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ANCIENT DNA STUDIES

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ABSTRACT: About three decades of scientific research confirmed ancient DNA as a powerful tool for anthropology, paleontology, molecular evolution, conservation genetics and forensics. A rapid evolution of the methodological approaches in the last ten years led to an important increase of data related both to the number of samples and to the informative genetic markers. Paleogenetics analysis has been applied to a wide spectrum of samples, answering to different kind of scientific and cultural questions. In this review, ancient DNA characteristics and methodological approaches are presented as well as recent results obtained from samples of bacteria, plants, animals, with a particular focus on the newest discoveries about the *Homo* genus.

Keywords: Ancient DNA, aDNA, Paleogenetics

1. INTRODUCTION

The first data obtained from ancient DNA samples were published in 1984, when Higuchi et al. (1984) presented the results obtained from a tissue samples of a museum specimen of the quagga, a zebra-like species (*Equus quagga*) extinct one hundred years before. In 1985 Svante Pääbo published the recovering of genetic material from an Egyptian mummy (Pääbo, 1985). The first results obtained from bone tissue date back to 1989 (Hagelberg et al., 1989). In thirty years from these first attempts, huge innovations in methodology, signed by the polymerase chain reaction (PCR) first and the Next Generation Sequencing (NGS) later, allowed researchers to understand the characteristics of the ancient DNA, to better recognize it from possible modern DNA contaminations and to obtain a large amount of data useful for different kinds of applications. After the first work by Higuchi where 299 nucleotides of the mitochondrial genome of the quagga were sequenced, data for the whole nuclear genome of this species were obtained (Jonsson et al., 2014). After the first attempt by Pääbo, the complexity of recovering authentic ancient molecular data from human remains was better defined and sophisticated experimental procedures and bioinformatics tools were developed in order to obtain reliable results.

2. ANCIENT DNA, DEFINITION AND CHARACTERISTICS

Despite its name, ancient DNA (aDNA) has no age. This expression defines degraded DNA, and degradation of genetic material starts immediately after the dead of the organism or when a biological trace is left in the environment. For this reason, both paleontological and historical samples and more recent traces analyzed in forensic contexts can be considered as aDNA. The starting material includes samples such as bones, hair, mummified tissues, coprolites, sediments, vegetable remains, and the temporal limits reached so far are about 400,000 years for samples recovered in caves from temperate regions (Meyer et al., 2014) and about 700,000 years for samples preserved in permafrost (Orlando et al., 2013).

aDNA has particular characteristics due to the degradation of the genetic material mainly occurring by hydrolysis and oxidation led by factors such as temperature, humidity and pH. Often, when the genetic material extracted from a degraded sample is analyzed, the majority of the DNA can be referred to a microbial origin and only a little percentage belongs to the organism from which the sample comes (Fig. 1). DNA degradation determines not only the loss of the genetic material, but also the high fragmentation of the surviving component

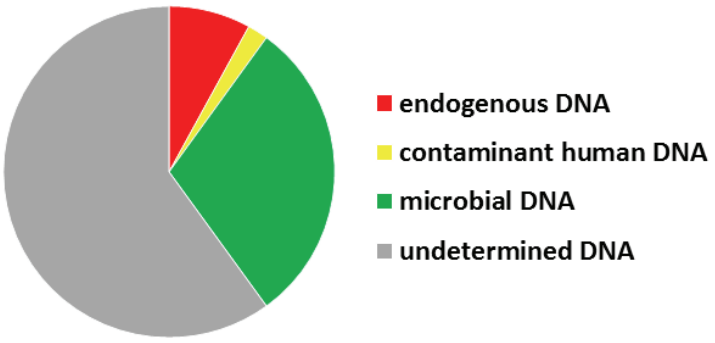

Fig. 1 - Graphical representation of the DNA content of an ancient sample (data unpublished).

