bone remains. Several studies[77] recommend hair as a more reliable source for studies on human ancient DNA, even if its presence is less common in ancient specimens.

Overall, the main source of sample contamination seems to be the human handling during and after excavation. Washing procedures and direct handling more often contaminate the both sample surfaces and depending on the porosity, also the interior of bones[78]. To avoid contamination, samples should be collected at the archaeological site using disposable gloves (changed between different samples), facemask, head-dress gown and lab coat. Another, important factors are the storage conditions (preferably at -20°C and as dry is possible), in

[67] RIZZI *et al.* 2012, 21–29.

[68] HOFREITER *et al.* 2001, 353–359; MAROTA *et al.* 2002, 310–318; IÑIGUEZ *et al.* 2003, 67–69.

[69] LINDAHL 1993, 709–715.

[70] DARZYNKIEWICZ *et al.* 1997, 1–20.

[71] LINDAHL 1993, 709–715; EGLINTON *et al.* 1991, 315–328.

[72] LINDAHL, ANDERSSON 1972, 3618–3623; LINDAHL, T., NYBERG, B. 1972, 3610–3618.

[73] LINDAHL 1993, 709–715; DIZDAROGLU 1992, 331–342.

[74] RIZZI *et al.* 2012, 21–29.

[75] KRINGS *et al.* 1991, 1166–1176; KOLMAN, TUROSS 2000, 5–23; HOFREITER *et al.* 2001, 353–359.

[76] GILBERT *et al.* 2005, 541–544.

[77] GILBERT *et al.* 2004, R463–R464.

[78] GILBERT *et al.* 2006, 156–164; MALMSTRÖM *et al.* 2005, 2040–2047; SALAMON *et al.* 2005, 13783–13788; SAMPIETRO *et al.* 2006, 1801–1807.

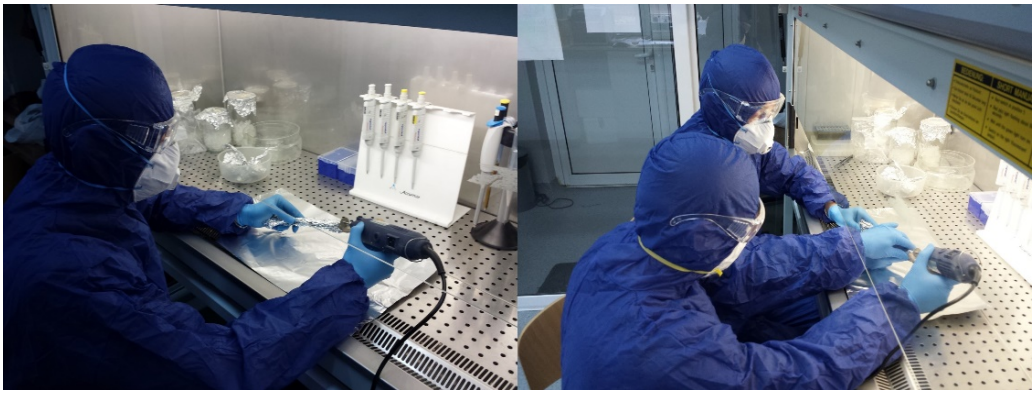

Figure 4. Researchers required dress code in aDNA analysis.

Figure 4. Samples preparation steps: (A) drying after sodium hypochlorite wash, (B) UV exposure, and (C) bone powder sampling.

