ANCIENT DNA:

A MULTIFUNCTIONAL TOOL FOR RESOLVING ANTHROPOLOGICAL QUESTIONS

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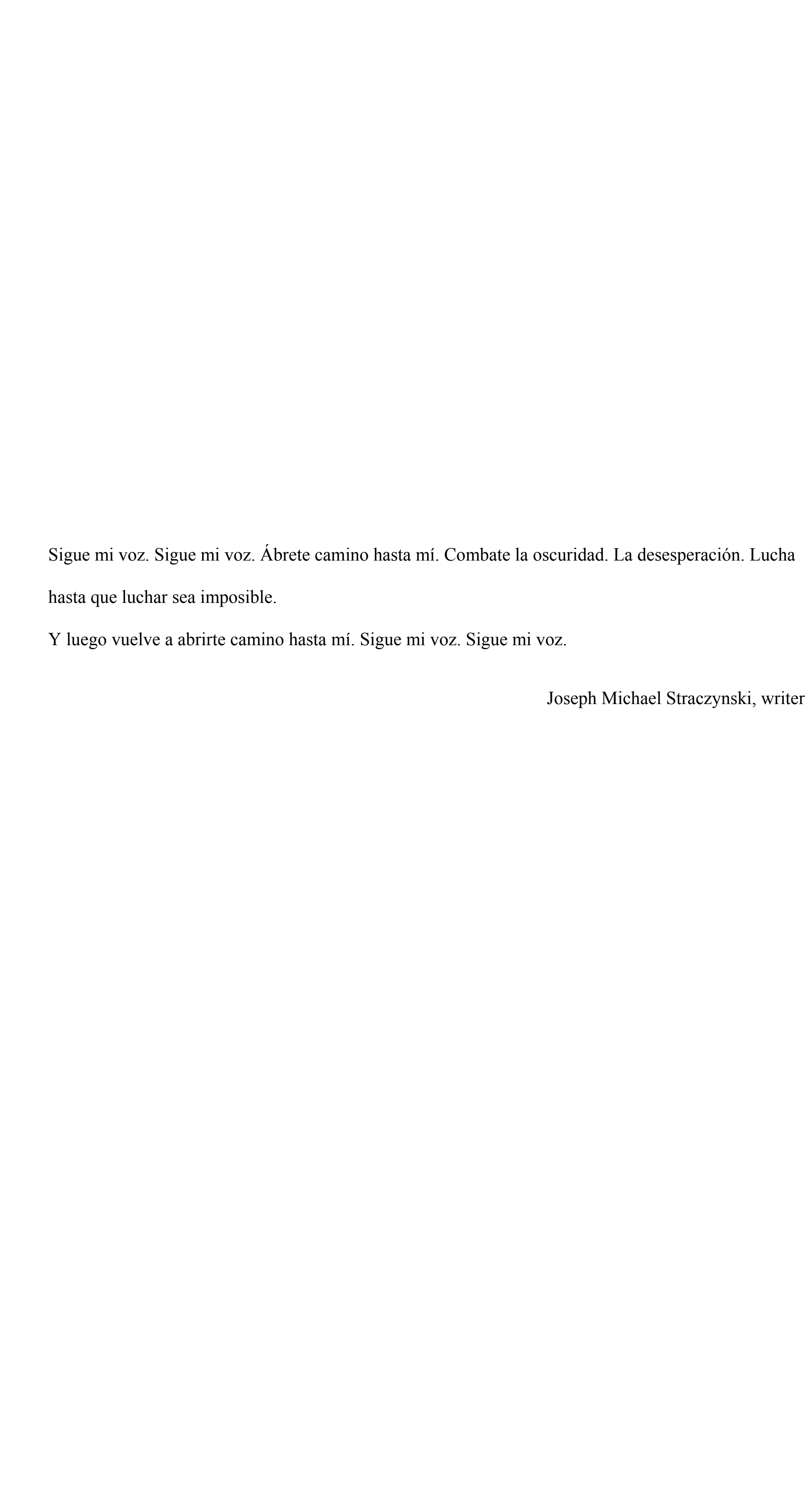

Sigue mi voz. Sigue mi voz. Ábrete camino hasta mí. Combate la oscuridad. La desesperación. Lucha hasta que luchar sea imposible.

Y luego vuelve a abrirte camino hasta mí. Sigue mi voz. Sigue mi voz.

Joseph Michael Straczynski, writer

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ABSTRACT

In the current thesis we have examined anthropological questions of ancient Catalonian and Balearic populations using DNA.

First, we have tried to improve our methodology applying a different protocol in the study of a site which had provided unexpected poor results. We have seen that under some circumstances the change of our protocol using phenol-clorophorm for the kit QIAamp DNA investigator which uses DNA affinity to silica particles is positive and delivers significantly better results, although it seems that the optimal method can vary at any given site and a consideration on a site by site basis should be made when deciding the most suitable protocol.

Next, we have aimed to go into the analysis of intrapopulational relationships in groups buried in close association to analyze the role of nuclear families in the antiquity. We examined a common burial cave from Catalonia in the Late Bronze Age, and proved that the hypothesis considering the individuals found in Catalonia's burial cave a nuclear family was erroneous. The high mtDNA variability inside the group and the fact that the only shared haplogroup (4 individuals) was uncommon in the region at that time, suggested the existence of a patrilocal mating system with the integration of foreign women and pointed to the kinship of some of the individuals. Our conclusion is that possibly the genetic ties were not the only determinant factor in close groups in the Late Bronze Age in contrast to the situation in current nuclear families in Western society, with cultural issues also playing an important role in what could possibly seen as an extended family structure.

Concerning ancient Balearic populations, we analyzed intra and interpopulational relationships. In general their frequency of haplogroup H was very important since at least the Iron Age, with the exception of one necropolis, Son Real, which has very particular characteristics. This fits with the high values from their contemporary populations from the Western Mediterranean as well as with the current ones, exception made of Current Minorca which may be influenced by the English colonization during the XVIIIth century.

Our results in the Minorcan necropolises proved the lack of sex bias in the interments and suggested different lifestyles between the populations living in the plain and those living in the rugged southern coast regarding inbreeding. In Majorca we proved a differential use of the necropolises. With the exception of Son Real, all the ancient Balearic necropolises seem to have an homogeneous European haplogroup pool so these differences evidence that the individual treatment of each necropolis makes sense as they all have their own uniqueness. The haplotipic analysis confirms that

they already belong to the European genetic variability and show a very similar genetic pool to the ancient Catalan population, reaffirming their already documented historic interactions, and rules out a direct relationship between the members of the Nuragic and the Talaiotic cultures regarding the feminine lineages.

Finally, we have made a first approach to the illnesses which accompanied these populations studying a widespread infection as caries. Studying the virulence factor dextranase of the cariogenic agent *Streptococcus mutans* we have been able to use direct data from the Bronze Age to the current era to propose that it has been evolving under neutral evolution at least since then and that a constraint of the selective pressure in this segment seems the most plausible explanation to understand its changes over time.

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1. INTRODUCTION

1.1. HISTORY OF ANCIENT DNA ANALYSIS

Since the first study that documented the retrieval of ancient DNA (aDNA) was published in 1980 (Pääbo 1986) -which was widely unknown as it was published in Chinese-, the recovery of mitochondrial DNA (mtDNA) from the extinct *Equus quagga* in 1984 (Higuchi et al. 1984) and of nuclear DNA (nuDNA) from a 2.400-year-old Egyptian mummy (Pääbo 1985), many advances have been made in paleogenetics. Owing to this, a huge amount of works that just used physical data have been now complemented with genetic analyses.

The most important step that propelled these improvements was the advent of the polymerase chain reaction (PCR) discovered by Mullis in 1983 (Mullis et al. 1986, Mullis and Faloona 1987). PCR could amplify preselected segments of DNA up to quantities which permitted direct sequencing, starting from extremely small amounts of DNA or even single molecules, also diminishing the time needed to retrieve useful information.

The first application of the PCR to ancient genetic material was carried out by Pääbo and Wilson in 1988. In this and the subsequent studies it was seen that for analysis of DNA from ancient samples, the application of this technique was an almost unavoidable prerequisite because of the low quantity of DNA extracted (Paabo et al. 1988, Pääbo 1989; Hagelberg et al. 1989; Lawlor et al. 1991). Moreover, PCR eliminated some of the problems caused by the low cloning efficiencies (Pääbo 1989). Using this technique, different groups were able to amplify and sequence DNA from soft tissues of natural or artificial human mummies as well as from animal species recently extinct (for example Thylacinus cynocephalus, Thomas et al. 1989).

However, these tissues represented very rare and geographically restricted remains whose conservation was strictly dependent on particular conditions. In addition, the fact that bones or teeth are the remains most currently found in archaeological contexts prompted scientists to study the presence of DNA in these samples. In this sense, three different studies came out in 1989 reporting the recovery of genetic material from bone (Hagelberg et al. 1989; Horai et al. 1989; Vargas 1989) encompassing antiquities ranging from 60 to 3.500 years BP. In 1990 Hanni et al. retrieved mtDNA fragments from teeth and bones of individuals ranging from 150 to 5.500 years BP. They amplified a

specific DNA fragment of 121 base pairs (bp) of human mtDNA which in the case of one bone of 150 years BP was also cloned and sequenced.

In 1991, an important milestone was achieved by Dr. Erika Hagelberg, who was responsible for the first forensic identification of a victim of murder accepted by an English court using a skeletal genetic analysis. In 1993 Kurosaki et al. used teeth to check for the first time the possible kinship of two sets of individuals from the 1st and 5th centuries AD using both mtDNA and short tandem repeats (STRs), proving that these markers could also be used to address this question in ancient remains.

As paleogeneticists realized that it was imperative to know under which conditions such experiments were optimized, different assays to check for the best conditions to carry them out appeared sporadically during those years. For instance, aiming to solve the subject of which kind of tissue was better for these type of studies, in 1994(a) Woodward et al. compared the ease of extraction, resulting quality of the DNA and ultimate reliability of data obtained from soft tissue and from teeth of ancient Egyptian mummies from a cemetery of the Greco-Roman period (200 BC-800 AD). They demonstrated that teeth provided the tissue of choice for recovering aDNA free of contaminating contemporary DNA and PCR inhibitors.

Likewise, in 1999, Burger et al. investigated teeth from 18 individuals from three archaeological sites of similar age (1st and 2nd millennium BC) and different diagenetic environments or different storing conditions to determine the effect of environmental factors on the preservation of DNA. In order to do so, they carried out the first multiplex approach on ancient specimens using several microsatellites and the sex-determining amelogenin gene, concluding that dryness, low temperature and absence of microorganisms favored the preservation of DNA.

While during the first years of the field mtDNA had been the molecule used because it has several characteristics that make it particularly amenable to genetic analysis, including maternal inheritance, absence of recombination and high mutation rate (Ramakrishnan and Hadly 2009), studies using this molecule have major drawbacks compared to analysis of the whole genome (Ballard and Whitlock 2004). First, mtDNA does not capture any information about the history of males (which may differ from that of females due to sex-biased demographic processes). More important, a study of a single locus has less statistical resolution for studies of history than do studies of the nuclear genome, as whole genome studies of an individual obtain information about hundreds of thousands of that individual's ancestors, not just those on a single lineage. It is thus important that the more advanced study designs of the mtDNA studies be combined with analysis of the more informative autosomal DNA.

On the other hand, not all kinds of DNA present in the cell are equally difficult to work with. Owing to its much higher copy per cell, mtDNA has been the first and most studied molecule in these kinds of samples. Although most aDNA studies rely on a somewhat ideal mitochondrial to nuclear genome ratio of 1.000:1 copies per cell (Zullo et al. 1993; Krings et al. 1997; Poinar et al. 2006; Kuch et al. 2007), being the typical range for different mammalian cell types from 110:1 to 860:1, there are no evidences that this ratio can be extrapolated to ancient tissues. Recently, Schwarz et al. in 2009

compared the copy numbers obtained for a 112 bp nuclear amplicon and for a similarly long one of the mitochondrial locus using both modern elephant and mammoth samples. The ratios derived from all mammoth samples were higher than the ones from the modern bone, suggesting a preferential preservation of mtDNA during diagenesis. This was the first empirical data indicating a preferential preservation of mtDNA in ancient samples, but further studies will be needed.

With regard to the impact caused by this new field focusing on ancient genomics in the scientific community, it should be acknowledged that during its first years it suffered from an excess of euphoria in relation to the information specialists thought could be obtained. This fact provoked a huge increase in the number of publications that supposedly recovered ancient genetic material from up to millions of years of antiquity (Sidow et al. 1991; Sykes 1993; Woodward et al. 1994(b); Henikoff 1995; Reese et al. 1996).

Unfortunately, though, things were proven to be not as easy as that (Wayne et al. 1994; Zischler et al. 1995; Mawk et al. 2002), and the difficulties to corroborate the authenticity of some of the works made some authors realize the problems inherent to working with aDNA. Consequently, they started proposing some criteria to follow in the methodology of the field and in the results obtained to be regarded as feasible (Handt et al. 1994; Richards et al. 1995; Austin et al. 1997).

Subsequent empirical and theoretical evidence placed the maximum survival time of DNA at under a million years (Lindahl 1993a, 1997; Smith et al. 2001; Willerslev et al. 2003) casting grave doubt on studies claiming the successful retrieval of “geologically ancient” or “antediluvian” DNA. Indeed, most or all of these sequences are now strongly suspected to be artefactual (Hebsgaard et al. 2005) and claims of geologically ancient DNA have become far less frequent in the XXIst century, although they continue appearing (Vreeland et al. 2006; Veiga-Crespo et al. 2007, 2008).

From the risk of contamination to the difficulty of the real recovery from the specimen own data, scientists are now fully aware that obtaining reliable results from aDNA is a tough question. Several papers specifying the criteria to accept the conditions that endorse the authenticity of these studies have continued being published (Cooper and Poinar 2000; Pääbo et al. 2004; Willerslev and Cooper 2005; Montiel et al. 2007) to try to guarantee their feasibility.

In spite of the difficulties, the possibilities opened by the emergence of this field were huge. Over the last twenty years this technology has been used for a wide range of purposes, from personal identification to human migrations and to study in depth the relationship with the Neanderthals.

Since the first forensic application of DNA fingerprints using highly polymorphic minisatellite loci in old samples (Gill et al. 1985) and the first successful identification of 8 year-old skeletal remains of a murder by bone DNA analysis (Hagelberg et al. 1991), the use of aDNA techniques started to be applied in the forensic context.

In 1995 Alt et al. pointed the necessity to use this information in archaeological contexts, as he considered it essential to confirm or refute hypotheses made by anthropologists about relations in ancient societies. In this sense, two years later, in 1997, Hummel and Herrmann followed the path started by Kurosaki et al. in 1993 providing the second proof of individual biological kinship at the

molecular level for prehistoric individuals in a burial where some of them were supposed to be genetically related.

In 1999, a Y chromosomal STR multiplex adapted to aDNA was presented by Schultes et al. making it possible for the first time to amplify STR loci of the Y chromosome from historical and prehistorical bones of up to 3.000 years old. From then on, multiple studies aiming to discern a possible kinship in ancient remains have been carried out (Scholz et al. 2001; Keyser-Tracqui et al. 2003; Gilbert et al. 2007a; Bouwman et al. 2008; Haak et al. 2008; Vanek et al. 2009; Simón et al. 2011; Baca et al. 2012; Deguilloux et al. 2014). Moreover, these studies have already been applied to deep into the relations of a Neanderthal population (Lalueza-Fox et al. 2011).

This application has also been used for personal identification. Regarding historical figures, it has been used to corroborate the documented data about their identity or to discern it whenever it was doubtful. For example, to disproof the alleged of a putative son of Marie-Antoniette (Jehaes et al. 1998) using mtDNA and to identify the remains of her true son Louis XVII (Jehaes et al. 2001), or to corroborate the Romanov family (Gill et al. 1994) and Nicholas Copernicus (Bogdanowicz et al. 2009) remains also using nuDNA information. In other cases like the one of the Italian poet and scholar Francesco Petrarca, the skull and the postcranial skeleton were confirmed to pertain to different individuals giving credibility to the historically documented profanation of his remains in 1.630 (Caramelli et al. 2007).

In relation to human migrations throughout our species history, aDNA has allowed the accession to the genetic make-up of populations living at archaeologically-known times and places, making it possible to directly track migrations and the population origins of their main actors (Pickrell and Reich 2014). Thus, the series of discoveries documenting the importance of mixture and migration in human history are increasingly coming from genetic analyses of ancient human remains (Skoglund et al. 2012; Raghavan et al. 2013), and many of the subjects that aDNA is beginning to shed light on are giving unexpected answers. For instance, focusing on the origin of the first people that arrived in America, the sequencing of DNA from two individuals who lived 24.000 and 17.000 years BP in the Lake Baikal region of Siberia showed that these ancient individuals are phylogenetically more closely related to present-day Native Americans than are present-day Siberians (Raghavan et al. 2013).

Another major debate in the last few decades among archaeologists and geneticists has been whether the arrival of agriculture in Europe involved the spread of people or an acculturation phenomenon. Ancient DNA studies have permitted to confirm that farming practices were brought by a group of people that were genetically distinct from resident hunter-gatherers (Haak et al. 2005, 2010; Bramanti et al. 2009; Brandt et al. 2013), and that gene flow between farmer and hunter-gatherer populations, possibly over a long period, eventually gave rise to the present pattern of genetic variation in Europe (Skoglund et al. 2012).

These successes in obtaining answers about two landmarks in human populational migrations set the stage to make more studies in the future without the necessity to use inferred data.

On a higher scale, aDNA is permitting to review our own history as a species, as aDNA studies have allowed us the possibility of recovering genetic material from *Homo neanderthalensis*, opening the possibility of knowing which genetic traits were specific of *Homo sapiens.* The first analysis of mtDNA from Neanderthals was published by Krings et al. in 1997 grinding up a small sample of bone from the first Neanderthal fossil discovered, in the Neander Valley, Germany. This and a subsequent study (Krings et al. 1999) proved that the Neanderthal mtDNA sequences were substantially different from modern human mtDNA. A second Neanderthal mtDNA sequence, announced in 2000, from a 29.000 year old Neanderthal found in Mezmaiskaya Cave, Russia (Ovchinnikov et al. 2000) proved to be similar to the former one, and both of them were distinct from those of modern humans, confirming a separation between the Neanderthal and modern human mitochondrial gene pools or with very low amounts of gene flow between them, at least between human males and Neanderthal females.

During the following years it was shown that mtDNA from anatomically modern *Homo sapiens* from Europe dating from the same time period as the Neanderthals (Caramelli et al. 2003) fit within the range of modern humans, while the Neanderthals remained consistently genetically distinct, showing that modern *Homo sapiens* and Neanderthals did not have more genetic similarities during the Pleistocene that were subsequently lost.

In 2005, with the advent of Next-generation sequencing (NGS) techniques (Margulies et al. 2005), a huge quality step was taken towards the retrieval of ancient genomes. By avoiding the capillary electrophoresis that limits the throughput of traditional Sanger sequencing, NGS platforms made it possible to perform tens of millions of sequence reactions per machine run, making it possible to generate whole eukaryote genome sequences in a few days (Kircher and Kelso 2010). This technology allowed confirming in 2008 that mitogenomes of *Homo sapiens* and *Homo neanderthalensis* had not mixed, when Green et al. reported the first complete mtDNA Neanderthal sequence from an individual dating from 38.000 years ago from Vindija Cave, Croatia. Finally, in 2009 the sequenciation of the entire mitochondrial genome of five Neanderthals proved that in spite of the wide geographic area covered by them, their mtDNA genomes were only one third as diverse as modern humans' (Briggs et al. 2009).

These achievements ran in parallel with the accomplishement of the retrieval of nuDNA. Two studies sequenced large amounts of Neanderthal nuDNA and their results were announced in 2006. Green and his collaborators announced the sequencing of one million bp of nuDNA of a Neanderthal specimen (Green et al. 2006) while the team lead by Noonan sequenced about 65.000 bp from the nuDNA of another specimen (Noonan et al. 2006), setting the stage to study nuDNA from this species. Finally, in 2010 Green's working group obtained a draft sequence of the Neanderthal genome (Green et al. 2010) that produced evidences consistent with an interbreeding between Neanderthals and anatomically modern *Homo sapiens* and pointed to aspects of the human genome that may have changed since the split between the two species.

Ancient DNA has also permitted to discover that in some places *Homo sapiens* genetic pool still holds the contribution of a previously unknown archaic population that was neither Neanderthal nor modern human and was present in Siberia less than 50.000 years ago (Krause et al. 2010; Reich et al. 2010). They were called Denisovans. In fact, there was gene flow from a population related to the Denisovans into the ancestors of present-day aboriginal people from New Guinea, Australia, and the Philippines (Reich et al. 2010, 2011; Meyer et al. 2012; Cooper and Stringer 2013; Prüferet al. 2014).

Apart from the extinct hominins, some other complete mitogenomes of extinct species have been obtained, such as the ones from moa (Cooper et al. 2001; Haddrath and Baker 2001), the woolly mammoth (Krause et al. 2006; Rogaev et al 2006), the mastodon (Rohland et al. 2007), the extinct cave bear (Bon et al. 2008; Krause et al. 2008, Stiller et al. 2009) and the woolly rhinoceros (Willerslev et al. 2009), as well as a draft sequence of the nuclear genome of the woolly mammoth (Miller et al. 2008).

Finally, a similar case of recovery of ancient genetic material could be obtained regarding the causing agents of ancient microbial infections (reviewed in Malgosa et al. 2005 and Anastasiou and Mitchell 2013). Among the most recent successes, it has been possible to shed light on the consequences that the evolving diet and behavior from the Stone Age to the modern day have had in human populations, using DNA preserved in calcified bacteria on the teeth of ancient skeletons (Adler et al. 2013). Moreover, it has been possible to suggest an European origin of leprosy in the Americas by reconstructing entire genome sequences of *Mycobacterium leprae* bacteria from five medieval skeletons (Verena et al. 2013), and to confirm that *Yersinia pestis* lineages that caused the Plague of Justinian and the Black Death were independent emergences from rodents into human beings (Wagner et al. 2014).

1.2 ANCIENT DNA CHARACTERISTICS AND POTENTIAL INTERACTIONS WITH ENVIRONMENTAL FACTORS

1.2.1 Ancient DNA damage

1.2.1.1 Decay of organic remains

The dynamics of the aged material is a necessary matter of study when aiming to work with aDNA. In brief, the onset of living beings decomposition is governed by a process called autolysis or self-digestion. As cells of the body are deprived of oxygen, carbon dioxide in the blood increases, pH decreases and wastes accumulate poisoning the cells. At the same time, unchecked cellular enzymes, such as lipases or proteases, begin to dissolve the cells from the inside out, eventually causing them to rupture, and releasing nutrient-rich fluids (Vass et al. 2001). After enough cells have ruptured, nutrient-rich fluids become available and the process of putrefaction can begin.

Putrefaction begins when bacterial enzymes cause the breakdown of tissues by breaking down proteins, carbohydrates and lipids into their basic components (amino acids, water and carbon dioxide, fatty acids and volatile substances) with gas formation (nitrogen, methane, etc.) causing the tissues to be digested to a fluid consistency, becoming moist and gas-ridden and liquefying down to the skeleton (Campobasso et al. 2001). At this point in the decay cycle both aerobic and anaerobic bacteria are present in large numbers, insect activity is very prominent and carnivores can contribute significantly to the decline of the corpse.

In some occasions, this process can be modified either by environmental or endogenous characteristics, which can provoke either the acceleration or the slowdown of the process. In the last case the cadaveric conservation phenomena appear, such as saponification, which inhibits bacterial putrefaction as a result of adipocere formation due to hydrolysis or hydrogenation from adipose tissues, and mummification, the characteristic ending result of a tissue that preserves its integrity when it undergoes desiccation or dehydration.

Finally, remains go through yet another complex process called diagenesis, which is any chemical, physical, or biological change undergone by organic remains from their initial deposition to their recovery.

1.2.1.2 Physical and chemical agents damaging DNA

In DNA molecules, the postmortem damage occurs (Figure 1) mainly as double-strand breaks and oxidative dinucleotide modification, both of which preventing subsequent enzymatic replication (Pääbo 1989; Lindahl 1993b; Höss et al. 1996), although minor sequence modifications, such as hydrolytic deamination and depurination, permit polymerase action and are manifested as limited amounts of base variation among sequenced clones (Krings et al. 1997). The action of alkylating agents can also alter the base composition to a variable degree of severity (Lindahl 1993b). Many studies have examined the prevalence of such postmortem damage in detail (Handt et al. 1996; Kolman and Tuross 2000; Hansen et al. 2001; Hofreiter et al. 2001; Gilbert et al. 2003a). Furthermore, some forms of postmortem damage, such as the so-called miscoding lesions characterized by Pääbo in 1989 can cause incorrect nucleotides to be incorporated during enzymatic amplification, resulting in amplified DNA sequences that are not a truthful copy of the original.

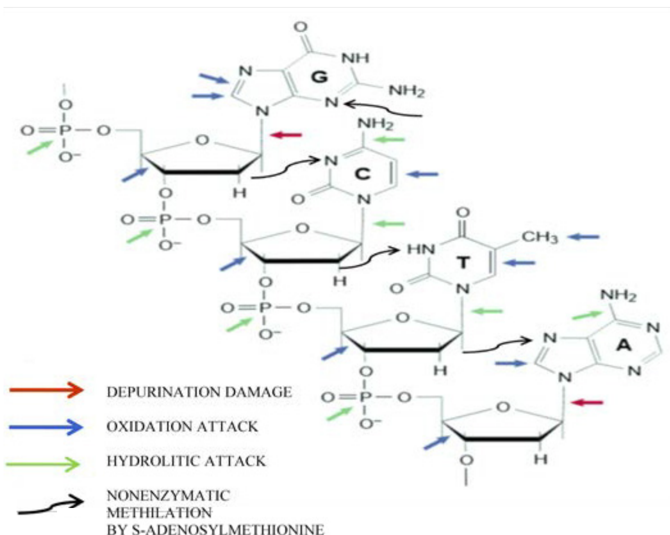

Figure 1. The principal sites of oxidative damage are marked with blue arrows, the green ones signal the principal points for hydrolitic damage, the red ones point the principal sites of depurination and the black signal methilation sites (modified from Hofreiter et al. 2001)

Another threat comes from free radicals. They are generated in cells as a by-product of respiration, by ionizing radiation or by the presence of water inside the body, interacting with the DNA molecules and modifying their composition. Moreover, water allows the presence of microorganisms that will use the cellular components as a resource to obtain energy (Teron AC 1990). A large variety of products are produced when DNA is attacked by these radicals (Cooke et al. 2003). For example, DNA can be attacked by some reactive oxygen species, such as peroxide radicals (O_2) and hydroxyl radicals (OH) as thymine glycol, shown in Figure 2, which can block replication.

Oxidation

Thymine Thymine glycol

Figure 2. Thymine glycol formation

While these damages can be produced by endogenous and exogenous factors, there are other ones among the exogenous that are in relation with the environment such as the climatology. There seems to be a consensus that for any given age, cold preserved samples are more likely to provide usable genetic material than those of a similar age that have been buried (or stored) at warmer temperatures (Smith et al. 2001, 2003). DNA preservation is best under cool, dry, anaerobic and slightly alkaline conditions (Bollongino et al. 2008). Moreover, for samples exposed to the sun, the ultraviolet radiation (UV) can chemically modify the nitrogenous bases that compose DNA's

backbone, specially by the formation of photodimeric lesions (Besaratinia et al. 2011) as well as causing the rupture of the molecule, either in a single strand or in both of them (Slieman and Nicholson 2000). Finally if the sample is in an acidic environment, the presence of H^+ cations will also increase the chance of a modification in the composition of DNA (Cano 1996).

1.2.1.3 Main types of damage

It has been demonstrated that the most common types of damage observed in aDNA are two complementary groups of transitions, termed "type 1" which covers the transitions A→G and T→C, grouped together in AT→GC as these substitutions are indistinguishable due to the complementarity of the sequence material, and "type 2" which covers the CG→TA transitions (Hansen et al. 2001). Each of these groups of transitions results from a single event, the deamination of adenine to hypoxanthine and of cytosine to uracil, respectively.

Owing to the advances in the knowledge in biochemical paths it is now possible to identify the originally damaged strand, increasing the number of detected jumping-PCR artifacts by up to 80% and providing a means to investigate the mode of DNA survival after death (Gilbert et al. 2003a, b). The first step in this sense was taken in 2001 when Hofreiter et al. demonstrated that the damage-driven modification of a G to an A analogue is highly unlikely, if not impossible. It could therefore be argued that any G→A transition that is observed on the L-strand due to damage must have originated as an H-strand C→T modification event. Conversely, any C→T transition observed on the L strand will reflect the genuine modification event.

In 2003(a) Gilbert et al. showed that damage-driven modification of a T→C analogue was also biochemically very unlikely. In this situation, any L-strand T→C modification will actually be due to an H-strand A→G event, whereas L-strand A→G events will be attributed to an original A→G damage event on the L strand (Figure 3).

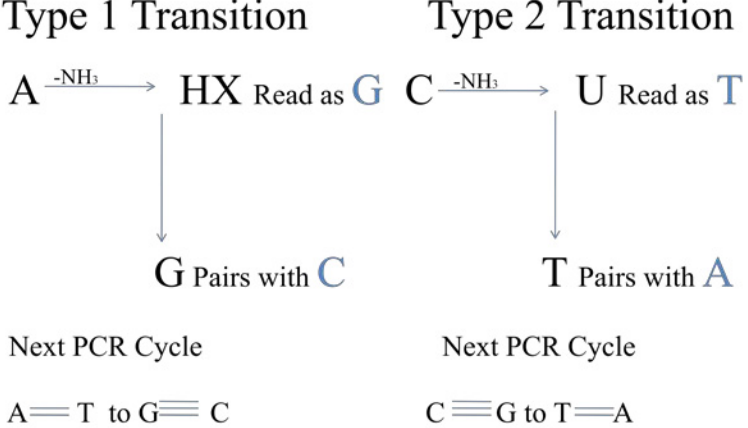

Figure 3. Type 1 and type 2 damage–induced transitions. Deamination of C→U (read as T) or A→HX (read as G). Changes introduced on the complementary strand when the damaged bases are subsequently copied are shown in blue. By convention, sequences are referred to in the L-strand orientation. Therefore, if an amplified sequence was initiated from an original H-strand template, then the type 1 and type 2 errors observed are expected to be T→C and G→A, respectively

To distinguish between postmortem damage and endogenous genetic material the simplest procedure appears to be a comparison of sequences before and after enzymatic treatment to reduce the number of templates with postmortem-damaged bases and the resulting possibility of misidentification. Different methods employ Uracil-N-Glycosilase (UNG) (Pääbo 1989; Hofreiter et al. 2001), Endonuclease IV (Endo IV) (Pääbo 1989), Alkyladenine DNA glycosilase (AAG) (Lau et al. 2000), or Pol I polymerase in conjunction with T4 ligase (Pusch et al. 1998; Di Bernardo et al. 2002). Among them the most widely used is the UNG treatment, which excises uracil caused by hydrolytic deamination of cytosine (Dinner et al. 2001). It reduces sequence artifacts caused by this common form of postmortem damage, resulting in an apparent C→T/G→A mutation (Pääbo 1989; Hofreiter et al. 2001), so after this treatment extracts containing multiple sources of DNA can be identified as those in which cloned sequences still either share no consensus sequence or retain sporadic C→T/G→A base changes that now may be recognized as being endogenous to the samples. The main drawback of this treatment is that it is expected to reduce the starting-template copy number, providing a check for authenticity but also limiting the number of samples suitable for study.

1.2.2 Inhibition

1.2.2.1 Nature of the inhibitors

The presence of inhibitory substances has also been a matter of concern in this field. Many substances have been identified as inhibitors from the PCR reaction, as they difficult or directly

prevent the possibility of the Taq polymerase interacting with the DNA material and carrying on the amplification (Fisher et al. 1993; Monteiro et al. 1997; Eckhart et al. 2000; Primorac 2004; Butler 2012).

The complex interaction of the biological, chemical, and physical taphonomic factors produces extensive variation within and among different burial sites. In fact, it has already been demonstrated that different parts of a single piece can bring radically different degrees of success (Lamers et al. 2008). This emphasizes the convenience of taking environmental samples when the sample of the study is recovered in the field, as it can shed light on the nature of some potential inhibitors which may difficult the recovery of the genetic material. These substances can be from very different origins, and ancient samples could display a single inhibitor or a combination of them (Montiel et al. 1997; Kemp et al. 2006; King et al. 2009).

Some of the most well-known ones are the Maillard products, which are the result of the reduction of sugars in an early stage with a compound bearing a free amino group (Pääbo 1989, 1990; Martins et al. 2001), and can block the PCR reaction due to the generation of cross-links in the DNA molecule (Pääbo 1989).

Other inhibitors include the humic substances (Montiel et al. 1997), coming from soil or water natural sediments (Tebbe 1993; Bourke 1999; Watson 2000) which can sometimes be easily detected as they may give a characteristic pigmentation to an extract (Stevenson 1982), tannic acid which seems to bind to both DNA and Taq (Opel 2010), calcium ions and chelating agents such as EDTA.

In the case of EDTA, though, its presence may have some advantages, because while it can inhibit the PCR reaction, it is useful in many DNA extraction methodologies to demineralize the bone or tooth permitting a higher DNA exposure and to inactivate nucleases that can provoke the degradation from the genetic material (Loreille et al. 2007). So equilibrium between its advantages and inconveniences must be found, a fact which is normally solved by the use of an adequate concentration of this substance in relation to each sample specific characteristics.

Finally, there can be residues of porphyrins or their breakdown products, which are present in many living tissues as blood and can be present in some cases in archaeological samples (Higuchi 1992). It has been postulated that they may not pass over the Centricon-30 membrane (Montiel 2003) because they tend to form aggregates with the nucleic acid molecules, and in a concentration of100 ng/ul are enough for complete PCR inhibition (Tuross 1994). Some of the main substances which can inhibit the PCR are shown in Figure 4.

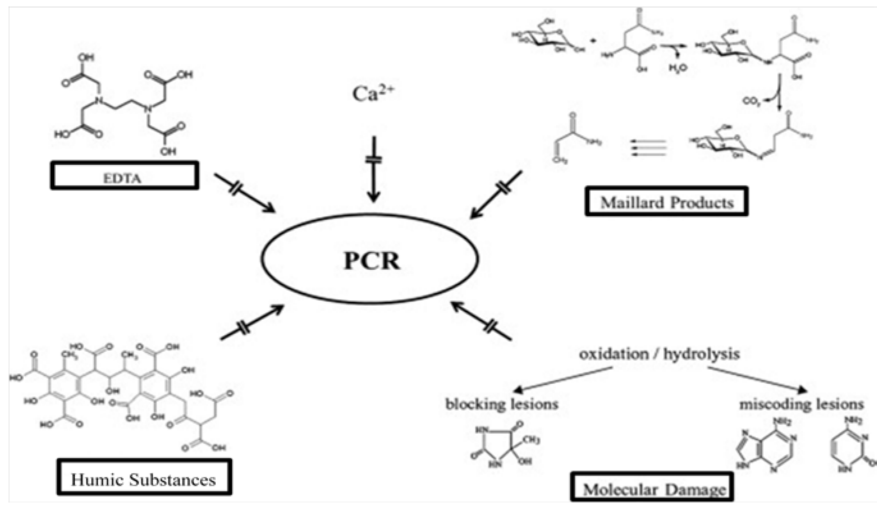

Figure 4. Some of PCR's main inhibitors (modified from Simón et al. 2012)

Other and more insidious PCR inhibitors are endogenous to the biological samples themselves and include calcium ions and collagen from bone (Scholz et al. 1998), blood components (billirubin, heme, immunoglobin, lactoferrin) (Akane et al. 1994), saliva, semen, cervical fluid, bile, feces (Monteiro et al. 1997; Butler 2012) or melanin and myoglobin from muscle tissue (Boom et al. 1990; Belec et al. 1998; Eckhart et al. 2000).

1.2.2.2 Avoiding inhibition

Some proceedings can eliminate or attenuate the inhibition, and can be carried out before the performance of the PCR in order to recover DNA with the best possible quality (Montiel et al. 1997; Montiel et al. 2001; Kermekchiev et al. 2003; Eilert et al. 2009). In fact, it has been shown that the widely used phenol–chlorophorm method allows the recovery of a higher DNA quantity (Rohland and Hofreiter 2007), but it can co-purify with some substances which can act as PCR inhibitors (Tsai et al. 1991; Tebbe 1993; Montiel et al. 1997) interfering in DNA amplification at several levels, leading to different degrees of attenuation and even to complete inhibition (Moreira 1998; Watson and Blackwell 2000; Alaeddini 2012). The methods to avoid this problem can be generally divided into two groups: (1) those that remove inhibitors during the DNA extraction and purification, and (2) those that diminish the effects of inhibitors by later manipulation of template DNA, PCR reagents, or by incorporating PCR additives.