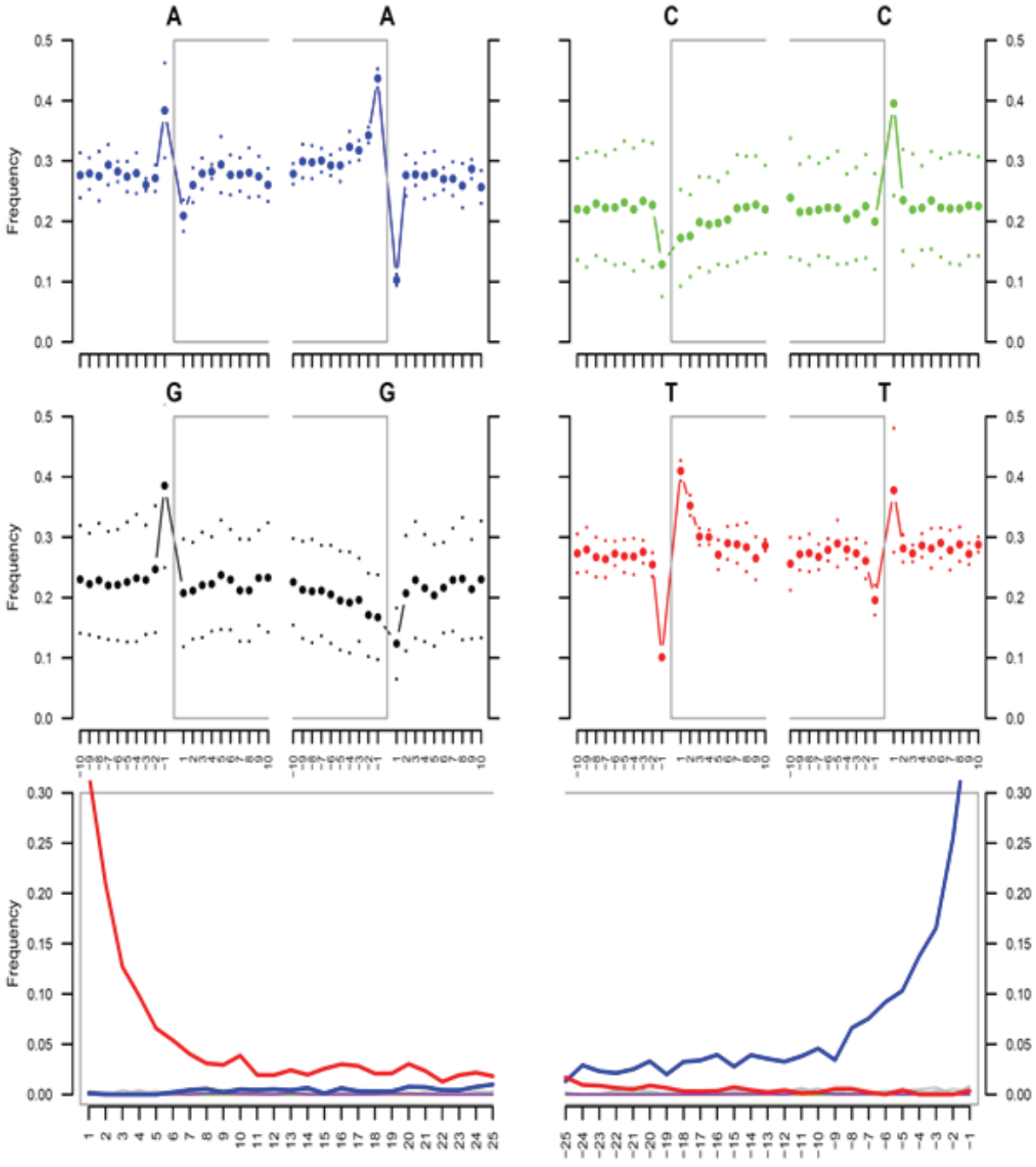

Fig. 2. Misincorporation pattern for a human sample dated 4700 BP (data unpublished). The four upper mini-plots show the base frequency outside and in the read (the open grey box corresponds to the read). The bottom plots are the positions' specific substitutions from the 5" (left) and the 3" end (right). The C>T substitutions are in red, the G>A substitutions are in blue. The estimation was performed using MapDamage 2.0 (Ginolhac et al., 2011).