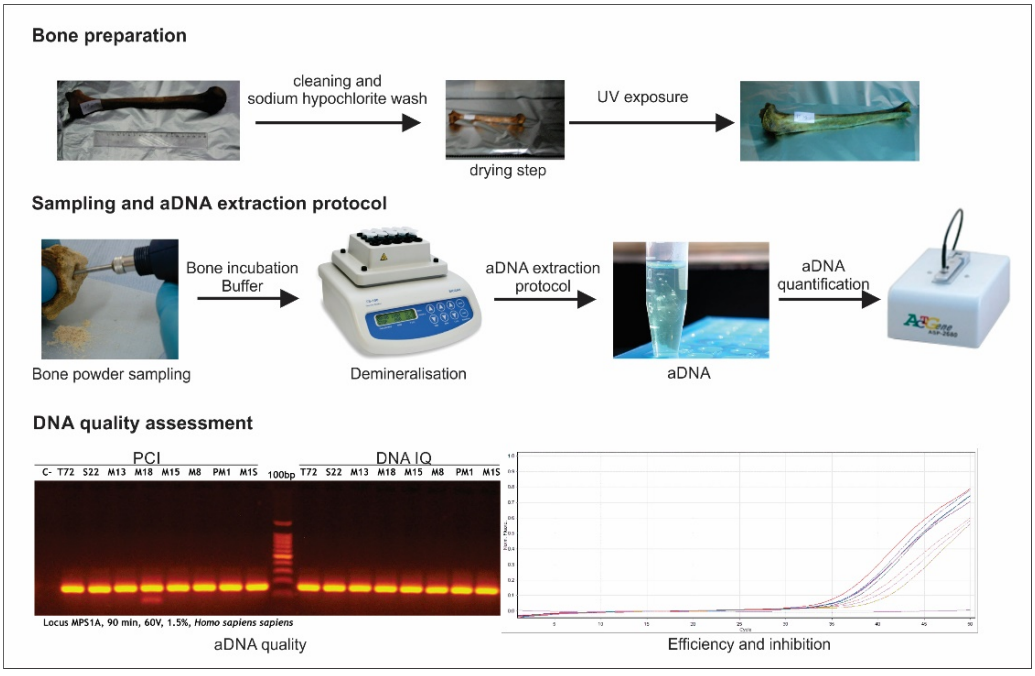

Figure 5. Graphical abstract of aDNA extraction steps.

order to minimize further DNA damage[79]. Finally, if a sample is susceptible to contamination, saliva samples should be taken from each person who has handled the material, as a control.

aDNA extraction: millstones and limitations

DNA extraction is probably the most crucial step in any ancient DNA analysis[80]. In this prior phase there is a huge risk to lose all potential genetic information. Even if the extraction is by far the most important step, there are few debates about the most appropriate method[81].

The extraction of aDNA from bone remains involves two main activities: samples preparation and extraction protocol. Behind the strict condition needed in the extraction room, like total isolation, positive pressure and UV decontamination overnight, the researcher himself could and most often represent a contamination source, therefore some measures must be followed (facemask, gloves, disposable lab coat and other accessories so that no layer of skin remains uncovered, Figure 4).

Sample preparation also requires specific steps to avoid the contamination. After the cleaning of the potential debris with a dry brush of the bones remains[82], two supplementary steps are needed: washing with sodium hypochlorite (NaOCl) and UV exposure. In a different study[83], was shown that the NaOCl treatment minimizes the risk of modern DNA contamination. UV irradiation for short time also minimizes the risk of contamination with modern DNA, damaging all potential exogenous DNA. Finally, with a dedicated bone drill in a small area the bone surface is removed and then bone powder can be sampled (Figure 5).

Extraction protocol of aDNA from bone remains has transformed over the years, known many variants that have been developed and used for accessing DNA from ancient material. Some methods utilize somewhat unusual reagents, for example Coca Cola[84], but the most prevalently used in recent years are the silica based approaches[85] and phenol/chloroform extraction and its derivatives. Regardless of the extraction method used, is necessary a prior demineralization step[86], total or just partially. After aDNA extraction one more supplementary step is required, DNA quality assessment (Figure 6). PCR application is a crucial step after extraction, being able to infer if the aDNA is or it is not contaminated. Furthermore, the inclusion of many blank controls in all processing stages is important in

[79] RIZZI *et al.* 2012, 21–29.

[80] HUMMEL 2003, 57–63.

[81] FISHER *et al.* 1993, 60–68; LALU *et al.* 1994, 160–163; CATTANEO *et al.* 1997, 1126–1135; VANDENBERG *et al.* 1997, 1624–1626; VINCE *et al.* 1998, 349–351.

[82] DASKALAKI 2014, 14–15.

[83] SALAMON *et al.* 2005, 13783–13788.

[84] SCHOLZ, PUSCH 1998, 283–286.

[85] DASKALAKI 2014, 15.

[86] JAKUBOWSKA *et al.*, 2012, 173–178.

order to control for potential lab contamination[87]. Even if all precautions are taken, periodically contamination can be observed in negative controls[88], in which case all the samples have to be discarded[89].

Exogenous DNA was an important issue since the beginning of archaeogenetics studies[90], leading to establishment of specific guidelines (Textbox 1) by the pioneers of the field[91].

"Ancient DNA: Do It Right or Not at All"

"**Physically isolated work area.** To avoid contamination, it is essential that, prior to the amplification stage, all ancient DNA research is carried out in a dedicated, isolated environment. A building in which large amounts of the target DNA are routinely amplified is obviously undesirable.