In the first group, there has been a development of new protocols that enhance the purity of the recovered DNA: the silica based purification methods (Boom et al. 1990, modified by Höss and Pääbo 1993) in which the silica virtually binds to every molecule of DNA (Höss 1994). The silica-based membrane is designed to bind DNA fragments that are larger than 100 bp but smaller than 10 Kb, while excluding the rest of nucleotides, proteins and salts. It appears to exclude PCR inhibitors and the substances that give rise to the brownish pigmentation observed in bone extracts (Ye et al. 2004).

However, methods that remove inhibitors during extraction are associated with DNA loss, especially fragments <200bp in length, which poses a significant problem when working with degraded samples (Kemp et al. meeting 2011). In addition, some purification methods may achieve the desired end of removing environmental or endogenous PCR inhibitors while unintentionally incorporating new ones as a result of reagent carry-over (Boom et al. 1999; Bassetti 2007).

In the second group, if the inhibitory substances have not been successfully eliminated during the extraction process, there are modifications which can be made in order to increase the possibilities of a successful amplification. For instance, a DNA sample can be diluted, thereby diluting the present PCR inhibitors (Montiel et al. 1997). However, aged and badly damaged bones contain limited amounts of DNA template, so this option is often not feasible (Ye et al. 2004).

In addition, the use of "hot start" Taq DNA polymerase in order to alleviate the problem of primer-dimers (D'Aquila et al. 1991; Birch et al. 1996) has also been successfully applied. This type of DNA polymerases remains inactive until high temperature is reached, requiring a pre-PCR heat activation step (95ºC for 11 min). Hot-start procedures provide other benefits apart from the dramatic reduction in primer-dimer artifacts, including room-temperature reagent assembly, increased yield and better specificity. It has also proven to be the most efficient against co-extracted PCR inhibitors (Monroe et al. 2013).

1.2.3 Contamination

1.2.3.1 The importance of contamination in aDNA work

Contamination with exogenous material either modern or ancient is the main problem scientists of this area have to deal with. The degradation of the endogenous DNA results in a susceptibility for specimens to become contaminated with higher levels of contaminant DNA, whether derived from contact with living tissues containing similar DNA or from previously PCR-amplified DNA (Sampietro et al. 2006). Moreover, it has been suggested that as the younger contaminant DNA is likely to be less degraded than the endogenous DNA, it may be preferentially amplified in subsequent PCR analyses (Handt et al. 1994).

Furthermore, there have been some instances when it has been demonstrated that the analyzed specimen has been contaminated prior to preparation for genetic analysis (Pääbo et al. 2004; Gilbert et al. 2005a; Willerslev and Cooper 2005), and several authors have shown how even stringent controls can fail to prevent or detect contamination (Handt et al.1996; Kolman and Tuross 2000). Furthermore, the fact that the people who handle the samples may hold exactly the same sequences as the ones from the studied subjects renders such studies particularly subject to criticism (Gilbert et al. 2005b), even when additional biochemical preservation data or analysis of associated fauna support the veracity of results (Caramelli et al. 2003).

Even relatively recent contaminating DNA can imitate sequence damage and also permits jumping PCR between endogenous and contaminant strands (Pääbo et al. 1990; Sampietro et al. 2006), which will increase the apparent number of damaged sites in cloned sequences by introducing positions that differ between the contaminant and the authentic DNA (Hofreiter et al. 2003).

With regard to the accession to the samples of contaminant subsrances, the likely route is through direct handling and washing, presumably due to DNA derived from the handler permeating throughout dentinal tubules into the pulp cavity (in teeth) and the Haversian system (in bone) (Gilbert et al. 2005b), although possibly not permeating as far as the osteocytes (Malmström et al. 2005; Salamon et al. 2005).

1.2.3.2 Reporting contamination

Thus far prelaboratory contamination has been reported in a number of studies dealing with it: several authors have reported the presence of modern human DNA in samples that are not expected to naturally contain it, as archaeological and historical specimens of calcified tissues from pigs (Richards et al. 1995), cave bears (Hofreiter et al. 2001), foxes (Wandeler et al. 2003), dogs (Malmström et al. 2005) and Neanderthals (Krings et al. 1997; Serre et al. 2004; Lalueza-Fox et al. 2005).

In other situations, prelaboratory contaminant DNA sequences have been directly identified in human remains, normally through observations of the presence of multiple DNA haplotypes among cloned sequences from one sample or inconsistent results between samples from one skeleton (Handt et al. 1996; Kolman and Tuross 2000; Gilbert et al. 2005a; Gilbert et al. 2006). This kind of contamination in bones and teeth does appear to depend upon sample preservation and porosity, with better preserved samples being more resistant than less well-preserved ones (Gilbert et al. 2005b).

Although the problem posed by contaminant sequences has long been recognized in the field of aDNA, its full magnitude was revealed during the initial high-throughput sequencing (HTS) analyses of Neanderthal and woolly mammoth samples (Green et al. 2006; Noonan et al. 2006; Poinar et al. 2006). It was shown that only a small percentage of the DNA in temperate-preserved Neanderthal bone was endogenous (Green et al. 2006). An even lower level of 0.27% was found in a *Myotragus* specimen, which was younger than the Neanderthal bone but had been preserved under warmer conditions in the Balearic Islands (Ramírez et al. 2009). Even in a seemingly well-preserved, deep-frozen mammoth bone, only 45.4% of the DNA could be mapped to the elephant draft genome (Poinar et al. 2006). Furthermore, recent studies on poorly preserved Neanderthal remains, where PCR amplification of conserved human genetic markers enables co-amplification of contaminant and authentic DNA, revealed that modern human mtDNA makes up a very large proportion of the DNA, and several studies report endogenous Neanderthal sequences constituting only around 5% of the total sequences retrieved (Serre et al. 2004; Lalueza-Fox et al. 2005).

For its part, other studies attempting to ‘‘freshly’’ contaminate human bones and teeth excavated many years before have noted that contamination seems to be most problematic in the

period immediately after excavation (Gilbert et al. 2005a; Gilbert et al. 2006). In 2006, Sampietro et al. demonstrated that the expected contaminants derived from those people who were involved in the initial washing and cleaning of the remains had a higher frequency than the expected frequency of contaminant DNA sequences derived from the other participants, supporting hypotheses from previous studies focusing on contamination that samples are most susceptible to suffer it when initially excavated and washed (Gilbert et al. 2005b; Gilbert et al. 2006). They also showed that contaminant sequences can undergo observable levels of miscoding lesion damage posthandling, being the damage level found among old (approximately 10 years old) sequences at levels indistinguishable from those in the believed endogenous DNA sequences, casting doubts on a commonly used argument for data authenticity, the accumulation of postmortem damage over time.

1.2.3.3 Decontamination methodologies

As of now, a universally accepted method to get rid of contamination has not been obtained although several methodologies have been tried (Watt 2005; Butler 2010; Kemp and Smith 2010; Champlot et al. 2010; Barta et al. 2013 among others). Moreover, most common decontamination methods are not efficient enough to decontaminate short DNA fragments of low concentration (Champlot et al. 2010).

Among the most widely used treatments before carrying out the extraction of a sample, it is believed that submersion of bone and tooth samples in bleach prior to it helps insure that the previous handling of ancient remains will not influence the results (Kemp and Smith 2005; Barta 2013), and it has also been suggested that longer exposure times to bleach may have the added benefit of minimizing co-extraction of PCR inhibitors (Watt 2005).

With regard to water, there are still discrepancies on whether washing the samples with it to clean the dirt can be a source of contamination (Sampietro et al. 2006). Some data seem to confirm previous hypotheses that washing the specimens is a critical step for contaminating the samples (Gilbert et al. 2006), and defend the necessity to exclude any sample washing, or if washing cannot be avoided, the need to do it with sterile water under controlled conditions. In 2008 Bollongino et al. defended that it can be the source of non-removable contamination.

The exposition of the samples to UV rays is also problematic because, as harmful for the contaminant material UV can be, it can also damage the endogenous genetic material, and while breaking the chains that compose DNA molecules, it is not the elimination but the reduction of the fragment lengths what it finally achieves. So its use continues to be a matter of controversy (Watt 2005).

It has even been proven that individuals preparing reagents and/or making and packaging lab ware pose a particular threat to the possibility of recovering authentic degraded DNA profiles. In fact, some cases of contamination have been found to have occured this way (Himmerlreich 2009; Jenkins et al. 2012; Barta et al. 2013), making it necessary to proceed with extreme caution when preparing

any material used in an aDNA laboratory and typing all the individuals involved in its preparation.

So, whatever the steps taken to avoid it, it should be noted that complete decontamination is not always possible (Barta 2014) and, thus, results from any aDNA study should be closely scrutinized. Maintaining high standards within the laboratory is compulsory, and many forensic DNA researchers and others working with aDNA samples have raised many papers about the points that must be accomplished to obtain reliable results, from Cooper and Poinar in 2000 to Gilbert et al. in 2005(b), Montiel et al. in 2007, Butler et al. in 2010 or Kemp and Smith in 2010.

1.3 AUTHENTICITY CRITERIA

1.3.1. Initial rules

As previously exposed, the difficulty of aDNA studies went hand in hand with the need of guaranteeing the feasibility of the obtained results. In 1989 Pääbo was the first to publish criteria of authenticity, which he focused on three points: testing of control extracts in parallel with extracts from old specimens to detect contamination introduced during the extraction procedure; preparing more than one extract from each specimen; and corroborating the existence of an inverse correlation between amplification efficiency and size of the amplified product, reflecting the degradation and damage in the aDNA template. However, serious problems of authenticity in this kind of works persisted: in 1993(a) Lindahl et al. already noted that some of the claims of very ancient DNA recovery were incompatible with the known biochemical properties of DNA (Golenberg EM et al. 1990). They could not be considered reliable and warned about the problem posed by trace amounts of contaminant DNA accidentally derived from laboratory glassware or the experimenter, and recommended the publication of both positive and negative results, the reproducibility of results, the use of negative controls and the chemical analysis of the samples to check the integrity of the biomolecules. Soon after Lindahl's reflections, Handt et al. published in 1994 the first set of integrated criteria which needed to be fulfilled before a sequence could be claimed as ancient. It comprised the following six points: the strict physical separation of the laboratory areas where the ancient samples were processed; specially dedicated laboratory clothing in order to avoid contamination carried by the researchers and accurate cleaning of the work areas with 5% sodium hypoclorite and UV radiation; routine monitoring of contamination -two extraction controls and one PCR control-; at least two extractions per sample performed at different moments and preferably from different parts of the sample, with the obligation to report incongruent results, if any, in the publication where the study is presented; consistency with the phylogenetic criterion and finally, the existence of an inverse relationship between amplification efficiency and the molecular length of the amplified fragment. In 1997, Béraud-Colomb et al. added more precautions, including the sterilization of all buffers by both autoclave and filtration, the use of dedicated pipettes sterilized by UV radiation and the use of aerosol-resistant pipette tips.

In spite of these trials, the fact that among the posterior studies that did not accomplish these demanded requirements there were some notorious ones that claimed to have recovered DNA from insects trapped in amber (Walden and Robertson 1997; Gutiérrez and Marín 1998) or from dinosaur bones (Allard et al. 1995; Hedges and Schweitzer 1995; Henikoff 1995; Zischler et al. 1995) reveals that not all the scientists in this field were convinced about the utility of these suggestions. In the end, however, the continuing accumulation of evidences regarding the difficulty to obtain feasible results and the different kinds of problems which continued to appear prompted the need to establish a definitive consensus made by specialists on which studies could be considered feasible, causing this subject to be pushed in the beginning of the XXIst century. The proposals went from the establishment of nine comprehensive and very stringent criteria (Cooper and Poinar 2000), since then considered the standard ones regarding authenticity, to the approaches suggesting that they should not be set in stone (Pääbo et al. 2004; Gilbert et al. 2005b; Montiel et al. 2007), allowing the investigators to adapt to the particularities of the samples studied and to apply the logic that each case might require.

One of the first studies that thoroughly applied the advice given in 2000 by Cooper and Poinar was the one of Di Benedetto et al. in the same year, but the accomplishment of these requisites represented such a great amount of effort that the authors concluded that sample sizes for human studies would remain small.

Moreover, it was observed that not just the source material used, but also some of the applied amplification strategies, could influence the outcome of an aDNA sequencing attempt in an adverse way. For instance, nested-PCR, in comparison to direct PCR, was more vulnerable to background noise and phantom mutations, possibly introduced by low-fidelity Taq polymerase in the first round of PCR (Grzybowski et al. 2003). In addition, the excess of PCR cycles and the handling of amplification products during the procedure introduced an increased risk of contamination (Brandstätter and Parson 2003). The addition of these two factors made a nested-PCR assay without independent replication in another laboratory bear a high risk of producing artefacts (García-Bour et al. 2004).

Another problem was that low copy number of partly preserved mtDNA also entailed an elevated risk of propagating sequencing artefacts due to suboptimal sequencing biochemistry and reading software. This actually constitutes not only a particular challenge to aDNA, but also remains a problem for modern mtDNA studies (Stenico et al. 1996; Di Benedetto et al. 2001; Bandelt et al. 2002; Vernesi et al. 2001, 2002).

While the rigidity proposed for the measures in Cooper's and Poinar's paper looked correct, they could not always be accomplished following the generic rules proposed in them as some authors argued later (Bandelt et al. 2005; Montiel et al. 2007) but had a high historical relevance. The main points they suggested to authenticate an aDNA study may be summed up in:

Sterility: all the instrumental required must be sterile, the gloves must be dispensable, a mask, hair coffin and uniform have to be worn, and the physical isolation of the laboratory where the treatment and extraction procedures will be carried out is compulsory. The laboratory should also be positively pressurized, and whenever possible the pieces used should present their full integrity.

Finally, it is essential to handle specimens, perform DNA extractions, and set up amplifications in dedicated laboratory facilities where no post-PCR work has ever been conducted (Pääbo 1990).

Controls of each step: all the steps carried out must have a control that checks for the absence of contamination. The processes that are carried out before the laboratory work should also be monitored and recorded, and the investigators that had entered in contact with the sample should be genetically characterized. In this sense it has been possible to track down the intralaboratory contamination on an aDNA study (Sampietro et al. 2006), setting a landmark for the future in this kind of studies.

Biochemical preservation: postmortem changes to tissues cause racemization of the L-amino acids (the only ones incorporated in protein synthesis) resulting in a mixture of L and D enantiomers. The rate of racemization started being used for dating purposes some decades ago (Hare and Mitterer 1967; Hare and Abelson 1968) and has been applied to geological dating in natural systems since then (Bada 1985, 1991). In 1996, Poinar et al. showed its utility in aDNA studies when proving that there was a relationship between the extent of amino acid racemization and the successful retrieval of authentic DNA from an ancient specimen. They demonstrated that higher values of D/L in an amino acid known to have a lower degradation rate than others were a sign of contamination by exogenous amino acids. Furthermore, later studies showed that the combination of total amount of amino acids, the frequency of each one and their extent of racemization was a useful approximation for DNA preservation in bones and teeth (Poinar et al. 1996; Krings et al. 1997; Serre 2004).

Appropriate molecular behavior: this point was first suggested by Pääbo et al. in 1988 as a valid authenticity criterion because it was observed that the size of the recovered fragments descended in frequency as the length of the amplicon increased. In fact, in 2011 Adler et al. showed that while the underlying kinetics of DNA degradation are complex enough to difficult accurately modelling it, the result is a reciprocal relationship between the size and number of remaining template molecules (Poinar et al. 2003, 2006; Deagle et al. 2006; Ottoni et al. 2009; Schwarz et al. 2009) (Figure 5).

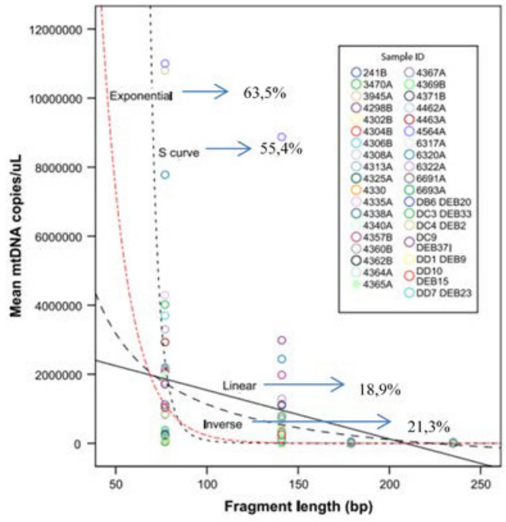

Figure 5. Percentages of variation explained under each model proposed for DNA degradation are shown (modified from Adler et al. 2011)

However, many problems have appeared with regard to the utility of this criterion for distinguishing between endogenous and contaminant DNA. For example, in DNA extracts of the El Sidron Neanderthal, mtDNA fragments retrieved that are known to be contamination because they carry nucleotide substitutions typical of current humans are as short as the endogenous Neanderthal mtDNA, and in the Vindija Neanderthal some contaminating fragments are longer whereas others are shorter (Briggs et al. 2009). Thus, it seems that fragment length *per se* is not a reliable estimator of contamination because in many cases, contaminating DNA may be both degraded (such as in skin fragments in dust particles) and deaminated (for example, when it has been deposited on a fossil or has been present in chemical reagents for a long time). Therefore, similar to fragment size, nucleotide misincorporations cannot easily be used to estimate contamination as endogenous and contaminant molecules may have similar characteristics with respect to this issue (Green et al. 2009).

Cloning: the damage that genetic material of dead corpses suffers and the possibility of a contamination, make the cloning of the sequences relevant. Having one sequence each time permits to see if some of the clones carry the expected kind of lesions under this aged material and, when some ambiguous position in a direct PCR is shown, it helps to clarify which one is the nucleotide originally present and which one is a point mutation caused by postmortem damage. Alternatively it can reflect either a true heteroplasmy or that there is more than one represented molecule with haplogroup defining mutations, thus indicating a contamination process with two or more sequences present from different sources.

Reproducibility and independent replication: it is advisable to independently replicate the results in both the same and different laboratories, and with both the same and different extracts from the same sample. The general rule is that the obtained results must be able to be replicated in case that there is enough sample to do so.

Quantitation: the copy number of the DNA target should be assessed using competitive PCR (Handt et al. 1996; Krings et al. 1997), being aware that 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. Also, the risk of nucleotide misincorporations will be higher if amplifications start from single molecules and DNA sequences are determined from a single amplification, provoking that any consistent misincorporation results in an incorrect base being determined (Handt et al. 1996; Pääbo et al. 2004).

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.

However, the debate about this subject was far from ending. In 2004 Pääbo et al. suggested that human aDNA analysis is only feasible in distinct and isolated populations but not in historic populations that are too similar to recent ones. Moreover, they warned about the need of being able to exclude the nuclear insertions of mtDNA and stated that the reproduction in a second laboratory was not warranted in each and every study, but rather when novel or unexpected results were obtained.

They also realized that a rigid adherence to each and every criterion in every case was not warranted because all sources of errors do not occur in all studies, and fulfillment of all the criteria cannot be taken as proof that a DNA sequence is genuinely ancient. There were examples where a specimen was contaminated with a certain DNA sequence and all the criteria, including repetition in second laboratory, were fulfilled but the result was still invalid (Hofreiter et al. 2001; Serre et al. 2004). Thus, the focus should be put on the scientific judgment of the reliability of results, which would be more of a necessity in the study of aDNA than in many other areas of genetics (Pääbo et al. 2004).

1.3.2 Phylogenetic and populational meaning: Refining the criteria

Despite these standard criteria were widely accepted some problems still remained, and in 2005 Bandelt and his collaborators added some new criteria to the nine proposed by Cooper and Poinar, suggesting that the targeted mtDNA fragments of a regional aDNA study should a priori be different from all of the potential mtDNA of the broad archaeological and laboratorial context when attempting to amplify ancient mtDNA, as most contamination would not be different from the expected authentic molecule. They also suggested the consideration of three indicators that could be used a posteriori: a) the principle of "phylogenetic expectation" (Richards et al. 1995) by which if the putative ancient mtDNA reflected mtDNA lineages of the human staff in contact with the samples rather than mtDNA lineages expected in the geographic area of the ancient population, contamination would likely have over-run any authentic DNA (Kolman and Tuross 2000); b) the mosaic structure, which argued that if the putative ancient mtDNA haplotype was composed by separate fragments that fit with modern mtDNA lineages from different branches of the mtDNA phylogeny, while showing an odd combination that did not come close to any point in it, the haplotype would most likely constitute some artificial recombinant (Bandelt et al. 2004); and c) the abnormal mutational spectrum, upon which if an agglomeration of unusual mutations was scattered across the mtDNA data deemed to be ancient, then postmortem changes and phantom mutations would have transformed the potentially authentic mtDNA to a degree that the resulting sequences would be virtually useless (Bandelt et al. 2005). Finally, in this work they criticized the data of García-Bour et al. in 2004 because they contained some haplotypes with clear phylogeographic inconsistencies, the data of Caramelli et al. 2003 and Vernesi et al. 2001 and 2004 because some of the sequences presented seemed to arise from recombination among cross-contaminants, and in the latter they also presented an abnormal mutational spectrum. Although some of these criticisms were the matter for later discussion (for example in Barbujani and Bertorelle 2003), Bandelt's work exemplified how a detailed analysis of data in the light of an edited worldwide database might be useful in authenticating ancient human sequences. On a final note, also with the aim of continuing to increase the feasibility of the works in this field, in 2007 Montiel et al. advised against the excessive clonation of the samples, since it might be expensive and generate more problems than it solves due to the increased risk of cross-contamination.

1.3.3 Decontamination: could it be achieved?

In 2005, Willerslev and Cooper highlighted the ubiquity of human contamination in bone and teeth stating that such contamination was impossible to clean despite extensive treatment with UV radiation and bleach, basing their arguments on unpublished results by M.T.P. Gilbert. However, some studies showing the feasibility of decontaminating ancient human samples (Kemp and Smith 2005; Salamon et al. 2005; Malmström et al. 2007) argued in favor of the real possibility of recovering authentic ancient human DNA. These two authors encouraged fulfilling the standard criteria proposed by Cooper and Poinar in 2000 and further proposed some additional ones, like a time-dependent pattern of damage and diversity, decontamination of reagents and specimens and uracil-N-glycosylase (UNG) treatment to eliminate some postmortem damage.

Soon after that, Gilbert et al. in 2006 and Montiel et al. in 2007 published two papers which relaxed to a certain degree the stringency of the criteria according to the nature and characteristics of the samples and gave more weight to scientific judgment of the reliability of results. Furthermore, they concurred with the assessment that all sources of errors do not occur in all studies so it was not strictly necessary to adhere to each and every criterion in every case (Pääbo et al. 2004; Gilbert et al. 2005b). Instead of planning or assessing studies by using criteria as checklists, Gilbert's paper suggested that consideration was given on a case-by-case basis as to whether the evidence presented is strong enough to satisfy authenticity criteria, given the specific problems of each case, thus placing the responsibility on authors to self-assess their work in light of the problems inherent to the field, adding a "tenth commandment" to the original nine points: "Thou shalt interpret the veracity of the data by a critical consideration of all available information." (Gilbert et al. 2005b).

Since then, some steps have been taken towards actualizing the criteria, which have been mainly focused on distinguishing between true and contaminant DNA molecules. In 2006 Bouwman et al. analyzed the spatial distribution of contaminant DNA in ancient bones, proposing that it presented different distribution in relation to authentic aDNA, so in the near future this could also be used to authenticate ancient sequences. Moreover, if aDNA is being protected from the chemical treatments to which the samples may be submitted by its association with hydroxiapatite crystal (Bouwman et al. 2006), it will be interesting to investigate whether this association provides protection to DNA from other treatments, like incubation with DNases, as it has already been proven that the binding of DNA to mineral surfaces may protect it against DNase I degradation (Lorenz and Wackernagel 1987; Romanowski et al. 1991; Álvarez et al. 1998). Decontamination with DNase I could be far more effective than chemical or UV light decontamination and may completely reduce any non-protected DNA segment to free nucleotides.

One of the main aspects not mentioned in the standard criteria was the necessity to know the probability of having undetected contamination and of introducing specific sequences. Before the arrival of NGS technology, this value might be estimated from the number of contamination events detected *a posteriori*, while the probability of introducing specific sequences could be estimated if one

knew the probable contaminants that could derive from people handling and analyzing the samples. Furthermore, if many studies like that of Sampietro et al. in 2006 were done the specialists would obtain prior information on this probability that would improve the models for general application. Finally, a mixture of different molecules may also be detected at the level of the whole mitochondrial or Y chromosome genomes by analyzing linked markers.

1.3.4 The arrival of High-Throughput Sequencing: new considerations

A turning point that prompted the revision of the authenticity criteria was the advent of the HTS technology (Margulies et al. 2005; Bentley et al. 2008). For example, it became clear that reproduction of results in the same or different laboratories as a prerequisite for publication was not practicable for large-scale DNA sequencing of random molecules because of constraints on time, costs, and sample materials.

The proof that contamination would continue being a problem with this technology came early on, when one of the first works using NGS for aDNA (Green et al. 2006) was proven to be affected by widespread contamination with larger-sized modern human DNA fragments and a high sequencing error rate by Wall and Kim (2007). They found that in Green et al. (2006) the post-mortem DNA damage, which often causes the deamination of cytosine to uracil, resulting in apparent C→T or G→A mutations, made up a significantly lower fraction of the Neanderthal-specific mutations in their work than the one from the same material studied by Noonan et al. (2006), suggesting that some other process (besides postmortem damage to Neanderthal DNA) was leading to the ‘‘Neanderthal-specific mutations’’ in the Green et al. data, pointing towards human contamination. This confirmed that contamination was introduced into this dataset during library construction outside the clean room, so a new criterion of authenticity should be the building of the library inside it.

NGS meant a revolution because of the knowledge it brought about aDNA diagenesis, and moreover it could be used to authenticate endogenous sequences. Thanks to this new technology, sequence patterns resulting from aDNA damage have been identified that help distinguish damaged ancient sequences from modern contaminants, because it has been observed that extensive degradation processes alter the base composition of aDNA sequence data through biased sequence substitution and fragmentation (Star et al. 2014). Specifically, fragmentation bias has been demonstrated through a relative increase of purines at the positions immediately preceding the 5’ termini (Briggs et al. 2007; Meyer et al. 2012; Overballe-Petersen et al. 2012) in aDNA sequences, while enhanced cytosine deamination occurs in single stranded 5’-overhangs that lead to C-to-T substitutions at 5’-ends (Briggs et al. 2007). The complementary G-to-A substitutions at 3’-ends that come with this latter phenomenon have been proven to be artefacts of the library preparation (Briggs et al. 2007; Green et al. 2009; Krause et al. 2010; Ginolhac et al. 2011). More features that come as a result of the next generation library creation are the higher GC content for fragments of shorter length (Green et al. 2008; Briggs et al. 2009; Meyer et al. 2010) possibly due to denaturation of short AT-rich fragments

during library preparation (Star et al. 2014), different polymerases affecting template length and GC content (Dabney et al. 2012), bias against fragments starting with thymine residues when using AT-overhang ligation protocols (Seguin-Orlando et al. 2013), hairpin loop formation of single-stranded DNA (ssDNA) that allows the proliferation of 3'-end terminal palindromes during the library creation (Star et al. 2014), and the absence of the artefactual complementary G-to-A substitutions at 3'- ends when using protocols targeting ssDNA (Orlando et al. 2011; Meyer et al. 2012; Jonsson et al. 2013). It is noteworthy that these protocols can also result in improved yield relative to background contaminant sequences (Orlando et al. 2011; Meyer et al. 2012; Gansauge and Meyer 2013) indicating that the widely used protocols for double stranded DNA (dsDNA) suffer from an inherent inefficiency during library creation (Star et al. 2014).

Finally, two advantages that enhance the recovery of authentic sequences when creating these libraries are given by the possibility of tagging them in clean labs with project-specific adaptors that allow tracking back downstream contamination sources that could result from library amplification and/or sequencing (Briggs et al. 2007), and generating such large amounts of DNA sequence that even very rare types of misincorporations can be detected.

As NGS techniques have come almost simultaneously with the possibility of genotyping extinct species, establishing a consensus about the authenticity criteria in these works has become as relevant as when Cooper and Poinar proposed their nine standard criteria in 2000. Thus far some suggestions have been:

1. In order to correctly estimate contamination, a two-phase approach has been suggested for sequencing ancient genomes, much as was done for the Neanderthal mitochondrial genome (Green et al. 2008, 2009) (Figure 6). Once a genome is determined to high coverage, the fixed positions showing differences between the extant and the extinct species shall be used to generate estimates of DNA contamination in sequencing of independent libraries from the same individual, such as in Briggs et al. 2009. It has to be considered that in these studies short DNA fragments will appear to carry derived alleles in the extinct species more rarely than long fragments because short fragments are more often lost in the analysis, resulting in an overestimate of contamination rates as calculated by Wall and Kim (2007), so this has to be taken into account for not biasing the results.

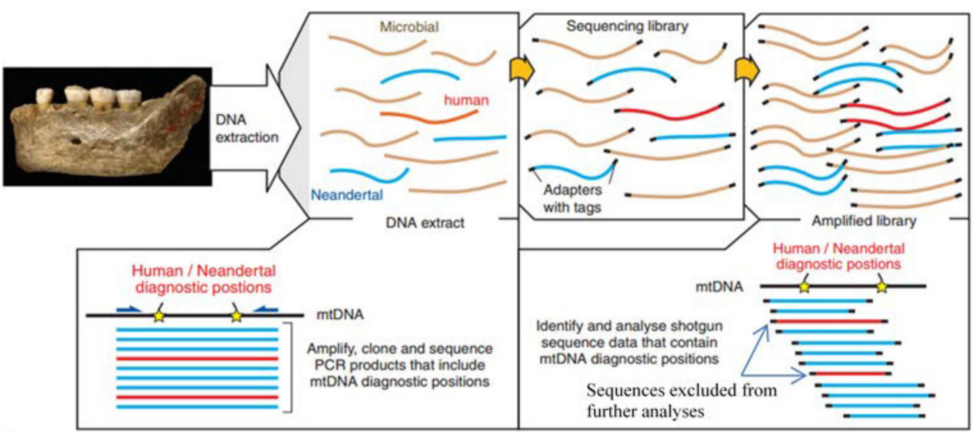

Figure 6. Process of amplification and distinction between Human and Neanderthal sequences (modified from Green et al. 2009)

2. Positions in which the fragment sequenced carries T or A residues and one of the two species carries C or G residues respectively, shall be excluded from the analyses because these may be caused by deaminated C residues in the extracted DNA (Hofreiter et al. 2001). In addition, when working with 454 sequencing insertions, the positions in which two or more of the same base exist in one of the species are excluded because homopolymer length is difficult to score by this method (Green et al. 2009).

3. Unless misincorporations occur, the number of any particular nucleotide differences between the extinct and the extant species should equal the number of reciprocal strand-equivalent differences, where the extinct species' sequence carries the complementary nucleotide. So this shall be used to detect nucleotide misincorporations. Moreover, reciprocal nucleotide differences should be equal in number if the rate and patterns of nucleotide substitutions along the evolutionary lineages leading to the extinct and the extant species were similar. Thus, any difference in number between pairs of such reciprocal differences would indicate either that the pattern of substitutions changed in one species (a fairly unlikely event when closely related species are studied) or that nucleotide misincorporations contribute to one of the two types of differences (Stiller et al. 2006) (Figure 7).

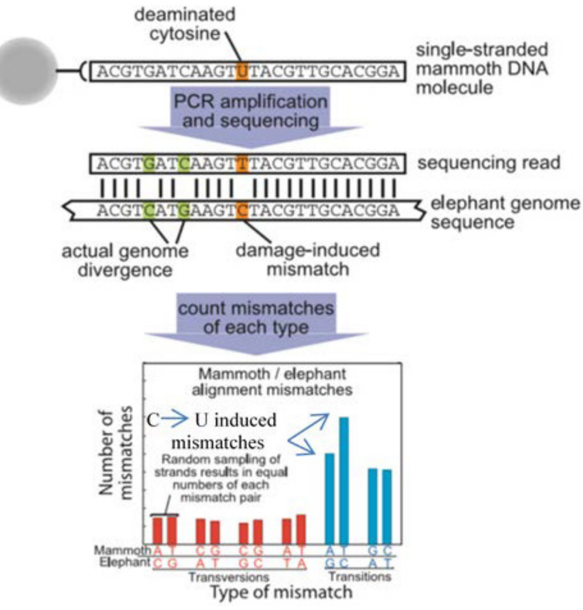

Figure 7. Identification of real differences between mammoths and elephants. Real differences between them are marked in red, whereas differences owing to nucleotide misincorporations are marked in blue (modified from Stiller et al. 2006)

1.4 TISSUES FROM WHERE THE DNA CAN BE EXTRACTED AND THEIR CHARACTERISTICS

There are many factors when considering the most adequate material to work with, encompassing from chronological age to storage conditions, depositional environments, piece integrity, burial depth, surrounding pH or water saturation (Allentoft et al. 2012), so a general formula to predict a priori DNA preservation is not plausible. Teeth have traditionally ranked in the first position among the tissues used as source material in paleogenetic studies, followed by bone material.

Teeth are considered the toughest components of the human body and have the highest resistance to most environmental effects like desiccation and decomposition (Patidar et al. 2010). In addition, their location within the jawbones largely protects them from the environmental and physical conditions that act to accelerate the processes of postmortem decomposition and DNA decay (Schwartz and Schwartz 1991; Álvarez García et al. 1996) while reducing the chance of contamination. It is also known that their mineral component preserves human DNA by the physical exclusion of microbes and contaminants (Milos et al. 2007) and enhances its preservation by adsorption to hydroxyapatite (HAP) (Lindahl 1993b; Okazaki et al. 2001). Adding all of these factors to HAP constituting a larger proportion of the overall material in teeth than in bones, the possibilities of successful recovery are usually higher in the former than in the latter, as confirmed in some works when DNA extracted from teeth was proven to be of higher quality (Alonso et al. 2001; Ricaut et al.

2005) and where the amounts of DNA yielded by teeth were higher (Kurosaki et al. 1993; Oota et al. 1995). Teeth are composed by different tissues (enamel, dentine and cementum) and regions (the root(s) and the crown) and DNA is not uniformly distributed among them (Higgins and Austin 2013) (Figure 8). The roots, which are composed of cementum, dentine and pulp, have been shown to yield more DNA than the crown which is predominantly composed of enamel (Dobberstein et al. 2008; Higgins et al. 2011), that is acellular and contains no DNA. Enamel is the hardest tissue in the skeleton and is composed of elongate crystals of HAP larger than the ones in bone (Carlson 1990) and shows a low porosity that makes it denser and less soluble than bone tissue, as well as less prone to contamination with modern DNA (Gilbert et al. 2005b; Sampietro et al. 2006). The dentine/pulp complex makes up the bulk of the tooth, with pulp being a richly vascularized connective tissue that contains numerous cell types, providing the richest source of DNA in teeth (Malaver and Yunis 2003), and dentine lacking nucleated cell bodies but being perforated by parallel tubules containing mtDNA-rich odontoblastic cell processes and nerve fibres (Mornstad et al. 1999). Finally, cementum covers the roots of teeth and is composed of (by weight) 45–50% inorganic mineral (HAP), collagen and non-collagenous matrix protein and is classified into two types based on the presence or absence of cells (cementocytes, which are a valuable source of DNA (Avery and Chiego 2006)).

Studies comparing DNA content among different tooth types have shown that teeth with the largest pulp volume provide the best source of DNA due to the presence of more pulp cells, and that more DNA is retrieved from multi-rooted teeth than from single-rooted teeth (De Leo et al. 2000; Rubio et al. 2009) most likely owing to the larger pulp volume and the increase in root surface area providing more cementum. These facts make pulp and cementum be the most valuable sources of nuDNA in the tooth and both these tissues and dentine good sources of mtDNA (Higgins and Austin 2013).

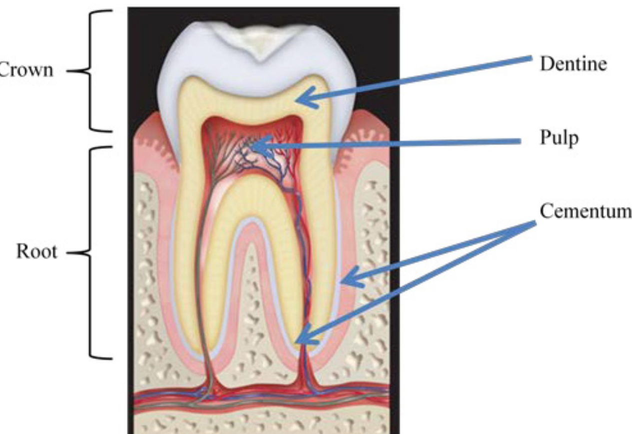

Figure 8. Inner and outer structure from teeth (modified from Matthew Chansky portfolio). Tissues from where DNA is obtained are marked with blue arrows

Bone is macroscopically composed of two main architectures. At the jointed ends of long bones and in flat sheet-like bones such as the sternum and skull vault, it comprises an outer layer of compact bone (cortical bone) that surrounds a load-bearing network of intersecting planes and buttresses called trabeculae (spongy bone), while the mid-shafts of long bones principally hollow tubes of cortical bone (Currey 2002).