that is usually characterized by DNA fragments of short length (Krause et al., 2010a). Another typical form of degradation that characterizes the aDNA is the deamination of the cytosine, that is translated in a misincorporation event during the determination of the DNA sequence where an original C (cytosine) is converted in T (thymine), or an original G (guanine) is substituted by a A (adenine) when the damage involves the opposite DNA strand (Brotherton et al., 2007). These misincorporations occur on the aDNA molecules following a particular pattern: their frequency increases at the ends of the molecules (Briggs et al., 2007) (Fig. 2).

These characteristics determine the need of a specific experimental workflow designed for recovering highly degraded molecules, but represent in the meantime an informative source that allow to discriminate between authentic ancient endogenous DNA from possible modern contaminants.

3. METHODS FOR THE STUDY OF ADNA

Specific criteria have been developed for working with aDNA. A first important advantage is provided by an appropriate behavior in sample recovering in order to avoid the contamination during handling of the remain wearing sterile gloves, masks and special or disposable clothes (Pilli et al., 2013). The laboratory where the molecular analysis is performed, has to be exclusively dedicated to aDNA with a physical separation between the pre-amplification and the post-amplification sections and devices for sterilizing the working areas.

Some years ago, the only methodological approach available for aDNA studies was based on PCR, cloning and Sanger sequencing. Following this strategy, a target DNA region is selected and amplified by PCR, obtaining an exponential increase of the number of molecules of interest. The PCR product is cloned using a plasmid vector and bacterial cells. Isolating and reproducing single molecules of the PCR product in the bacterial cells, it is possible to sequence independently each amplicon that consists on a bacterial colony carrying several copies of a single original DNA molecule obtained by PCR. In this way the sequence of numerous molecules selected by PCR is determined, giving the possibility to observe the possible presence of multiple biological sources and of misincorporations (Fulton and Stiller, 2012). Using this methodological approach, it is necessary to have a prior knowledge of the target sequence in order to design the primers needed for the amplification and the whole target sequence (primers annealing regions included) have to be preserved intact in the ancient sample. Furthermore a minimum fragment length of about 60bp is required for the molecules to be recovered by PCR. This requirements represent a limitation for highly degraded samples with really short DNA fragments. The so-called golden criteria were developed to assess the authenticity of the results obtained by PCR (Cooper and Poinar, 2000).

With the more recent NGS approach, universal adapters are ligated to the DNA fragment, independently from their sequence or length (Briggs and Heyn, 2012). Every kind of DNA molecule can be potentially recovered, without modifying its original characteristics that can be then used for assessing its authenticity as ancient. Barcodes can be added to the DNA fragments together with the adapters, creating the so-called DNA library. With this strategy is possible to process numerous samples in parallel producing a large amount of sequence data, even whole genomes, and consequently more information.

Since the endogenous DNA could be represented in a small percentage in the sample, usually the NGS approach is flanked by a strategy of target enrichment using baits complementary to the sequences of interest, in order to increase their relative amount. The hybridization between baits and target molecules is usually performed in solution with a bead-based capture approach (Carpenter et al., 2013; Maricic et al., 2010).

The sequencing of the whole library (shotgun approach) or of the enriched one produces a big amount of data and specific bioinformatics pipelines have been developed for handling them taking into account the characteristic of the aDNA. Software for filtering the data according to quality and length such as FastQprocessing (Kircher, 2012), mapping and assembling procedures as MIA (Green et al., 2008), specific procedures for misincorporation pattern analysis (Ginolhac et al., 2011) and contamination estimation (Fu et al., 2013), as well as more complete pipelines like Paleomix (Schubert et al., 2014) or Schmutzi (Renaud et al., 2015) are available. Guidelines for NGS work on ancient DNA were also proposed (Knapp et al., 2015).