Control amplifications. Multiple extraction and PCR controls must be performed to detect sporadic or low-copy number contamination, although carrier effects do limit their efficacy. All contaminated results should be reported, and positive controls should generally be avoided, as they provide a contamination risk.

Appropriate molecular behavior. PCR amplification strength should be inversely related to product size (large 500- to 1000-base pair products are unusual). Reproducible mitochondrial DNA (mtDNA) results should be obtainable if single-copy nuclear or pathogen DNA is detected. Deviations from these expectations should be justified; e.g., with biochemical data. Sequences should make phylogenetic sense.

Reproducibility. Results should be repeatable from the same, and different, DNA extracts of a specimen. Different, overlapping primer pairs should be used to increase the chance of detecting numts or contamination by a PCR product.

Cloning. Direct PCR sequences must be verified by cloning amplified products to determine the ratio of endogenous to exogenous sequences, damage-induced errors, and to detect the presence of numts. Overlapping fragments are desirable to confirm that sequence variation is authentic and not the product of errors introduced when PCR amplification starts from a small number of damaged templates.

Independent replication. Intra-laboratory contamination can only be discounted when separate samples of a specimen are extracted and sequenced in independent laboratories. This is particularly important with human remains or novel, unexpected results.

Biochemical preservation. Indirect evidence for DNA survival in a specimen can be provided by assessing the total amount, composition, and relative extent of diagenetic change in amino acids and other residues.

Quantitation. The copy number of the DNA target should be assessed using competitive PCR. When the number of starting templates is low (<1,000), it may be impossible to exclude the possibility of sporadic contamination, especially for human DNA studies.

Associated remains. In studies of human remains where contamination is especially problematic, evidence that similar DNA targets survive in associated faunal material is critical supporting evidence. Faunal remains also make good negative controls for human PCR amplifications."

Textbox 1. The golden rule — guidelines for aDNA good laboratory practice by Cooper and Poinar 2000.

[87] DASKALAKI 2014, 14–15.

[88] IZAGIRRE, de la RÚA 1999, 199–207; LEONARD *et al.* 2007, 1361–1366; MALMSTRÖM *et al.* 2005, 2040–2047; YANG *et al.* 2003, 355–364.

[89] DASKALAKI 2014.

[90] HIGUCHI *et al.* 1987, 283–287; HOSS *et al.* 1996, 1304–1307.

[91] COOPER, POINAR 2000, 1139–1139; HANDT *et al.* 1996, 368.

Even when all the golden rules are respected and all laboratory restrictions are satisfied, still the contamination can appear. In this case the contamination occurred during excavation and post-excavation and this is a limit that cannot be exceeded in the lab. The communication between geneticists, archaeologists and anthropologists seems to be the only solution able to overcome the limits of the archaeogenetics.

As Mende considered in 2006[92], the application of scientific results in the interpretation of historical processes is frequently influenced by major difficulties and limitations. It is therefore necessary to analyse these limitations which determine the direct applicability of results obtained by archaeogenetic research:

- limitations on conclusions and difficulties of interpretation and chronology, mainly due to the fact that samples subjected to genetic analysis already represent an archaeological-historical, consequently also chronological, preconception.

- limitations posed by the ”inaccuracy“ of databases – during the results evaluation, it is also important to consider the relationships between the available databases, which shall be evaluated according to geographical and chronological aspects. The majority of reference databases are built on ”modern“ samples, which means that the information concerning the population of a given area either has little or no time depth, or offers possibilities of interpretation of extremely long time spans, as is the case of mtDNA haplogroups.

- limitations raised by diachronic and taphonomic processes on DNA preservation, depending on the microenvironment and the significance of sampling problems either should not be underestimated. These aspects are mostly important when there are no opportunities to select the best of several samples. Previous studies have shown that the relationship between the DNA preservation potential of bones (and the related success of analysis) and time of deposition is not as important as the effects of micro- and macro-environmental factors, burial rite and isolation that all influence the success of the PCR reaction. It is also problematic to evaluate the remains of children, because in the case of juvenile skeletons, which already contain only a negligible amount of cortical bone, it is more difficult to take non-contaminated samples, being a fundamental issue upon the success of viable DNA isolation and amplification. Meanwhile, in order to clarify internal kinship relations and chronological sequences within groups of burials, one should know their genetic affiliations as well. An additional difficulty is posed by the unfortunate fact that many physical anthropologists are hesitant when destructive sampling techniques must be used, even on a relatively small scale.