Microscopically, bone consists of a hard, apparently homogeneous intercellular material, within or upon which there are a number of characteristic cell types including either active or inactive osteoblasts, their osteoprogenitors, osteocytes and osteoclasts. Osteoblasts are bone cells responsible for bone formation that secrete osteoid, a protein mixture that subsequently mineralises with carbonated HAP to become the rigid, load-bearing solid that is bone mineral and produce hormones that have a role in the mineralisation of bone (Ortner and Turner-Walker 2003). Osteocytes are cells that originate when osteoblasts become trapped within the matrix they produce, occupying spaces in the bone known as lacunae. Osteoclasts are responsible for bone resorption (Nijweide et al. 1986; Ortner and Turner-Walker 2003). These cells are interconnected inside a network of pores represented by the Haversian and Volkmann canals which also carries blood vessels, nerves and that also hold small canaliculi (Figure 9).

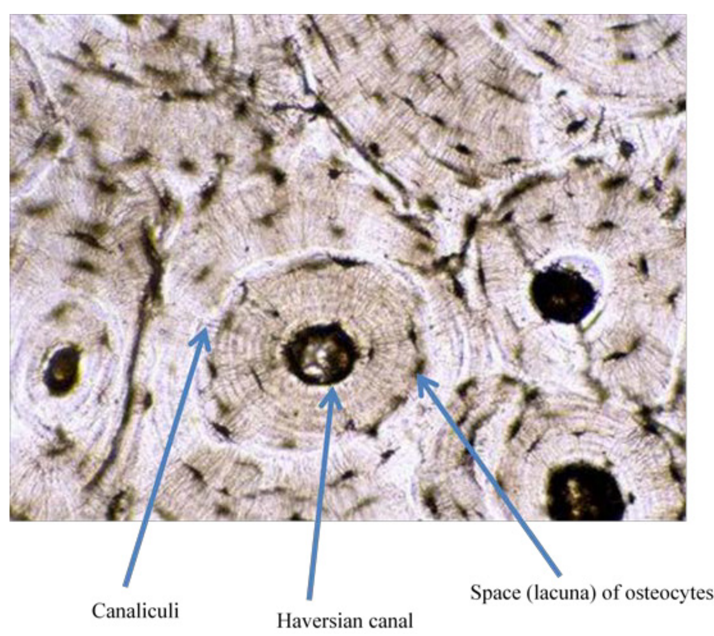

Figure 9. Inner structure from bone (modified from pixshark.com)

Dried, fully mature bone tissues comprise approximately 46% collagen, 46% HAP and 8% water by volume (Todoh et al. 2009) and show a porous structure which can facilitate the presence of high levels of contaminants in some extracts (Gilbert et al. 2005b; Green et al. 2006, 2008). The bone matrix, which constitutes the majority of bone, consists of both organic and inorganic fraction. The inorganic fraction is composed of cryptocrystalline carbonated HAP to which DNA may adsorb (Lindahl 1993b) and the organic fraction is composed principally of Type I collagen as well as various

non-collagenous proteins and glycoproteins (Tuross 2003). Tropocollagen molecules self aggregate extra-cellularly into fibrils (fibrillogenesis), with its molecules interdigitating in such a way that there are gap zones and overlap zones where the molecules are well aligned and more closely packed. The initial mineralisation of collagen takes place in the gap zone and progresses along the fibrils, and full mineralisation is accomplished by the replacement of water between fibrils by mineral (Campos et al. 2012) (Figure 10).

Focusing in the possible locations of the genetic material, in the first place DNA can be adsorbed by the HAP and influence crystal growth as previously explained (Lindahl 1993b; Okazaki et al. 2001). It can survive within bone bioapatite crystals (Salamon et al. 2005), presuming that this adsorption or encapsulation takes place in vivo during the growth and remodelling of bone, and it can also be relatively well conserved in intergrown crystal aggregates within fossil bones, where also remnants of collagen and probably other proteins are preserved (DeNiro and Weiner 1988). In vitro experiments and theoretical models (Kitamura et al. 1997; Mrevlishvili and Svintradze 2005) suggest that nuDNA not only binds to collagen but can act as a scaffold or matrix in the collagen fibrillogenesis, so short fragments of either nuDNA or mtDNA can become trapped in aggregating or mineralising fibrils. Furthermore, the gap zones may also be sites where smaller DNA fragments can bound to collagen molecules, being later encapsulated within HAP crystallites as mineralisation proceeds (Campos et al. 2012) which would fit with the sizes of aDNA fragments usually recovered from ancient bone (60–150 bp) (Prüfer et al. 2010) equivalent to 22–54 nm (Figure 10). Moreover, during bone resorption and its new formation, large amounts of mtDNA are released into the forming osteoid matrix following the apoptosis of osteoclasts or osteoblasts, becoming available to bind to the outer surfaces of collagen fibrils in the mineralising osteoid or to the surfaces of developing HAP crystallites. Finally there is also the potential release of tissue decomposition products, including DNA and collagen fragments released by chemical and/or microbial degradation of unmineralised osteoid.

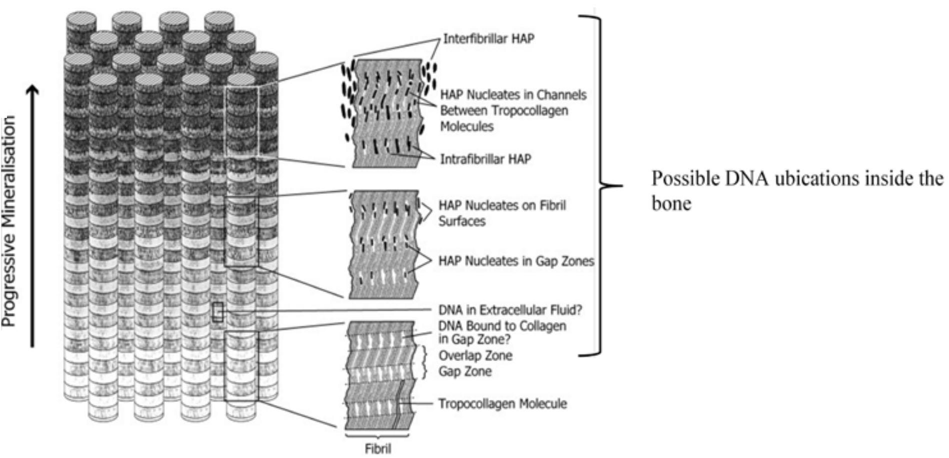

Figure 10. Tropocollagen structure (modified from Campos et al. 2012)

In accordance to previous studies (Schwarz et al. 2009 and Ottoni et al. 2009), recent data (Campos et al. in 2012) have suggested that the HAP component is at least, or even more, important than the organic component for DNA recovery. For the majority of ancient samples the proportion of mtDNA in each of the extraction fractions was sufficiently high (ratios of between 1:4 and 1:1) that the discarding of either one of the fractions would lead to a loss of a considerable proportion of the total mtDNA. Ottoni et al. (2009) also observed that DNA preservation in archaeological bone is not related to the presence of intact collagen fibrils, so damaged collagen fibrils do not necessarily mean that DNA is poorly preserved. As in teeth, the results showed that larger and better preserved fragments were obtained by using crystal aggregates within fossil bones compared with those from whole bone powder, which inhibited the PCR by 5 orders of magnitude more. Studies using the oxidant NaOCl to clean the surfaces of macroscopic pieces of bone (Kolman and Tuross 2000; Yang et al. 2003; Gilbert et al. 2005a) have proven that the possibility of obtaining well preserved DNA assuming that the intergrown crystal aggregates serve as protection was correct for some fossil bones. In addition this strong oxidant substance removes modern contaminants as well as many of the inhibitors that interfere with the PCR. Thus, reproducible sequences of well preserved DNA from a NaOCl-treated sample can be used as a reliable criterion for authenticity (Salamon et al. 2005) and to maximize DNA recovery from both mineral and organic fractions.

Apart from these two widely used hard tissues, there are occasionally other kinds of tissues available for study such as keratinous tissues like hair and nail (Gilbert et al. 2007a, b; Willerslev et al. 2009) as well as horn, feather and scales in vertebrates. These tissues are derived from living progenitor cells that undergo cell-death during their biogenesis, implying that the DNA present is not just at low level but heavily fragmented (Forslind and Swanbeck 1966; Olsen et al. 2012). As they undergo natural desiccation during keratinization, DNA survival may be prolonged due to the lack of free water in the keratinized cells, as suggested by the low numbers of miscoding lesions observed among sequenced clones and within second-generation sequencing data (Gilbert et al. 2004, 2007b).

With regard to hair, its metabolically active region is the “root” while the dead, fully keratinized component is the “shaft”. Over the past 2 decades scientists have demonstrated that, within limits, PCR amplifiable mtDNA and nuDNA can be retrieved from almost all sources of “fresh” hair, including the one coming from the human head, eyebrow, pubis and torso (Baker et al. 2001). The metabolically active state of the root allows recovering high quality DNA from it, while in the hair shaft the keratinocytes which ultimately compose it are dead cells, leading to the catabolic breakdown of cell organelles and nucleic acids. Molecular damage caused by the keratinisation process likely has significant consequences for DNA survival and partly explains why DNA analyses on hair shaft invariably report the recovery of much poorer quality fragments than those on root (Higuchi et al. 1988). However, recent microscopic imaging of hair shafts have shown that nuclear and mitochondrial remnants can be observed between macrofibrils within the keratinized hair medial layers that form the majority of the shaft (cortex), which makes it probably the most important structure with regard to DNA content (Bengtsson et al. 2012). Consequently, while in the beginning most hair-based genetic

studies had used roots instead of shafts as a DNA source (Pilkington et al. 1987), later ones started reporting shafts as a viable source of modern (Higuchi et al. 1988) and ancient (Gilbert et al. 2004) mtDNA. One of the advantages provided by hair is that it tends to come in large quantities, allowing sampling to be performed with less destructive methods than with tooth or bone. Moreover, hair shafts are resistant to contamination from exogenous DNA sources such as bacteria, blood or skin cells, easier to decontaminate than bone (Jehaes et al. 1998; Gilbert et al. 2004, 2006, 2007b) and have an apparently lower DNA rate of damage (Gilbert et al. 2004, 2007b). In 2007(b) Gilbert et al. finally proved that hair shafts surpassed comparably stored bone (Poinar et al. 2006) as aDNA source for use in terms of preservation and concentration of mtDNA relative to nuDNA. A further general observation is that mtDNA appears to survive in better condition than nuDNA in hair as confirmed through analyses of the length distributions of identifiable mtDNA and nuDNA generated through second-generation sequencing (Gilbert et al. 2008a; Rasmussen et al. 2010). While this may be due to the larger number of copies of mtDNA in each cell or the high energetic demands in hair shafts increasing the number of mitochondria per cell (Robin and Wong 1988), Linch and Prahlow suggested in 2001 that the smaller mitochondrial membranes might remain relatively intact during the process of hair shaft formation, conferring some protection on the mtDNA molecules located inside, whereas the nuDNA may be more exposed to endogenous endonucleases during nuclear fragmentation and lysis.

The advent of NGS has largely overcome the problem regarding the length of the fragments, provoking that hair shafts receive renewed interest as a source for HTS and recently ancient mitogenomes of taxa including mammoths, humans, thylacines and extinct and extant rhinos (Gilbert et al. 2007b, 2008a,b; Miller et al. 2009; Willerslev et al. 2009) have been obtained, culminating in 2010 in the publication of the first high-quality ancient nuclear genome of an approximately 4.500 year old extinct “Saqqaq” Greenlander (Rasmussen et al. 2010). A drawback to consider is potential mtDNA heteroplasmy (Sekiguchi et al. 2004 and Tully et al. 2004), maybe owing to either the enrichment of keratinocyte mitochondrial numbers through donation from adjacent melanocytes (Linch and Prahlow 2001), as errors can come associated with the rapid rate of mitochondrial replication in the hair root, or simply as an effect of the large mitochondrial population.

Regarding nail, while sharing its composition of keratinized cells with hair and also permitting the recovery of mtDNA and nuDNA in fresh samples (Tahir and Watson 1995; Cline et al. 2003), it provides a lower obtainment of DNA. In a comparison between both tissues, Willerslev et al. (2009) obtained a lower yield of endogenous mtDNA from century-old toenail samples from black and Javan rhinoceroses compared with those from the hair shaft of an ancient woolly rhinoceros, although the small sample size recommends more studies before drawing a final conclusion.

Preserved ancient fecal matter, or coprolites, can also be a rich source of molecular data (Poinar et al. 1998; Hofreiter et al. 2003) and aDNA sequences have already been retrieved from coprolites preserved in desert caves and rodent burrows (Küch et al. 2002; Poinar et al. 2003), where characteristically dry conditions are thought to be critical for the molecule's long term survival (Lindahl 1993a). They can also be a source of keratinous tissues because many mammals orally groom

themselves, their offspring, and conspecifics using the tongue and teeth, and moreover the carnivores may intake large amounts of hair from mammalian preys. In both cases, hair can be excreted in organism's feces, together with a keratinous casing making fecal hairs exceptionally durable and resilient on a macroscopic and molecular level (Gilbert et al. 2004). In fact, recent studies evaluating modern feces and fecal hairs as a source of mitochondrial and microsatellite DNA for South China tigers found that hairs sampled from feces are actually a more reliable source of DNA than the bulk fecal matter itself (Zhang et al. 2009; Clack et al. 2012). Finally, a drawback is that coprolites are a genetically heterogeneous set of materials, containing a diversity of processed food matter along with the defecator's own sloughed tissue (Poinar et al. 1998, 2001). Thus, without initial sorting of visually identifiable constituents, a final extract is likely to be a mixture of various sequence templates, making them especially adequate for species-specific shotgun sequencing.

The rest of aDNA samples from animal origin come from tissues which are more unlikely to be recovered but that can be preserved under exceptional conditions (Prats-Muñoz et al. 2013). In this sense, the mummification process (whether artificial or carried out by human action) helps the overall preservation of organic samples and raises the possibility of finding such tissues (De Marinis and Brillante 1998). Mummification can occur under a wide range of desiccating or anoxic conditions, but only mummification by desiccation generally provides high quality DNA. Notable DNA from human mummified tissues from samples with favorable conditions for DNA preservation have been recovered, such as the ones from two Upper Palaeolithic *Homo sapiens* from Italy (Caramelli et al. 2008), a Mesolithic individual from Cheddar in England (Sykes et al. 2001) or the whole genome of the Ötzi mummy, a 5.300 year-old Copper Age individual (Keller et al. 2012). However, there is not a consensus on whether true endogenous DNA can be recovered from ancient Egyptian mummies. In 2005(c) Gilbert et al. casted doubts about the authenticity of works dealing with them, predicting that the thermal history of most, if not all, ancient Egyptian material argued against the recovery of endogenous DNA from them. However, new studies reporting results from this kind of material have continued being published (Hawass et al. 2010, 2012; Khairat et al. 2013). Recently, a study that could shed light on this controversy was done applying molecular methodologies to the investigation of postmortem alterations in a human experimental mummification setting (Shved et al. 2014). A lower limb was desiccated in a container of natron salt, simulating the desiccation phase of ancient Egyptian mummification, and the evolution of skin and muscle tissue was compared taking samples at multiple time points over a period of 322 days. Analyses showed that slight postmortem DNA degradation was present in both muscle and skin tissues, pointing to most aDNA degradation occurring either during putrefaction or in the long-term exposure to environmental conditions, and showing that salt desiccation is an effective method for medium to long-term preservation of human tissues at the molecular level (Shved et al. 2014). However, later time points during mummification will have to be analyzed in order to determine the threshold beyond which DNA fragmentation prevents DNA recovery.

Regarding bacterial DNA, the studies are highly appreciated as they constitute the basis of molecular paleopathology. Up to the arrival of techniques using aDNA, the studies from ancient illnesses had been focused on the appearance and prevalence of pathological lesions in human skeletal remains, an approach which had many limitations (Wood et al. 1992) because it was restricted to the individuals with diseases causing pathognomonic lesions. This field has been largely driven to the analysis of three organisms, *Yersinia pestis* (Bos et al. 2011), *Mycobacterium leprae* (Suzuki et al. 2010), and *Mycobacterium tuberculosis* (Donoghue 2011), for which pertinent samples are easily identifiable through historical records or pathological markers. While bacteria such as Mycobacteria are considered to be particularly suitable targets for aDNA research because of their protective waxy, hydrophobic and lipid-rich cell wall (Zink et al. 2002; Donoghue et al. 2004), the work by Donoghue et al. in 2013 focusing on ancient metagenomics proved that, under the right conditions, sequences can be recovered from organisms with more fragile cell envelopes at least hundreds of years postmortem. So for example, in spite of the difficulty in obtaining ancient treponemal DNA as a result of the fragility of the bacterium (von Hunnius et al. 2007) a recent work showed that, in particular cases such as neonates with congenital syphilis, it could be recovered as the number of spirochetes in the bones was high enough to allow its preservation (Montiel et al. 2012).

In recent years, shotgun metagenomics has been a powerful new tool for paleogenetics to shed light to the emergence, evolution, and spread of microbial pathogens, using contemporaneous, historic and even prehistoric strains (Whatmore 2014). The HTS technologies like microarray-based hybridization capture have permitted to unveil the full bacterial genome of the causative agent of the medieval Black Death epidemic (Schuenemann et al. 2011; Bos et al. 2012) as well as of historical leprosy strains (Schuenemann et al. 2013).

Finally, a comparative study done by Willerslev et al. in 2004 to know which bacterial species preserved their genetic material the best demonstrated that the DNA degradation rates were close to theoretical calculations (Hofreiter et al. 2001), indicating a limit of approximately 400 thousand years. This fact contradicted claims of the obtainment of multi-Ma (million years) DNA sequences or of the recovery of putative viable cells of endospores and Proteobacteria from amber and halite (Cano and Borucki 1995; Vreeland et al. 2000; Fish et al. 2002) as well as from bacteria from many Ma old permafrost samples (Shi et al. 1997). In this study sequences of non-sporeforming gram-positive (GP) Actinobacteria consistently persisted for the longest time, followed by GP endospore-forming Bacillaceae and Clostridiaceae, and finally gram-negative (GN) bacteria, mostly Proteobacteria. While the superior persistence of GP over GN bacterial DNA was in agreement with in vitro based predictions, the superior persistence of DNA from nonspore-forming-GP Actinobacteria was unexpected because endospores are generally regarded as the hardiest of all cell types, but the lack of metabolic activity or active repair of their DNA seems to be the reason for the results obtained.

Focusing on DNA from plant remains, many small-scale plant aDNA studies have been undertaken (Jaenicke-Després et al. 2003; Palmer et al. 2009; Kistler et al. 2014 among others) and recently HTS has been started to be used in the analysis of this type of remains (Ávila-Arcos et al.

2011; Bunning et al. 2012; Palmer et al. 2012). Among the problems to overcome when dealing with these kinds of samples there are the possible presence of polysaccharides and polyphenols like tannins, which have already been found in modern plant materials and can pose significant problems for the extraction of nucleic acids (Japelaghi et al. 2011). In addition, archaeological plant materials are often rich in humic acids and despite the possibilities of current genotypation technologies, assembling complete plant genomes is a challenge even for modern samples owing to their large, highly repetitive and heterozygous genomes, confounded by varying ploidy-levels (Wales et al. 2014).

In spite of these problems, recent successes include the obtainment of RNA preserved in some ancient seeds, presenting an opportunity to directly test evolutionary changes in gene expression at a key developmental stage (Fordyce et al. 2013), and the genomic characterization of ancient genomes of the plant pathogen *Phytophthora infestans*, the oomycete responsible for the Irish potato famine (Martin et al. 2013). Larger and more in-depth studies using ancient plant genomes are expected in the coming years given the economic importance of major crops and the possibility of reintroducing alleles involved at various stages of the domestication process (Wales et al. 2014).

1.5 EVOLUTION OF THE EXTRACTION METHODS

Obtaining DNA from ancient remains is a process which usually needs a pretreatment process that includes removal of the dirt and of possible contaminating substances as well as the solubilization of the genetic material. There are several techniques to carry out these processes but consensus on which is the best technique has not been reached by scientists. In this sense, the most frequently reported decontamination method for teeth has been the use of bleach (Kemp and Smith 2005). Until recently, it was thought that bleach destroyed exogenous DNA while leaving endogenous DNA unaffected. However, a recent study suggests that with this treatment the exogenous DNA is simply degraded making it difficult to be discerned from degraded endogenous DNA (García-Garcera et al. 2011). Thus, as the true impact of decontamination techniques on the DNA content of organic remains has yet to be established, some authors argue that it is potentially better to avoid the use of bleach or other chemicals that can destroy DNA, and suggest simple cleaning techniques involving brushing or light scraping to remove debris and/or wiping with DNA free water instead (Higgins and Austin 2013). Another disagreement appears in the first step of the DNA extraction because currently there are at least three different methodologies which continue to be used: a) there are cases specially in museum samples, where the powder is obtained without breaking the piece in a non-destructive extraction method just involving incubation in a specially prepared extraction solution (Bolnick et al. 2012); b) there are also groups that rely on a total breakdown of the piece using liquid nitrogen (Fernández et al. 2014); and finally there are cases where the piece is cut by its amelocementary limit in the case of teeth and the diaphysis in the case of bones, and the powder is obtained with a diamond bur from either the radicular or the medullary canal.

Focusing on the efficiency of the extraction methodologies, there are also different strategies. Phenol-chloroform method allows the recovery of a higher DNA quantity, but it has some problems like the possible co-purification with some substances which can act as PCR inhibitors (see section 1.2.2.2 "Avoiding inhibition") or the toxicity of the reagents used. The interest in both trying to use new methodologies to avoid the risk for the investigators and optimizing the genetic material recovery prompted specialists to look for alternatives to efficiently recover DNA, leading to the creation of the silica-based purification protocols (Boom et al. 1990, modified by Höss and Pääbo in 1993), which enhanced the purity of the recovered DNA as the silica was virtually bound to every single molecule (Höss 1994). Soon afterwards, the first kits aimed specifically at the recovery of aDNA using silica-based spin columns started being used (Yang et al. 1997), followed by several commercial systems based on the capture of DNA using them. The method was characterized by the bind/release of DNA depending on salt concentration and had the advantage of being faster, using selective binding and being amenable to automation and miniaturization. Since then, other methods using the selective affinity between DNA and silica have continued appearing, as the ones based on silica-coated magnetic beads to separate DNA from the rest of the lysed cell such as Promega's DNA IQ (Promega, USA) or Invitrogen's Chargeswitch (Invitrogen, USA). Optimizations of these to suit robotic systems (Greenspoon et al. 2004; Frégeau et al. 2010) have also been applied.

Silica gel membranes have also been used to purify the solutions after having carried out the PCR by removing salts, ions and unused dNTPs and primers from the reaction (for example, in the MinElute PCR Purification Kit columns (QIAgen, Netherlands)) as in Roeder et al. in 2009 or the JetQuick Spin Column Technique (Genomed, Germany) as in Simón et al. in 2011. Other methods used to purify DNA have been filtration using Microcon filter columns (Forster et al. 2008; Simón et al. 2012) or enzymatic hydrolysis using ExoSAP-IT (Smith and Ballantyne 2007). However, while being safer for the investigators, the problem of inhibition could not be totally overcome with the inception of the silica-based methods as some inhibitors were not effectively removed from the most challenging samples (Kemp et al. 2006; Lee et al. 2010). In 2005 though, a novel technology denominated SCODA ("Synchronous coefficient of drag alteration") (SCODA, Boreal Genomics, Vancouver) appeared that removed inhibitors from different types of samples at levels not possible by a commonly used silica column-based purification (Marziali et al. 2005; Broemeling et al. 2009) and avoided the notable loss of nucleic acids as a result of its automated, minimal-step approach (Schmedes et al. 2013). SCODA employs alternating electric fields that concentrate DNA into the center of the electrophoretic field while driving non-nucleic substances out of it (Marziali et al. 2005; Pel et al. 2009), enabling large volumes of samples to be extracted, which would facilitate recovering DNA from challenging samples such as diluted stains. In order to know if a single extraction method was better than the others many comparative studies have been carried out over the years, from which no single method has been unanimously concluded to be the best (Yang et al. 1998; Bouwman and Brown 2002; Rohland and Hofreiter 2007; Simón et al. 2012).

1.6 EVOLUTION OF THE SEQUENCIATION METHODS

To understand the improvement in sequencing technologies one has to look also at the improvement in the amplification methods. Since 1988, when the PCR was first applied to exponentially increase the recovered genetic material from ancient remains by Pääbo and Wilson, until the end of the XXth century, this technique has been the one applied to obtain viable quantities of genetic material from this kind of samples. Possibly, the greatest success obtained with the study of material recovered via a traditional PCR was the obtainment of the first ancient mitochondrial genomes entirely sequenced in 2001, when two independent groups of researchers presented the mitogenomes of four extinct moa (Cooper et al. 2001; Haddrath and Baker 2001). Five years later, in 2006, Krause et al. and Römpler et al. improved significantly on the conventional method by demonstrating that the initial simplex PCR amplifications could be replaced with a small number of multiplex PCRs. Later, some other notorious studies appeared, such as the ones presenting the obtainment of the first mammoth and mastodon mitogenomes (Krause et al. 2006 and Rohland et al. 2007), the cave bear mitogenome (Krause et al. 2008) and part of those of the Romanov family (Rogaev et al. 2009).

However, recovering genome-wide sequence information from ancient remains clearly required other approaches as the extensive fragmentation of aDNA molecules was not optimal for PCR investigation. With a majority of molecules shorter than 50–100 bp, the genetic information retrieved excluding PCR primers was rather limited. Longer fragments could sometimes be recovered, however at higher risks of contamination by fresh DNA, and in addition the number of samples available for destructive sampling was often limited. The arrival of the so-called “Next-Generation Sequencing” (Margulies et al. 2005) would soon prove to overcome many of the traditional amplification limitations.

1.6.1 Next-Generation Sequencing

The first decade of the XXIst century witnessed the arrival of HTS. It was a fast and cheap way to sequence and analyze large genomes that generally involved the amplification of DNA templates by PCR and the physical binding of template DNA to a solid surface or to tiny beads called microbeads. These techniques are often referred to as massively parallel DNA sequencing, because thousands or millions of sequencing reactions are run at once to greatly speed up the process. Besides these advantages, these technologies transformed the sample preparation process in a way that is eminently suitable for the sequencing of aDNA.

The most widely used high-throughput technologies required the conversion of DNA fragments into DNA libraries before sequencing (Margulies et al. 2005; Bentley et al. 2008; McKernan et al. 2009; Rothberg et al. 2011). This is achieved by attaching artificial DNA segments

(adaptors) to both ends of the fragments, providing PCR and sequencing primer ligation sites, leading ultimately to the sequencing of hundreds of thousands, to millions, of template molecules in parallel per sequencing reaction (Ho and Gilbert 2010). The extremely large number of sequences that are generated means that, for the most part, the content of any PCR product will be sequenced in depth, mimicking conventional molecular cloning and DNA sequencing. In addition to the extremely large increase in sequences that can be generated through massive parallel sequencing of the libraries, a key benefit of this technique with regard to degraded DNA is that extremely short DNA fragments can be sequenced. Moreover, aDNA rarely requires fragmentation prior to library construction, which represents a stage in which very large amounts of DNA are lost and the amount of starting material used can also be diminished as more sensitive ways of building and measuring the libraries are proposed (Meyer et al. 2008; Maricic and Pääbo 2009). Finally, by amplifying all fragments that were successfully converted into library molecules, the library is effectively immortalized. The high-throughput DNA sequencers which exist so far are the Roche FLX (Roche, Switzerland) and the Illumina GA (Illumina, USA), which make it possible to sequence multiple PCR products rapidly and efficiently. Both use double-stranded DNA (*ds*DNA) and were originally developed for HTS of modern DNA. The methods are fundamentally based on the principle of shotgun sequencing, which involves the random selection of large numbers of DNA molecules from those available in an extract. The frequency of any resulting sequence is a direct function of its original frequency. The first method was developed by 454 Life Sciences and is based on the ligation of two different adapters to blunt end–repaired *ds*DNA (Margulies et al. 2005). The second method, first suggested by Illumina, uses a single, Y-shaped adapter with a T-overhang that is ligated to both ends of DNA fragments that have been manipulated to carry A-overhangs (Bentley et al. 2008) (Figures 11a and 11b).

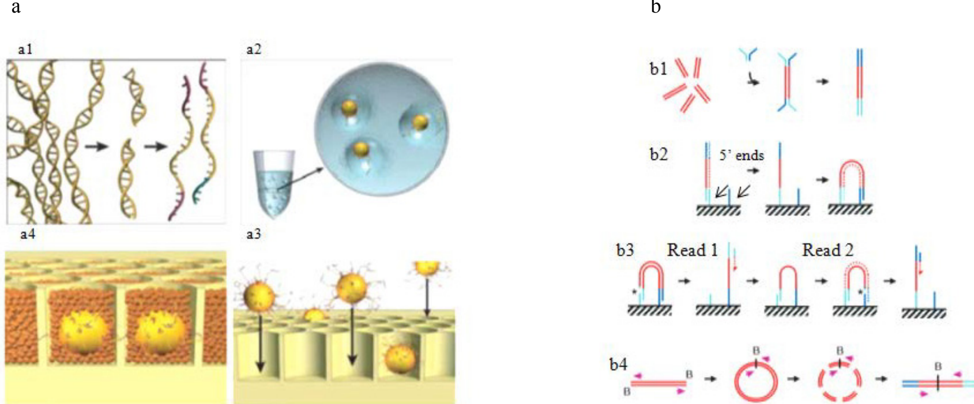

Figure 11. Main methods used by high-throughput DNA sequencers a: 454 Life Sciences method (modified from Margulies et al. 2005). b: Illumina Sequencing method (modified from Bentley et al. 2008)

Figure 11a. Method used in 454 Life Sciences: genomic DNA is isolated, fragmented, ligated to adaptors and separated into single strands (a1). Fragments are bound to beads captured in the droplets of a PCR-reaction-mixture-in-oil emulsion and PCR occurs within each droplet starting from a unique DNA template (a2). The emulsion is broken, DNA strands are denatured, and beads carrying *ss*DNA clones are deposited into wells of a

firag-optic slide (a3). Smaller beads carrying immobilized enzymes required for pyrophosphate sequencing are deposited into each well (a4)

Figure 11b. Method used in Illumina Sequencing: DNA fragments are generated by random shearing and joined to a pair of oligonucleotides and then amplified resulting in *ds* blunt-ended material with a different adaptor sequence on either end (b1). These DNA fragments are denatured and *ss*DNA anneals to complementary oligonucleotides. A new strand is copied from the original strand and the original strand is then removed by denaturation. The adaptors' sequence at the 3' end of each copied strand is annealed to a new surface-bound complementary oligonucleotide, forming a bridge and generating a new site for synthesis of a second strand. Multiple cycles of PCR are carried out (b2). The DNA in each cluster is linearized by cleavage within one adaptor sequence and denatured, generating an *ss* template for sequencing and obtaining a sequence read (read 1). Next, the products of read 1 are removed , the template is used to generate a bridge, the second strand is re-synthesized and the opposite strand is cleaved to provide the template for the second read (read 2) (b3). To sequence the ends of a long DNA fragment, the ends of each fragment are tagged by incorporation of biotinylated (B) nucleotide and then circularized. Circularized DNA is randomly fragmented and the biotinylated junction fragments are recovered and used as starting material in the standard sample preparation (b4). (: orientation of the sequence reads relative to the DNA; : oligonucleotides; : genomic DNA; : newly synthesized strands during cluster formation or sequencing: dotted lines)

While direct PCR amplification can just reconstruct sequences from fragments that are long enough to permit the hybridization of two PCR primers in library-based techniques, even shorter DNA fragments will be able to be sequenced in their entirety because the priming sites required for amplification and sequencing are added externally and finally the short overlapping sequences are assembled into the final consensus sequence. In the context of mitogenomics, the FLX sequencer was first used in this way to sequence the simplex products of the mitogenome of the Tyrolean Iceman (Ermini et al. 2008). Moreover, the approach was taken further with the publication of the first ancient mitogenomes that were directly FLX-sequenced from the products of multiplex reactions (Stiller et al. 2009), and in doing so the authors of the study successfully obtained near-complete mitogenome sequences from 31 cave bears. However, at that time these methods continued being both time- and resource-consuming, as they required many hundreds of PCRs, each targeting a small DNA fragment. Therefore, maximising DNA extraction efficiency was of vital importance (Hofreiter et al. 2014) and the next step was on the way: enriching genomic regions of interest in the libraries of aDNA.

1.6.2 Enrichment techniques

In 2009, Briggs et al. proposed an appealing solution to the target-enrichment problem of HTS that was published in their analysis of five Neanderthal mitogenomes named "primer-extension-capture" (PEC), which involved large numbers (>600) of 5′-biotinylated oligonucleotide probes,

designed to bind specifically to small mitogenomic fragments present in HTS libraries prepared on aDNA extracts. Following a series of enzymatic elongation, magnetic streptavidin bead capture and amplification steps, they obtained a significant enrichment of the target in the final library to be sequenced, (from 1% to 40% in Neanderthal samples). With such enrichment, libraries from multiple individuals would be able to be sequenced simultaneously by HTS yielding extremely well-covered mitogenomic sequences. The method was able to recover very small targets, because the probes only required a small capture region (approximately 20 bases) and primer-binding sites were not needed. This extended the range of material from which aDNA could be retrieved. This way, the ratio of endogenous to contaminating hominid DNA could be substantially shifted in favor of the former, allowing for authentic consensus sequences to be obtained even from samples that were relatively strongly contaminated when judged by PCR (Krause et al. 2010a). Other in-solution enrichment methods relied on biotinylated baits to target complementary library inserts, either prepared from modern DNA extracts (Maricic et al. 2010) or designed from known sequences and commercially manufactured (Ávila-Arcos et al. 2011), often delivering complete mitochondrial genome sequences with high depth of coverage (Maricic et al. 2010; Krause et al. 2010b; Horn et al. 2011; Fu et al. 2013; Orlando et al. 2013; Thalmann et al. 2013; Vilstrup et al. 2013; Zhang et al. 2013). Another option, named “Microarray-based hybridization capture” (Hodges et al. 2009) (Figure 12) also performed well in enriching Neanderthal DNA libraries (Burbano et al. 2010) containing very low endogenous DNA content, and delivering the full bacterial genome of the causative agent of the medieval Black Death epidemic (Schuenemann et al. 2011; Bos et al. 2012) as well as of historical leprosy strains (Schuenemann et al. 2013).

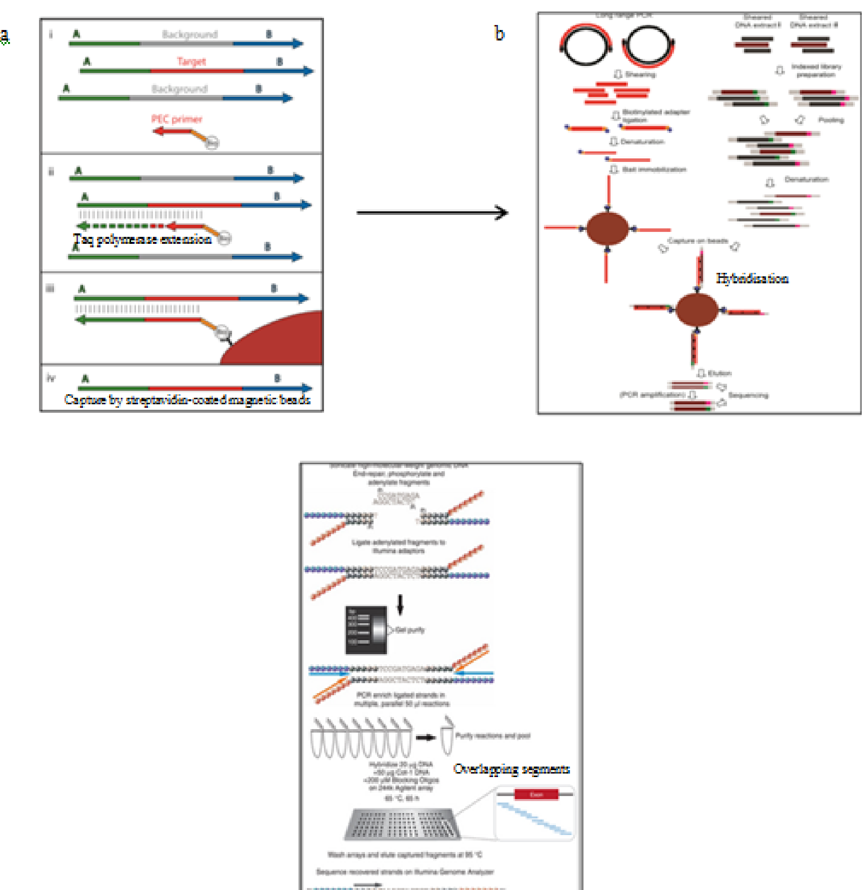

Figure 12. Setting of enrichment methods. a) It shows the setting (top left) and improvement (top right) of PEC capture methods (modified from Briggs et al. 2009 and Maricic et al. 2010). b) (bottom) shows the microarray capture method (modified from Hodges et al. 2009).

