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Review Synthetic Report

Ancient DNA investigations: A review on their significance in different research fields

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Abstract - In 1984, the extraction of DNA from dried muscle of the extinct quagga specie (*Equus quagga*) was a first in the analysis of ancient DNA (aDNA). Since, many studies have highlighted the feasibility of analyzing DNA from all type of ancient tissues including bones, hair, teeth and even coprolithes. Today, aDNA is widely investigated in several research fields. It is a useful tool in archeaozoology to elucidate the biology and the evolution of extinct species. In paleobotany, aDNA is used to reconstruct the paleoenvironments and to determine the time and place of the domestication of many varieties. It is also a powerful tool in forensic science to identify undetermined remains. In anthropological research aDNA provide important information to trace geographic distribution of human genetic variation and to reconstitute settlements history. However, its degradation and post-mortem chemical alteration make difficult its quantification and amplification. Moreover the study of aDNA is challenging due to the contamination by exogenous current DNA. Recently, the progress of molecular techniques and the use of sophisticated approaches greatly improved the ratio of endogenous DNA to contaminant DNA and allowed a better and more powerful aDNA investigation.

Keywords: ancient DNA, authentication, zooarcheology, paleobotany, anthropology, forensic, paleopathology

Discovery of ancient DNA:

Germination of 550 years old peanut seed (*Canna* species) (Sivori et al, 1968) is one of the first experiments demonstrating the conservation of genetic material in archaeological specimens. However, it was not until the 80s that analysis of the DNA in ancient remains started.

In 1984, Higuchi and his team have succeeded in isolating a 228 base pair (bp) segment of DNA from a fragment of dried quagga muscle (*Equus quagga*): a species close to the current zebra and missing since 1883 (Higuchi et al 1984). This ancient DNA (aDNA) was obtained by cloning. Positive clones were detected using a radiolabeled probe recognizing the mitochondrial DNA of the zebra (*Equus zebra*) (Higuchi et al., 1984).

A year later, Pääbo et al obtained a 3400pb long DNA fragment from dried tissue of a 2430 years old Egyptian mummy (Pääbo et al., 1985). The extraction of DNA from an 8000 years old human brain found in a swamp in Florida showed that aDNA is not only conserved in dried tissue but also in soft tissues (Doran et al., 1986). Improved techniques of molecular biology and in particular PCR (Polymerase Chain Reaction) (Saiki et al., 1985) gave new impetus to the aDNA studies that have multiplied. It became possible to isolate aDNA from any type of tissue and specimens. Indeed, the aDNA was extracted from bone (Hagelberg et al 1991, Bon et al 2008), dried tissue of animals or plants (Rollo et al 1988), hair (Higuchi et al 1988), teeth (Hanna et al, 1990, Campos et al, 2010), soft tissues (Pääbo et al 1988), mummies (Kim et al, 2010) and even coprolithes (fossil feces) (Poinar et al 2003, Lele et al, 2008).

Under favorable conditions, DNA can survive in tissue remains for several millennia and in some cases over 100,000 years. Indeed,, aDNA has been isolated from the remains of the Neanderthals (Krings et al 1997, Briggs et al 2009) and mammoth remains (Hagelberg 1994, Hoess et al 1994). However, DNA extracted from archaeological remains of a few million years was revealed to be the results of contamination. The DNA sequence supposed to belong to a dinosaur of 65 million years was revealed to be the nuclear copy of a human mitochondrial gene (Zichele 1995).

Properties of ancient DNA:

aDNA has many characteristics among them its alteration, difficult amplification and quantification and need of authentication to distinguish it from potential recent DNA contaminations.

An altered DNA:

The ancient DNA is degraded into fragments which size does not exceed 300 bp. The alteration of aDNA is a consequence of post-mortem chemical processes. Indeed, degradation begins within hours or days after the death of the organism by autolysis produced by endonucleases. In the long term, the phenomenon of oxidation due to radiation alters nitric bases and the complex sugar-phophate DNA. In addition, hydrolysis leads to deamination of bases, depurination and depyrimidination (Hofreiter et al 2001, Briggs et al, 2007, Briggs et al, 2010).