4. ANCIENT DNA STUDIES

aDNA data represents an informative source that have application in different fields. The range of possible starting material is wide, and the questions that can receive a contribution from the Paleogenetics involve topics related for example to the study of evolution and phylogenesis contributing to anthropological, archaeozoological and archaeobotanical studies and of archaeology, cultural heritage and fauna and flora conservation.

aDNA studies on Bacteria

As previously described, most of the DNA preserved in an ancient sample usually belongs to microorganisms. Characterization of the microbial diversity in horse bones from a time span between 200 and 13,000 years ago, consisted in site-specific microbial profiles that allow to characterize the environment from which the samples were recovered (Der Sarkissian et al. 2014). However, the majority of data belonged to a recent bacterial colonization of the bones. This observation implies that the characterization of authentic ancient microbial remains is a complex process that requires specific methodological devices.

In the last years, a particular interest was focused on the analysis of the oral microbiota in human samples, in order to reconstruct its possible variations during time linked to specific subsistence and cultural shifts occurred in human history. The starting material for this kind of studies is the dental calculus (plaque), that showed to preserve a record of the microorganism composition of the oral cavity, including bacteria involved in

local and systemic diseases, as well as molecular information about host immunity and diet (Warinner et al., 2014; Weyrich et al., 2015). The Neolithic Revolution, began about 10,000 years ago, with the shift toward a carbohydrate-rich diet, and the recent employment of industrial processed sugar and flour started around 1850, determined major changes in the microbial oral community. Disease-associated configuration remained constant between Neolithic and Medieval times, then the cariogenic bacteria became dominant with the Industrial Revolution, with a general less diverse microbial profile in modern populations (Adler et al., 2013).

Another research field is about the reconstruction of ancient pathogens' genomes in order to clarify the phylogenesis of such species, to understand the relationship between different strains, and to identify possible polymorphisms related to virulence. The whole genome of *Mycobacterium leprae* was determined from medieval leprosy cases and its comparison with modern strains revealed remarkable genomic conservation during time (Schuenemann et al., 2013). Differently, the analysis of whole genomes of the medieval strain of *Yersinia pestis* recovered from victims of the Black Death revealed that this variant of the bacterium may no longer exists, but it is probably ancestral to the modern strains. The epidemiological differences in time seem to be not related to genetic differences, but to other factors such as environment, host susceptibility or vector dynamics (Bos et al., 2011; Schuenemann et al., 2011).

aDNA studies on plant remains

Data about aDNA from vegetable remains are not so numerous, since it is difficult to extract genetic material from ancient plants that often also contain inhibitory substances that interfere with some experimental procedures. Despite these difficulties, archaeobotanical samples can provide information about species identification, domestication and evolutive processes.

Genetic material can be found in different kind of samples: wood, fruits, leaves, seeds and pollen in archaeological or herbarium specimens. DNA of vegetable origin can be preserved also in feces (Wood et al., 2012) and dental calculus (Weyrich et al., 2015) providing information about diet. Also sediments are a source for plant DNA revealing the vegetation distinctive of past times even in absence of macrofossil evidences (Willerslev et al., 2003). Paleoenvironmental studies on sedimentary ancient DNA permit to reconstruct the plant cover history and to understand the changes during time due to climate or human activities (Pansu et al., 2015). With the same approach, it is possible to attest the presence of domestic plants in sediments, better defining the chronology of the Neolithic transition (Smith et al., 2015). Comparing genetic data between different wild and domestic forms, it is possible to identify the original variants from which the domestication occurred, to follow routes of diffusion related with the anthropic exploitation and to give information about both the vegetal and human history. It is also possible to identify genetic loci under selection during domestication for adaptation to climatic and cultural contexts, such as drought and sugar content (Allaby et al., 2014; Allaby et al., 2015; da Fonseca et al., 2015; Palmer et al., 2012).