1.6.3 Passing from second to third generation sequencing technologies: advantages of single-stranded library preparation

In the work that signaled the encounter between second (based on *ds*DNA and having their representatives in Illumina and FLX sequencers) and third generation sequencing technologies (based on *ss*DNA, with the ability of sequencing an actual single strand of DNA and having its representative in the Heliscope Single Molecule Sequencer (Helicos Bioscience, USA)), an early Middle Pleistocene horse genome was sequenced at approximately 1.1-fold coverage using a combination of the former (Illumina) and the latter (Helicos) (Orlando et al. 2013). Helicos sequencing, based on true single DNA molecule sequencing (tSMS), appeared to be advantageous when targeting short and damaged molecules for several reasons: first, through the initial biotinylation of the ancient molecules, all reaction steps are carried out while the DNA is tightly bound to streptavidin-coated beads and the only enzymatic treatment that templates require is poly-adenylation of *ss*DNA templates, limiting the number of DNA purification steps needed and avoiding the loss of molecules that happens in DNA purification steps using silica spin columns or carboxylated beads (DeAngelis et al. 1995) which are integral parts of the *ds* library preparation methods described above. Second, by targeting *ss*DNA, the

presence of any single strand break gives rise to three library templates versus one for approaches targeting *ds* templates as the sequencing reaction is primed at any 3'-OH terminus available, yielding higher incorporation rates for aDNA than for fresh DNA molecules, particularly in cases where DNA templates are extremely damaged. Otherwise such molecules would be entirely lost in *ds* library preparation, whereas with the *ss* method they are disassembled into multiple fragments upon heat denaturation, and each fragment has an independent chance of being recovered in the library (Figure 13). Third, end modifications located on one strand of a *ds* molecule may completely inhibit adaptor ligation during *ds* library preparation. In the *ss* library approach, on the other hand, the strand opposite to that containing such a modification can still be retrieved. Finally, all the other methods for preparing DNA libraries cause the loss of the original ends of molecules, because they either involve blunt-end repair or enzymatic fragmentation of DNA (Adey et al. 2010). With the *ss* method, adapters are joined without removing nucleotides from the DNA strands, which enables the determination of DNA fragmentation patterns in high resolution and in principle even enables the reconstruction of both strands of DNA fragments. However, a general limitation of the method is that it is suitable only for the sequencing of relatively short molecules, because the efficiency of *ss* ligation decreases for molecules longer than 120 bp (Li and Weeks 2006). The majority of DNA fragments in ancient specimens are typically well below this size, but for samples preserved under very favorable conditions, for instance in the permafrost environment, *ds* library preparation may be a more suitable approach. It should also be noted that *ss* library preparation is more costly and time-consuming than *ds* library preparation. Thus, if there are few restrictions on the amount of material that can be used for destructive sampling, it may be preferable to proceed with *ds* library preparation at the expense of using more material.

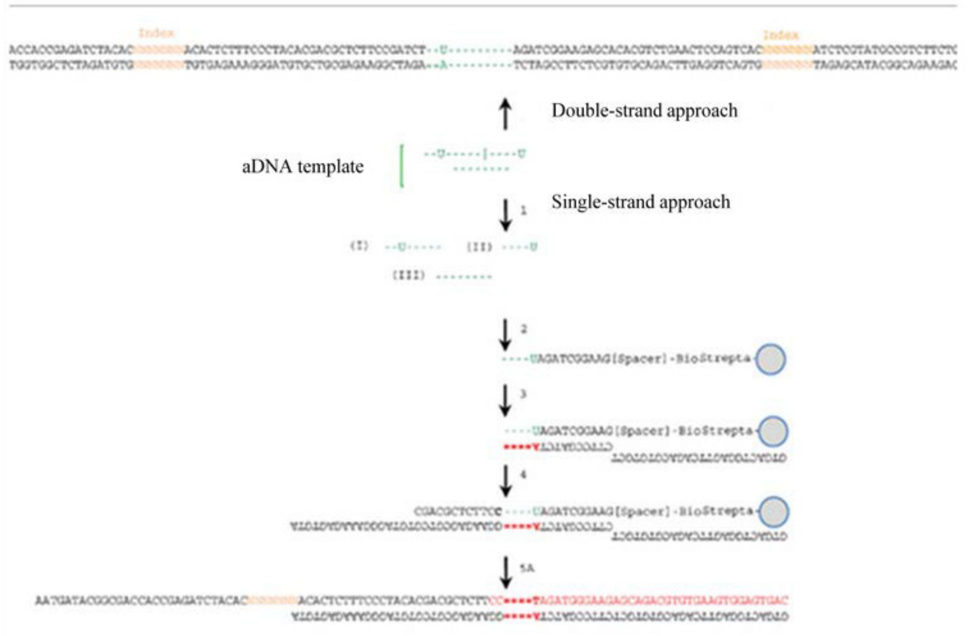

Figure 13. Whereas only one insert will be incorporated following library preparation methods based on *dsDNA* templates (top), the single strand approach (bottom) will result in the formation of three library inserts, increasing the contribution of ancient and nicked templates to the final molecular complexity of the DNA library. 1: Heat denaturation into three *ss*DNA pieces (I, II and III). 2: Single Strand DNA ligation to a first biotinylated adapter. 3: Synthesis of the complementary strand, showing a G>A misincorporation following elongation through an uracil residue. 4: DNA ligation to a second adapter. 5A: PCR amplification, 1st cycle (modified from Orlando et al. 2014)

Earlier observations using the Heliscope had already shown that the ability to access aDNA molecules could be improved with true Single Molecule DNA sequencing (Orlando et al. 2011), so this suggested that targeting single strands could benefit ancient genomics (Orlando et al. 2011; Ginolhac et al. 2012). In 2013, Gansauge and Meyer developed an *ss*DNA library preparation method specifically developed for the sequencing of aDNA compatible with HTS using Illumina technology. Using this type of library and improving a silica-based DNA extraction technique optimized for ancient bones and teeth (Rohland and Hofreiter 2007), Dabney et al. in 2013 managed to attenuate the bias against ultra-short templates present in aDNA sequencing libraries. This enabled the efficient retrieval of phylogenetically informative sequences from samples in which virtually all DNA was diminished to fragments shorter than 50 bp, reconstructing the mitochondrial genome sequence from a Middle Pleistocene cave bear (*Ursus deningeri*) bone excavated in the Sierra de Atapuerca, Spain. Finally, the recovery of material of an hominin from the same site and period was obtained in 2014 when Meyer and collaborators developed a method compatible with Illumina sequencing that targeted *ss*DNA rather than *ds* molecules, enriched their libraries for human-like DNA and selected in silico those reads that exhibited typical aDNA damage patterns, selecting reads short enough to potentially originate from highly fragmented ancient templates and ultimately obtaining the mitochondrial genome of a 400 thousand year-old hominin.

In both Dabney's and Meyer's studies, targeting ultra-short fragments also reduced the relative impact of contamination, as 75 bp fragments mainly consist of fresh exogenous DNA while for shorter fragments the proportion of endogenous DNA material increases (Orlando 2014) and the lack of purification steps avoided the loss of molecules in DNA purification using silica spin columns (DeAngelis et al. 1995). However, as it stands now, the in silico filtering procedure described in Meyer et al. (2014) would simply discard too many of the sequences produced, being prohibitive given the scale of the whole nuclear genome (Orlando 2014). It could be that more sophisticated models of DNA damage over time could help improve the selection of reads that show the expected damage signature. Such approaches are emerging and have shown some success with younger Neanderthal material that was extensively contaminated (Skoglund et al. 2014). However, as antiquity of the sample increases the damage of most, if not all, DNA molecules increases too, so developing a method directly enriching for highly damaged DNA templates prior to sequencing seems to be the next step to

achieve, as well as developing read mapping procedures against reference genomes that integrate the specific misincorporation patterns of aDNA sequences.

Current methodology has allowed the reconstruction of the oldest mitochondrial genomes from non-permafrost samples to date, a 400 thousand year-old cave bear and a 400 thousand year-old hominin (Dabney et al. 2013 and Meyer et al. 2014), and a 700 thousand year-old horse genome (Orlando et al. 2013), demonstrating that the genomic era has breached the Middle Pleistocene. With molecular tools now truly tailored to the chemical specificities of aDNA molecules, it could be possible to access genetic information over the last million years (Millar and Lambert 2013), also potentially from warm regions of the planet where DNA decays at much faster rates (Smith et al. 2003) and where previous approaches mostly returned negative results. Furthermore, knowing that in some cases only a small portion (or even none) of the endogenous DNA can be solubilized into extraction buffer (Geigl 2002) and that most methods recover only a small proportion of the aDNA present (Barta et al. 2014), improvements in DNA extraction techniques are probably still possible for all ancient substrates.

2. AIMS

The principal aim of this thesis is to use information provided by aDNA to solve problems that commonly appear in Physical Anthropology laboratories, trying to give answers to archaeological questions such as the identity of the people studied, the type of relationship that existed between them, their origin or what illnesses they suffered during their life. Therefore, this work has numerous specific purposes and has taken advantage of the different levels of analysis that aDNA allows to develop. Thus, it has permitted covering from the analysis of possible existing kinship ties inside a close group to the performance of population analyses.

The first question which was studied in this thesis was of methodological nature. We find it important to know the impact of the chosen methodology in DNA retrieval and, as a consequence, in the results of aDNA analysis, and the motivation to carry out this study was the low extraction rate of success in relation to the DNA retrieval from individuals from La Cova des Pas, a cave in Minorca with exceptionally well preserved skeletons dating from the Late Bronze Age, even with some of them still presenting soft tissues (Armentano et al. 2010). To elucidate if the extraction method was the cause of this poor success, we have assayed in parallel two different extraction processes, the modified phenol-clorophorm method usually used in our laboratory (Malgosa et al. 2005) and a new silica-based extraction process (QIAamp DNA Investigator kit, QIAgen, USA) to see if the second one could improve our results.

The second question we wanted to focus on was related with familial ties. Our study has aimed to elucidate whether the structure of small groups buried in the same site having similar or common burial patterns can be related with the degree of genetic kinship between the individuals present, and to make inferences about the evolution of the concept of family in such reduced groups during the Late Bronze Age. In this sense, a collective burial with 8 individuals found at the Catalan PrePyrenean Cave of Sant Joan de Montanissell in Lleida, Catalunya, has provided a very good chance to test if some assumptions commonly made by anthropologists when these kinds of burials are found agree with the genetic data obtained, even more in this case as the age and gender distribution of the individuals seemed to ideally represent what is currently known as a “nuclear family”.

On a larger scale, the focus has also been put in the relationship between populations that are time or geographically close. We put the focus on small populations from the Balearic Islands encompassing from the Late Bronze Age to the Early Middle Ages. Comparing the mitochondrial genetic pools from single populations from both Minorca and Majorca in intra and interpopulational analyses, the aim has been to unveil if they showed homogeneity and thus could be seen as a single

population, or if each one had to be considered different on its own right. In case that the latter possibility was correct to some degree, we also wanted to know whether genetic differences were reflected in some particular characteristics, either morphological or behavioral (for example different funerary patterns) based on data supported by Font in 1974 (Son Real), Malgosa in 1992 (S'Illot des Porros), Ortega in 2005 (Can Reiners), Plantalamor et al. in 2008 (Son Olivaret) and Armentano et al. in 2012 (La Cova des Pas). Next, our aim has been to carry out a more general study encompassing the population dynamics of ancient populations from the Balearic Islands, adding the new data obtained in the current thesis from the mitochondrial genetic pool of the island of Minorca to the work carried out by Díaz in 2009 studying the island of Majorca. The purpose is having a complete genetic characterization of the feminine lineages in the populations that have inhabited the region from the Late Bronze Age period to the current days. Finally, we have intended to know the geographic origins of these people in the islands and the possible connection they might have with different contemporary regions from the European continent or other islands from Western Mediterranean. The new information can shed light on the long-term debate about the possible relation between the inhabitants of Nuragic Sardinia and Talaiotic Balearic Islands (Rosselló 1979; Guerrero et al. 2002) and the long-storied relationship between these islands and Catalunya (Pericot 1975).

Finally, the fourth aim is focused on ancient people health. Diseases that population suffers are one of the most interesting informations for the interpretation of particular ancient populations, but also for the study of current and future illnesses. One of the most spread and ancient illnesses in humans is dental caries. We focused the last aim of this thesis in the understanding of this infection in the ancient world, specifically whether one of the cariogenic agents, *Streptococcus mutans,* a bacterium perennially related with caries presence, was exactly the same over time and space. We will focus our interest on the virulence factor of this bacterium to give some data about the evolution of cariogenicity over time. In this study, genetic material belonging to a known virulence factor of *Streptococcus mutans*, from the Late Bronze Age to the beginning of the XXth century, will be checked to know whether it has deviated from neutral selection over the centuries in response to some adaptive requirement. Thus, we have intended to make for the first time deductions about its evolution based on real obtained data instead of hypothetical inferences.

3. MATERIALS AND METHODS

The materials used in the present work come from different archaeological sites, each one being specified in its corresponding paper. In all the cases the source material has been a hard tissue, either teeth or bone, because as specified in the introduction they are by far the best conserved and the most commonly found in ancient remains (see Section 1.4 "Ancient tissues from where the DNA can be extracted and their characteristics"). The genetic material of interest has been the mtDNA, as its higher number of copies in relation to the nuDNA combined with the big number of individuals needed to do population analyses, made it more time- and cost-efficient to put the focus on this molecule. In the cases where it could bring relevant information the genetic sex has also been checked using a segment of the first intron of the amelogenin gene (Nakahori et al. 1991) and a segment of SRY gene (Santos et al. 1998) that accounts for cases where in a masculine individual the band signaling the Y chromosome presence is not correctly amplified.

Two different extraction protocols have been used with the aim of improving the results in a specific site (Simón et al. 2012). While the extraction methodology normally used has been the one based on a modified version of the phenol-clorophorm method (Malgosa et al. 2005), in some instances a kit based on the binding of DNA to a silica-based membrane method named QIAamp DNA Investigator kit (QIAgen, USA) (Simón et al. 2012) has been employed. All the sequence reactions have been carried out using the sequencing kit BigDye Terminator v.3.1 (Applied Biosystems, USA) according to the manufacturer's specifications and run in an ABI 3130XL sequencer (Applied Biosystems, USA). Whenever a clonation process has been carried out, the TOPO TA Cloning Kit for Sequencing (Invitrogen, USA) has been used. Moreover, the amplification methodology has evolved from using the traditional PCR (Simón et al. 2011, 2012) to the application of the real-time PCR using the Qiagen Rotor-Gene Q (QIAgen, Turnberry Lane, USA) and the Type-it HRM PCR kit (400) (QIAgen, USA) (Simón et al. 2014), which provides the possibility of following the whole amplification process. Also the use of HRM PCR allows the incorporation of a High-resolution melting step that permits to know if the desired fragment has been obtained checking its melting temperature and to avoid the necessity of verifying the amplification in an agarose gel. In consequence, the purification kit for the amplified samples has changed from the JetQuick PCR Purification kit (JetQuick, Germany) (Simón et al. 2011, 2012) to the PCRapace kit (Invitrogen, USA)

(Simón et al. 2014), better suited to eliminate the dyes that bind to the DNA during the real-time PCR. Moreover,

4. RESULTS: PUBLICATIONS

The aims of the current thesis have been covered in three published and two submitted peer-reviewed publications and one requested paper.

1. Simón M, González-Ruiz M, Prats-Muñoz M, Malgosa A. 2012. **Comparison of Two DNA Extraction Methods in a Spanish Bronze Age Burial Cave.** Quaternary International 247:358-362. DOI:10.1016/j.quaint.2011.04.026. Impact factor: 1.962 (2012); Q (SSCI 2012): 2º, 58/172, Geosciences, Multidisciplinary.

2. Simón M, Jordana X, Armentano N, Santos C, Díaz N, Solórzano E, López JB, González-Ruiz M, Malgosa A. 2011. **The Presence of Nuclear Families in Prehistoric Collective Burials Revisited: The Bronze Age Burial of Montanissell Cave (Spain) in the Light of aDNA.** American Journal of Physical Anthropology 146:406-413. DOI:10.1002/ajpa.21590. Impact factor: 2.824 (SSCI, 2011); Q (SSCI, 2011): 1º, 6/81 Anthropology.

3. Simón M, Malgosa A. 2014. **Caracterització genètica de les restes humanes talaiòtiques del jaciment de Son Olivaret, Ciutadella, Menorca.** Revista de Menorca, 93: 239-258.

4. Simón M, Malgosa A. **La Menorca talayótica desde el punto de vista genético: el yacimiento de la Cova des Pas.** Trabajos de Prehistoria. Impact factor: 0.682 (2013, 2014); Impact factor: 0.682 (SSCI, 2013); Q (SSCI, 2013): 3º, 42/83 Anthropology. Submitted

5. Simón M, Montiel R, Francalacci P, Díaz N, Malgosa A. 2015. **Dissecting Balearic populations at the molecular level from the Bronze Age to the current era.** American

Journal of Physical Anthropology. Impact factor: 2.514 (2013); Q (SSCI, 2013): 1º, 5/82 Anthropology. Submitted

6. Simón M, Montiel R, Smerling A, Solórzano E, Díaz N, Álvarez-Sandoval BA, Jiménez-Marín AR, Malgosa A. 2014. **Molecular analysis of ancient caries.** Proceedings of the Royal Society B: Biological Sciences 281(1790):20140586. DOI: 10.1098/rspb.2014.0586. Impact factor: 5.292 (2013); Q (SSCI, 2013): 1º, 9/83 Biology.

4.1 Comparison of two DNA extraction methods in a Spanish Bronze Age burial cave

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Comparison of two DNA extraction methods in a Spanish Bronze Age burial cave

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ABSTRACT

The site from La Cova des Pas, located in Menorca, is a funerary cave with a collective burial dating from the Late Bronze Age. This cave presents a good conservation of the remains of both humans and other organic elements. In spite of the good preservation, the first DNA amplification tests showed a low amplification success rate, probably owing to the presence of substances from the process of decomposition of the bodies and substances of the soil present in the samples. To overcome this problem, 41 samples were processed by means of two different extraction methods, following sterility criteria used in ancient DNA research. Two fragments of different sizes of mitochondrial DNA were amplified in order to evaluate the success rate of both methods. The results did not show a statistically significant better recovery of DNA from either teeth or bones. For the length of the retrieved DNA, the recovery was significantly better with the QIAamp kit procedure than with the phenol–chloroform method.

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1. Introduction

The archaeological site of La Cova des Pas, located in Menorca (Spain), dates from the Late Bronze Age (14th– 8th centuries BC) and is a collective burial site with 66 individuals that belonged to an ancient population (Armentano et al., 2010). Externally, some of the skeletons are exceptionally well-preserved in spite of their antiquity, with a few retaining some soft tissues. Thus, the expected percentage of genetic material retrieval was supposed to be extremely high. Nevertheless, the retrieval percentage turned to be moderate, with varying degrees of success depending on the extraction protocol used. The aim of this study is to test which of the two extraction methods employed is better to overcome the problems seen in the recovery of genetic material of the individuals from La Cova des Pas site.

1.1. Geographic localization and relevance of the site

La Cova des Pas site is considered the most important talaiotic necropolis from Menorca, and was used as a funerary cave approximately 3000 years ago. It is a karst cave placed in the municipal term of Ferreries (Fig. 1), located in the southeast cliff-wall of the gorge of Trebalúger, about 15 m above the canyon floor. The cave is rather small, maximum 6.5 m depth, 4.5 m wide and never higher than 1.7 m, and it was active for approximately three or four centuries. Despite their antiquity, the conservation of the individuals is outstanding. This degree of preservation is not easily explained. Many factors have contributed to this preservation. One of them could be related to the funerary ritual, by which the bodies were transported to the Cave in funerary bunk beds and covered with a shroud made of fur. They were placed in foetal position and tied with ropes (Fig. 2). The most relevant soft tissues found are intrathoracic mass, muscular and cerebral tissues, as well as hair and faecal remains. Some other very relevant organic elements are present, including wood and vegetation tissues. The presence of small branches and leaves under the deceased indicates complexity in the treatment of the corpse (Cabanes and Albert, 2011) (Fig. 3).

This high degree of preservation can also be due to other reasons, including the microclimate of the cliffs that somehow protected the integrity of the individuals inside the cave, or the acidity of the sediment. The presence of highly soluble minerals such as sodium nitrate and gypsum suggests a stable dry environment inside the cave that preserved the organic material. These minerals absorbed the low humidity present in the sediments and enhanced the natural mummification of the corpses by the partial inhibition of bacterial activity (Cabanes and Albert, 2011). This degree of natural preservation is only matched in Europe by that of the Otzi mummy (De Marinis and Brillante, 1998), preserved by the cold conditions, and the two semi-mummies from the Galer site in Castellón Alto (Granada), with an antiquity of 3500 years. Altuna (1991) stated that the annual thermal profile inside the caves is less variable than outside, providing a less variable environment.

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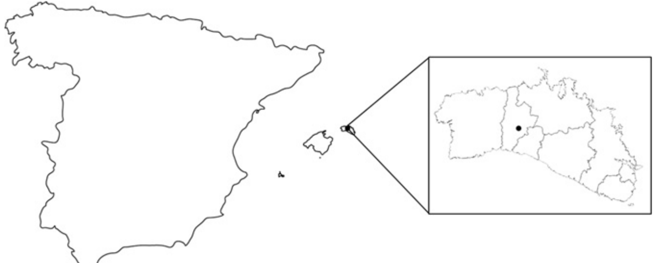

Fig. 1. Geographical localization of the archaeological site Cova des Pas, Menorca.

2. Materials and methods

DNA was extracted from 31 individuals from La Cova des Pas. In 7 of them both extraction methods were used. Samples for ancient DNA (aDNA) analysis were taken in the field following sterility criteria (only non-fragmented pieces were selected and the archaeologists wore face masks and sterile gloves at the time of the collection), and were stored in cold conditions.

Genetic analysis was carried out at the laboratory of aDNA of the Biological Anthropology Unit of Universitat Autònoma de Barcelona (UAB). In accordance with previously suggested aDNA procedures (Cooper et al., 2001), the DNA extractions were performed in an isolated pre-PCR area that is dedicated to ancient DNA, and equipped with both positive air pressure and UV–light exposure systems. Sterile gloves, face masks, sterilised reagents, pipette filter tips and frequent washing with bleach and ethanol of the working surfaces and the equipment were some of the precautions adopted during the process. Moreover, the researchers worked first in the pre-PCR and afterwards in the post-PCR area, which can be as effective as positive pressure chambers to avoid contamination (Willerslev and Cooper, 2005). Three of the samples could be fully replicated, giving coincident results. Also, three of the samples were cloned, indicating that they do not give any additional information to the sequence obtained in the PCR reaction.

The selected pieces were well-preserved teeth, or bone (phalanges and metacarpus) as a second option. Pieces that showed any kind of fracture were discarded. DNA extraction was performed in batches of 5 teeth and/or bones with one blank extraction in each batch to monitor the eventual presence of contaminant DNA. Teeth samples were washed in 50% bleach for 5 min, and each side exposed to UV light for 15 min to eliminate inhibitors or contaminant substances on dental surfaces. In the case of bones, their surface was brushed with a toothbrush with 50% diluted bleach and the rest of the procedure was as explained above. Teeth were cut by the amelo-cementary limit to expose the pulp chamber. In the case of bones, surface removal with a dental drill was carried out before transversal cutting to expose the medullar canal. The spongy tissue was removed before working on the compact tissue. Between 25 and 50 mg of the selected piece were obtained from either the pulp chamber or the compact tissue of the medullar canal of the diaphysis.

Fig. 2. Picture from the inside of the cave, showing distribution of some of the skeletons over the soil of the cave.

With all these tools, and all the posterior amplifications including a blank control to prevent contamination, the veracity of the data was confirmed by a critical consideration of all available information, taking into account the problems pointed out by Gilbert et al. (2005). An integrative approach for human population studies was used, where the flexibility and the intelligent use of authentication criteria was applied (Montiel et al., 2007).

Two different kinds of extraction protocols were carried out. For phenol–chloroform extraction, DNA was isolated using a standard phenol–chloroform extraction protocol. Briefly, the powder was incubated overnight in 5 ml of extraction tampon (Tris–HCl 1 M (pH 8.0–8.5), SDS 10%, EDTA 0.5M and sterile deionised water) and 50 μl of 0.01 g/ml proteinase K. After incubation, the DNA was subsequently extracted with phenol–chloroform and the aqueous phase was concentrated using a Centricon-30 filter column (Millipore®) up to a 30 μl volume (Malgosa et al., 2005). For QIAamp DNA extraction, the DNA was isolated using the QIAamp DNA investigator kit (Qiagen) according to the manufacturer's recommendations.

Following the laboratory criteria, a 230 bp long fragment of Hypervariable region I (HVRI) was amplified. If this did not work, two shorter fragments ranging from 120 to 150 bp that encompassed this region were used to amplify this zone, between positions 16,191 and 16,420. Also, multiple pairs of primers around 120 bp in length were used to amplify the coding region to carry out enzymatic restriction in the areas that define haplogroups (Supplementary data). One microlitre of template was subjected to 39 cycles of amplification in a 50 μl-reaction volume. The amplification consisted in a first step of denaturation of 5 min at 94 °C, 39 cycles of 50 s at 94 °C, 1 min at the adequate annealing temperature depending on the region to amplify and an elongation phase of 1 min at 72 °C. There was a final elongation step of 5 min at 72 °C. Amplified fragments were then visualized with Ethidium Bromide staining in a 3% agarose gel. The rate of success of DNA recovery was classified into three categories: null, medium and high. Moreover, other tests were carried out, considering the absence or presence of positive results, whatever the length of the obtained fragments. With this aim, two-tailed Chi-square tests and Fisher exact tests were performed when the size of the samples required it, using the program SPSS v 15.0 and assuming a 95% confidence interval.

3. Results

DNA was extracted from 41 samples belonging to 31 individuals from La Cova des Pas, 7 of them with both extraction methods. All the samples have been taken into consideration and considered as independent to carry out the statistical analysis because, as seen in individuals 21 and 22, the behaviour of the samples under different extractions are not necessarily coincident (Supplementary data).

Genetic material could be obtained from 30 samples (73.17%). The rate of success was high in 10 samples (33.33%) and medium in the remaining 20 (66.6%).

Nineteen samples were extracted from teeth, and the rate of success in DNA retrieval was high in 4 (21.05%) and medium in 12 (63.15%). Concerning the extraction from bone (22 samples), this rate was high in 6 (27.27%) and medium in 8 (36.36%) (Table 1).

Using the phenol–chloroform method, 15 samples were processed. The 10 amplified had a medium rate success amplification (66.66%). Obtained amplicons were not longer than 150 bp in any case. A total of 26 samples were extracted with the QIAamp

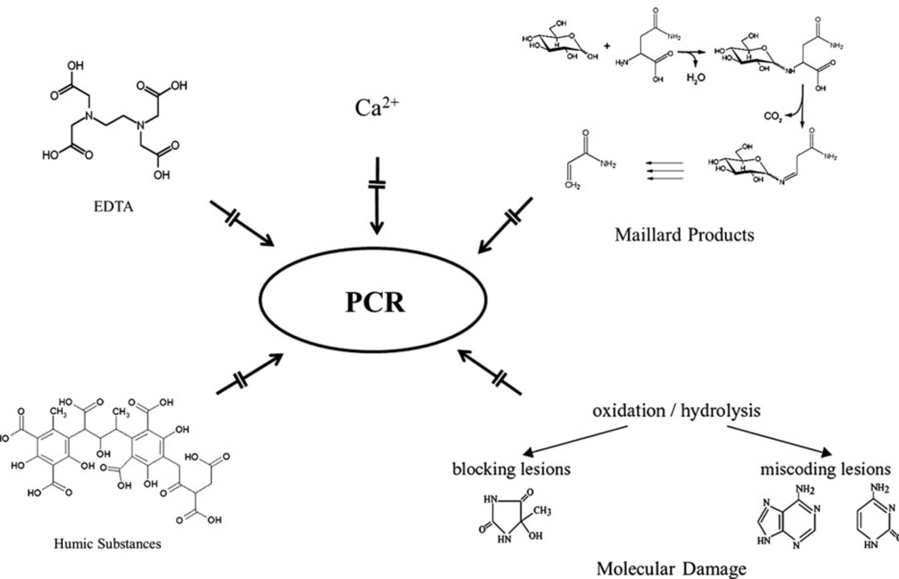

Fig. 3. Main inhibitory elements. This is a simple scheme of the inhibitors most likely to have influenced this study.

method. Ten samples showed a high rate of success (38.46%), and the same percentage presented a medium rate of success (Table 2).

3.1. Statistical analysis

Longer fragments could be amplified using the QIAamp method than using the phenol–chloroform method (Fisher exact test p: 0.011). There is a significant difference in the kind of piece selected depending on the method used (Chi-square test p: 0.011) but not in the rate of success obtained in DNA recovery (Chi-square test p: 0.274) and the presence or absence of results (Chi-square test p: 0.138). The methods show significant differences in the overall rate of success (null, medium or high) (Fisher exact test p: 0.006) but not in the absence or presence of results, whatever the length of the recovered segments (Chi-square test p: 0.327).

To properly compare both methods, the same 7 individuals were compared. The overall rate of success did not show significant differences between both methods, either in the presence or absence of genetic material (both Fisher exact tests p: 0.462), although a tendency towards better recovery results with the QIAamp method can be seen. The QIAamp method allowed the recovery of the majority of the required fragments in the 7 individuals, using in one case the primers to amplify the full-length sequence and, in the remaining six, the ones to amplify the shorter fragments. Finally in the phenol–chloroform method, 2 samples did not work and in the remaining 5, middle-sized fragments could be recovered.

4. Discussion

It is already known that the preservation of DNA can be more linked to the temperature and the environmental conditions of the site than to its age (Poinar, 2002). Moreover, the complex interaction of the biological, chemical, and physical taphonomic factors produces extensive variation within and among different burial sites (Lamers et al., 2008). Some of the substances found in the environment of each particular site can act as inhibitors for the DNA amplification. In the case of La Cova des Pas, in spite of the remarkable external preservation of the bodies and artefacts, the state of conservation of the bone material was very deficient as it contained little and heavily deteriorated collagen (Van Strydonck et al., 2010). When the routine protocol, the phenol–chloroform method (Malgosa et al., 2005) was applied, the results were disappointing, and an inhibition of the amplification of the samples seemed to be a real possibility. In order to improve results, another extraction method known to be a better technique to eliminate some of the inhibitors that these kind of samples might carry was applied. Although phenol–chloroform allows the recovery of a higher DNA quantity (Rohland and Hofreiter, 2007), it can co-purify with some substances which can act as PCR inhibitors (Montiel et al., 1997). Some proceedings can eliminate or attenuate the inhibition, and they can be carried out before the performance of the PCR in order to recover DNA with the best possible quality (Montiel et al., 1997; Montiel et al. 2001). In this sense, there has been a development of new protocols that enhance the purity of the recovered DNA: the silica based purification methods (Boom et al., 1990, modified by Höss and Pääbo, 1993) in which the silica virtually binds to every molecule of DNA (Höss, 1994). In this study, one of these, the QIAamp kit (QIAgen) was applied to try to improve the results and to compare the results obtained with both procedures. With regards to both extraction methods used, significant differences in favour of the use of the QIAamp method were found, because it permits to recover longer size fragments in La Cova des Pas site. Therefore, the substances co-purified with the phenol–chloroform method could have interfered in DNA amplification at several levels, leading to different degrees of attenuation and even to complete

Table 1
Rate of success.

Material	Null	Frequency (%)	Medium	Frequency (%)	High	Frequency (%)	Total
Tooth	3	15.78	12	63.15	4	21.05	19
Bone	8	36.36	8	36.36	6	27.27	22
Total	11		20		10		41

Table 2
Results obtained for each extraction protocol used.

Extraction method	Results	Frequency (%)	Medium	Frequency (%)	Large	Frequency (%)	Total
Phenol–chloroform	5	33.33	10	66.66	0	0	15
QIAamp	6	23.07	10	38.46	10	38.46	26
Total	11		20		10		41

inhibition (Moreira, 1998). The QIAamp kit action removing the inhibitors could have compensated for the decrease in the total amount of DNA recovery.

As DNA shows a high affinity to the hydroxyapatite molecules present inside them, dental and bone tissues were the material used in this study (Freilfelder, 1982; Sambrook et al., 1989). The enamel, considered the hardest tissue in the organism, constitutes an additional layer of protection for teeth. Moreover, the durability and resistance of hydroxyapatite crystals in dental tissue is higher than in bone tissue due to their higher concentration (DeGusta et al., 1994). Thus, they are chosen as the main source of DNA in ancient anthropologic studies. Nevertheless, statistical analysis carried out in Cova des Pas study do not show significant differences between teeth and bones. Moreover, in both kinds of tissues, the size of recovered fragments descends in frequency as the length of the amplicon increases, showing an appropriate molecular behaviour for ancient DNA, although this subject is still a matter of discussion (Krings et al., 2000; Kolman and Tuross, 2000).

When the two methods are compared on the same 7 individuals, no statistical differences are found although a tendency to the recovery of longer fragments with QIAamp method can be seen. Nevertheless, when all the samples in the study are considered, the QIAamp extraction method has proven to improve the length of the recovered genetic material. Therefore, QIAamp offers better results for this analysis.

Focusing on the inhibitors' nature, these substances can be from a very different origin, and it is likely that ancient samples could display a single inhibitor or a combination of them (Montiel et al., 1997). They can be present in almost all kinds of ancient tissues. Some of the most well-known inhibitors are the Maillard substances related to a chemical reaction between an amino acid and a reducing sugar (Pääbo, 1989), the humic substances, calcium ions and chelating agents such as EDTA. According to Higuchi (1992), in some cases they could be residues of porphyrins or their breakdown products, which are present in many living tissues as blood.

Maillard substances, humic substances, calcium ions, porphyrins, or breakdown products in general, could be present in the samples of La Cova des Pas given the dynamics in this burial cave. The fact that the bodies were wrapped with leather and piled up over a deathbed composed of wood and other vegetation suggests a great amount of decomposition products. Maillard products, which are the result of the reduction of sugars in an early stage with a compound bearing a free amino group (Pääbo, 1990; Montiel et al., 1997; Martins et al., 2001), can block the PCR reaction due to the generation of cross-links to the DNA molecule (Pääbo, 1989). Therefore, the recovery of long fragments would not be possible if these substances were present. This pattern can be seen in this study because fragments no longer than 150 bp could be recovered with the phenol–chloroform method; in contrast, a 230 bp fragment could be amplified approximately in half the samples with the QIAamp method. If the problem generically affected DNA, the damage would equally affect the results whatever the employed method was.

The colour of the recovered samples could also indicate the presence of humic substances, which tend to form aggregates with the nucleic acid molecules, and in a concentration of 100 ng/μl are enough for complete PCR inhibition (Tuross, 1994). Tannins are another possible source for inhibitory substances, especially in the case of bones (Hagelberg and Clegg, 1991; Cooper et al., 1992). In this sense, in the samples extracted with the phenol–chlorophorm method, the colour ranged from light to dark yellow in most cases, while in the samples extracted with the QIAamp method a transparent colour was the norm. Humic substances can give a characteristic pigmentation to an extract (Stevenson, 1982). Finally, it is thought that the porphyrins or their by-products might not pass over the Centricon-30 membrane because they tend to form aggregates (Isidro and Malgosa, 2003). Nevertheless, analytical tests will have to be done in order to determine the exact nature of the inhibiting substances.

However, there are some kinds of inhibitory substances that can be discarded as possible sources of the inhibition, like EDTA and calcium ions. The EDTA molecule is used to remove the mineral component because it is a powerful kidnapper of calcium and thus it is used for the demineralization of the bone matrix. This permits a higher exposure of the DNA and an efficient contact between the buffer components and the DNA molecules and it diminishes contaminant components in the extract. In addition, EDTA offers advantages as acting in the inactivation of nucleases by the capture of divalent cations, Mg^{2+} or Ca^{2+}, which act as cofactors in cell reactions and can therefore still be present in the recovered remains, avoiding the degradation from the recovered DNA (Loreille et al., 2007). Despite these advantages, it tends to precipitate with the DNA and cause PCR inhibition (Jiménez and Morera, 1999), so it has to be fully removed before the amplification procedure. With regards to the present study, the habitual procedure carried out indicates that the phenol–chloroform protocol used (Malgosa et al., 2005) is suitable for ancient DNA studies, as both EDTA and the calcium ions are correctly removed during the extraction procedure (Solórzano, 2006; Díaz, 2009).