The accumulation of these degradation processes over time reduces the amount of DNA in archaeological specimens. However, conservation of aDNA does not only depend on the age of the specimen, but also mainly on environmental conditions (Pääbo et al 1989, Burger et al 1999). The aridity, low temperature and neutral or slightly alkaline pH promote the preservation of DNA (Burger et al 1999, Hoess et al 1996). It was also shown that the presence of a mineral layer such as hydroxyapatite on the bone consequently protects and preserves its DNA (Lindahl, 1993, Bon et al 2008). But the most important factor affecting the proper conservation of the DNA in ancient remains is the amount of micro-organisms found in contact with them. Micro-organisms degrade the aDNA by mechanical action and the substances they release. Empirical studies on the physical and chemical processes damaging the DNA showed that it can not survive more than 100,000 years even in extreme cold conditions or in anoxic environment (Lindahl 1993, Hoess 1996).

A difficult DNA to quantify:

After extraction, the solution containing the aDNA presents a color that varies from yellow to dark brown making its quantification difficult or impossible. This coloration is due to the presence of compounds which nature is still unclear although some studies could identify fulvic acid and humic extracts in the aDNA (Burger et al 1999). Therefore, the technique of spectrophotometry, conventionally used to quantify DNA, is ineffective.

In recent years, new techniques have been developed such as quantification by hybridization with a probe and Quantitative Real Time PCR that gave satisfactory results (Alonso et al 2004, Kefi et al 2003, kefi et al 2005, Haak et al, 2010).

A difficult DNA to amplify:

Amplification of aDNA is not an easy task. The compounds that coexist with aDNA such as humic and fulvic acids and tannins inhibit the action of Taq polymerase enzyme (ensuring the amplification of DNA during PCR). Techniques are becoming more and more sophisticated to reduce the amount of inhibitors and successfully amplify aDNA (Kefi et al 2003). Less than 50pb aDNA fragment cannot be retrieved by classical PCR. New approaches such as Multiplex PCR require 22–25 times less aDNA template and proved to be a powerful tool for analyzing highly degraded aDNA (Haak et al 2010). Moreover, the use of single primer extension-based approaches and high-throughput DNA sequencing allow DNA fragments of short lengths to be completely sequenced (Krause et al 2010, Briggs et al 2010).

aDNA authentication :

Genetic analysis of archeological remains is very challenging because of the contamination by current DNA. Several studies have focused on the identification of the sources of contamination and on the determination of means of aDNA authentication. These studies led to the publication of ‘‘criteria of authenticity’’ and the “Guidelines” to ensure the quality of aDNA. In order to avoid contamination with contemporary DNA particular precautions must be taken (Cooper 2000, Poinar 2003, Paabo et al, 2004, kefi et al 2003, Bon et al 2008, Haak 2010, Deguilloux et al 2011). One of the most important rules is the use of a physically isolated clean room (pre-PCR area) dedicated to aDNA experiments for all manipulations prior to DNA amplification. Some authors recommend to clean the work-top with bleach, ethanol and “DNA away” (solution destroying DNA, commercialized by Molecular Bio Product, Inc) to store solutions used for DNA extraction in another isolated room separated by sieve system and to test extraction buffer by PCR before utilisation. Pre-PCR and Post- PCR manipulations should be carried out in separate laboratory.

Second, the use of mask, coat, gloves and sterile materials for all manipulation even during excavation is mandatory as well as the use of extraction and PCR negative controls (reagent without sample) in parallel with samples on all occasions. Cloning and sequencing of the amplification products along with genotyping all individuals who have come into contact with a sample are also key steps in avoiding contamination.

In addition, the inverse relationship between DNA quantity and fragment length and the reproducibility of results can support the presence of endogenous DNA. Also results must present a phylogenetic and phylogeographic coherence. For example for mitochondrial DNA analysis, obtained sequences must be consistent with existing mtDNA haplotypes and haplogroups (Haak et al 2010, Kim et al 2011).

It is important to note that the application of new technologies such as multiplex approaches, quantitative real time PCR, SNaPshot Typing, the adaptor-ligation and high-throughput DNA sequencing greatly improves the ratio of endogenous DNA to contaminant DNA and identifies new source of human contamination (Krause et al 2010, Haak et al 2010, Kim et al 2011, Deguilloux et al 2011).