aDNA studies on animals

Genomes of extinct species is a powerful tool to understand their phylogenetic relationships with extant forms. Many paleogenomics studies have been conducted on faunal remains. The nuclear genome of several woolly mammoths was sequenced, defining with higher resolution the phylogenetic tree of the Elephantids. Some differences with functional effects were found between mammoth and elephant and in some cases variants were probably positively selected in the mammoth lineage. Intra-population differences, that were not evident from the fossil records, were also highlighted (Miller et al., 2008).

The most ancient genome determined so far is from a horse sample preserved in permafrost dated between 560-780 thousand years BP. By comparing this sequence with those of other ancient and modern horse and donkey samples, the most recent common ancestor of the genus *Equus* was dated to 4.0-4.5 million years BP. Interestingly, multiple population size fluctuations related to climate changes have been attested in the history of this taxa, as well as particular genomic regions have been found to be possibly selected during domestication (Orlando et al., 2013).

About domestication process, several researches have been conducted in order to identify the area where it took place, the possible routes followed by the spread of the livestock and the possible admixture with wild local forms. While the history of the domestication of sheep and goat is rather simple to be reconstructed since the wild forms were limited to the Fertile Crescent before Neolithic, the dynamics related to the domestication of cattle and pig could be more complex because the wild forms of *Bos taurus primigenius* and *Sus scrofa* were spread in a wider area. Ancient and modern DNA data collected from Europe, Western Anatolia and Iran, shown that the domestication of cattle occurred as a single process in the Near East in the 9th millennium BC. With the Neolithic transition, the domestic form spread into Europe with no significant interbreeding with the local wild individuals (Scheu et al., 2015) even if some haplotype sharing between pre- and post-Neolithic samples in southern Europe suggests a possible contribution from the wild local forms in particular areas (Beja-Pereira et al., 2006; Lari et al., 2011; Mona et al., 2010).

Several studies on ancient and modern DNA were conducted in order to understand the dynamics related to the swine domestication. A phylogeographic approach suggested the origin of the domestic form in the Near East, its spread in Europe during the Neolithic diffusion, then a local domestication of the European wild boar. A later replacement of the eastern lineages by the European ones occurred not only in Europe but also in Asia Minor during the Bronze Age (Larson et al., 2007; Ottoni et al., 2013). A recent contribution demonstrated that also natural population dynamics, occurred independently of domestication, can determine the variation in frequency and distribution of such genetic lineages. A so -called Near Eastern haplotype, previously considered as marker of the neolithization wave from Near East, was found in Italian samples two thousand years before the arrival of the Neolithic culture in that area (Vai et al., 2015). This example shows that the reconstructions of