5. Conclusion

The context of burials is very important to understand the possible interference of substances from soil and the biochemical and molecular processes. The Cova des Pas is a funerary cave with at least 66 bodies deposed through a hundred years. Decomposition of these bodies and soil components created an environment that produced PCR inhibitors that resulted in a decrease in the quality of the results when applying the phenol–chloroform method. A method based in silica-columns has been useful to partially solve this problem. Testing the best extraction procedure for each site seems the most reasonable way of proceeding, as there is not a universal method which can be carried out to obtain the highest degree of success for all the samples and archaeological sites.

This work supports the results obtained in a previous study (Jiménez, 2009), stating that the best method for DNA purification is the usage of the kit QIAmp (QIAgen) when inhibitors are present. This also means that there is not necessarily a positive correlation between the presence of organic remains and the integrity of the cellular components, as stated by Poinar (2002).

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.quaint.2011.04.026.

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4.1.1 Supporting Information

SUPPLEMENTARY DATA

Table 1: Description of primers used and their references

	Region	Sequence	References
HVRI	16190 - 16420	5'-CCCCATGCTTTACAAGCAAGT -3'	Montiel et al. 2001
		5'-TGATTTCACGGAGGATGGTG -3'	Vigilant et al. 1991
	16190 - 16339	5'-CCCCATGCTTTACAAGCAAGT -3'	Montiel et al. 2001
		5'-GTGCTATGTACGGTAAATGG-3'	Díaz 2010
	16292 - 16420	5'- CACCCTTAACAGTACATAGTAC-3'	Montiel et al. 2001
		5'- TGATTTCACGGAGGATGGTG -3'	Vigilant et al. 1991
Coding Region	-7025 *Alu I*	5'-CCGTAGGTGGCCTGACTGGC-3'	Montiel et al. 2001
		5'-TGATGGCAAATACAGCTCCT-3'	
	-13704 *Bst*0I	5'-TCACCCTAACAGGTCAACC-3'	Montiel et al. 2001
		5'-ATGAGAAATCCTGCGAATAG-3'	
	-9052 *Hae* II	5'-ACGCCTAACCGCTAACATTAC-3	Montiel et al. 2001
		5'-AGATGATAAGTGTAGAGGGAAG-3'	
	+11718*Hae*III	5'-AGTAACAGCCATTCTCATCC-3'	Díaz, 2009
		5'-GAGTGCGTTCGTAGTTTGAG-3'	
	+15606 *Alu*I	5'-CCCACATCAAGCCCGAATG-3'	Montiel et al. 2001
		5'-GATGAGGATGGATAGTAATAGG-3'	
	+12308 *Hinf* I	5'-CACAAGAACTGCTAACTCATGC-3'	Izagirre, 1998
		5'-ATTACTTTTATTTGGAGTTGCACCAAGATT-3'	
	-8994 *Hae*III	5'-TTCTTACCACAAGGCACACC-3'	Montiel et al. 2001
		5'-AGGTGGCCTGCAGTAATGT-3'	
	+14465 *Acc*I	5'-ACACTCACCAAGACCTCAA-3'	Díaz, 2004
		5'-GGGAGGTTATATGGGTTTAA-3'	

Table 2: Amplification results of each individual

Extraction Method	Individual	Long Fragment	Short Fragment	Tissue
Phenol-Chloroform	4	-	+	Tooth
	6	-	+	Tooth
	11	-	-	Tooth
	22	-	-	Bone
	22	-	-	Bone
	23	-	-	Tooth
	25	-	+	Tooth
	25	-	+	Tooth
	26	-	+	Tooth
	35	-	+	Tooth
	41	-	+	Tooth
	46	-	+	Tooth
	47	-	+	Tooth
	47	-	+	Bone
	50	-	-	Bone
QIAamp kit	2	+	+	Bone
	4	+	+	Tooth
	6	-	+	Bone
	9	+	+	Bone
	17	-	+	Tooth
	21	+	+	Bone
	21	-	+	Bone
	22	-	+	Bone
	22	-	-	Tooth
	26	-	+	Tooth
	27	-	-	Bone
	30	+	+	Tooth
	32	-	-	Bone
	34	-	-	Bone
	37	`+	+	Bone
	41	-	+	Bone
	43	-	+	Bone
	46	-	+	Tooth
	50	-	+	Bone
	53	-	-	Bone
	57	+	+	Tooth
	57	+	+	Bone
	61	-	-	Bone
	62	+	+	Tooth
	62	+	+	Bone
	69	-	+	Bone

4.2 The Presence of Nuclear Families in Prehistoric Collective Burials Revisited: The Bronze Age Burial of Montanissell Cave (Spain) in the Light of aDNA

The Presence of Nuclear Families in Prehistoric Collective Burials Revisited: The Bronze Age Burial of Montanissell Cave (Spain) in the Light of aDNA

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KEY WORDS mtDNA; ancient remains; haplogroup J; amelogenin

ABSTRACT Ancient populations have commonly been thought to have lived in small groups where extreme endogamy was the norm. To contribute to this debate, a genetic analysis has been carried out on a collective burial with eight primary inhumations from Montanissell Cave in the Catalan pre-Pyrenees. Radiocarbon dating clearly placed the burial in the Bronze Age, around 3200 BP. The composition of the group—two adults (one male, one female), one young woman, and five children from both sexes—seemed to represent the structure of a typical nuclear family. The genetic evidence proves this assumption to be wrong. In fact, at least five out of the eight mitochondrial haplotypes were different, denying the possibility of a common maternal ancestor for all of them. Nevertheless, 50% of the inhumations shared haplogroup J, so the possibility of a maternal relationship cannot be ruled out. Actually, combining different analyses performed using ancient and living populations, the probability of having four related J individuals in Montanissell Cave would range from 0.9884 to 0.9999. Owing to the particularities of this singular collective burial (small number of bodies placed altogether in a hidden cave, the evidence of non-simultaneous interments, close dating and unusual grave goods), we suggest that it might represent a small group with a patrilocal mating system. Am J Phys Anthropol 146:406–413, 2011. © 2011 Wiley-Liss, Inc.

Funerary practices during the Catalan Bronze Age were characterized by collective burial, either in caves, in megalithic structures or in common graves with different morphologies. The lack of grave goods also seems to be a characteristic of these collective inhumations. Coastal sites mainly show megalithic constructions to protect these burials, while the use of caves, caverns or dolmens was more usual in mountain areas (Armentano and Malgosa, 2002, 2003, 2004; Rodríguez and Palomo, 2004; Francès, 2007; Armentano et al., 2010). A debate emerged about the relationship between individuals buried in close association, and nuclear family ties are the main hypothesis proposed so far (López et al., 2005). In fact, there are many studies devoted to understanding family relationships in ancient times (Heyd, 2007; Bentley et al., 2008).

In 2005, an interdisciplinary team recovered eight human skeletons from a natural hidden cave in the Catalan Pre-Pyrenees. The archaeological context and radiocarbon dating (Table 1) pointed towards a prehistoric Bronze Age burial (Armentano et al., 2007).

The cave is located in Sant Joan de Montanissell, Lleida (Fig. 1), Spain. This cave seems to be an exception in the context of the other burial sites found in the region, mainly constituted by megalithic structures and graves with an easy access (Armentano et al., 2007). The Montanissell Cave is hidden and it is difficult to reach the funerary chamber, since a narrow passage that is 40 m long must be crossed and afterwards descend a well that is 28 m deep. In the base of the well, the skeletons were placed directly into the ground. No sediments covered them, with the exception of those nearest to the walls, which were covered by a thin layer of calcite (Fig. 2). In general, the skeletons were well preserved and showed a high percentage of anatomic articulations. The individuals were placed in a flexed position throughout the whole burial chamber.

Anthropological diagnoses during the fieldwork showed that the group comprised two adults (a man and a woman), and six non-adult individuals (Table 1) (Armentano et al., 2007).

Some individuals were ornamented with bronze bracelets, necklaces and dentalia tubular pieces. An excep-

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TABLE 1. Age, sex and radiocarbon dating of the individuals

Ind.	Age (years)	C^{14} dating	Amelogenin size	SRY	Sex (genetic data)	Sex (morphological data)
E1	35–39	3180 ± 40 BP	106 bp	No	♀	♀
E2	6–8	3190 ± 40 BP	106,112 bp	Yes	♂	IND
E5	6–8	3200 ± 40 BP	106 bp	No	♀	IND
E7	40–49	3180 ± 40 BP	106,112 bp	Yes	NR	♂
E8	19–21	3180 ± 40 BP	NR	NR	♀	♀
E9	13–15	3240 ± 40 BP	106 bp	No	♀	♀
E10	9–11	3260 ± 40 BP	NR	NR	NR	IND
E11	11–13	3140 ± 40 BP	106 bp	No	♀	IND

NR, No Results; IND, Indeterminate.

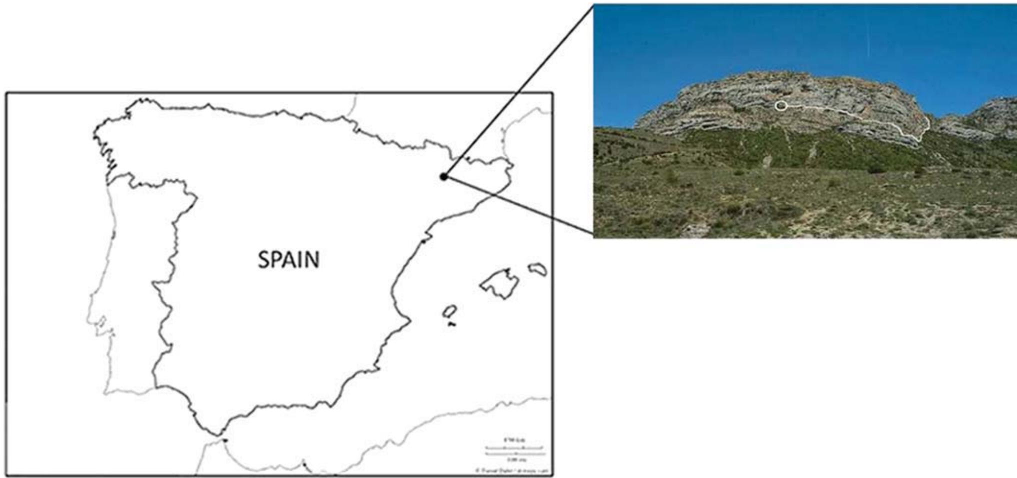

Fig. 1. Situation of Montanissell Cave in the Northeast of Spain. The location of the cave and the path to achieve it is showed on the right image. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Fig. 2. Picture of individual E11, recovered with a thin layer of calcite. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

tional bronze diadem was also found between the two skeletons, the woman and a child, placed in the middle of the chamber. These ornaments seem to be connected to the high development of Blechkreis bronze metallurgy, which, during the early Bronze Age, took place in East France, Western Switzerland and Northern Italy. The diadem found in Montanissell presents affinities with another from the Rhone culture (López et al., 2005). Remains of caprinae and fragmentary pottery were also found near the skeletons.

Some occasional superposition of the bones observed during the fieldwork and radiocarbon dating indicate that the burials were not simultaneous, but most probably over a short period of time (maximum 100 years). Moreover, the repetition of some of the funerary patterns seems to demonstrate that some kind of "identity" is shared among the group or community that carried out the burials.

This funerary cave is so singular in relation to the funerary ritual and the individuals' age structure that the most likely explanation seems to be that it was a funerary place for close-relatives. It could be interpreted as a nuclear family structure (a family group consisting of a father and mother and their children), like some other small prehistoric burials, where the adult male and female might have been buried together with their descendants, as has occurred in the scarce number of genetically tested cases (Haak et al., 2008).

Although, until recently, women and children have traditionally been neglected in archaeological interpretations (Conkey and Spector, 1984; Scott, 1997; Olsen, 1998; Perry, 2005; Sofaer, 2006; Baxter, 2008), to understand small groups or family dynamics in ancient times, it is important to know gender and age specific roles. It is essential to know the sex of non-adult individuals to discard a sex-bias related to special conditions inside the group. In relation to the role of women, archaeological data can support specific status, for example, a special grave good for an adult woman would indicate the spe-

TABLE 2. Primers used to amplify and sequence the HVRI of mtDNA

Fragment 1 (404 bp)	L-15996: 5′-CTCCACCATTAGCACCCAAAGC-3 (Vigilant et al., 1991)
	H-16401: 5′-TGATTTCACGGAGGATGGTG-3′ (Vigilant et al., 1991)
Fragment 2 (156 bp)	L-16030: 5′-CATGGGGAAGCAGATTTGGG-3′ (Present study)
	H-16230:5′-GATAGTTGAGGGTTGATTGCTG-3′ (Present study)
Fragment 3 (106 bp)	L-16060: 5′-AGATTTGGGTACCACCCAAG-3′ (Knight et al., 2004)
	H-16187: 5′-GGGGTTTTGATGTGGATTGG-3′ (Knight et al., 2004)
Fragment 4 (191 bp)	L-16209: 5′-CCCCATGCTTACA€€€AGCAAGT-3′ (Montiel, 2001)
	H-16401: 5′-TGATTTCACGGAGGATGGTG-3′ (Vigilant et al., 1991)
Fragment 5 (102 bp)	L-16209: 5′-CCCCATGCTTACAAGCAAGT-3′ (Montiel, 2001)
	H-16339: 5′-GTGCTATGTACGGTAAATGG-3′ (Montiel, 2001)
Fragment 6 (87 bp)	L-16313: 5′-CACCCTTAACAGTACATAGTAC-3′ (Montiel, 2001)
	H-16401: 5′-TGATTTCACGGAGGATGGTG-3′ (Vigilant et al., 1991)

cial relevance of this individual inside the group. Moreover, this could be reflected in genetic terms as the motherhood of some of the individuals. Finally, the difficult access to the cave (unusual in the area of Montanissell) and the ornaments found pointed to a foreign group and/or with contacts with the other side of the Pyrenees Mountains.

To investigate these points, genetic tools are required. Despite the improvements in aDNA technology for using nuclear genetic information in terms of kinship (Hansen and Gürter, 1983; Alt et al., 1995; Hummel and Herrmann, 1997; Schultes et al., 2000; Scholz et al., 2001; Keyser-Tracqui et al., 2003; Gilbert et al., 2007; Bouwman et al., 2008; Haak et al., 2008; Vanek et al., 2009; Lalueza et al., 2011) and sex determination (Vaňharová and Drozdová, 2008; Hisham et al., 2009), mitochondrial DNA (mtDNA) is still the main resource of genetic information in ancient contexts since it is easier to recover (Shuster et al., 1988).

Taking into account the facts exposed previously, the genetic analysis of the individuals of Montanissell Cave was performed and our main goals were: (i) to understand the genetic affiliation of the group members and, eventually, their family relationship; (ii) to find out whether there was a common female lineage inside the Cave; and (iii) to get more in depth information about the origin of the group.

MATERIALS AND METHODS

Samples and DNA extraction

DNA was obtained from the teeth and bones of all the individuals buried in Montanissell Cave. Well-preserved dental samples were selected. Moreover, when teeth were not available, bone samples were collected. During the excavation process, samples were taken using gloves and face masks to minimize the risk of contamination; they were then kept in cold conditions. Afterwards, samples were carried to the laboratory dedicated to paleogenetic studies at the Universitat Autònoma de Barcelona where they were processed.

Two samples from each individual were used in independent extractions and by different investigators to ensure that the results were fully duplicated. Moreover, individuals E2 and E9 underwent a third extraction which also brought coincident results with the previous ones. For DNA extraction, 0.5 g of powder was extracted from teeth pulp cavities; when bones were used, 1 g of powder was collected from the internal compact tissue. After DNA treatment and extraction (as described in Malgosa et al., 2005), purification of the samples was performed with a JetQuick PCR Purification kit (Genomed, Löhne, Germany) to remove any possible inhibitors that the samples might carry.

Sex determination

For genetic sex determination, X and Y amelogenin loci and the SRY gene (sex-determining region Y gene) were analyzed using primers and conditions described respectively by Sullivan et al. (1993) and Santos et al. (1998).

Mitochondrial DNA analysis

For each sample, the mtDNA hypervariable region I (HVRI) was amplified and sequenced and coding region informative polymorphisms for haplogroup assignment were analyzed by PCR-RFLPs.

The PCR reactions were carried out in a final volume of 50 μl and AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Foster City, USA) was used. Each PCR reaction consisted in an initial denaturation step (5 min at 94°C) followed by 39 cycles of PCR (50 s at 94°C, 1 min at annealing temperature depending on the region to amplify, and 1 min at 72°C) and a final extension step of 5 min at 72°C, or of 10 min if the amplified segment must be cloned. Amplified fragments were then visualized with Ethidium Bromide staining in a 3% agarose gel. To analyze the HVRI, fragments ranging from 87 to 404 bp were amplified using six overlapping fragments (Table 2). These were subsequently sequenced and, in some samples, cloned. Sequence reactions were carried out using the sequencing kit BigDye Terminator v.3.1 (Applied Biosystems, Carslbad, USA) according to the manufacturer's specifications and run in an ABI 3130XL sequencer. The clonation process was carried out using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carslbad, USA).

For coding region analysis, 10 coding region segments, determining the 10 European haplogroups, were submitted to PCR-RFLPs. Restriction sites and the primers used to amplify each specific fragment of the coding region are shown in Table S1 (Díaz, 2004, 2009; Izagirre, 1998; Montiel, 2001).

Samples were assigned to haplogroups using the combined information of HVRI and coding region variation following the phylogenetic classification updated by van Oven and Kayser (2009).

Authentication of results

To authenticate the results, the recommended criteria concerning sterility, reproducibility, clonation, characterization of the investigators' haplotype, coincidence of associated markers and diversity of the results were ful-

TABLE 3. Results obtained from the mtDNA in both extractions and inferred consensus haplotype

	Extract I		Extract II		
Ind	HVRI (16,210–16,400)	Haplog	HVRI (16,051–16,400)[a]	Haplog	Consensus
E1	224C; 311	K	224C; 311	K	K
E2	CRS	U	CRS	U	U
E5	CRS	J	069T; 126C	J	J/J1c
E7	CRS	J	Unable to obtain results	J	J
E8	298C	V	298C	V	V
E9	CRS	J	069T; 126C	J	J/J1c
E10	Unable to obtain results	U	192T; 256T; 270T; 320T; 399G	U	U5a1c
E11	CRS	J	069T; 126C	J	J/J1c

[a] This sequence was obtained with the combination of five overlapping segments, except in individual E11 where a sixth segment permitted the recovery of a segment encompassing from position 15,997 to 16,400 (see fragments 1 to 6 in TABLE 2).

filled. An integrative approach for human population studies was used, where the flexibility and the intelligent use of authentication criteria was applied (Pääbo et al., 2004; Gilbert et al., 2005; Montiel et al., 2007).

Data analysis

For comparative purposes haplogroup frequencies of 10 extinct and 27 extant European populations were compiled (populations, sample size, and references are listed in Table S2 (Alfonso-Sánchez et al., 2008; Álvarez et al., 2007; Baasner et al., 1998; Bertranpetit et al., 1995; Chandler et al., 2005; Corte-Real et al., 1996; Díaz., 2009; Dimo-Simonin et al., 2000; Dubut et al., 2004; Francalacci et al., 1996; García et al., 2011; Haak et al., 2005; Handt et al., 1994; Hofmann et al., 1997; Izagirre, 1998; Lutz et al., 1998; Mogentale-Profizi et al., 2001; Montiel, 2001; Parson et al., 1998; Pereira et al., 2000; Pfeiffer et al., 1999; Picornell et al., 2005; Pult et al., 1994; Richard et al., 2007; Richards et al., 1996, 2000; Sampietro et al., 2005, 2007; Tagliabracci et al., 2001). For our data and for those ancient populations for which complete data was available (five populations), a correction of the haplogroups frequencies for the possible existence of kinship was performed. A similar procedure to that described by Vernesi et al. (2004) was undertaken: when an haplotype is represented by two or more individuals buried in the same tomb or in collective burial, only one of them was considered. Haplogroup frequencies were used for Gene diversity ($\hat{H}$) calculation (Nei, 1987). Moreover, the exact test of population differentiation, as described in Rousset and Raymond (1995) was used to compare the haplogroup frequencies among pairs of populations. The previously mentioned analyses were performed using Arlequin v. 3.11. (Excoffier et al., 2005).

Bayesian 0.95 credible region (95% CR) for haplogroup frequencies was calculated using the SAMPLING program (Macaulay, personal communication).

RESULTS

We were able to retrieve ancient DNA and reproduce unambiguous results for all the individuals from Montanissell Cave. The genetic sex determination was possible in six individuals (Table 1). Morphological and molecular genetic sex diagnoses were concordant in the three individuals where the morphological sex estimation was possible (E1, E7, and E9). The individual E8 could only be assigned to the female gender owing to the morphological data. In addition, we were able to determine the sex of the three non-adult skeletons: the genetic analysis showed a boy (E2) and two girls (E5 and E11). Combining genetic and morphological data, in the cave there were two adults (one man and one woman), a young woman and five non-adults: three girls, one boy, and one whose sex could not be established.

Table 3 shows the results of the HVRI sequences and the haplogroup obtained by PCR-RFLPs analysis for each individual. In the first extraction, sequences of 191 bp were obtained for seven of the individuals, while individual E10 could just be characterized using the RFLPs analysis. In the second extraction, using the combination of four overlapping fragments, sequences of 404 bp were obtained in seven individuals, and a sequence of 191 bp was obtained in individual E7, which could only be sequenced without ambiguity between positions 16,210 and 16,400. Finally, a third extraction was carried out in individuals E2 and E9 giving coincident results with previous extractions.

Sequence and PCR-RFLPs coincided in seven individuals. In individual E7, whose HVRI haplotype could not be fully determined, the PCR-RFLPs study in two independent extractions confirmed that it belonged to haplogroup J and also denied the possibility that it belonged to any of the other nine European haplogroups (the whole PCR-RFLPs study was carried out in both extractions).

The clonation process was applied to the most informative HVRI regions of all individuals except for individual E7, because the region with the two defining mutations of its haplogroup (J: 069T, 126C) could not be amplified. The results further verify that the data of the sequences obtained represent the consensus in each individual as shown in Table S3 (Supporting Information).

No mitochondrial profiles of the individuals from the cave matched those of any of the investigators involved in the study, which is known to constitute the main source of natural contamination (Herrmann and Hummel, 1994; Keyser-Tracqui et al., 2002)

Four different mtDNA haplogroups across these eight individuals were identified (Table 3): four individuals belong to haplogroup J, two to haplogroup U, one to haplogroup V and one to haplogroup K. Moreover, five different haplotypes were found. Concerning the individuals that belong to J haplogroup, for one of them we were not able to obtain the HVRI sequence between positions 16,051 and 16,209, while positions ranging from 16,051 to 16,400 (404 bp) show that the other three individuals (an adult male and two subadults) share the same haplotype (16069T, 16126C) and can be maternally related. On the other hand, individuals E2 and E10, both bearing haplogroup U, present different HVRI haplotypes, and are therefore not maternally related.

Individuals from Montanissell Cave share their sequences with ancient and recent populations consid-

ered for comparison (Table S2, Supporting Information) with the exception of individual E10, which belongs to subhaplogroup U5a1c and bears transitions at positions 16,192, 16,256, 16,270, 16,320, and 16,399. This haplotype, however, was previously reported in other European and North African populations not used for comparison (Empop database).

The haplogroup frequencies obtained for Montanissell and in populations used for comparison are presented in Table S2 (Supporting Information). The haplogroup diversity (Nei, 1987) of Montanissell is 0.7500 ± 0.1391 and it would increase to 0.9000 ± 0.1610 if we excluded those individuals that could be maternally related. These values are similar to the average obtained in ancient (0.7577 ± 0.1201 using uncorrected frequencies and 0.8160 ± 0.0708 using corrected frequencies) and modern (0.7499 ± 0.0572) populations considered for comparison. Considering uncorrected haplogroup frequencies, the exact test of population differentiation indicates that Montanissell presents significant differences with 33 of the populations used for comparison and is only similar to the Catalan Neolithic necropolis from Granollers (Gra) and the Basque Bronze Age burial site of Cueva Urratxa (CU), the Majorcan Iron Age necropolis of Son Real (Sre) and the Catalan Modern Age necropolis of Plaça Vella (PV). If corrected frequencies were used, Montanissell presents a similar haplogroup composition to more than half of the populations used for comparison.

DISCUSSION

Archaeological studies in antiquity show that populations were organized in small groups, which were generally family groups. On the basis of historical evidence, epigraphy (Saller and Shaw, 1984; Rawson and Weaver, 1999), iconography (Olsen, 1998) or ethnographical studies, it seems a reasonable hypothesis that the continuity of the nuclear family goes back much further in time and that it was characteristic of many regions in Western Europe (Martin, 1996).

In prehistoric contexts, the concept of family is often associated with the nuclear family in terms of an occidental model of society, with very close blood ties, without knowing whether this concept from modern western societies was suitable to the people of antiquity. In fact, few studies have been devoted to investigating genetic kinship in ancient populations (Alt et al., 1995; Hummel and Herrmann, 1997; Schultes et al., 2000; Scholz et al., 2001; Keyser-Tracqui et al., 2003; Gilbert et al., 2007; Bouwman et al., 2008; Haak et al., 2008; Vanek et al., 2009; Lalueza et al., 2011), and even less have proven a direct child-parent relationship. The oldest molecular genetic evidence of a nuclear family was detected in one Neolithic burial discovered near Eulau, Germany (Haak et al., 2008).

Taking into account osteological and archaeological data, the collective Bronze Age burial from Montanissell Cave also seemed to fit perfectly into the structure of a nuclear family. There were two adults, a man and a woman, a young woman, and five non-adult individuals, all of which had the same burial process and common grave goods. Nevertheless, genetic analyses have proven the assumption that this group represented a family nucleus to be wrong, since mtDNA analysis reveals that the adult female could not be the mother of any of the non-adults buried in the cave. Although three of the non-adult individuals and the adult male share the same mtDNA haplogroup, at least five different mtDNA lineages were found in the cave. In fact, the study by Haak et al. (2008) states that while kinship relationships were very important in that period in the treatment of the dead, there were also cases where the emphasis was also put on the supragenetic social connections between the individuals buried together. For example, in that study, one of the four multiple burials contained two siblings with an adult female that were not maternally related. Our results, therefore, in agreement with other studies (Gilbert et al., 2007; Haak et al., 2008), suggest that the nuclear family, as we know it today, was not a universal model among ancient populations. Notwithstanding, as is to be discussed later, some of the individuals inside the group could share genetic ties, in what can be seen as an extended family structure.

Montanissell Cave holds some particular aspects: fifty percent of the individuals bear haplogroup J, while haplogroup H, the most frequent haplogroup in all European populations nowadays, is absent. The high frequency of haplogroup J observed in Montanissell is also a characteristic of all the Catalan and Basque prehistoric populations analyzed so far (Table S2, Supporting Information) (Izagirre, 1998; Sampietro et al., 2005, 2007). If we pool the data of prehistoric Catalan populations, the frequency of J in ancient populations would be 14.3 or 12.5% considering respectively uncorrected and corrected haplogroup frequencies. Considering this range for J frequency, the probability of having four unrelated J individuals in the cave would range from 0.0002 to 0.0040.

The existence of close kinship in ancient burials would compromise all the population comparisons. In this sense, to remove possible effects of kinship on the estimated population statistics, some authors introduce a correction for the possible existence of kinship (see for example Vernesi et al., 2004; Alzualde et al., 2005). They consider that when an haplotype is represented by two or more individuals interred in the same burial group, only one of them should be considered. Although we corrected our data using a similar criterion, in our opinion the systematic application of this approach is questionable, since other issues, such as the type of burials and haplotype frequencies should be taken into account. When considering the possible existence of kinship inside a necropolis, we should differentiate between individuals buried together or in close proximity inside a cemetery with several tombs, and individuals buried altogether in a collective burial that represents the necropolis of a particular population at a given time, as in the case of Montanissell Cave. Kinship should probably be greater between the individuals in the former case than in the latter. Moreover, the presence of kinship would depend on whether an haplotype is common or uncommon in the studied population. In this sense, the true frequency of haplotypes is unknown for ancient populations, and it makes it even more complicated to apply such simple criteria to remove the effect of possible kinship.

For the current Catalan population, different studies gave very different estimations of haplogroup J frequency (Table S2, Supporting Information); the majority of studies (Côrte-Real et al., 1996; Álvarez et al., 2007; García et al., 2011) report low values of J haplogroup (mean value of 3.75%) while Montiel (2001) identifies 13% of the individuals as belonging to this haplogroup (Table S2, Supporting Information). If haplogroup J is infrequent in the current Catalan population, as reported by the majority of studies, it would suggest that the presence of four J individuals in the Montanissell Cave could be a sign that they share a genetic link since

the probability of having four unrelated J individuals in the cave would be 0.0001. However, in accordance with the kind of ornaments associated to the burials (Armentano et al., 2007), the group buried in Montanissell Cave appears to have cultural connections with populations from the South of France at least, more precisely from the Rhone region (López et al., 2005). In that region, the frequency of J is 13.04% in the present day population, and the probability of having four unrelated J individuals in the Cave would increase to 0.0116.

Combining the different analysis performed using ancient and current populations, the probability of having four related J individuals in the Montanissell Cave would range from 0.9884 to 0.9999.

Thus, and despite the high diversity of mtDNA haplogroups found in the cave, the high frequency of J is probably an evidence of kinship. Unfortunately, nuclear DNA was retrieved with limited success, only for genetic sex determination, and there is no additional relationship information that would help to clarify the existence of kinship ties.

Our molecular analysis permits an insight into prehistoric social behavior, in relation to parent-offspring relations (whether or not nuclear family burials were the norm), kinship ties (whether or not the prehistoric communities followed an extended or a nuclear family model) and the marriage system (patrilocal vs. matrilocal). Montanissell collective burial shows high mtDNA variability inside a small group. Some individuals are probably relatives, although we do not know the degree of kinship. Therefore, together with the evidence of non-simultaneous interments, the cave, most likely, was used as an "attritional" cemetery (accumulated deaths of a population over a period of time, see Margerison and Knüsel, 2002) of an extended family. Moreover, the high diversity of such an ancient cluster and the short time spent among the successive burials suggests two possible scenarios: on the one hand, a small group with a patrilocal mating system, in which the integration of women was necessary to incorporate new haplotypes into the population; on the other hand, this could be a numerous and stable group. Attending to the unusual characteristics of the burial, the most likely hypothesis seems to be the former.

In conclusion, our study points towards a group of individuals that shared their existence and were not just connected by kinship, but also by culture, as shown by the common funerary ritual. So while the currently predominant structure is the nuclear family strongly conditioned by genetic ties in the Bronze Age, it could have been somewhat a looser structure, where cultural issues might have played an important role too. This is in accordance with the hypothesis suggested by Hill et al. (2011), in which our species' biological success would find its origin in cooperation with non-kin that would finally imply a higher degree of cultural transmission.

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4.2.1 Supporting information

Supplementary Information

TABLE I. Restriction sites studied for haplogroup determination

Restr.Site	Primers	Coordinates	Primer sequences
-7025 Alu I	L6968/H7052	6949-6968	5’-CCGTAGGTGGCCTGACTGGC-3’
	(Francalacci, p.c.)	7071-7052	5’-TGATGGCAAATACAGCTCCT-3’
-1715 Dde I	L1698/H1776	1678-1698	5’-TAGCCCCAAACCCACTCCAC-3’
	(Montiel, 2001)	1797-1776	5’-CTTTCCCTTTGCGGTACTATATC-3’
-13704 Bst0I	L13640/H13720	13621-13640	5’-TCACCCTAACAGGTCAACC-3’
	(Montiel, 2001)	13739-13720	5’-ATGAGAAATCCTGCGAATAG-3’
-9052 Hae II	L9020/H9082	9000-9020	5’-ACGCCTAACCGCTAACATTAC-3
	(Montiel, 2001)	9103-9082	5’-AGATGATAAGTGTAGAGGGAAG-3’
+11718 Hae III	L11650/H11771	11669-11650	5’-AGTAACAGCCATTCTCATCC-3’
	(Díaz, 2009)	11791-11771	5’-GAGTGCGTTCGTAGTTTGAG-3’
+15606 Alu I	L15561/H15625	15543-15561	5’-CCCACATCAAGCCCGAATG-3’
	(Montiel, 2001)	15646-15625	5’-GATGAGGATGGATAGTAATAGG-3’
+12308 Hinf I	L12237/H12309	12216-12237	5’-CACAAGAACTGCTAACTCATGC-3’
	(Izagirre, 1998)	12279-12308	5’-ATTACTTTTATTTGGAGTTGCACCAAGATT-3’
-4577NlaIII	L4538/H4621	4519-4538	5’-CACTCATCACACAGCGCTAAGC-3’
	(Izagirre, 1998)	4638-4621	5’-TGGCAGCTTCTGTGGAAC-3’
-8994 HaeIII	L8927/H9014	8908-8927	5’-TTCTTACCACAAGGCACACC-3’
	(Montiel, 2001)	8995-9014	5’-AGGTGGCCTGCAGTAATGT-3’
+14465 AccI	L14399/H14533	14417-14398	5’-ACACTCACCAAGACCTCAA-3’
	(Díaz, 2004)	14513-14533	5’-GGGAGGTTATATGGGTTTAA-3’

TABLE II. Populations used in comparative analysis. For each population, the haplogroup frequencies are displayed.