Applications of aDNA:

In recent years, the proliferation of studies on aDNA shows the great interest that this molecule may have. Investigation of ancient DNA helps to shed light on several historical events in various research fields.

aDNA and Zooarchaeology:

aDNA has been extensively used in the archeozoology field to resolve evolutionary, ecological and palaeogeographical questions. To this point, ancient mitochondrial DNA has been investigated to understand the evolution and history of Pleistocene saiga (*Saiga tatarica)* and to assess the level of genetic variation that has been lost during the last 50 000 years (Campos et al 2010). aDNA is a powerful tool to elucidate the biology and the evolution of extinct species such as the quagga: *Equus quagga* (Higuchi et al, 1984), the marsupial wolf: *Thylacinus cynocephalus*, the saber tooth tiger: *Smilodon fatalis* (Janczewski et al, 1992), the mammoth: *Mammuthus primigenus* (Höss et al, 1994), the moas of New Zealand (Cooper et al, 2001, Huynen et al 2010), the sparrow: *Xenipus longipes* (Cooper, 1994), the Ground Sloth: *Mylodon darwinii* (Höss et al,

1996), the cave bear: *Ursus spelaeus* (Bon et al 2008). In addition, aDNA has been used to better understand the animal domestication. It was shown that domestication of the horse was made from several different ancestral species (Jansen et al 2002) and that the domestication of the dog would have occurred 15,000 years ago (Dayton 2003).

The study of aDNA extracted from pig skeletons from the Neolithic period helped identify the geographical origin of pigs of Europe and to estimate the time of domestication of wild boars in Europe (Larson et al 2007). aDNA and Phylogeographic analysis of turkey (*Meleagris gallopavo)* bones and coprolites from archaeological sites (200 BC–AD 1800) in Southwestern United States revealed a unique domesticated breed originating from outside the region. This turkey rules out the South Mexican domestic turkey (*Meleagris gallopavo gallopavo*) as a progenitor. The low genetic diversity of turkey reflects intensive human selection and breeding. This study points to at least two occurrences of turkey domestication in precontact North America and highlights the intensity and sophistication of New World animal breeding practices (Speller et al 2010).

aDNA and paleobotany:

The study of ancient plant species has enabled the reconstruction of paleoenvironments and the comparison to current varieties to determine the time and place of domestication. In this context aDNA fragments were isolated from 4,700 years old *Zea mays* (Goloubinoff et al, 1993), wheat *Triticum spelta* (Brown et al, 1994) and from 5,300 years old herbs found on the "Iceman" Tyrol (Rollo et al, 1994). The domestication of maize took place 6,300 years ago in Mexico (Jeanick-Despres 2003).

aDNA and paleopathology:

The aDNA was very useful for understanding the origin and nature of diseases. The analysis of aDNA of individuals with congenital dislocation of the hip of the medieval population of Notre Dame du Bourg in Digne Les Bains in France, has shown that this disease, with higher rates in this population, is not due to a genetic (hereditary) factor but to mechanic factor (Kefi et al 2003, Mafart et al 2006).
The discovery of Mycobacterium tuberculosis in a 1,000 years old pre-Columbian mummy confirms the presence of tuberculosis in America before the arrival of Europeans (Salo et al 1994).

The same bacterium was also found in the remains of Egyptian individuals dated to 2,500 to 5,000 years ago (Zink et al 2001), in British medieval skeletons (Mays et al 2001) and in the remains of Hungarian individuals dated between 300 and 1,300 years (Haas et al 2000). The evolution of Mycobacterium tuberculosis and its interactions with its hosts has been made possible by the information provided by aDNA (Donoghue et al 2004.

The presence of *Yersinia pestis* has been shown in the teeth of 400 years old plague victims (Drancourt et al, 1998) and *Mycobacterium leprae* (agent of leprosy) has been found on the remains from archaeological German and Hungarian sites dated between 300 and 1,100 years (Haas et al, 2000). Finally, DNA from the roundworm *Ascaris lumbricoides* (intestinal parasite of humans) has been isolated from the coprolite of a prehistoric population of Brazil and Chile, dated from 8800 years (Lele et al 2008).

aDNA and forensics:

The study of ancient DNA has identified many undetermined remains such as victims of the Vietnam War (Holland et al, 1993), victims of criminal incidents (Hagelberg et al 1991, Bender et al , 2000), victims of air disasters (Olaisen et al 1997) and victims of natural disasters like Tsunamis (Deng et al 2005). Through aDNA analysis it was possible to trace the parents of abandoned newborns (Seo et al, 2000). Some historical mysteries were also unveiled thanks to aDNA analysis. It was possible to identify the remains of the Romanov family (the last tzars of Russia missing in 1918 and discovered in 1991) by analysis of STR (Short Tandel Repeats) and mitochondrial DNA (Gill et al, 1996). The analysis of mtDNA from the body of the dead child in the Temple in 1795 under the name of Louis XVII does not exclude his filiation to Queen Marie-Antoinette (Jehaes et al 2001).

aDNA and anthropology:

The application of aDNA has proved very useful in studying genetics of human populations making it possible to trace and reconstitute their history in some regions of the world. With aDNA analysis it was possible to uncover the genetic heritage of prehistoric man (the Iberomaurusian) from the Taforalt grotto in Morocco (13,000 years BP) consisting of a North African and a Eurasian component.