- limitations of technology and financing research.

[92] MENDE 2006, 29–33.

Applications of DNA analysis in archaeology

One of the most intriguing questions in archaeology is the possible reconstruction of *phylogeny* and the elucidation of the *evolutionary processes*. The genetic relationship between extinct hominins, mostly Neanderthals and modern humans represents a very contentious subject in archaeology[93]. Even if some inferences about the human population's history were done already by the pioneering study of Cann and co-workers[94], the determination of phylogenetic relationship between modern humans and extinct hominins can get more reliable results using aDNA analysis. Because there are no Pleistocene anatomically modern human's genomes, the detection and quantification of levels of interbreeding between anatomically modern humans and Neanderthals is approached by comparing modern human DNA and Neanderthal aDNA[95]. The limits of this approach are due to the fact that *Homo sapiens sapiens* and *Homo sapiens neanderthalensis* had a close evolutionary relationship and the number of the expected differences between the two is small[96]. In order to clarify the phylogenetic relationship and possible patterns of interactions or degrees of affinity between hominin groups , mitochondrial and nuclear DNA have been used to estimate population and genomic divergences between Neanderthals, early modern humans and the archaic hominins of the Denisova cave[97]. Even if most of the physical evidence of hominin evolutionary history lies irretrievably beyond the limits of aDNA molecular survival, palaeogenetic analysis of non-Neanderthal extinct hominins has the potential to extend our knowledge on the hominin evolutionary history[98]. Palaeogenetic data have also influenced the study of hominin philogeography, offering important information about the ecological and geographic context of archaic hominins. mtDNA data have been used to determine the eastward extent of the Neanderthals[99], while Denisovans aDNA data has been used to compare the putative range of these archaic hominins with that of the Neanderthals[100]. For a better image on the spatial distribution of the modern human genetic lineages it was used also the mitochondrial sequence data obtained from archaeological modern human specimen such as the Tirolean Iceman (*Ötzi*) even if the informational content obtained from a single human lineages in space and time are very limited[101].

[93] KIRSANOW, BURGER 2012, 122.

[94] CANN *et al.* 1987, 31–36.

[95] KIRSANOW, BURGER 2012, 122.

[96] GREEN *et al.* 2006, 330–336; GREEN *et al.* 2008, 416–426; GREEN *et al.* 2009, 2494–2502; GREEN *et al.* 2010, 286–288.

[97] GREEN *et al.* 2006, 330–336; GREEN *et al.* 2008, 416–426; KRAUSE *et al.* 2010, 231–236; NOONAN *et al.* 2006, 1113–1118; REICH *et al.* 2010, 1053–1060.

[98] KIRSANOW, BURGER 2012, 122.

[99] KRAUSE *et al.* 2010, 894–897.

[100] REICH *et al.* 2010, 1053–1060.

[101] ERMINI *et al.* 2008

aDNA recovered from specimens representing a diachronic sequence can be used to interpret the development of population structure[102], changes in diversity[103], population movements[104] and the relationship of these factors to cultural development and socioeconomic exchange patterns[105]. Data obtained from aDNA were used in the debate concerning the nature of the transition from foraging to agriculture in Europe. Depending on the model proposed for the transmission of agricultural technology, different models for the genetic relationships between hunter-gatherers and farmers were inferred. The *demic diffusion model* considers that farming may have become established through the replacement of indigenous hunter-gatherers by immigrant farmers, while the *cultural diffusion model* does not take into consideration population replacement and is compatible with genetic continuity from pre-Neolithic times to present[106]. Based on the aDNA analysis it has been argued that modern Central and Northern Europeans bear regionally variable proportions of ancestry from both original hunter-gatherers and early farmers[107]. Recent data from the Iberian Peninsula found similar haplogroup compositions in the Neolithic populations and modern Iberians, which is compatible with the *demic diffusion model* of agriculture to region[108]. mtDNA from Scandinavian hunter-gatherers reveals a genetic discontinuity between prehistoric hunter-gatherers and modern Scandinavians, supporting a population shift with the adoption of agriculture, which, also, supports the *demic diffusion model*[109]. Recent data obtained for the Neolithic from Central Europe indicate a demic influence from the steppe and point to a more complex and dynamic process of the Neolithic transition in some regions of Europe[110]. The complexity and dynamism of the *demic diffusion model* in South-eastern Europe is supported, also, by the recently published mtDNA from Romania[111]. If the Neolithic agricultural transition was more similar to a *mosaic* which supports also the *demic diffusion model* and *the cultural diffusion model*, this has to be supported by future regional aDNA datasets.