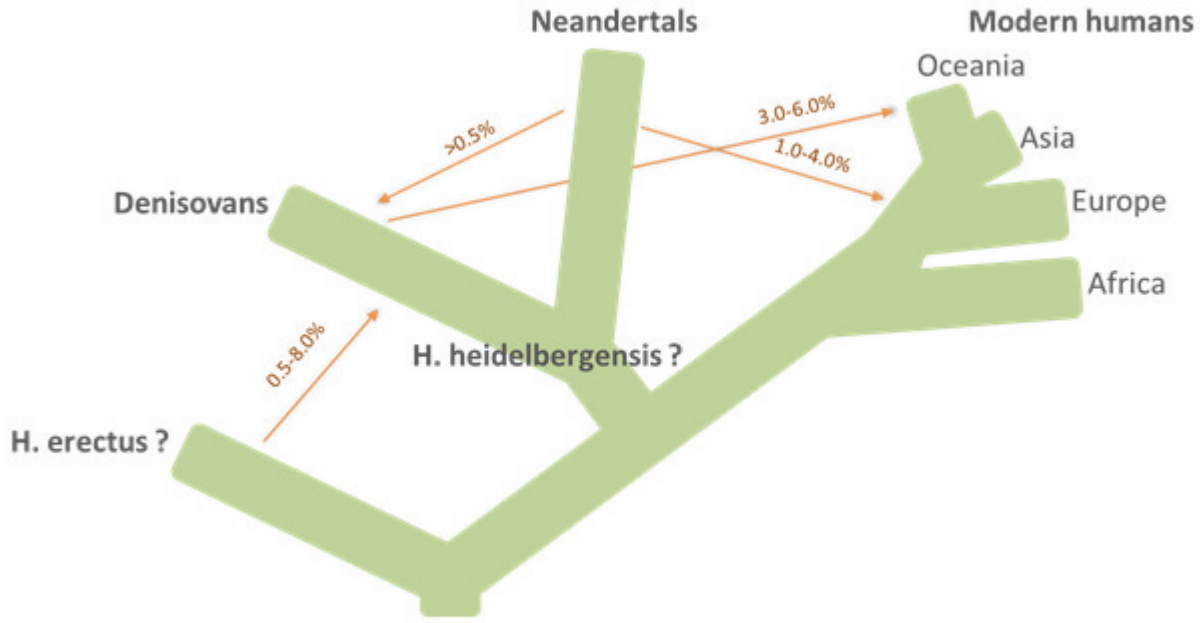

Fig. 3 - Schematic reconstruction of the possible gene flow events occurred between human lineages during Late Pleistocene (data from Prüfer et al., 2014).

past events are always open to new developments as new samples are analyzed covering new geographical areas and new chronological periods, as well as more genetic markers are considered.

***aDNA studies on genus* Homo**

Particular interest focused on the study of human species, in order to clarify the relationships between different forms that in some cases shared the same geographical areas in the same period, such as Neandertals and early anatomically modern humans (EMHs). Genetic variation of Neandertals has been explored in several studies, starting with the methodological approach based on PCR for studying particular loci followed by the sequencing of the whole genome by NGS. Genetic discontinuity between this extinct human form and extant and ancient modern humans was already attested analyzing a portion of the mitochondrion (mtDNA) (Krings et al., 2000; Caramelli et al., 2003) and was confirmed when the whole mtDNA genome was determined in 2008, confirming that the Neandertal mtDNA falls outside the variation of modern humans with an estimation of the divergence time around 660,000 years ago (Green et al., 2008). Analyzing more Neandertal individuals, it was possible to identify a possible genetic structure at intra-population level, probably related with the geographical and/or temporal distribution of the samples, with a general low mtDNA diversity that may reflect a low effective population size (Briggs et al., 2009). In addition, some nuclear genes were studied, since they are involved in phenotypic traits that could highlight possible differences between modern humans and Neandertal. For example, a private variant of the MC1R gene was found in two Neandertal remains, coding for pale skin color and red hair (Lalueza-Fox et al., 2007), representing an example of convergent evolution for adaptation to environments with low solar radiation. With the NGS approach, it was then possible to obtain the whole nuclear genome from Neandertal specimens, and to find that a portion around 1-4% of the genome of the extant inhabitants of Eurasia is shared with Neandertals. The most parsimonious explanations for this sharing are two: i) the Neandertals exchanged genes with the ancestors of all non-Africans or ii) the presence of an old substructure in Africa, whose traces persisted both in the Neandertal lineage and in the modern human one (Green et al., 2010). Recently, the genome of a modern human sample, found in Romania and dated to 39,000-42,000 years ago, carrying morphological traits that could indicate admixture with Neandertals, was determined. 6-9% of the genome of this individual showed Neandertal ancestry compatible with an admixture occurred four to six generation back (Fu et al., 2015).