Population	Reference	Code	N (corrected)	Period	Haplogroup Frequency (corrected frequencies)								
					H	I	J	K	TX[1]	U	V	W	OT
Montanissell, Catalonia, Spain	Present study	Mont	8 (5)	Bronze Age	0.00 (0.00)	0.00 (0.00)	50.00 (20.00)	12.50 (20.00)	0.00	25.00 (40.00)	12.50 (20.0)	0.00 (0.00)	0.00 (0.00)
Granollers, Catalonia, Spain	Sampietro et al. 2007	Gra	11(8)	Neolithic	36.36 (25.00)	9.09 (12.50)	18.18 (12.50)	0.00 (0.00)	18.18 (25.00)	9.09 (12.50)	0.00 (0.00)	9.09 (12.50)	0.00 (0.00)
Pre-Roman Iberian, Catalonia, Spain	Sampietro et al. 2005	Pri	17(16)	Iron Age	52.94 (50.00)	0.00 (0.00)	11.76 (12.50)	5.88 (6.25)	5.88 (6.25)	17.65 (18.75)	0.00 (0.00)	0.00 (0.00)	5.88 (6.25)
Plaça Vella, Catalonia, Spain**	Montiel, 2001	PV	24	Modern Age	33.33	4.17	12.50	16.67	8.33	8.33	12.50	0.00	4.17
Catalonia 1, Spain	Montiel, 2001	SpCat1	90	Current	44.44	0.00	13.33	5.56	15.56	11.11	3.33	0.00	6.67
Catalonia 2, Spain	Corte-Real et al., 1996; Álvarez et al., 2007; García et al., 2011	SpCat2	80	Current	30.00	1.25	3.75	10.00	13.75	21.25	7.50	5.00	7.50
Pico Ramos, Basque Country, Spain*	Izagirre, 1998	Pra	24	Neolithic	37.50	0.00	16.67	16.67	16.67	12.50	0.00	0.00	0.00
SJAP Latinam, Basque Country, Spain*	Izagirre, 1998	Sjap	61	Neolithic	37.70	0.00	16.39	22.95	4.92	18.03	0.00	0.00	0.00
Cueva de Urratxa, Basque Country, Spain*	Izagirre,1998	CU	5	Bronze Age	40.00	0.00	20.00	0.00	0.00	40.00	0.00	0.00	0.00
Basque Country 1, Spain	Richards et al., 2000; Côrte-Real et al., 1996; Bertranpetit et al., 1995;	SpB1	436	Current	52.75	0.00	2.75	5.05	8.94	16.28	11.24	0.69	2.29

	Izagirre, 1998; García et al., 2011												
Basque Country 2, Spain	Alfonso-Sánchez et al., 2008	SpB2	55	Current	50.91	1.82	14.55	3.64	5.45	18.18	5.45	0.00	0.00
Son Real, Majorca, Spain**	Díaz, 2009	Sre	37	Iron Age	24.32	0.00	21.62	8.11	10.81	27.03	0.00	5.41	2.70
Can Reiners, Majorca, Spain**	Díaz, 2009	Cre	28	Medieval Age	42.86	3.57	3.57	3.57	3.57	28.57	3.57	7.14	3.57
Majorca, Spain	Picornell et al. 2005	Maj	45	Current	31.11	2.22	8.89	11.11	15.56	11.11	11.11	0.00	8.89
Valencia, Spain	Picornell et al. 2005	Val	42	Current	47.62	0.00	9.52	2.38	11.90	11.90	4.76	0.00	11.90
Bretons (Finistère), France	Richard et al., 2007; Dubut et al., 2004	Fbf	142	Current	35.21	4.23	9.15	8.45	7.04	21.13	6.34	5.63	2.82
Bretons (Loire Atlantique), France	Richard et al., 2007	Fbrl	75	Current	53.33	2.67	2.67	5.33	8.00	16.00	4.00	2.67	5.33
Bretons (Morbihan), France	Richard et al., 2007; Dubut et al., 2004	Fbm	81	Current	44.44	0.00	6.17	17.28	14.81	9.88	3.70	1.23	2.47
Baie de Somme, France	Richard et al., 2007	Fbs	79	Current	35.44	1.27	8.86	11.39	13.92	10.13	3.80	1.27	13.92
Basques (Pyrénnées Atlantiques), France	Richard et al., 2007	FBPy	81	Current	58.02	0.00	17.28	3.70	6.17	8.64	4.94	0.00	1.23
Calvados, France	Richard et al., 2007	Fca	46	Current	45.65	4.35	10.87	13.04	2.17	13.04	4.35	4.35	2.17
Hérault, France	Richard et al., 2007	Fhe	85	Current	35.29	1.18	8.24	5.88	16.47	17.65	2.35	2.35	10.59
Maine-Loire, France	Richard et al., 2007	Fma	55	Current	50.91	1.82	5.45	1.82	18.18	7.27	10.91	0.00	3.64
North East France,	Richards et al.,	Fne	47	Current	36.17	2.13	8.51	10.64	8.51	12.77	4.26	4.26	12.77

France	2000												
Normandy, France	Dubut et al., 2004	Fno	39	Current	48.72	0.00	0.00	7.69	17.95	23.08	0.00	2.56	0.00
Perigord-Limousin, France	Dubut et al., 2004	Fpe	72	Current	41.67	2.78	8.33	15.28	12.50	9.72	2.78	2.78	4.17
Rhône, France	Richard et al., 2007	Frh	46	Current	32.61	2.17	13.04	13.04	2.17	6.52	10.87	0.00	19.57
Sarthe, France	Richard et al., 2007	Fsa	36	Current	47.22	0.00	5.56	13.89	8.33	19.44	0.00	0.00	5.56
Var, France	Dubut et al., 2004	Fva	37	Current	37.84	5.41	0.00	5.41	8.11	10.81	13.51	0.00	18.92
Vendee, France	Richard et al., 2007	Fve	80	Current	57.50	2.50	7.50	7.50	5.00	8.75	6.25	0.00	5.00
Vienne, France	Richard et al., 2007	Fvi	44	Current	45.45	0.00	11.36	13.64	0.00	11.36	4.55	2.27	11.36
Neolithic Germany*	Haak et al. 2005	Gne	22	Neolithic	13.64	0.00	4.55	18.18	22.73	4.55	4.55	0.00	31.82
Germany	Hofmann et al., 1997; Richards et al., 1996; Baasner et al., 1998; Pfeiffer et al., 1999; Lutz et al., 1998	Ger	1311	Current	46.76	1.98	8.77	6.71	11.67	14.80	4.12	1.83	3.36
Austria	Parson et al., 1998; Handt et al., 1994	Au	116	Current	47.41	2.59	11.21	9.48	9.48	12.07	0.86	0.86	6.03
Italy	Richard et al., 2007; Richards et al., 2000; Tagliabracci et al., 2001; Mogentale-	It	397	Current	39.04	1.76	9.82	8.82	16.62	11.08	3.27	1.01	8.56

	Profizi et al., 2001; Francalacci et al., 1996												
Neolithic Portugal*	Chandler et al. 2005	Pne	23	Neolithic	73.91	0.00	0.00	0.00	0.00	21.74	4.35	0.00	0.00
Portugal	Côrte-Real et al., 1996; Pereira et al., 2000	Pt	294	Current	43.20	0.68	6.80	5.78	12.59	9.52	5.78	1.02	14.63
Swiss	Richard et al., 2007, Pult et al., 1994; Dimo-Simonin et al., 2000	Sw	429	Current	43.82	1.40	11.19	5.36	12.35	15.62	4.43	1.63	4.20

[1] Haplogroups T and X were joined, since they were hold together by the authors in the ancient Basque populations.

*Data not available to perform correction for the existence of kinship.

**all the individuals analysed were buried in different tombs

TABLE III. Results of clones of the individuals of the Cave

Individual E1

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          000000000000000000000000000000000000000000000000000000000000000000000000000001111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222233333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333334
          222222233333333334444444444555555555566666666667777777777888888888899999999990000000000111111111122222222223333333333444444444455555555556666666666777777777788888888889999999999000000000011111111112222222222333333333344444444445555555555666666666677777777778888888888999999999900000000001111111111222222222233333333334444444444555555555566666666667777777777888888888899999999990
          345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
          GTTCTTTCATGGGGAAGCAGATTTGGGTACCACCCAAGTATTGACTCACCCATCAACAACCGCTATGTATTTCGTACATTACTGCCAGCCACCATGAATATTGTACGGTACCATAAATACTTGACCACCTGTAGTACATAAAAACCCAATCCACATCAAAACCCCCTCCCCATGCTTACAAGCAAGTACAGCAATCAACCCTCAACTATCACACATCAACTGCAACTCCAAAGCCACCCCTCACCCACTAGGATACCAACAAACCTACCCACCCTTAACAGTACATAGTACATAAAGCCATTTACCGTACATAGCACATTACAGTCAAATCCCTTCTCGTCCCCATGGATGACCCCCCTCAGATAGGGGTCCCTTGAC
Clone 1                                                                                                                                                                                            L16209...A..........C..............................A..................A....................................CH16339
Clone 2                                                                                                                                                                                                  ..............C..........................................TT..........................................C
Clone 3                                                                                                                                                                                                  ..............C......................................................................................C
Clone 4                                                                                                                                                                                                  ..............C.......................T..............................................................C
Clone 5                                                                                                                                                                                                  ..............C......................................................T...............................C
Clone 6                                                                                                                                                                                                                                                                        ..............................T...............................C
Clone 7                                                                                                                                                                                                                                                                                                             .........................C
Clone 8                                                                                                                                                                                                  ..............C......................................................................................C
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Individual E2

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          111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
          666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666
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          222222233333333334444444444555555555566666666667777777777888888888899999999990000000000111111111122222222223333333333444444444455555555556666666666777777777788888888889999999999000000000011111111112222222222333333333344444444445555555555666666666677777777778888888888999999999900000000001111111111222222222233333333334444444444555555555566666666667777777777888888888899999999990
          345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
          GTTCTTTCATGGGGAAGCAGATTTGGGTACCACCCAAGTATTGACTCACCCATCAACAACCGCTATGTATTTCGTACATTACTGCCAGCCACCATGAATATTGTACGGTACCATAAATACTTGACCACCTGTAGTACATAAAAACCCAATCCACATCAAAACCCCCTCCCCATGCTTACAAGCAAGTACAGCAATCAACCCTCAACTATCACACATCAACTGCAACTCCAAAGCCACCCCTCACCCACTAGGATACCAACAAACCTACCCACCCTTAACAGTACATAGTACATAAAGCCATTTACCGTACATAGCACATTACAGTCAAATCCCTTCTCGTCCCCATGGATGACCCCCCTCAGATAGGGGTCCCTTGAC
Clone 1                                                                                                                                                                                            L16209..............................................................................................................................................................................................H16400
Clone 2                                                                                                                                                                                                  ......................................................................G.......................................................................................................................
Clone 3                                                                                                                                                                                                  ...............................................................................................................................................................................................
Clone 4                                                                                                                                                                                                  ..............C.......................................................................................C.......A.................................................................................
Clone 5                                                                                                                                                                                                  ..............................................................................................................................................................................A................
Clone 6                                                                                                                                                                                                  ..............................G................................................................................................................................................................
Clone 7                                                                                                                                                                                                  ...............................................................................................................................................................................................
Clone 8                                                                                                                                                                                                  ...............................................................................................................................................................................................
Clone 9                                                                                                                                                                                                  ...............................................................................................................................................................................................
Clone 10                                                                                                                                                                                                 ...............................................................................................................................................................................................
Clone 11                                                                                                                                                                                                 ...............................................................................................................................................................................................
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Individual E5

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          666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666
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          222222233333333334444444444555555555566666666667777777777888888888899999999990000000000111111111122222222223333333333444444444455555555556666666666777777777788888888889999999999000000000011111111112222222222333333333344444444445555555555666666666677777777778888888888999999999900000000001111111111222222222233333333334444444444555555555566666666667777777777888888888899999999990
          345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
          GTTCTTTCATGGGGAAGCAGATTTGGGTACCACCCAAGTATTGACTCACCCATCAACAACCGCTATGTATTTCGTACATTACTGCCAGCCACCATGAATATTGTACGGTACCATAAATACTTGACCACCTGTAGTACATAAAAACCCAATCCACATCAAAACCCCCTCCCCATGCTTACAAGCAAGTACAGCAATCAACCCTCAACTATCACACATCAACTGCAACTCCAAAGCCACCCCTCACCCACTAGGATACCAACAAACCTACCCACCCTTAACAGTACATAGTACATAAAGCCATTTACCGTACATAGCACATTACAGTCAAATCCCTTCTCGTCCCCATGGATGACCCCCCTCAGATAGGGGTCCCTTGAC
Clone 1                         L16030..............................................T..................................T..TT..T..............C......................................................H16230
Clone 2                               ..............................................T........................................................C......................................................
Clone 3                               ...............................G..............T........................................................C.......T..............................................
Clone 4                               ..............................................T........................................................C......................................................
Clone 5                               ..............................................T......T.....TT.T..................T.....................C.T....................................................
Clone 6                               ..............................................T..................................T..TT..T..............C...........................................
Clone 7                               ..............................................T........................................................C......................................................
Clone 8                               ..............................................T........................................................C......................................................
Clone 9                                                                                       .................................................C.......T..............................................
Clone 10                              ..............................................T........................................................C......................................................
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Individual E8

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          111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
          666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666
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          345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
          GTTCTTTCATGGGGAAGCAGATTTGGGTACCACCCAAGTATTGACTCACCCATCAACAACCGCTATGTATTTCGTACATTACTGCCAGCCACCATGAATATTGTACGGTACCATAAATACTTGACCACCTGTAGTACATAAAAACCCAATCCACATCAAAACCCCCTCCCCATGCTTACAAGCAAGTACAGCAATCAACCCTCAACTATCACACATCAACTGCAACTCCAAAGCCACCCCTCACCCACTAGGATACCAACAAACCTACCCACCCTTAACAGTACATAGTACATAAAGCCATTTACCGTACATAGCACATTACAGTCAAATCCCTTCTCGTCCCCATGGATGACCCCCCTCAGATAGGGGTCCCTTGAC
Clone 1                                                                                                                                                                                            L16209........................................................................................C.....................................................................................................H16400
Clone 2                                                                                                                                                                                                  ...................................T....................................................C.................................................................................T....................
Clone 3                                                                                                                                                                                                  ........................................................................................C......................................................................................................
Clone 4                                                                                                                                                                                                  ........................................................................................C......................................................................................................
Clone 5                                                                                                                                                                                                  ...A...................G................................................................C......................................................................................................
Clone 6                                                                                                                                                                                                  ....T........T..........................................................................C......................................................................................................
Clone 7                                                                                                                                                                                                  ....................................................................................TT..C......................................................................................................
Clone 8                                                                                                                                                                                                  ........................................................................................C......................................................................................................
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Clone 9                                                                                                                                                                                                                    ......................T.........T.....T.T...........T...TTT.....................T.......C......................................................................................................
Clone 10                                                                                                                                                                                                                   .....................................................................T..................C......................................................................................................
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Individual E9

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          111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
          666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666
          000000000000000000000000000000000000000000000000000000000000000000000000000001111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111112222222222222222222222222222222222222222222222222222222222222222222222222222222222222222222223333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333334
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          345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
          GTTCTTTCATGGGGAAGCAGATTTGGGTACCACCCAAGTATTGACTCACCCATCAACAACCGCTATGTATTTCGTACATTACTGCCAGCCACCATGAATATTGTACGGTACCATAAATACTTGACCACCTGTAGTACATAAAAACCCAATCCACATCAAAACCCCCTCCCCATGCTTACAAGCAAGTACAGCAATCAACCCTCAACTATCACACATCAACTGCAACTCCAAAGCCACCCCTCACCCACTAGGATACCAACAAACCTACCCACCCTTAACAGTACATAGTACATAAAGCCATTTACCGTACATAGCACATTACAGTCAAATCCCTTCTCGTCCCCATGGATGACCCCCCTCAGATAGGGGTCCCTTGAC
Clone 1                                 L16041........T........................................................C.............................................................H16187
Clone 2                                       ........T........................................................C.............................................................
Clone 3                                       ........T........................................................C.............................................................
Clone 4                                       ........T........................................................C.............................................................
Clone 5                                       ........T........................................................C.............................................................
Clone 6                                       ........T........................................................C.............................................................
Clone 7                                       ........T........................................................C.............................................................
Clone 8                                       ........T........................................................C.............................................................
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Individual E10

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          111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
          666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666
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          345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
          GTTCTTTCATGGGGAAGCAGATTTGGGTACCACCCAAGTATTGACTCACCCATCAACAACCGCTATGTATTTCGTACATTACTGCCAGCCACCATGAATATTGTACGGTACCATAAATACTTGACCACCTGTAGTACATAAAAACCCAATCCACATCAAAACCCCCTCCCCATGCTTACAAGCAAGTACAGCAATCAACCCTCAACTATCACACATCAACTGCAACTCCAAAGCCACCCCTCACCCACTAGGATACCAACAAACCTACCCACCCTTAACAGTACATAGTACATAAAGCCATTTACCGTACATAGCACATTACAGTCAAATCCCTTCTCGTCCCCATGGATGACCCCCCTCAGATAGGGGTCCCTTGAC
Clone 1                                                                                                                                                                                                       L16209..............................................T.............T.................................................T.......T......................................................................G.H16400
Clone 2                                                                                                                                                                                                             .........................G....................T.............T.................................................T..............................................................................G.
Clone 3                                                                                                                                                                                                             ..............................................T.............T.................................................T..............................................................................G.
Clone 4                                                                                                                                                                                                             ..............................................T.............T.................................................T..............................................................G...............G.
Clone 5                                                                                                                                                                                                             ..............................................T.............T.................................................T..............................................................................G.
Clone 6                                                                                                                                                                                                             ..............................................T.............T.................................................T..............................................................................G.
Clone 7                                                                                                                                                                                                             ..............................................T..T.T..T.....T...................................T.C............T...............T........................................T.T...................T
Clone 8                                                                                                                                                                                                             ..............................................T.............T...............................................G.T..............................................................................G.
Clone 9                                                                                                                                                                                                             ......................T.........T.............T.............T.................................................T..............................................................................G.
Clone 10                                                                                                                                                                                                            ..............................................T.............T................................A......A.........T...............A...............................................A....AAA.......G.
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Individual E11

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          666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666
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          222222233333333334444444444555555555566666666667777777777888888888899999999990000000000111111111122222222223333333333444444444455555555556666666666777777777788888888889999999999000000000011111111112222222222333333333344444444445555555555666666666677777777778888888888999999999900000000001111111111222222222233333333334444444444555555555566666666667777777777888888888899999999990
          345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
          GTTCTTTCATGGGGAAGCAGATTTGGGTACCACCCAAGTATTGACTCACCCATCAACAACCGCTATGTATTTCGTACATTACTGCCAGCCACCATGAATATTGTACGGTACCATAAATACTTGACCACCTGTAGTACATAAAAACCCAATCCACATCAAAACCCCCTCCCCATGCTTACAAGCAAGTACAGCAATCAACCCTCAACTATCACACATCAACTGCAACTCCAAAGCCACCCCTCACCCACTAGGATACCAACAAACCTACCCACCCTTAACAGTACATAGTACATAAAGCCATTTACCGTACATAGCACATTACAGTCAAATCCCTTCTCGTCCCCATGGATGACCCCCCTCAGATAGGGGTCCCTTGAC
Clone 1  L15996..............................................T........................................................C.......T......................................................C........................................................................................T.....................................................................................
Clone 2           ..............................................T........................................................C..................................................................................................................................................................................................................................................................................H16400
Clone 3           ..............................................T........................................................C..AA..............................................................................................................................................................................................................................................................................
Clone 4           ..............................................T........................................................C..................................................................................................................................................................................................T...............................................................................
Clone 5           ..............................................T........................................................C..................................................................................................................................................................................................................................................................................
Clone 6           ..............................................T......................C.................................C...........................................................................................................................................................................................
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Clone 8           ..............................................T........................................................C..................................................................................................................................................................................................................................................................................
Clone 9           ..............................................T........................................................C............................G...............................................................................................................................................................................................................................................T.....
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4.3 Caracterització genètica de les restes humanes talaiòtiques del jaciment de Son Olivaret, Ciutadella, Menorca

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Caracterització genètica de les restes humanes talaiòtiques del jaciment de Son Olivaret, Ciutadella, Menorca

Marc Simón, Assumpció Malgosa

RESUM

S'ha dut a terme l'estudi genètic de 22 individus del jaciment prehistòric de Son Olivaret. Els resultats mostren la presència exclusiva d'haplogrups mitocondrials europeus. D'altra banda, el conjunt d'individus estudiats no es diferencia significativament de les poblacions prehistòriques i modernes de les illes Balears, amb l'excepció de la Menorca actual, tot i que ambdues presenten un bagatge genètic plenament integrat dins l'àmbit europeu. La freqüència de l'haplogrup H del jaciment apunta a una possible relació genètica entre aquests primers pobladors de la illa de Menorca i els de la Península Ibèrica, sense que es pugui descartar un flux genètic amb altres zones de la Mediterrània més oriental.

Paraules-clau: ADN mitocondrial, haplogrups, seqüència, talaiòtic

RESUMEN

Se ha llevado a cabo el estudio genético de 22 individuos del yacimiento prehistórico de Son Olivaret. Los resultados muestran la presencia exclusiva de haplogrupos mitocondriales europeos. Por otra parte, el conjunto de individuos estudiados no se diferencia significativamente de las poblaciones prehistóricas y modernas de las Islas Baleares, con la excepción de la Menorca moderna,

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pese a que ambas muestran un bagaje genético plenamente integrado en el ámbito europeo. El estudio de las frecuencias del haplogrupo H del yacimiento y de otros yacimientos antiguos apunta hacia una posible relación genética entre estos primeros pobladores de la isla de Menorca y los habitantes de la Península Ibérica, sin descartarse un cierto flujo genético con otras zonas del Mediterráneo oriental.

Palabras-clave: ADN mitocondrial, haplogrupos, secuencia, talayótico.

SUMMARY

A genetic study has been carried out from the mitochondrial genome of 22 individuals from the prehistoric site of Son Olivaret. Results show the presence of fully European population haplogroups. In addition, the group does not differentiate in a significant way from both the prehistoric and the modern populations from the Balearic Islands, exception made of current Minorca, despite both of them being fully integrated in the European scope. The study of the haplogroup H frequency from this site and other ancient sites points towards a possible genetic relationship between these first settlers from the island of Minorca and the inhabitants of the Iberian Peninsula, without ruling out a certain genetic flow with other regions from the Eastern Mediterranean.

Key-words: mitochondrial DNA, haplogroups, sequence, talayotic.

INTRODUCCIÓ

El monument funerari de Son Olivaret està situat al sud-oest de l'illa de Menorca, a ponent de la carretera de Ciutadella a Cap d'Artrutx, al Km. 6 (Fig. 1). Gràcies al projecte «Hipogeisme i Megalitisme a les Illes Balears i les Pitiüses en el marc de la Mediterrània Occidental» (Plantalamor *et al.* 2009) es va iniciar el reconeixement i estudi d'aquest sepulcre de tipus col·lectiu d'època pretalaiòtica (segona meitat del III mil·lenni aC) i talaiòtica (1500-500 aC). Aquest projecte, iniciat l'any 2000, es va finalitzar l'any 2005 i a partir d'aleshores es van portar a terme diferents actuacions antropològiques.

El monument es troba envoltat per un mur aproximadament globular amb elements comuns a d'altres sepulcres menorquins (grup sud-est i Ses Arenes de Baix a Ciutadella). La cambra interior té forma ovalada i l'accés es troba al SO a través d'un Corredor (Figs. 2 i 3). L'interior de la cambra estava cobert

de lloses planes. Pel fet de tractar-se d'una estructura anterior a la naveta, que va evolucionar partint del model de sepulcres megalítics fins a les navetes, el sepulcre de Son Olivaret s'ha anomenat protonaveta, com el de ses Arenes de Baix i el de Torreta de Tramuntana (Plantalamor *et al.* 2007). S'hi van distingir 5 períodes diferents d'ús separats per fases d'abandonament. El material esquelètic més antic trobat data de 3640±40BC (KIA - 27133) (Van Strydonck i Boudin 2008).

Les restes van ser recuperades per l'equip d'arqueologia del Museu de Menorca durant les campanyes que comprenen del 2003 al 2005 (Plantalamor et al, 2007). Es van recuperar una gran quantitat de restes humanes pertanyents a tots dos períodes, pretalaiòtic (unitats estratigràfiques (UEs 15 i 16) i talaiòtic (UEs 6 a 11), però la majoria d'elles estaven fragmentades i mal conservades (Fig. 2). L'estudi dut a terme sobre totes les dents va determinar un nombre mínim de 160 individus (NMI), 30 pertanyents al període pretalaiòtic i els altres 130 a l'època talaiòtica (Carrascal et al, 2008a). L'estudi morfològic de les restes òssies va proporcionar unes dades lleugerament menors a causa de l'estat del material i la impossibilitat de determinar individus. El càlcul del NMI a partir de dades òssies indica la presència d'un mínim de 25 enterraments pel període pretalaiòtic (7 individus diferents en l'estrat 15 , i 10 en l'estrat 16; Carrascal *et al.* 2008b) i 90 pel Talaiòtic (1 individu a la UE 1, 5 individus diferents a la UE 6, 2 a la UE 7, 4 a la UE 8, 27 a la UE 9 i 51 a la UE 11) (Carrascal, Nociarová i Malgosa 2012; Carrascal *et al.* 2011) . La fragmentació i el deteriorament de les restes són responsables de la diferència entre els valors de recuperació dentals i ossis.

Aquest registre antropològic és un dels més complets de l'illa tot i estar en un estat de fragmentació molt elevat. L'estat de conservació impedeix conèixer la població, les seves característiques morfològiques, per poder-les comparar amb altres poblacions contemporànies de Menorca (Armentano *et al.* 2010, 2012) i també de Mallorca (Font 1974, Malgosa 1992, Ortega 2005). Per poder caracteritzar amb més detall la població, tenim però altres opcions com l'estudi genètic. Però, per a l'anàlisi genètica a partir de restes antigues, habitualment sorgeixen dos grans inconvenients, que en aquest cas poden ser importants: l'obtenció del propi material genètic, l'ADN, i la seva autentificació.

L'ADN és la molècula biològica que conté la informació genètica de cada organisme en cada cèl·lula del cos. Aquesta informació és única per a cada

individu de manera que no hi ha dos individus iguals, excepte en el cas de bessons homozigòtics. L'ADN està distribuït en dos orgànuls cel·lulars, el nucli i els mitocondris. El primer, l'ADN nuclear (ADNn), conté la major part de la informació genètica de l'individu i només n'hi ha una sola còpia per cèl·lula, composta per les còpies d'ADN que rebem del pare i de la mare. No obstant això, els estudis paleogenètics es basen habitualment en l'anàlisi de l'ADN mitocondrial (ADNmt), que és el material genètic dels mitocondris, els orgànuls que generen l'energia per la cèl·lula. L'ADNmt és molt més usat que l'ADNn en treballs amb restes antigues pel fet que en cada cèl·lula hi ha un elevat nombre de mitocondris, cadascun dels quals conté diverses còpies d'ADNmt, per la qual cosa a cada cèl·lula hi ha milers de còpies d'ADNmt idèntiques entre si. Aquest elevat nombre de còpies n'incrementa la probabilitat de preservació (O'Rourke et al, 2000). D'altra banda, l'herència de l'ADNmt és exclusivament materna, de manera que l'ADNmt de qualsevol persona és absolutament idèntic al de la seva mare; per això és possible reconstruir llinatges femenins.

En certs casos l'ADN es pot recuperar de restes d'organismes que van morir fa desenes, centenes o milers d'anys; és el que s'anomena ADN antic i s'ha pogut recuperar de plantes i animals extints, mòmies i fins i tot d'algunes restes fossilitzades (Montiel *et al.* 2007). Aquest ADN extret de restes antigues ens permet accedir a la informació directa de l'organisme, i, per tant, aprofundir en el coneixement de l'individu. No hem d'oblidar, però, que la informació que es pot obtenir és molt parcial ja que, fins al moment, només és possible recuperar petits fragments d'ADN que sovint van acompanyats de substàncies inhibidores de la reacció en cadena de la polimerasa (PCR), i poden estar contaminats amb ADN actual. Així doncs, el fet que un material sigui susceptible de ser analitzat genèticament depèn de les condicions d'enterrament, emmagatzematge, preservació, de la temperatura, humitat i pH, i de la presència d'inhibidors com els àcids húmics i fúlvics, entre d'altres. En casos especialment ben conservats i d'enorme interès científic, però, les noves tècniques de *Next Generation Sequencing* estan començant a proporcionar genomes complets (Green *et al.* 2010, Fua *et al.* 2013, Raghavan *et al.* 2013, Prüfer *et al.* 2013 Rasmussen *et al.* 2010, entre altres).

La caracterització genètica ens apropa al coneixement de les poblacions antigues i el nostre interès és poder conèixer a nivell genètic la població que es va inhumar al jaciment de Son Olivaret. En un estudi preliminar (Simón i

Malgosa 2008) es van poden caracteritzar parcialment 9 individus que mostraven un ventall d'haplogrups. En aquest treball es pretén analitzar les dades que ha proporcionat l'anàlisi de l'ADNmt en el conjunt de restes humanes del jaciment de Son Olivaret, a partir de l'estudi de l'haplogrup H, el més comú a Europa i a la conca Mediterrània en general.

MATERIAL I MÈTODES

La caracterització genètica dels individus de Son Olivaret (Ciutadella, Menorca) es va realitzar al laboratori d'ADN antic de la Unitat d'Antropologia Biològica de la Universitat Autònoma de Barcelona. Atès que l'estudi genètic està altament condicionat per l'estat de conservació del material, es van triar peces dentals per dur-lo a terme. La capa d'esmalt dental actua com una protecció enfront de les possibles contaminacions amb DNA exogen (Montiel *et al.* 2007, Díaz 2009), la qual cosa ajudarà a preservar el material genètic. En el procés de selecció de les peces dentals, es van escollir les dents més ben conservades a fi de maximitzar la possibilitat d'obtenir ADN, i es van tenir en compte tant l'absència de patologies evidents com la integritat de la peça, i es van descartar totes aquelles dents que presentessin una petita fractura o esquerda, o qualsevol altra anomalia. La peça que millor es va ajustar a aquests requisits va ser la dent 24 (FDI, primer premolar superior esquerre) del qual es conservaven 84 espècimens, encara que només una petita fracció d'aquest nombre va ser susceptible de ser analitzada. Aquest petit grup es va quantificar en 37 peces.

Les mostres van ser analitzades en el laboratori de paleogenètica de la Unitat d'Antropologia Biològica de la Universitat Autònoma de Barcelona. La fase experimental es va realitzar seguint la metodologia descrita per Malgosa i col·laboradors (2005). En tots els procediments de laboratori, es van establir criteris d'autentificació. A més, tots els processos d'extracció i amplificació mitjançant la reacció en cadena de la polimerasa (PCR per les seves sigles en anglès) es van realitzar en un laboratori dedicat exclusivament al treball amb ADN antic, físicament separat de les àrees en què s'havien de realitzar els procediments posteriors a la PCR (post-PCR), restringint així la contaminació per transport d'amplicons (carryover). Tot i tenir en compte tots els criteris d'esterilitat i d'autenticitat possibles i atès l'estat general de les restes i la impossibilitat en la majoria dels casos de demostrar que diferents peces dentals

pertanyessin al mateix individu, va ser totalment impossible seguir dos dels criteris requerits habitualment: la confirmació amb diferents extractes de la mateixa persona i l'anàlisi en un laboratori extern. Tanmateix, es van complir tots els altres criteris (Montiel *et al.* 2007).

Les dents van ser tractades per a netejar-les i descontaminar-les abans de realitzar l'extracció. Per a l'obtenció d'ADN, es van treure 0,5 grams de pols de la dentina de dins de la cavitat polpar utilitzant material odontològic. L'extracció d'ADN es va realitzar seguint la metodologia descrita a Malgosa *et al.* 2005 i els extractes obtinguts es van emmagatzemar a 4 º C durant un mínim de 3 dies (Montiel *et al.* 1997).

La 1a zona hipervariable de l'ADNmt obtingut (HVRI) es va amplificar mitjançant la tècnica PCR (Taula 1). Es van amplificar fragments petits, entre 87 i 191 parells de bases (pb), amb encebadors específics, i es van obtenir fragments solapants, a fi d'aconseguir un fragment major –aproximadament 300pb. D'altra banda, de la zona codificant es va amplificar el fragment que conté el polimorfisme determinant de l'haplogrup H. Per tal de detectar-ne la seva presència o absència, aquest fragment es va sotmetre a digestió enzimàtica (RFLPs). Així, els 2 resultats obtinguts amb metodologies diferents verificaven l'autenticitat de l'altre. En aquells individus que podien pertànyer a l'haplogrup H mitjançant la zona amplificada de la HVRI, i també en aquells on no s'havien pogut obtenir resultats amb el procés de seqüenciació, es va comprovar la pertinença o no a l'haplogrup H a partir de l'anàlisi de la posició 7025 de la zona codificant que conté una diana per l'enzim AluI en els individus no-H (Taula 2).

Les reaccions de PCR es van dur a terme en un volum final de 50 ul i es va utilitzar AmpliTaq Gold® ADN polimerasa (Applied Biosystems, Foster City, EUA). Cada reacció de PCR va consistir en una etapa inicial de desnaturalització, seguida per 39 cicles de PCR i un pas d'extensió final de 5 min a 72 º C. Els fragments amplificats es van visualitzar després de tinció amb bromur d'etidi en un gel d'agarosa al 3 %. La purificació de les mostres es va realitzar amb un kit de purificació de PCR anomenat Jetquick (Genomed, Löhne, Alemanya) per eliminar la resta dels reactius de l'amplificació.

És interessant comentar que cada procés d'extracció va incloure diversos controls de la contaminació abans i durant aquest procés. A més, els investigadors implicats en l'estudi van ser analitzats per controlar l'eventual contamina-

ció durant els processos d'anàlisi morfològica i al laboratori.

RESULTATS I DISCUSSIÓ

De l'estudi realitzat sobre 37 peces dentals pertanyents a la dent 24 (FDI), només 22 van donar resultats concloents, totes elles d'època talaiòtica. No va ser possible obtenir material analitzable de les mostres més antigues, d'època pretalaiòtica. De les mostres obtingudes, 9 es van determinar com pertanyents a l'haplogrup H per la seva seqüència i la corroboració amb PCR-RFLPs, mentre que 13 pertanyien a altres haplogrups (Taula 3).

L'haplogrup H és un llinatge mitocondrial típic d'Euràsia occidental i és el més freqüent a Europa (Torroni *et al.* 2006; Roostalu, 2007). Es deuria originar probablement al Pròxim Orient fa uns 30.000 anys (Metspalu *et al.* 1999). S'ha trobat en restes antigues europees en baixa freqüència des del Neolític antic, fa 5450 anys, i a partir d'aquí ha anat augmentant la seva presència en les poblacions analitzades (Brotherton *et al.* 2013).

Les majors freqüències es troben a Europa (40%, Roostalu *et al.* 2007), però també és comú al Pròxim Orient, Àfrica del Nord, Àsia Central, Sibèria Occidental i, fins i tot, a Mongòlia. A la Península Ibèrica es troba en altes freqüències, per exemple a Catalunya és present en un 46,2% (Eupedia, 2014), i ja es trobava entre els ibers (53%, Sampietro et al, 2005).

Al jaciment de Son Olivaret, el percentatge d'individus H és d'un 40,9%; és a dir que gairebé coincideix amb la freqüència europea actual. A fi d'analitzar l'impacte d'aquest haplogrup en aquesta sèrie i en la població menorquina antiga, s'ha comparat amb altres sèries properes en l'espai i en el temps (taula 3). En aquest sentit, s'ha analitzat el percentatge d'una altra sèrie talaiòtica menorquina, la constituïda per les restes humanes de la Cova des Pas (Armentano *et al.* 2012, Simón *et al.* 2012). En aquest darrer cas, el percentatge d'individus H és més alt, i assoleix un 60%. La diferència entre ambdós és difícil d'interpretar. Cal tenir en compte que en jaciments antics i col·lectius, com són els que aquí s'analitzen, és molt difícil poder comprovar el grau d'endogàmia del grup; aquesta característica podria ser la clau de la discrepància entre ambdós grups ja que el nombre d'individus analitzats és molt semblant. Amb tot, es pot observar com aquest haplogrup és present en una elevada freqüència a la Menorca talaiòtica.

D'altra banda, la freqüència de Son Olivaret és molt propera als percentatges de les sèries antigues de Mallorca i de les Balears en general, amb excepció de

la sèrie de Son Real (23,7%). Caldria tenir en compte les peculiaritats d'aquesta sèrie mallorquina que correspon a una necròpolis de característiques úniques, tant a nivell constructiu com genètic. La presència d'haplogrups no gaire habituals a la Mediterrània occidental i també d'haplotips únics, actualment desapareguts (Diaz 2009), li confereix singularitat dins del conjunt de poblacions balears antigues. L'alt percentatge de l'haplogrup H a la Cova des Pas i el baix percentatge a Son Real determinen que la freqüència d'aquest haplogrup a la Menorca antiga sigui més alta que la de la Mallorca antiga.

Si es compara la freqüència de H de Son Olivaret amb les dades actuals de les Balears, s'observa una clara diferència entre les dades de la Menorca prehistòrica, en relació a la població moderna. Com havien descrit Picornell i col·laboradors (2005), les freqüències actuals d'haplogrups de l'illa de Menorca són bastant diferents dels trobats a les illes de Mallorca i Eivissa, i, pel que fa a l'haplogrup H, s'assembla a la que presenten els xuetes.

En relació a altres sèries antigues contemporànies a Son Olivaret, es pot observar que difereix clarament dels sards a causa de l'elevada presència de H en aquests territoris des d'antic. La sèrie antiga de Turquia presenta únicament aquest haplogrup, cosa que planteja diferents incògnites sobre la metodologia o bé sobre la representativitat d'aquest grup com a població. En canvi, s'assembla molt als valors trobats entre el grecs antics i és una mica més elevat que els ibers catalans i els valencians, i molt més que els sirians.

Son Olivaret, doncs, té un percentatge d'haplogrup H molt semblant a la mitjana actual europea i no difereix de les mitjanes balears antigues. En canvi, sí que és realment diferent dels grups europeus que es consideren més antics com són els sards, tal i com era d'esperar. En relació a la pròpia illa, les diferències entre les dues sèries antigues podrien molt bé ser degudes a la composició de la població. Habitualment es considera que les poblacions antigues estaven constituïdes per grups relativament petits i endogàmics. El nombre d'enterraments calculat a Son Olivaret (NMI) supera els 160 individus, mentre que a la Cova des Pas s'hi van trobar 66 enterraments. En ambdós casos, el lloc es va utilitzar per enterrar durant els períodes pretalaiòtic i talaiòtic, per la qual cosa es pot argumentar que el grup humà que enterrava a la protonaveta de Son Olivaret era més gran i potser més divers que el que utilitzava la Cova des Pas, ambdues situades a la zona sud-oest de l'illa, tot i que la mostra que s'ha pogut utilitzar és molt semblant en ambdós jaciments

(20 a Cova des Pas/22 a Son Olivaret).