Conclusions

There are several reasons why the mtDNA markers have been used extensively in archaeological investigations. First of all, mtDNA is relatively easy to work with. Its small size,

[102] IZAGIRRE, de la RÚA 1999, 199–207; CHILVERS *et al.* 2008, 2707–2714; de-la-RUA *et al.* 2015, 306–311; BRANDT *et al.* 2015, 73–92.

[103] BROTHERTON *et al.* 2013, 1764; 1–6; ERMINI *et al.* 2015, 4–20.

[104] HAAK *et al.* 2015, 207–211.

[105] SZÉCÉSNYI-NAGY *et al.* 2015, 1–9.

[106] HAAK *et al.* 2005, 1016–1018; PINHASI *et al.* 2012, 496–505; KIRSANOW, BURGER 2012, 123.

[107] PINHASI *et al.* 2012, 496–505

[108] SAMPIETRO *et al.*, 2007, 2161–2167; OLALDE *et al.*, 2015, 1–11.

[109] MALMSTRÖM *et al.* 2009, 1758–1762.

[110] HAAK *et al.* 2015, 207–211.

[111] HERVELLA *et al.* 2015, 1–20.

coupled with the conserved arrangement of genes, means that many pairs of universal primers will amplify regions of the mitochondria in a wide variety of vertebrates and invertebrates. This means that data often can be obtained without any a priori knowledge about a particular species' mitochondrial DNA sequence. Second, although the arrangement of genes is conserved, the overall mutation rate is high. The rate of synonymous substitutions in mammalian mtDNA has been estimated at $5{:}7 \times 10^{-8}$ substitutions per site per year[112], which is around ten times the average rate of synonymous substitutions in protein-coding nuclear genes. The non-coding control region, which includes the displacement (D) loop, evolves particularly rapidly in many taxa. The high mutation rate in mtDNA may be due partly to the by-products of metabolic respiration and also to less-stringent repair mechanisms compared with those acting on nuclear DNA[113]. Regardless of the cause, these high mutation rates mean that mtDNA generally shows relatively high levels of polymorphism and therefore will often reveal multiple genetic lineages both within and among populations.

The third relevant property of mtDNA is its general lack of recombination, which means that offspring usually will have (barring mutation) exactly the same mitochondrial genome as the mother. As a result, mtDNA is effectively a single haplotype that is transmitted from mothers to their offspring. This means that mitochondrial lineages can be identified in a much more straightforward manner than nuclear lineages, which, in sexually reproducing species, are continuously pooling genes from two individuals and undergoing recombination. The effectively clonal inheritance of mtDNA means that individual lineages can be tracked over time and space with relative ease, and this is why, as we will see later, mtDNA sequences are commonly used in studies of phylogeny and phylogeography.

Finally, because mtDNA is haploid and uniparentally inherited, it is effectively a quarter of the population size of diploid nuclear DNA. Because there are fewer copies of mtDNA to start with, it is relatively sensitive to demographic events such as bottlenecks. These occur when the size of a population is temporarily reduced, e.g. following a disease outbreak or a catastrophic event. Even if the population recovers quickly, it will have relatively few surviving mitochondrial haplotypes compared with nuclear genotypes. As we will see in later, inferring past bottlenecks can make an important contribution towards understanding the current genetic make-up of populations.

The ability to genotype from bone and teeth remains depends entirely on whether DNA can be recovered from these calcified tissues. It is often the case that only small fragments of bone or teeth can be recovered and thus methods of extraction must be efficient and nondamaging to the endogenous DNA available. The anthropology and archaeology alike have benefited from the genetic analyses of DNA contained in fragments of well-preserved

[112] BROWN *et al*., 1982, 225–239.

[113] WILSON *et al*., 1985, 375–400.

bone or tooth. Such DNA can provide invaluable links to the past and help to determine indigenous foundations and reveal ancestral flow.

aDNA data can be used to clarify the evolutionary relationships between hominin species and to understand the evolutionary processes affecting the hominin lineage, answering to the previously formulated questions by the archaeologists. Except this, aDNA analyses are very useful for exploring the affinities and interactions between members of the same populations, and between hominin populations and their hypothetical ancestors and descendants. By adding aDNA data to archaeological and linguistic evidences we can investigate the relationship between population dynamics and sociocultural change.

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