The absence of Sub-Saharan polymorphisms suggested that Taforalt individuals did not originate from sub-Saharan region. The presence of the Sub-Saharan component in the genetic structure of current North Africans populations (Chaabani et al. 1989, Cherni et al. 2005) would be due to the gene flow from the sub-Saharan region after 13,000 years BP (Kefi et al 2005). This agrees with a recent analysis of STR/Alu combination polymorphisms that suggest that the sub-Saharan component of current North Africans is rather an ancient original component, which could be traced back to the first stage of Neolithic (around 9,000 YBP) characterized by an ethnic contribution from present-day Sudan (El Moncer et al. 2010).

aDNA and phylogeographic analysis of a population of the earliest farming culture in Central Europe; the Linear Pottery Culture (from 5.500 to 4.900 Calibrated BC) allowed the understanding of the Neolithic transition (8,000–4,000 B.C.) in Europe which is a matter of continuous scientific debate in archaeology, anthropology and human population genetics (Haak et al 2010). One of the most remarkable uses of aDNA is its contribution to solving the puzzle of Neanderthal Man. Neanderthals lived in Europe and western Asia between 150,000 and 30,000 years. They had specific morphological characters (elongated skull, the presence of a supraorbital Taurus, high cranial capacity ...) that distinguish them from anatomically modern humans. The Neanderthals coexisted with anatomically modern humans before disappearing 30,000 years ago (Grimaud-Hervé, 2001). Many questions were raised regarding the role of Neanderthals in the evolution of modern humans. Is the Neanderthal man our ancestor? Or is he a separate species from ours? Had he contributed to our genetic heritage? Or had he disappeared without leaving a trace in our genome?

A comparison of mitochondrial DNA sequence of the Neanderthal with 994 modern human mitochondrial sequences from the five continents shows that the difference between modern man and Neanderthal man is greater than the intra-specific diversity of the human species today. Indeed, the average difference between two modern human sequences is 8 ± 3, while the difference between an actual human sequence and that of Neanderthal man was 25.6 ± 2. This difference is maintained even in cases where only the sequences of one continent are considered. The differences are in the range of 27.4 ± 1.8 to 28 ± 3.7 by continent. This proves that Europeans are no closer to Neanderthals than individuals from other continents (Krings et al 1997).

The phylogenetic tree constructed from mitochondrial DNA of modern humans (986 sequences), 16 chimpanzee sequences and the sequence of the Neanderthal suggests that this sequence occupies a distinct position in the group comprising all modern human sequences. The divergence of mitochondrial sequence of modern human from Neanderthal sequence would have occurred between 550,000 and 690,000 years BP which is 5 times older than the common ancestor of modern humans (120,000 to 150,000 BP). These results show that the Neanderthal is not an ancestor of modern humans which was confirmed by other studies analyzing aDNA extracted from other Neanderthal skeletons and comparing it with sequences of aDNA from first anatomically modern humans (dated to 30,000 years) and the sequences of modern humans (Caramelli et al 2003, Serre et al 2004, Briggs et al 2009).

Recently the use of high-throughput DNA sequencing technology allowed large-scale genome-wide sequencing of more than 4 billion nucleotides from three Neanderthal individuals. The comparisons of the resulting draft sequence of the Neanderthal genome to the genomes of five current humans (one San from Southern Africa, one Yoruba from West Africa, one Papua New Guinean, one Han Chinese and one Frenchman from Western Europe) showed that Neanderthals shared more genetic variants with present-day humans in Eurasia than with those in sub-Saharan Africa.

These results suggest that Neanderthal genome is likely to have had a role in the genetic ancestry of present-day humans outside of Africa. However this contribution was relatively minor given that only a few percent of the genomes of present-day people outside Africa are derived from Neanderthals (Green et al 2010).

In conclusion, the study of aDNA during the past twenty years offers a new avenue of research in different areas and providing answers to many questions. The development of more sophisticated techniques either for amplification or aDNA sequencing encourages researchers to launch new challenges such as determining the sequence of authentic ancient whole genome (Millar et al 2008, Mardis et al 2008, Green et al 2010). Very thorough studies can then be made to assess positive selection of genes over time and the evolution of biological mechanisms in relation to environmental changes.

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