De les dades presentades fins al moment, el que sorprèn més és la diferència a nivell de l'haplogrup H entre la sèrie de Son Olivaret i la de la Menorca actual. La sèrie antiga és molt més propera a la freqüència de l'haplogrup H europeu i peninsular actual que no pas la sèrie moderna. És possible que el baix nombre mostral de la sèrie moderna sigui responsable de la baixa freqüència de l'haplogrup H, tot i que és més alt que en la sèrie antiga (46:22). Cal dir, però, que manté una variabilitat haplotípica elevada, igual que Mallorca i Eivissa (Picornell *et al.* 2005), però que els esdeveniments històrics dels darrers segles a Menorca i els canvis que van provocar en el si de la població, com la formació d'una important colònia anglesa, haurien pogut tenir una important rellevància en la composició de la població actual. Així, les freqüències de les poblacions antigues semblen indicar un substrat amb una elevada proporció d'haplogrups H a Menorca, com a la majoria de poblacions europees i, per tant, suggereixen una important relació amb les poblacions europees i peninsulars. Pel que fa a les poblacions més orientals de la Mediterrània, mostraria una major semblança amb la sèrie grega i allunyada en dos extrems diferents de les dues del Pròxim orient per raons oposades i que més aviat semblen mostrar poblacions amb una important endogàmia (Turquia) o una alta diversitat (Síria).

En conclusió, la sèrie de Son Olivaret mostra una composició de l´haplogrup mitocondrial H molt semblant a les poblacions antigues i actuals europees i no sembla que hi hagi una continuïtat amb la població actual pel que fa a aquest haplogrup. Possiblement els esdeveniments més recents de la història de Menorca estan a la base d'aquestes diferències.

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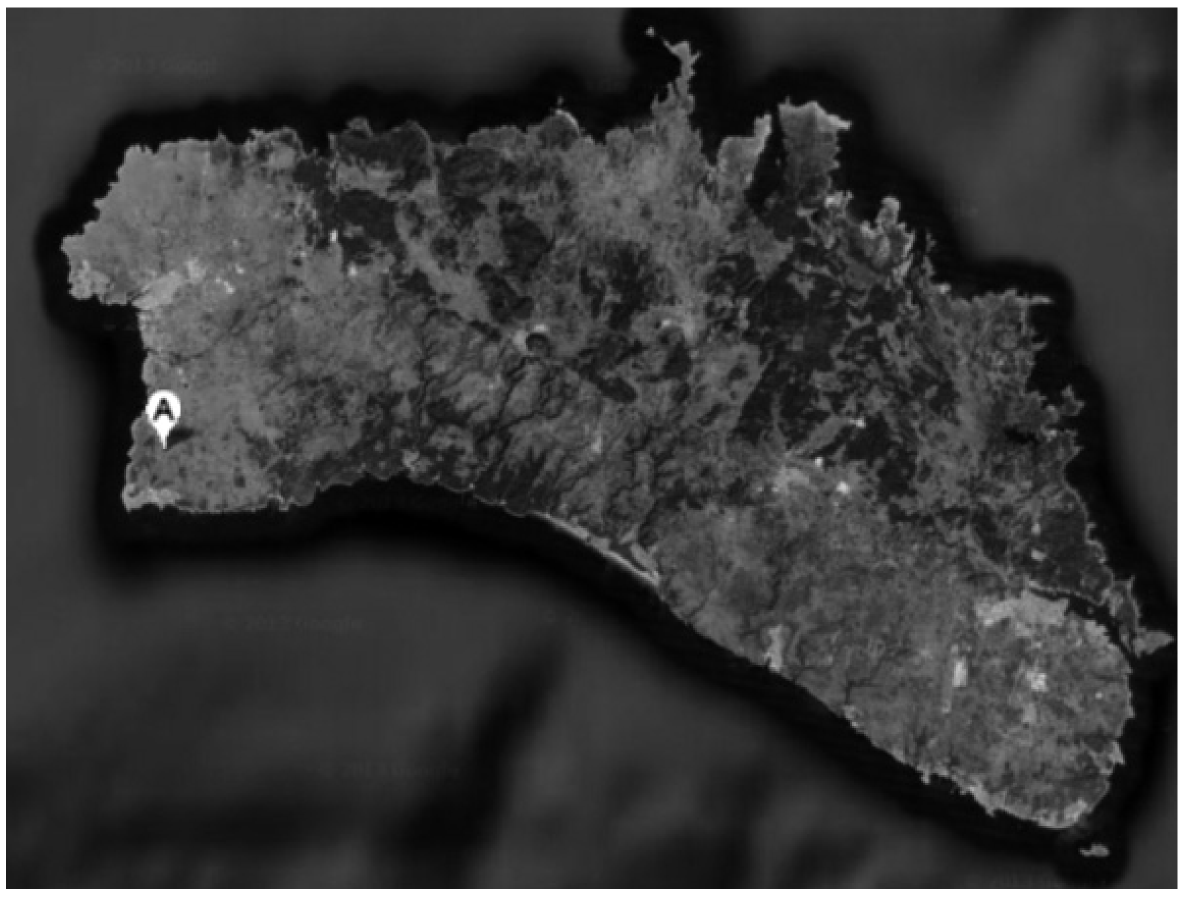

Fig. 1: Mapa de Menorca on s'assenyala la situació del jaciment prehistòric de Son Olivaret

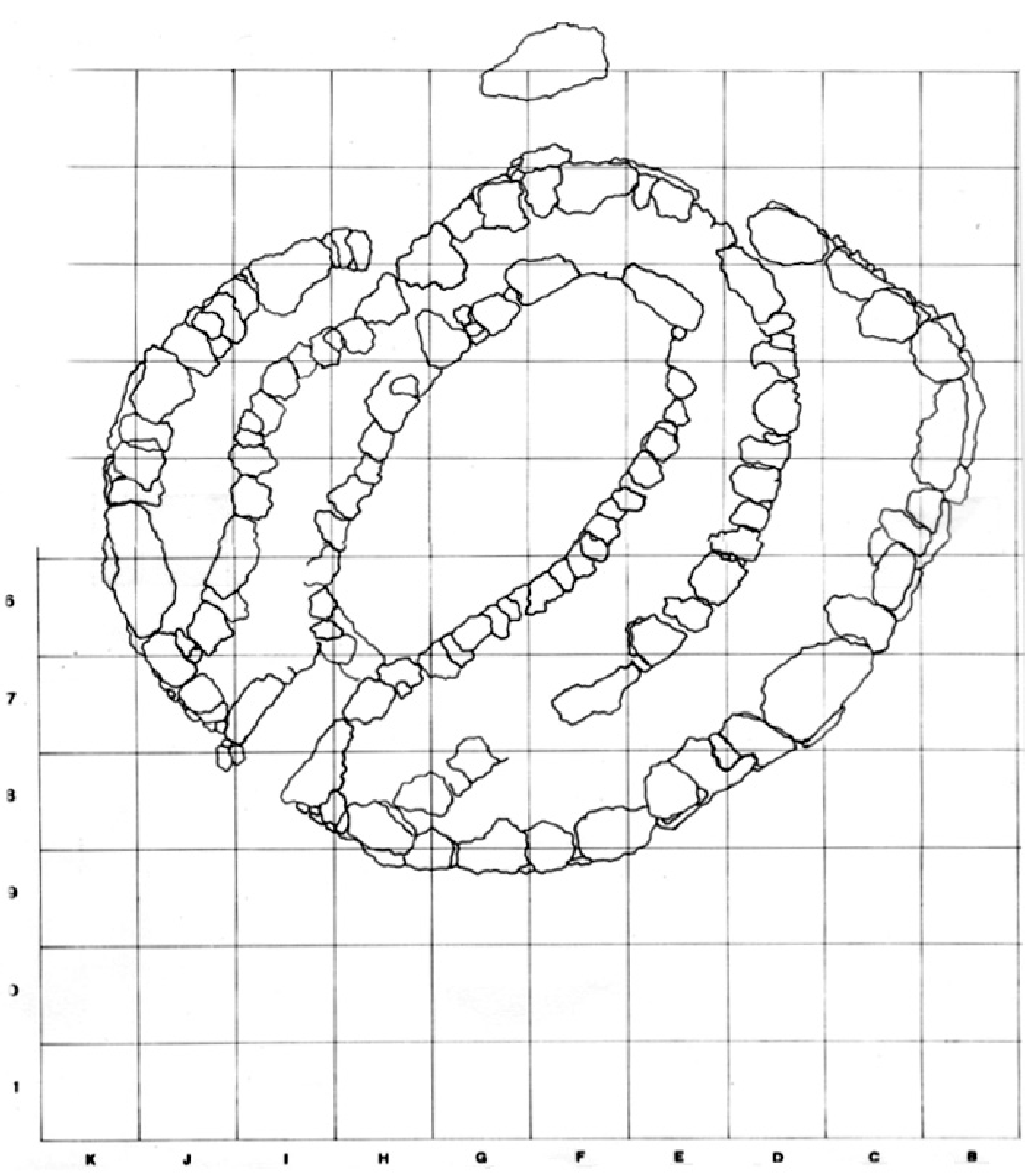

Fig. 2. Planta del jaciment de Son Olivaret (imatge cedida per L. Plantalamor)

Fig. 3. Interior de la cambra del sepulcre de Son Olivaret on s'observen algunes restes òssies (Fotografia de L. Plantalamor).

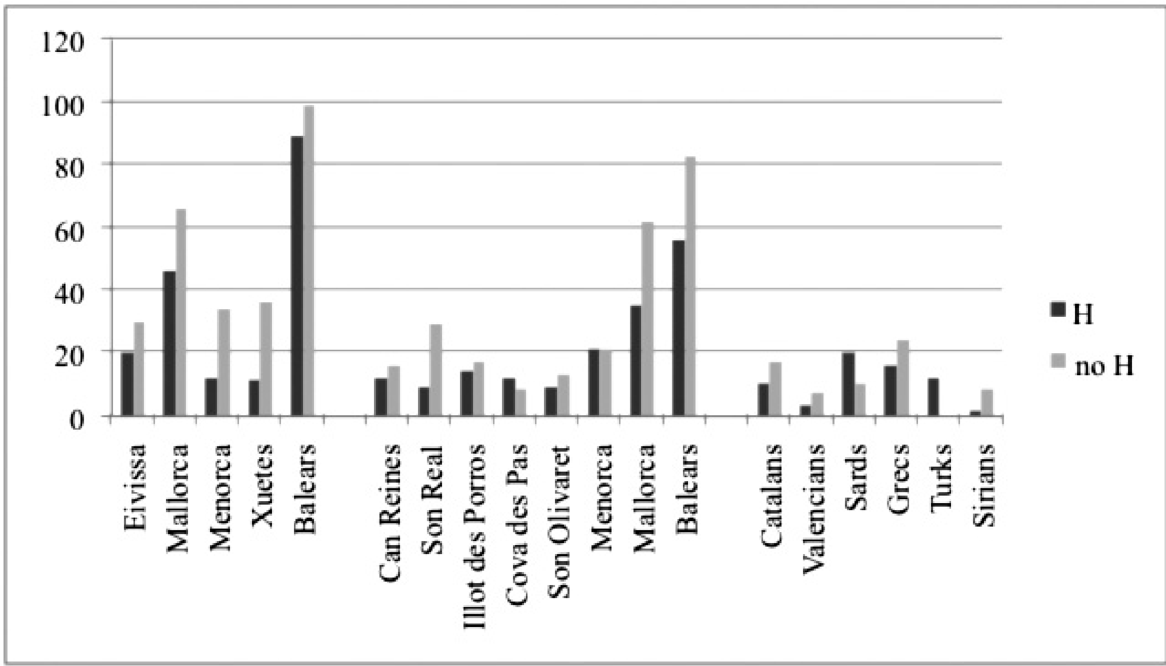

Fig. 4.- Freqüències relatives de l'haplogrup H en relació amb els altres haplogrups en diverses sèries: actuals de les Balears (Eivissa, Mallorca, Menorca, xuetes i el conjunt de les Balears), antigues de les Balears (Son Real, S'Illot des Porros, Can Reiners, Cova des Pas, Son Olivaret, els tres jaciments de Mallorca en conjunt, els 2 jaciments de Menorca junts i el conjunt dels diferents jaciments de les Balears) i altres jaciments antics de la Península i la Mediterrània (catalans, valencians, sards, grecs, turcs i sirians). Les referències es troben a la taula 3.

TAULES

TAULA 1.
Primers utilitzats per amplificar i seqüenciar la regió hipervariable I de l'ADNmt (HVRI).

Fragments	Descripció dels primers i els autors que els van publicar
Fragment 1 (405bp)	L-15996: 5'-CTCCACCATTAGCACCCAAAGC-3 (Vigilant et al, 1991) H -16401: 5'-TGATTTCACGGAGGATGGTG-3' (Vigilant et al, 1991)
Fragment 2 (200bp)	L-16030: 5'-CATGGGGAAGCAGATTTGGG-3' (Simón et al, 2012) H-16230:5'-GATAGTTGAGGGTTGATTGCTG-3 (Simón et al, 2012)
Fragment 3 (231bp)	L-16209: 5'-CCCCATGCTTACAAGCAAGT-3' (Montiel, 2001) H -16401: 5'-TGATTTCACGGAGGATGGTG-3' (Vigilant et al, 1991)

TAULA 2.
Lloc de restricció estudiat per a realitzar la determinació de l'haplogroup H.

Lloc de restricció per l'haplogrup H	Primers	Posicions	Seqüència dels primers
-7025 Alu I	L6968/H7052 (Francalacci, comunicació personal)	6949-6968 7071-7052	5'-CCGTAGGTGGCCTGACTGGC-3' 5'-TGATGGCAAATACAGCTCCT-3'

TAULA 3.
Freqüències absolutes de l'haplogrup H en diverses sèries modernes i antigues de les Balears, i antigues de la Península Ibèrica i la Mediterrània

Sèries	Localització geogràfica	Referència	n	H	no H	%H
Modernes de les Balears	Eivissa	Picornell *et al.* 2005	50	19	31	38,0
	Mallorca	Picornell *et al.* 2005, Falchi *et al.* 2006	112	46	66	41,1
	Menorca	Picornell *et al.* 2005	46	12	34	23,9
	Xuetes	Picornell *et al.* 2005	47	11	36	18,8
	Balears	Picornell *et al.* 2005, Falchi *et al.* 2006	188	89	99	45,2
Antigues de les Balears	Son Real	Díaz 2009	38	9	29	23,7
	Illot des Porros	Díaz 2009	31	14	17	45,2
	Can Reines	Díaz 2009	28	12	16	42,9
	Cova des Pas	Present estudi	20	12	8	60,0
	Son Olivaret	Present estudi	22	9	13	40,9
	Menorca ant	Present estudi	42	21	21	50,0
	Mallorca ant	Díaz 2009 i present estudi	97	35	62	36,1
	Balears ant	Díaz 2009 i present estudi	139	56	83	40,3
Altres poblacions mediterrànies antigues	Catalans ant	Sampietro *et al.* 2005, 2007; Simón *et al.* 2011; Gamba *et al.* 2008	36	13	23	36,1
	Valencians ant	Fernández 2006, Arroyo *et al.* 2006, Gamba *et al.* 2008	10	3	7	30
	Sards ant	Caramelli *et al.* 2007 Present estudi	29	19	10	65,5
	Grecs ant	Hughey *et al.* 2013, Bouwman *et al.* 2008	37	15	22	40,5
	Turcs ant	Matney *et al.* 2012	12	12	0	100
	Sirians ant	Fernández 2005, Witas *et al.* 2013, Tomczyk *et al.* 2011	9	1	8	11,1

4.4 La Menorca talayótica desde el punto de vista genético: el yacimiento de la Cova des Pas

La Menorca talayótica desde el punto de vista genético: el yacimiento de la Cova des Pas

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Resumen: En el presente artículo se estudian las características genéticas del yacimiento Talayótico de la Cova des Pas, que revela una importante endogamia a nivel de ADN mitocondrial. La comparación con otras series contemporáneas de Baleares permite hipotetizar sobre la existencia de grupos cerrados en sí mismos, con limitados linajes femeninos y necrópolis de difícil acceso como este yacimiento, frente a grupos más abiertos con un mayor intercambio de linajes femeninos situados en las zonas llanas centrales de la isla.

Palabras claves: Talayótico, ADN mitocondrial, endogamia, Mediterráneo.

The Minorcan Talayotic Era from genetic point of view: The Cova des Pas site.

Abstract: In the current article the genetic characteristics of the Talaiotic site of la Cova des Pas are studied, the results of which reveal an important level of endogamy at the mitochondrial DNA level. The comparison with other contemporary series from the Balearic Islands permits us to hypothesize about the existence of groups closed in on themselves, with a limited amount of feminine lineages and difficult to access necropolises as the present site, opposite to the existence of more open groups with higher interchange of femininie lineages placed in the central flat zones of the island.

Key words: Talaiotic, mitochondrial DNA, endogamy, Mediterranean Sea.

Durante el Bronce final, se produjeron una serie de cambios en las comunidades que habitaron Mallorca y Menorca a nivel sociológico, económico y de relación con otras islas y territorios. Afectaron también a la concepción espacial y territorial, así como al ámbito simbólico, religioso y funerario (Calvo y Guerrero, 2011). El resultado fue el desarrollo de una nueva cultura de gran carga simbólica, caracterizada por una arquitectura monumental. En ella destacan las grandes construcciones en forma de torre, llamadas de forma genérica Talayots, que dan nombre a esa cultura (Guerrero *et al.* 2006 a, b). La función principal de los talayots era el dominio del territorio, además de servir como cohesionador social. El marco temporal de estos cambios se inicia en la denominada Edad de Hierro en el Oeste Mediterráneo (Guerrero *et al.* 2002).

En relación a las prácticas funerarias, es bien conocido que en las comunidades talayóticas hasta el 800-700 a.C. se siguieron utilizando las antiguas necrópolis originarias del Bronce Final (cuevas naturales con muro de cierre ciclópeo en las estribaciones de los barrancos, o navetas funerarias en las zonas más llanas). Se constata, igualmente, la aparición de nuevos fenómenos funerarios, como el enterramiento en cal o las necrópolis de hipogeos de planta sencilla en Menorca. Existe pues una gran diversidad de estrategias funerarias que corroboran la enorme complejidad de las primeras fases de la Cultura Talayótica (Calvo y Guerrero, 2011). En este horizonte cronológico (900-800 a.C.) se conocen en Menorca distintos complejos funerarios como los monumentos circulares de Son Olivaret (Plantalamor *et al.* 2008), navetas como la de Rafal Rubí (Fadrique y Malgosa, en prensa) en las zonas centrales de la isla, o las cuevas excavadas en los acantilados de calas y barrancos como la de Calascoves (Alaior). Las más antiguas de estas cuevas tienen la planta circular u ovalada, son de pequeñas dimensiones y se encuentran en sitios elevados de difícil acceso como la Cova des Càrritx (Lull *et al.* 1999a), la Cova del Mussol (Lull *et al.* 1999b) o la Cova des Morts de Mongofre Nou (Bergadà y Nicolàs 2005).

En este contexto, se sitúa la Cova des Pas, uno de los yacimientos funerarios más importantes de Menorca. Se trata de una cueva kárstica situada al sur de la isla, en el término municipal de Ferreries (Menorca, España. Figura 1). Está enmarcada en un paisaje de barrancos que desembocan en los acantilados de la costa meridional de la que le separan solamente cuatro kilómetros en línea recta. Concretamente, la cueva se encuentra en la pared de un acantilado en la zona sureste del barranco de Trebalúger (39 ° 57 ‘50’’E, 80 msm), a unos 15 m por encima del suelo del cañón. La cueva no es muy grande, presentando aproximadamente unos 6.5 m de anchura, 4.5 m de longitud y con una altura máxima de 1.7 m (Fullola *et al.* 2008).

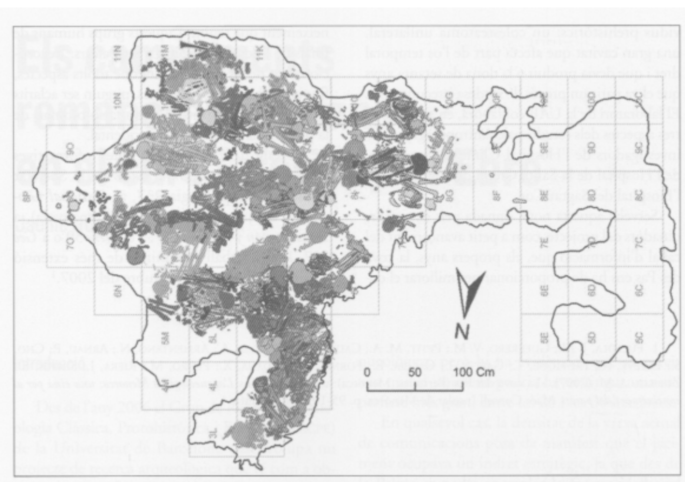

Figura 1. Esquema de la distribución de individuos en La Cova des Pas.

En la Cova des Pas se encontraron al menos 66 individuos que, atendiendo al patrón común de enterramiento y las características morfológicas, pertenecieron a una misma población que, según las dataciones de C^{14}, la utilizó aproximadamente durante unos tres o cuatro siglos (Armentano *et al.* 2010, 2012). A pesar de que los primeros enterramientos fueron depositados aproximadamente el 1.100 cal. a.C., al final de la Edad de Bronce, el mayor número de inhumaciones se realizaron entre el 900 y el 800 cal. a.C. (Van Strydonck *et al.* 2010), durante el periodo Talayótico (Cabanes y Albert 2011). Los individuos encontrados pertenecían a todas las clases de edad y ambos sexos, lo que indica que probablemente reflejaban la situación demográfica de la población que le dio origen (Armentano *et al.* 2010).

Los restos se encontraron excepcionalmente bien preservados a pesar de su antigüedad, conservando incluso tejidos blandos, hecho muy inusual en el contexto mediterráneo norte. Entre los factores que podrían haber contribuido a su preservación se encuentran tanto el ambiente y las características geomorfológicas de la cavidad, como el propio ritual funerario. En la cueva se encontraron minerales altamente solubles como el nitrato de sodio y el yeso. La estructura microcristalina del yeso indica que su origen es diagenético y ha sido considerado el resultado de la oxidación del azufre orgánico de los cuerpos enterrados. Los análisis micromorfológicos y químicos sugieren que predominó un ambiente seco estable dentro de la cueva que favoreció la preservación del material orgánico. Así pues, tanto la ubicación, como la morfología y la litología del yacimiento contribuyeron a las condiciones ambientales áridas, lo que evitaría la lixiviación de yeso y favorecería la conservación de los restos y materiales de origen orgánico (Bergadà *et al.* 2015). Estos minerales absorberían la baja humedad presente en los sedimentos e inducirían la conservación natural de los cuerpos mediante la inhibición parcial de la actividad bacteriana (Cabanes y Albert 2011).

El ritual funerario por su parte, implicaba el enterramiento de los individuos en posición flexionada muy forzada, envueltos con pieles y atados con cuerdas formando fardos funerarios (Armentano et al. 2012). En esta forma fueron transportados en literas funerarias hasta la cueva, donde se han encontrado restos de diversas parihuelas. Debajo de algunos cuerpos se hallaron depósitos de pequeñas ramas y hojas en forma de lecho mortuorio (Servera et al. 2008) lo que confirma la complejidad de la práctica sepulcral. El estudio histológico y químico de los tejidos humanos conservados muestra que el mecanismo de preservación fue, en un primer momento, la formación de adipocera (Prats-Muñoz *et al.* 2013) favorecida por la acumulación de cuerpos. Así pues, en conjunto, las diversas evidencias indican que el tipo de ritual funerario y las condiciones ambientales actuaron conjuntamente a favor de esta conservación.

La excelente preservación de los diversos tejidos orgánicos humanos y pieles, cuerdas y maderas, hacía prever una excelente conservación del material genético. El principal objetivo de este estudio fue caracterizar la población inhumada en La Cova des Pas con la finalidad de concretar la composición de la población confirmando el sexo de los individuos subadultos mediante el análisis genético, interpretar las relaciones intra e interpoblacionales mediante la determinación de los haplogrupos mitocondriales, y conocer el origen de la población comparando los resultados obtenidos con poblaciones actuales y antiguas de las Islas Baleares.

Material y métodos

Se estudiaron muestras procedentes de 50 individuos enterrados en la Cova des Pas. En aquellos casos en los que fue posible, se escogieron piezas dentales que no presentasen ningún tipo de fractura y pudiesen ser asignados sin duda a un individuo determinado. Cuando no fue posible, se escogió una pieza del esqueleto postcraneal. Debido a la diferente estructura de huesos y dientes, el protocolo de descontaminación fue ligeramente distinto en ambos casos. Los dientes se sumergieron en lejía al 5% durante 5 min en un tubo Falcon previamente esterilizado con luz ultravioleta (UV en adelante) durante 30 min agitándolo de forma intermitente con la intención de eliminar los restos de suciedad y minimizar el ADN contaminante. Posteriormente, cada una de sus caras fue expuesta durante 15 min a luz UV y se llevó a cabo la extracción en sets de 4 o 5 muestras y un blanco de control. Cada pieza fue cortada por su límite amelocementario usando para ello material odontológico, y se extrajeron 0.5 gr de polvo procedente de la dentina presente en la cavidad pulpar usando un micromotor de alta velocidad al que se le acopló una fresa de diamante (Solórzano 2006; Díaz 2010).

En lo que se refiere a los huesos, debido a su estructura porosa, no se usó lejía y no se expusieron a luz UV ya que podría afectar la integridad del material genético y disminuir la eficiencia de los resultados. Cada hueso fue cortado con una fresa serrada en la parte medial de la diáfisis, se eliminó la capa de hueso esponjoso con una fresa en punta, y se extrajo aproximadamente 1gr de polvo de tejido compacto interno del

canal medular. La cantidad de polvo extraída fue mayor en huesos ya que la eficiencia en la extracción es menor en las piezas óseas (Schultes *et al.* 2000; Díaz 2010).

Se utilizaron dos métodos de extracción del ADN a fin de maximizar el éxito en la recuperación del material genético. Uno de los métodos está basado en la metodología clásica del fenol-cloroformo, mientras que el otro utilizó un kit comercial de Qiagen específico para muestras degradadas. El método de extracción del fenol-cloroformo modificado (Malgosa *et al.* 2005), consistió en la incubación del polvo de dentina o hueso durante toda la noche en 5 ml de buffer de extracción (TrisHCl 1M (pH 8.0-8.5), SDS 10%, EDTA 0.5M y agua estéril desionizada) y 50 ml de proteinasa K a 0.01 gr/ml. Después de la incubación, el ADN fue extraído con el método del fenol-cloroformo y la fase acuosa fue concentrada usando las columnas de filtración Centricon-30 (Millipore) eluyendo un volumen final de 30 ml. No obstante, el porcentaje de éxito obtenido fue escaso. Por ello, se ensayó con un kit comercial especialmente preparado para muestras forenses: el QIAamp DNA Investigator (Qiagen), con el cual los resultados mejoraron notablemente (Simón *et al.* 2012). Siguiendo esta nueva metodología, las muestras se incubaron con EDTA 0.5M en agitación constante durante toda la noche a 37ºC. Al día siguiente, se prosiguió con la extracción tal y como especifica el protocolo del kit. En aquellos casos en que no se obtuvieron resultados después de 3 reacciones de amplificación, se realizó un paso extra de purificación para tratar de eliminar el remanente de inhibidores que pudieran quedar. Las muestras que seguían proporcionando resultados negativos se descartaron. Globalmente, este segundo protocolo proporcionó un mayor porcentaje de éxito que el del fenol-cloroformo (Simón *et al.* 2012).

Debido a que suele haber varios centenares de copias de ADN mitocondrial (ADNmt en adelante) por cada copia de ADN nuclear (Poinar *et al.* 2006; Kuch *et al.* 2007), y a que parece conservarse mejor en los procesos de diagénesis (Schwarz *et al.* 2009), se ha usado esta molécula en este trabajo como principal fuente de información. Entre sus ventajas se incluyen una herencia exclusivamente materna sin recombinación y una alta tasa de mutación, por lo que se puede seguir su huella en períodos de tiempo relativamente cortos (Ramakrishnan y Hadly, 2009).

Para su análisis se tuvo en cuenta tanto la secuencia de la primera región hipervariable I del D-loop (RHVI en adelante) a fin de determinar el haplotipo, como los polimorfismos de restricción (RFLPs en adelante) de su zona codificante para determinar el haplogrupo. En primer lugar se amplificó un fragmento de 230 pares de bases (pb en adelante) de la RHVI entre las posiciones 16210 y 16400. En los casos en los que no se obtuvo amplificación, se analizaron 2 fragmentos más pequeños solpantes, de 89 y 193 pb que abarcan esta región (Tabla 1). Para amplificar las fragmentos de la región codificante que contienen las posiciones que definen los haplogrupos y llevar a cabo la restricción (Torroni *et al.* 1996; Richards *et al.* 2000), se usaron pares de primers de aproximadamente 120 pb (Tabla 2).

Tabla 1. Primers usados para secuenciar la primera región hipervariable del DNAmt (HVRI)

Fragmento 1 (193pb)	L-16209 : 5'-CCCCATGCTTACAAGCAAGT-3' (Montiel et al. 2001) H -16401 : 5'-TGATTTCACGGAGGATGGTG-3' (Vigilant et al. 1991)
Fragmento 2 (131pb)	L-16209 : 5'-CCCCATGCTTACAAGCAAGT-3' (Montiel et al. 2001) H-16339 : 5'-GTGCTATGTACGGTAAATGG-3'(Díaz 2010)
Fragmento 3(89pb)	L-16313 : 5'-CACCCTTAACAGTACATAGTAC-3' (Montiel et al. 2001) H -16401 : 5'-TGATTTCACGGAGGATGGTG-3' (Vigilant et al. 1991)

Las reacciones de PCR se llevaron a cabo en un volumen final de 50 ul usando la ADN polimerasa *AmpliTaq Gold*® (Applied Biosystems). La amplificación consistió en un primer paso de desnaturalización de 10 min a 94ºC, seguido de 39 ciclos de amplificación (50 s a 94ºC, 1 min a la temperatura adecuada de annealing dependiendo de la región a amplificar y una fase de elongación de 1 min a 72ºC) y un paso final de elongación de 5 min a 72ºC, o de 10 min cuando la región amplificada iba a ser clonada. La purificación de las secuencias amplificadas se realizó mediante el kit JetQuick PCR Purification kit (Genomed). Los productos amplificados fueron visualizados con bromuro de etidio en un gel de agarosa al 3%. Las reacciones de secuenciación se llevaron a cabo utilizando el kit BigDye Terminator v.3.1 (Applied Biosystems) siguiendo las instrucciones del fabricante y se analizaron en un secuenciador ABI 3130XL (Applied Biosystems). El proceso de clonación se llevó a cabo usando el TOPO TA Cloning Kit (Invitrogen).

Tabla 2. Primers de la región codificante y sitios de restricción

Restr.Site	Primers	Coordinates	Primer sequences
-7025 Alu I	L6968/H7052	6949-6968	5'-CCGTAGGTGGCCTGACTGGC-3'
	(Francalacci, p.c.)	7071-7052	5'-TGATGGCAAATACAGCTCCT-3'
-9052 Hae II	L9020/H9082	9000-9020	5'-ACGCCTAACCGCTAACATTAC-3
	(Montiel, 2001)	9103-9082	5'-AGATGATAAGTGTAGAGGGAAG-3'

+11719 Hae III	L11650/H11771	11669-11650	5'-AGTAACAGCCATTCTCATCC-3'
	(Díaz, 2009)	11791-11771	5'-GAGTGCGTTCGTAGTTTGAG-3'
+12308 Hinf I	L12237/H12309	12216-12237	5'-CACAAGAACTGCTAACTCATGC-3'
	(Izagirre, 1998)	12279-12308	5'-ATTACTTTTATTTGGAGTTGCACCAAGATT-3'
-8994 HaeIII	L8927/H9014	8908-8927	5'-TTCTTACCACAAGGCACACC-3'
	(Montiel, 2001)	8995-9014	5'-AGGTGGCCTGCAGTAATGT-3'
+14465 AccI	L14399/H14533	14417-14398	5'-ACACTCACCAAGACCTCAA-3'
	(Díaz, 2004)	14513-14533	5'-GGGAGGTTATATGGGTTTAA-3'

Cuando fue posible, las muestras se asignaron a haplogrupos usando la información combinada de la RHVI y los polimorfismos de la región codificante siguiendo la clasificación filogenética actualizada que iniciaron van Oven y Kayser en el 2009.

Para la determinación del sexo se analizó el polimorfismo de longitud en el primer intrón del gen que codifica para la amelogenina (Nakahori *et al.* 1991). El tamaño del gen en ambos cromosomas sexuales difiere a causa de la deleción de 6 pb en el cromosoma X. La diferencia en la longitud del fragmento se detectó en un gel de Nusieve al 3% a 120V durante 30 minutos.: Al existir la posibilidad de que en algunos casos sólo amplificase la banda de amelogenina de un tamaño de 106 pb correspondiente al cromosoma X, pudiendo ser un falso positivo para el sexo femenino, a modo de control se realizó una amplificación para cada individuo para un fragmento de 93 pb del gen SRY del cromosoma Y (Santos *et al.* 1998) (Tabla 3) que amplifica exclusivamente en los individuos masculinos.

Tabla 3. Primers de amelogenina y SRY utilizados

Primers Amelogenina (58 y 64 pb) (Nakahori et al. 1991)	AMEF: 5'- CCCTGGGCTCTGTAAAGAATAGTG-3' AMER: 5'–ATCAGAGCTTAAACTGGGAAGCTG-3'
Primers SRY (49 pb) (Santos et al. 1998)	SRYF: 5'- ATAAGTATCGACCTCGTCGGAA-3' SRYR: 5'- GCACTTCGCTGCAGAGTACCGA-3'

Para enmarcar la población inhumada en la Cova des Pas en su contexto geográfico e histórico, se compararon los resultados obtenidos con los de otras poblaciones antiguas de las Islas Baleares y con sus homólogas modernas (Tabla 4). El tratamiento estadístico de los datos en lo concerniente a haplogrupos y haplotipos se realizó mediante el programa Arlequin v3.1 (Excoffier *et al.* 2005). Aparte de comprobar la presencia o ausencia de diferencias significativas entre las poblaciones en estudio, se realizó un escalamiento multidimensional para el análisis de secuencias usando un fragmento de 156 pb de la RHVI, que abarcaba las posiciones 16210 a 16365, a partir de los valores transformados de FST en la matriz de Slatkin (Slatkin 1995). Además, se realizó el análisis de correspondencias de haplogrupos usando el paquete estadístico SPSS (v.15.0).

Tabla 4. Muestras usadas para la comparación de haplogrupos y haplotipos

Poblaciones	**Abreviatura**	**N haplogrupos**	***N secuencias***	**Periodo**	**Región**	**Referencias**
Antiguas						
Cova des Pas	CPHI	20	*17*	Edad de Bronce-Hierro	Menorca, Islas Baleares	Presente estudio
Son Olivaret	SOVHI	22	*15*	Edad de Hierro	Menorca, Islas Baleares	Simón y Malgosa, 5/5/2014
Son Real	SRHI	37	*22*	Edad de Hierro	Mallorca, Islans Baleares	Díaz 2010
S'Illot des Porros	SIPHI	31		Edad de Hierro	Mallorca, Islas Baleares	Díaz 2009
Can Reiners	CRMA	28	*15*	Edad Media	Mallorca, Islas Baleares	Díaz 2010
Modernas						
Mallorca	MALL	112	*112*	Actual	Islas Baleares	Picornell et al. 2005 y Falchi et al. 2006
Menorca	MEN	46	*46*	Actual	Islas Baleares	Picornell et al. 2005
Chuetas	CHU	47	*48*	Actual	Islas Baleares	Picornell et al. 2005
Ibiza	IB	50	*50*	Actual	Islas Baleares	Picornell et al. 2005

Controles de autenticidad

Uno de los problemas principales del trabajo con ADN antiguo (en adelante ADNa) viene dado por la posible contaminación con material genético exógeno. En este tipo de estudios, al estar el material genético tan degradado, cualquier exposición a fuentes mejor conservadas, principalmente ADN actual, incrementan la posibilidad de que éstas últimas sean las observadas en los resultados en lugar del ADN perteneciente a los propios restos arqueológicos. En este sentido, la fuente más probable de contaminación es la de los investigadores que manipulan las muestras desde su hallazgo por parte de los arqueólogos, pasando por la manipulación a la que son sometidas en el laboratorio para el estudio de su material genético (Herrmann y Hummel 1994). En concordancia con procedimientos sugeridos anteriormente en trabajos con ADNa (Cooper *et al.* 2001; Pääbo *et al.* 2004; Montiel *et al.* 2007) las extracciones se llevaron a cabo en un laboratorio aislado, exclusivo para los procesos prePCR. El laboratorio está equipado con presión positiva y un sistema de exposición de luz UV. Además se utilizó material de un solo uso (guantes estériles, mascarillas, reactivos, pipetas con filtro, etc), y lavados frecuentes con etanol y lejía de las superficies de trabajo y del equipo. Finalmente, los investigadores, cuyo perfil genético es conocido, trabajaron siempre primero en el área de prePCR y después en el área de postPCR, lo que ha demostrado ser una medida tan efectiva como el uso de las cámaras de presión positiva para evitar contaminaciones (Willerslev y Cooper 2005).