A new human species was identified in 2010 thanks to aDNA analysis: samples from the Denisova Cave, in southern Siberia, dated to 50,300-62,200 years ago, showed a genetic variation that falls outside the range of both modern humans and Neandertals. According to the mitochondrial genome, the Denisova repre-

sents a human lineage that diverged about 1 million years ago from the branch that carried to *H. sapiens* and Neandertal (Krause et al., 2010b). Data from the nuclear genome showed that probably Denisovans contributed to the 4-6% of the genome of present-day Melanesians (Reich et al., 2010). The phylogenetic tree reconstructed using nuclear data place Denisovans as sister group of Neandertals. The difference between the phylogenesis obtained from mitochondrial and nuclear genomes can be explained considering that the mtDNA is only maternally inherited, while the nuclear genome carries information from both parents. For this reason the two genetic markers can tell different population histories.

The nuclear data from human forms closely related to modern humans represent an important informative source also for understanding which genetic variants characterize the *Homo sapiens* lineage becoming fixed after its separation from the ancestors of Neandertals and Denisovans. Genes that showed derived variants only in the modern humans are involved with brain function and nervous system development, as well as the physiology of skin and eye and dental morphology (Meyer et al., 2012; Prufer et al., 2014).

Thanks to the reconstructed ancient genomes, it was possible to recognize several gene flow events, with general low extent or at least with low effect on the extant populations, that have involved Neandertals, Denisovans and EMHs during their coexistence in Eurasia during Late Pleistocene (Fig. 3).

The research on past human forms is still developing, since new specimens are discovered. A new protagonist of the human history provided genetic information to be added to the already complex reconstruction, representing also the most ancient sample from temperate environment that yielded endogenous DNA. The sample, attributed to *Homo heidelbergensis* according to morphology but with some Neandertal-derived characteristics, was date over 300,000 years ago. The already determined mitochondrial genome showed to be closely related to the lineage of Denisovans, increasing the expectation for the next nuclear data (Meyer et al., 2014).

aDNA data gave a big contribution also for understanding the more recent population history of *Homo sapiens*, especially for Europe. The presence of EMHs in Europe is attested since 45,000 BP and particular climatic and cultural changes characterized the population dynamics during time: Last Glacial Maximum, Mesolithic with mobile groups and Neolithic transition with the appearance of a sedentary lifestyle, Copper, Bronze and Iron Ages with the emergence of complex societies. With the NGS technology, several prehistoric human genomes were sequenced, leading to a deeper understanding of such dynamics (Olalde and Laluez-Fox, 2015).

The first genome to be sequenced was obtained from the the Tyrolean Iceman (Ötzi) lived during the Neolithic-Copper Age transition: the nuclear genomes permitted to determine some phenotypic characteristic and pathogens' presence as well as information useful for determining the relationship of the sample with modern European, North African and Middle Eastern populations (Keller et al., 2012).

Analyzing other ancient and modern genomes, it was highlighted that most present-day Europeans derive from at least three highly differentiated populations: West European hunter-gatherers who probably contributed ancestry to all Europeans, Upper Palaeolithic north Eurasians who contributed to Europeans and Near Easterners and early European farmers of Near Eastern origin with signs also of European hunter-gatherer ancestry (Lazaridis et al., 2014).

Analysis on 101 ancient humans from Eurasia confirmed that the Bronze Age was a period of big social changes. Genomic data showed that large-scale population migrations and replacements occurred, and that probably they are responsible for the major parts of present-day demographic structure in both Europe and Asia. These migrations could also be related with the spread of Indo-European languages hypothesized to be occurred during the Early Bronze Age (Allentoft et al., 2015).

5. CONCLUSIONS

For its huge informative power, aDNA represents a precious resource to support all those disciplines that have the aim to reconstruct past biological events and to use this knowledge for better understand and manage the present variability. Major limits are due to different degree of DNA preservation in ancient samples, but fast and continuous developing in methodologies, leading to discoveries that were unimaginable three decades ago at the beginning of this discipline, are promising to push beyond the current limitations.

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