Como controles directos, se replicaron totalmente tres de las muestras obtenidas y otras siete de forma parcial, obteniéndose idénticos resultados en todos los casos. También se realizó la clonación de la secuencia obtenida en 3 de los individuos, con el objetivo de verificar que la secuencia directa era el resultado consenso de esa región para dicho individuo, y no el de una molécula afectada por el daño postmortem o el de una molécula exógena no perteneciente al individuo.

Con estas herramientas, y todas las amplificaciones posteriores incluyendo los blancos de extracción y de amplificación, se confirmó la procedencia de las secuencias con una consideración crítica de toda la información disponible. Finalmente se llevó a cabo una aproximación integrada para las poblaciones humanas, donde se aplicaron la flexibilidad y el uso inteligente de los criterios de autentificación (Montiel *et al.* 2007).

Resultados

Se obtuvieron resultados genéticos en 20 de los 50 individuos analizados, 10 en lo concerniente a la determinación del sexo genético, y 20 para ADNmt. En lo referente a la determinación del sexo, de los 10 individuos determinados con éxito, 6 habían sido diagnosticados ya a nivel morfológico, existiendo una

correspondencia clara entre ambos diagnósticos (Tabla 5). Los 4 restantes corresponden a individuos con exigua preservación o de corta edad y escaso dimorfismo sexual, por lo que el diagnóstico morfológico del sexo no se realizó, no pudiendo ser contrastada la determinación genética. Se confirmó así la presencia de dos individuos masculinos y uno femenino adultos, así como 4 subadultos masculinos y 3 femeninos.

Tabla 5. Diagnóstico sexual de los individuos inhumados en la Cova des Pas

Individuo	Edad (años)	Sexo morfológico	Amelogenina	SRY	Determinación consenso
2	30-40	♂	XY	+	♂
4	15-16	♂?	XY	+	♂
9	12±1	♀?	XX	-	♀
25	9-10	♂?	XY	+	♂
26	13±1	Indeterminado	XX	-	♀
37	2±8 meses	Indeterminado	XY	+	♂
41	35-45	♀	XX	-	♀
50	45-50	♂	XY	+	♂
57	8±1	Indeterminado	XX	-	♂
69	12±1	Indeterminado	XY	+	♀

En relación al ADNmt, se obtuvieron secuencias de la RHVI en un 38% de las muestras (19 individuos secuenciados a partir de 50 muestras). Se recuperó un fragmento de 193 pb de 17 individuos, mientras que de dos individuos, #17 y el #22, sólo fue posible recuperar fragmentos de 131 pb y 89 pb respectivamente.

A nivel de haplogrupos, la eficiencia fue de un 40% habiéndose obtenido información genética en 20 de los 50 individuos analizados (a los resultados de las secuencias debemos añadir el del individuo #15, del cual no se pudo recuperar ningún fragmento de la secuencia RHVI, pero se pudo analizar por RFLPs). De los 20 individuos, 12 pertenecían al haplogrupo H, 5 al U, 2 al K y 1 al W (Tabla 6, Fig. 2).

Tabla 6. Caracterització de los individuos inhumados en la Cova des Pas mediante análisis de RFLPs de la región codificante.

Individuo	Secuencia (16210-16400), -16000	7028 Alu I	12308 HinfI	11719 HaeII	8994 HaeIII	9052 Hae II	Hg RHVI	RFLPs
Ind 2	362C	+	-				H	H
Ind 4	rCRS	-	+				U	U
Ind 9	362C, 390A	-					H	H
Ind11	rCRS		+				U	U
Ind 15	n.d	-					H	H
Ind 17	223T, 301C(1°)	+	-		-		W	W
Ind 22	399G(2°)	+	+				U	U
Ind 25	rCRS	-					H	H
Ind 26	rCRS	-					H	H
Ind 29	rCRS	-	-	-			H	H
Ind 30	rCRS	-					H	H
Ind 31	rCRS	-					H	H
Ind 35	rCRS	+	+				U	U
Ind 37	256T, 270T, 389A, 399G		+				U5	U5
Ind 41	224T, 311C		+			-	K	K
Ind 47	rCRS	-					H	H
Ind 50	224 T, 311C	-					K	K
Ind 57	rCRS	-					H	H
Ind 62	rCRS.	-					H	H
Ind 69	rCRS.	-					H	H

De los 11 individuos H de los que se pudo determinar la secuencia, 9 pertenecían al haplotipo rCRS para el fragmento amplificado (Andrews *et al.* 1999), mientras que uno presentaba una transición en la posición

16362, y el otro añadía a ésta una transición en la posición 16390. En referencia al haplogrupo U, 3 de los 5 individuos pertenecientes a dicho grupo mostraban la secuencia de referencia (rCRS), mientras que otro presentaba transiciones en las posiciones 16256, 16270, 16389 y 16399, perteneciendo por lo tanto al haplogrupo U5, y el último individuo del cual sólo se pudo obtener una secuencia parcial de 89 pb, mostró una transición en la posición 16399. La secuencia perteneciente al haplogrupo U5 no es compartida con ningún individuo de las poblaciones actuales y antiguas de las Baleares; se trata pues de una secuencia única. Por su parte, los 2 individuos K pertenecían a su haplotipo basal (16224T, 16311C), mientras que el individuo determinado con el haplogrupo W presentaba 2 transiciones, habiendo sufrido una retromutación en una de sus 2 posiciones definitorias, la 16292, que ha sido definida sin embargo como un hotspot mutacional (Santos *et al.* 2010).

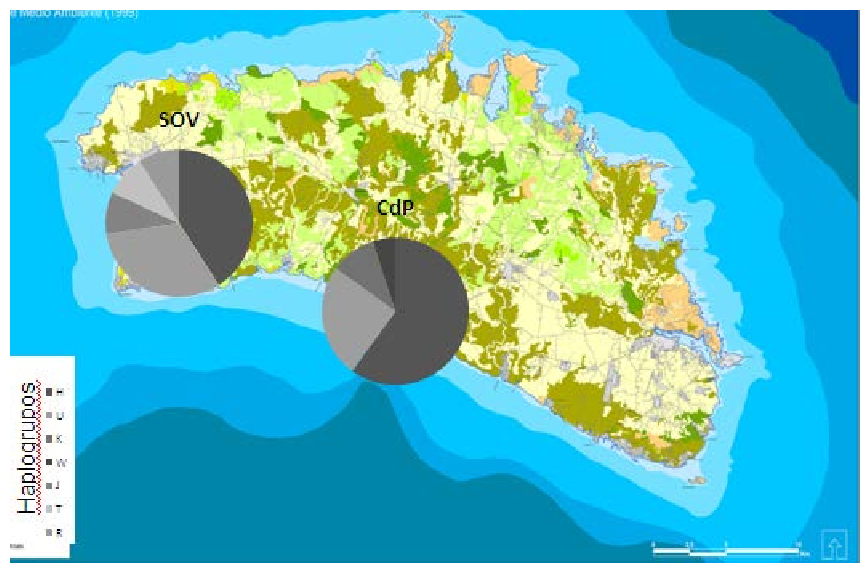

Figura 2: Distribución de haplogrupos mitocondriales en los dos yacimientos talayóticos de Menorca

Estos resultados se replicaron mediante diversos análisis a fin de autentificarlos: a) se duplicaron totalmente los análisis de los individuos 57 y 62 con extracciones independientes tanto por secuencia como por restricción, b) se obtuvo por duplicado el fragmento de 131 pb en los individuos 2, 4, 11 y 41, c) se duplicó el fragmento de 89 pb en el individuo 30, y d) se determinaron por restricción también por duplicado los haplogrupos de los individuos 4, 25, 26, 41 y 47. Además se realizó la clonación de tres muestras obteniendo entre cinco y ocho clones de los individuos 17, 37 y 62 (Tabla 7). En todos los casos, se confirmaron los resultados obtenidos previamente.

Tabla 7. Clones obtenidos de 3 individuos de la Cova des Pas. rCRS (revised Cambridge Reference Sequence) muestra los nucleótidos que se encuentran en las posiciones señaladas en la secuencia de referencia

	Posiciones ADNmt									
INDIVIDUO	16210	16223	16256	16270	16271	16301	16322	16389	16399	16401
rCRS	A	C	C	C	T	C	A	G	A	C
17.1	.	T	.	.	.	T	.			
17.2	.	T	.	.	.	T	.			
17.3	.	T	.	.	.	T	.			
17.4	.	T	.	.	C	T	.			
17.5	.	T	.	.	.	T	.			
62.1	.	.	.	.	.	.	.	.	.	.
62.2	.	.	.	.	.	.	.	.	.	.
62.3	.	.	.	.	.	.	.	.	.	.
62.4	.	.	.	.	.	.	.	.	.	.
62.5	.	.	.	.	.	.	.	.	.	.
62.6	.	.	.	.	.	.	.	.	.	.
62.7	.	.	.	.	.	.	.	.	.	.
62.8	.	.	.	.	.	.	.	.	.	.
37.1	.	.	T	T	.	.	.	.	G	.
37.2	.	.	T	T	.	.	G	A	G	.
37.3	.	.	T	T	.	.	.			
37.4	.	.	T	T	.	.	.	A	G	.
37.5	.	.	T	T	.	.	.	A	G	.
37.6	.	.	T	T	.	.	.	A	G	.
37.7	.	.	T	T	.	.	.	A	G	.
37.8	.	.	T	T	.	.	.	A	G	.

El análisis de diversidad de Nei, mostró que la población inhumada en la Cova des Pas tenía un grado de diversidad genética muy bajo (0.5947) relacionada con el hecho de que sólo están representados 4 haplogrupos. Esto la sitúa en el nivel más bajo de diversidad de las poblaciones analizadas. En este contexto, el haplogrupo más frecuente es el haplogrupo H (60%) con el porcentaje más alto entre las poblaciones baleares actuales y antiguas, mientras que el haplogrupo U (25%) se encuentra dentro del rango normal de las poblaciones utilizadas para hacer las comparaciones. El tercer haplogrupo en frecuencia, el haplogrupo K, muestra un elevado porcentaje (10%, 2 individuos), aunque menor que en las poblaciones actuales de Mallorca y Menorca. Por su parte, el único individuo del haplogrupo W (5%) representa un porcentaje muy similar al encontrado en la actual isla de Menorca (4,87%). Dicho haplogrupo no se ha encontrado en la actual isla de Mallorca (Picornell *et al.* 2005, Falchi *et al.* 2006) pero sí en las 3 necrópolis mallorquinas antiguas estudiadas (Díaz 2010).

El análisis de correspondencias (Fig. 3), sitúa La Cova des Pas junto a dos de las necrópolis mallorquinas, la población Talayótica de S'Illot des Porros y la tardorromana de Can Reiners, debido al alto porcentaje de los dos haplogrupos H y U que presentan las tres series (las frecuencias de H y U tomadas en conjunto no son inferiores al 85%). También, la presencia del haplogrupo W en sus mayores frecuencias en las necrópolis antiguas de La Cova des Pas (5%) y la tardorromana de Can Reiners (7,14%) contribuye a su proximidad genética. Respecto a Son Olivaret, la otra serie Talayótica de Menorca, la serie de la Cova des Pas comparte también una elevada frecuencia de los haplogrupos H y U (72,71%), pero Son Olivaret está fuertemente influenciada por la presencia de los haplogrupos J, T y R0 (9,09% cada uno) ausentes en la serie de estudio. De las series antiguas, la población talayótica mallorquina de Son Real es la más alejada debido a su bajo porcentaje en los haplogrupos H y U (51,34%) y la mayor importancia de haplogrupos poco frecuentes o directamente ausentes en el resto de necrópolis, como puedan ser J (21,62%) o X (8%).

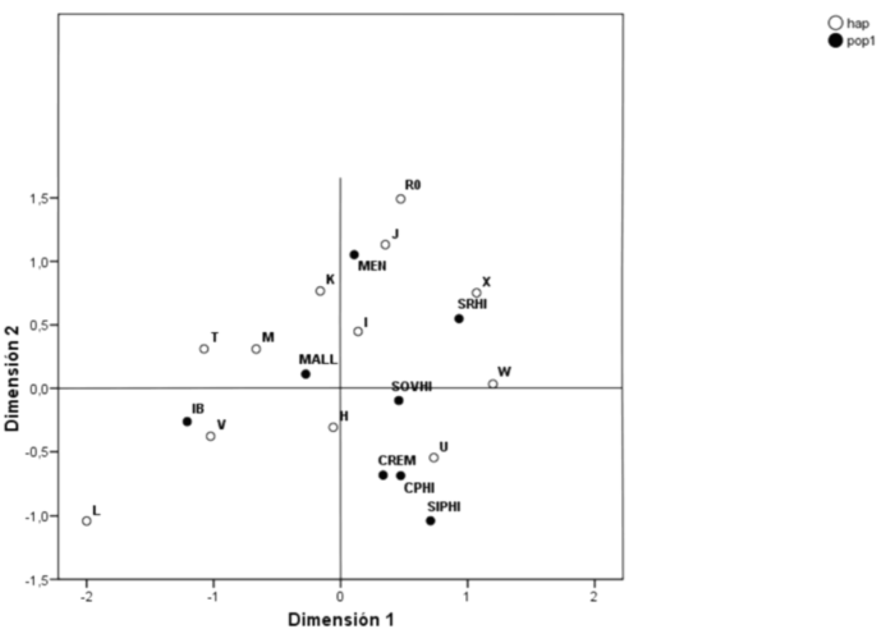

Figura 3. Análisis de correspondencias de las poblaciones baleares actuales y antiguas

En relación a las poblaciones modernas, la Cova des Pas tampoco muestra una dotación particularmente similar. Las dos islas mayores, Menorca y Mallorca, muestran actualmente una frecuencia sensiblemente menor de H (26,08% y 41,07% respectivamente), a la vez que presentan otros haplogrupos como J (17,39% vs 8,92%) excluidos de la dotación de la Cova des Pas. Por otra parte, la población de Ibiza también se aleja de Cova des Pas debido a la alta incidencia de los haplogrupos V (12%) y T (26%) y al hecho de ser la única población balear moderna que presenta un haplogrupo de origen africano (L, 6%), lo cual tiene un gran peso específico en la ubicación lejana de Ibiza respecto a sus contemporáneas, pero también respecto a las series antiguas.

El escalamiento multidimensional (Fig. 4), muestra la Cova des Pas dentro del mismo cuadrante que las poblaciones antiguas de Mallorca (SRHI, CREMA, la necrópolis de S'Illot des Porros se ha excluido en los análisis al no haberse recuperado secuencias de un tamaño suficiente) y las actuales de Mallorca y Menorca que presentan los haplotipos y frecuencias más habituales en las poblaciones actuales plenamente europeas. Las poblaciones en las que se percibe una mayor influencia de poblaciones exteriores, como la africana en Ibiza (Picornerll *et al.* 2005) o del Próximo Oriente en Son Olivaret (Simón y Malgosa 2014) son las más alejadas. Una mención especial merece la población de Son Real (Díaz 2010) que, a pesar de tener una composición bastante distinta de la Cova des Pas no queda demasiado apartada de ella en el escalamiento multidimensional. Son Real cuenta con haplotipos de origen no muy bien definido pero atípicos no encontrados en la actualidad en el continente, y una elevada frecuencia de haplogrupos J y X; sin embargo

muestra una elevada diversidad haplotípica y en su pool genético están representados la gran mayoría de los polimorfismos que se encuentran en las poblaciones europeas, compensando así parcialmente su especificidad.

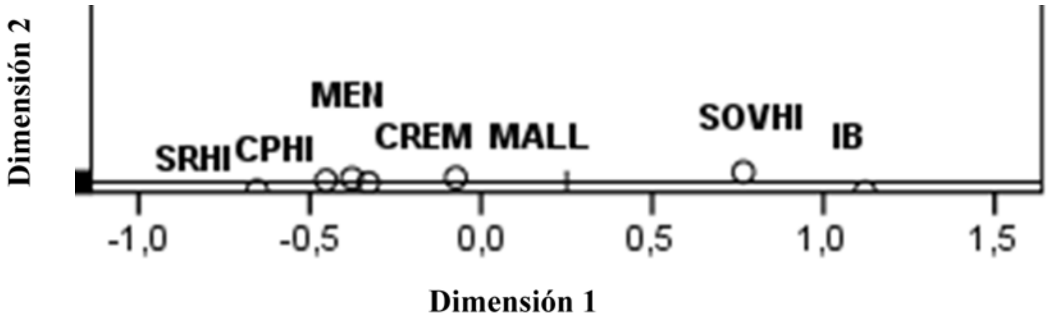

Figura 4. Escalamiento multidimensional de las poblaciones actuales y antiguas de las Baleares a partir de la secuencia de la HVRI (región 16210-16365).

Las distancias genéticas haplotípicas (Tabla 8) con respecto a la Cova des Pas sitúan la población prehistórica menorquina de Son Olivaret, la mallorquina medieval de Can Reiners y las actuales de Menorca y Mallorca con valores negativos, existiendo en promedio mayores diferencias entre los individuos de la Cova des Pas que entre los individuos de dicho yacimiento y dichas poblaciones.

Tabla 8. Distancias genéticas entre la Cova des Pas y el resto de poblaciones baleáricas.

Población	datación	F_{ST}
Son Olivaret	Talayótica	-0,0081
Can Reiners	Tardoromana	-0,00807
Mallorca	Actual	-0,00776
Menorca	Actual	-0,0007
Son Real	Talayótica	0,0244
Ibiza	Actual	0,046

Discusión

Los resultados del análisis molecular de 50 individuos del yacimiento talayótico de la Cova des Pas ha proporcionado información genética de 20 individuos. Así pues, la eficiencia en la recuperación del DNA resultó ser moderada, con distintos grados de éxito dependiendo del protocolo de extracción utilizado (Simón

et al. 2012). Estos resultados concuerdan con el bajo grado de conservación bioquímica y molecular de los restos esqueléticos demostrado en los análisis de C^{14} e isótopos estables (Van Strydonck *et al.* 2010), que contrasta con la excelente conservación de tejidos blandos (Prats-Muñoz *et al.* 2013). La conservación pasó por cierto grado de sapofinicación posiblemente responsable de la conservación estructural y la escasa preservación molecular. A pesar de este sesgo en la preservación, los datos obtenidos son claros y coincidentes entre sí. El hecho de que el análisis del ADN mitocondrial se haya enfocado hacia dos zonas distintas de la molécula, dando en todo momento resultados coincidentes, que haya sucedido lo propio con el análisis de la amelogenina para la determinación sexual en relación al análisis morfológico, la coincidencia en la replicación parcial o total del ADN mitocondrial en extracciones independientes, así como la clonación de tres de los individuos, dan veracidad a la afirmación de que los resultados son robustos y que el ADN obtenido es de origen endógeno.

En referencia a la determinación sexual, los resultados también avalan el estudio morfológico y aportan nuevas identificaciones. Se ha identificado el sexo en 4 individuos subadultos y se ha confirmado en otros 3, ampliando la identificación sexual que hasta el momento era de un 34,8 % (23) de individuos masculinos (Armentano et al 2010) a 36,4 % (24), de un 36,4% (24) de individuos femeninos a 40,9% (27), y disminuyendo el número de indeterminados de 28,8 (19) a 22,7% (15). Aunque en la nueva determinación se altera ligeramente el porcentaje de sexos, continúa habiendo un elevado porcentaje de individuos no diagnosticados a ese nivel. En todo caso, en la necrópolis se constata la presencia de individuos de ambos sexos y distintas edades, confirmando un trato igualitario al menos en el acceso al ritual mortuorio. De todas formas el ritual funerario es habitualmente un reflejo de la situación y posición social, por lo que se deduce que en esta población talayótica todos los grupos de edad y ambos sexos tenían una consideración similar y eran considerados integrantes del grupo desde corta edad.

En relación al ADN mitocondrial los haplogrupos más representados en la necrópolis de la Cova des Pas fueron precisamente los más frecuentes en la mayoría de poblaciones europeas desde la época del Calcolítico, H y U (Ricaut *et al.* 2012) como era de esperar. El hecho de que sólo se hayan encontrado 4 haplogrupos (H, U, K y W) refleja una baja diversidad genética (0.5947) y apunta a una alta endogamia, aunque también podría estar influenciada por la baja medida muestral. A pesar de ello, la composición de haplogrupos en la Cova des Pas no se diferencia significativamente de ninguna otra población balear antigua con la excepción de Son Real, que tiene una baja frecuencia del haplogrupo H y alta de J y X en comparación al resto (Díaz 2009). Lo mismo sucede con la composición de haplotipos, en la que esta necrópolis mallorquina presentó hasta 5 haplotipos únicos.

También en lo que se refiere a la composición haplotípica, la diversidad en la Cova des Pas es baja (0.5) y muy inferior a la de las otras poblaciones baleares antiguas (0.909 en la población talayótica mallorquina de Son Real, 0.714 en la menorquina de Son Olivaret y 0.933 en la población tardoromana mallorquina de Can

Reiners). Combinando este dato con el elevado porcentaje de haplotipos basales encontrados (rCRS en los haplogrupos H y U; y 224T, 311C en el haplogrupo K), se reafirma la percepción de una cierta identidad grupal con un alto nivel de endogamia. Esta identidad está en consonancia con los patrones funerarios comunes que también se han observado en este enterramiento colectivo sucesivo (Armentano *et al.* 2010).

En total, se encontraron 4 haplotipos diferentes entre las secuencias con la longitud suficiente para ser incluidas en el análisis estadístico. El haplotipo rCRS se encuentra ampliamente representado en las Baleares, tanto en las poblaciones antiguas como en las modernas. En cambio los haplotipos correspondientes a los dos individuos H que presentaron mutaciones (#2 i #9) no se corresponden con ningún haplotipo reportado en las poblaciones baleares antiguas (Díaz 2009; Simón y Malgosa 2014) pero sí con un individuo actual de Mallorca y otro de Menorca, y con uno de Mallorca respectivamente (Picornell *et al.* 2005). Estos 2 haplotipos están presentes con una frecuencia moderada en el continente europeo.

A partir de los datos moleculares, se establece un sustrato genético típicamente europeo para la población inhumada en la Cova des Pas. Sin embargo, la comparación con series actuales y antiguas de Menorca y Mallorca, muestran la serie de la Cova des Pas como una población bastante más endógama desde el punto de vista de los linajes femeninos, mucho más que cualquiera de las otras necrópolis antiguas y naturalmente que las poblaciones que habitan las islas en la actualidad. La baja diversidad, manifiesta a partir de la presencia de sólo 4 haplogrupos y 5 haplotipos de ADN mitocondrial, sugiere una población de raíces más matriarcales y poca exogamia femenina, en contraste con otra necrópolis talayótica de la isla (Son Olivaret), y con las grandes necrópolis mallorquinas (Son Real, S'Illot des Porros y Can Reiners).

Este tipo de comportamiento podría estar relacionado con la idiosincrasia del grupo. Los enterramientos talayóticos en Menorca parecen distribuirse en cuevas alejadas (Cova des Mussol, Cova d'es Càrritx), hipogeos (Torre del Ram), y estructuras tipo dolmen (Alcaidús) o sepulcros circulares (Son Olivaret) y finalmente navetas de enterramiento (Rafal Rubí). En algunos casos se buscan espacios alejados de los centros de hábitat, en barrancos y cerca del mar (Javaloyas *et al.* 2008), como es el caso de las cuevas como la Cova des Pas. Estos grupos buscan espacios cerrados, aislados y de muy difícil acceso para sus enterramientos, una forma muy distinta a la ostentosidad de las navetas o estructuras tipo dólmenes. Los ritos de los grupos que enterraban en cuevas parecen mucho más elaborados como demuestran los elementos simbólicos relacionados con tintes del cabello (tintes como en la Cova des Mussol o Es Càrritx; Lull *et al.* 1999a,b), los recipientes complejos de madera y piel para guardar pelo (Cova des Pas, Fullola *et al.* 2008), o el tratamiento del cuerpo (cadáveres envueltos con pieles y transportados en parihuelas en la Cova des Pas; Armentano *et al.* 2012 y Prats-Muñoz *et al.* 2013). No se puede descartar la pérdida de evidencias sobre el rito en las zonas más visitadas, sin embargo no se conserva ningún tipo de elementos rituales más allá de la certeza de enterramientos secundarios en algunas navetas (por ejemplo en la Naveta de Rafal Rubi septentrional; Fadrique y Malgosa, en prensa). Así pues, el rito practicado por el pueblo que dio origen a la

Cova des Pas denota un comportamiento diferente al de las poblaciones del interior de la isla y en este caso, la composición genética explica también este comportamiento recluido y aislado. Esta hipótesis es una invitación al estudio genético de nuevos conjuntos de la isla que permitan testar estas inferencias.

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4.5 Dissecting Balearic populations at the molecular level from the Bronze Age to the current era

Dissecting Balearic Populations at the Molecular Level from the Bronze Age to the Current Era

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The Balearic Islands have been inhabited since the fifth millennium BC. During the Iron Age the communities that inhabited both Majorca and Minorca introduced in both islands constructive elements so majestic that they ended being part of their landscape in a perennial way. The most characteristic among them are the big tower-shaped monuments, generically called talaiots, which give their name to the Talaiotic culture. Their origin can be traced back to the Bronze Age, when the maritime route that joins the Balearic Islands with Catalonia and the Gulf of Leon was one of the most important paths of contact of the Balearic Islands with the continent and Central and Nordic Europe through the Rhône basin (Guerrero et al., 2006a,b).

Between 900 and 800BC, factors such as the high Phoenician influence, the beginnings of a climate crisis provoked by one of the cold episodes of the Holocene, and possibly endogenous reasons of economic and social origin, caused that the archeological entity known as the Talaiotic culture arose in the islands (Guerrero et al., 2006b). At that point in time the naviform settlements used during the Late Bronze Age were being depopulated, and taking profit of those houses as foundation ground, talaiots were built. They had a high symbolic value for the community and signalled their territory, phenomenon which was unprecedented in the islands. The importance given to these places was reflected by their consideration as sacred places throughout the centuries, even after the original constructions had been abandoned, and the reuse of many of these structures (Liliu, 1960; Chapman and Grant, 1995; Calvo, 2009). This moment coincided with the establishment of the Iron Age in the Western Mediterranean façade (Guerrero et al., 2002).

Nevertheless, in spite of a common background in the funerary practices of the Talaiotic populations, in Minorca, between 900 and 750 BC, a great variety of burial methods could be seen: interments in hypogea of either simple or complex floor, natural caves with a cyclopean wall, and an intensive use of the “navetas” and the “taules”, as well as the practice of complex rituals of staining and hair cutting (Lull et al., 1999). All of this drew a scenario different enough to the one documented in Majorca, indicating the need to study these ancient necropolises in each of the islands individually. In this study, we also included a Late Antiquity Majorcan necropolis, which is the biggest ancient cemetery of the island. Thus, our analysis encompasses approximately 1,000 years of history, from the VIth century BC to the VIIth century AD providing the opportunity to gain insights into genetic aspects of the transition to the classical Age.

Another largely discussed subject regarding Balearic prehistory is the origin of the people that generated this culture. Until recently, its emergence was regarded as a direct consequence of the establishment on the islands of battle-hardened people coming

from the East (Rosselló, 1979) better known as Sea peoples (Sandars, 1978), or from populations displaced by them, that would expand throughout the Western Mediterranean and would settle the islands of Corsica, Sardinia, Minorca and Majorca, successively giving rise to the Torreana, Nuragic and Talaiotic cultures (Grosjean, 1955). However, the new dating seems to discard this possibility (Waldren, 1992; Mestres and Nicolás, 1999; Gornés et al., 2001, among others), as the military confrontations in the Nile delta are thought to have occurred between 1,520 and 1,370 BC, in the period when the naviform constructions in the two main Balearic Islands were at their peaks. Moreover, taking into consideration that the Talaiotic culture appeared some decades after 1,000 BC (Trias et al., 2002), the assumed relationships in the origins of the Torreana, Nuragic and Talaiotic cultures seem unlikely (Rosselló, 1979, Plantalamor, 1991, 1997).The two latter cyclopean tower-shaped constructions, Nuragic and Talaiotic, were contemporaneous only between the third phase of the Nuragic and the first phase of the Talaiotic periods, being the Torreana culture more ancient. However, to try to shed some light to this widely discussed subject using molecular tools, the comparison with people from the other western islands is interesting, reason why the sample of ancient Sardinian population has been increased in the present study. Unfortunately, there is no genetic data from the ancient Corsican population.

From this premises, the aims of this work are: a) at territorial level, to compare the mitochondrial genetic pool held by the inhabitants of Majorca and Minorca during the boundary between the Pretalaiotic and the Talaiotic periods to assess whether they share a common origin; b) at temporal level, to assess if there is a genetic continuity in the Balearic islands or if some kind of replacement has happened; and c) to place the ancient Balearic populations in the mitochondrial genetic context of both ancient and current populations from the Mediterranean basin, in order to analyze whether a close genetic relationship between the members of the Talaiotic and Nuragic cultures can be established.

MATERIALS AND METHODS

Location of the studied necropolises

The samples from Majorca analyzed in this paper belong to three necropolises placed at the Bahia de Alcudia: Son Real (ISR, Font, 1974), Illot des Porros (IIP, Malgosa, 1985) and Can Reiners (MCR, Ortega, 2005), at the northeast of the island. They are the only three big necropolises of the island with more than 200 skeletons each one and encompass different moments in time, from the Talaiotic period (ISR and IIP) to the late Roman period (MCR) (Fig.1a, the detailed characteristics of each necropolis can be found at the Supplementary Information). It is important to highlight that IIP and ISR are in front of one another. The main question is, owing to their similarity and proximity, whether the entire ensemble represents one funerary set or if IIP could have been used for the burial of individuals of the high hierarchy inside the Talaiotic Majorcan world, as has been suggested by some authors (Malgosa, 1992; Díaz, 2005).

Concerning the samples from Minorca, they are located in the southern part of the island (Fig.1b), and represent two different kinds of burial practice, both of them being first used during the Late Pretalaiotic period: Son Olivaret (ISOV), a necropolis that was used until the IVth century BC (Plantalamor et al., 2008), and La Cova des Pas (ICP), which was used during the Late Bronze-Early Iron Age (Armentano et al., 2010, 2012). Son Olivaret can offer an overall picture of the genetic pool in the island during the Talaiotic period, taking into account its location in an open landscape and expanded use, while La Cova des Pas is a close necropolis hidden on the wall of a cliff, quite inaccessible and time-bound.

Fig. 1. Location of the ancient Balearic sites. a) Majorca and Minorca island in the context of the Mediterranean Sea. b) Location of the Iron Age necropolises of Son Olivaret (ISOV), Cova des Pas (ICP), Son Real (ISR) and Illot des Porros (IIP), and the Late Roman necropolis of Can Reiners (MCR).

The teeth and fragments of diaphyseal bones from 239 individuals were used: 54 from ISR, 40 from IIP, 40 from MCR, 37 from ISOV, 50 from ICP and 18 from Santa Teresa di Gallura. Whenever possible, teeth that did not show any kind of fracture and could be unambiguously assigned to a specific individual were chosen. Alternately, an intact bone from the postcranial skeleton was selected. However, remains from ISOV were very damaged and mixed, and therefore isolated teeth were used; to avoid the coincidence of the same individual, only the well preserved teeth #24 were processed.

Procedural work

All the samples were analyzed at the laboratory of paleogenetics at the Universitat Autònoma de Barcelona (UAB). All the sterility and authenticity criteria required for aDNA studies were taken into consideration. Sample preparation, DNA extraction and PCR set-up were performed in a laboratory dedicated specifically to ancient DNA (aDNA), positively pressurized and physically isolated from the laboratory for post-PCR procedures, thus restricting the possibility of carryover contamination; additionally, full-body suits, breathing masks and protective lenses were used.

Owing to their different structures, the decontamination protocol was slightly different in teeth and bones. The teeth samples were cleaned and immersed in 5% bleach during 5 min to eliminate contaminant DNA, and finally embedded in distilled sterile water to eliminate the possible remnants of bleach, which could act as PCR inhibitors and/or degrade the DNA samples (Sankaranarayanan et al., 2011). Finally, each of their sides was exposed 15 min to UV light and the extraction was carried out. Each tooth was cut by its amelocementary limit and 0.5 g were extracted from the dentine present inside the pulp cavity using a rotor and a high-speed dental diamond bur (Solórzano, 2006; Díaz, 2009).

Bones were cleaned with a brush. Owing to their porous structure, bleach was not used and they were not exposed to UV light as it could affect the integrity of the genetic material and diminish the efficiency of the results. Each bone was cut in the medial part of the diaphysis and the layer of spongy tissue was eliminated with the

diamond bur. Approximately 1g of powder of internal compact tissue was extracted. The quantity of powder used was bigger in the bone samples because the DNA present is usually not as well preserved as it is in dental pieces (Díaz, 2009).

DNA extraction was performed as described in Malgosa et al. (2005), using a variant of the classic method of phenol-clorophorm, documented as the most effective for this kind of samples (Rohland and Hofreiter, 2007). However, in the case of ICP, the obtained results were very scarce, and a kit (QIAamp DNA Investigator, Qiagen, Carlsbad, USA) was used (Simón et al., 2012) to try to improve results. Sets of both four or five teeth and a blank control were processed in each extraction set.

PCR reactions were carried out in a final volume of 50 ul and *AmpliTaq Gold*® DNA Polymerase (Applied Biosystems, Foster City, USA) was used. Each PCR reaction consisted of an initial denaturation step (10 min at 94ºC) followed by 39 cycles of amplification (50 s at 94ºC; 1 min at the adequate annealing temperature depending on the region to be amplified; and 1 min at 72ºC) and a final extension step of 5 min at 72ºC, or of 10 min if the amplified segment had to be cloned. Amplified fragments were then visualized with Ethidium Bromide staining in a 3% agarose gel.

To analyze the Hypervariable Region I (HVRI), fragments ranging from 87 to 191 base pairs (bp) were amplified. Purification of the amplified samples was performed using JetQuick PCR Purification kit (Genomed, Löhne, Germany).

Sequence reactions were carried out using the sequencing kit BigDye Terminator v.3.1 (Applied Biosystems, Carslbad, USA) according to the manufacturer's specifications and run in an ABI 3130XL sequencer (Applied Biosystems, Carslbad, USA). The clonation process was carried out using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carslbad, USA). In order to detect jumping-PCR artifacts, the original strands from the clones carrying what seemed punctual postmortem damage were identified following the reasoning of Gilbert and collaborators (Gilbert et al. 2003).

Mitochondrial haplogroups were preliminarily inferred from HVRI sequences using human-specific primers (Supplementary Table S1, Supporting Information), and then polymorphic positions of the coding region of the mtDNA associated to the inferred haplogroups (Torroni et al., 1996; Richards et al., 2000) were amplified and analyzed byRFLPs, except for haplogroup R0, whose assignment was corroborated by sequencing the region that included nucleotide position 11,719. Restriction sites and the primers used to amplify each fragment of the coding region are shown in

Supplementary Table S2 (Supporting Information). Samples were finally assigned to haplogroups using the combined information of HVRI and coding region polymorphisms following the phylogenetic classification updated by van Oven and Kayser (2009) whenever possible, as some of them were resolved only by RFLPs owing to the null or partial obtaining of the HVRI sequence.

In order to place the five Balearic necropolises and the Sardinian individuals in the Mediterranean genetic context, an interpopulational statistical analysis was carried out comparing their haplogroup and haplotype frequencies with those of ancient and current populations (Supplementary Table S3, Supporting Information) by Principal Components Analysis (PC) and Correspondence Analysis (CA). To assess the degree of intra and interpopulational variance to determine the possible population internal structure, Analyses of Molecular Variance (AMOVA) were carried out.

Population-specific pairwise genetic distances (F_{ST}) were calculated using 156-bp HVRI sequences (np 16,210–16,365) from each of the populations used in this study, using Arlequin version 3.5. Sequences for comparisons were extracted from worldwide databases such as Human Genome Mitochondrial Database (Ingman and Gyllenstein, 2006 updated), EMPOP CR Database (Parson and Dür, 2007), and GenBank (Benson et al. 2012). Two studies encompassing 24,912 haplotypes were also checked (Richards et al., 2000; van Oven et al., 2009 updated) to look for phylogeographic inconsistencies, abnormal mutational spectra, mosaic (chimeric) sequences that could arise from *in vitro* recombination of different DNA molecules, mainly through jumping-PCR (Bandelt, 2005) and unique haplotypes. An integrative approach for human populations supported by an intelligent use of authentication criteria was applied (Pääbo et al., 2004; Gilbert et al., 2005; Montiel et al., 2007).

RESULTS

The RFLP haplogroup determination from 138 individuals and the region encompassing positions 16,210 to 16,400 of the HVRI sequence from 69 individuals from ancient Balearic necropolises were obtained, as well as 6 new sequences and haplogroups from the Nuragic necropolis from Santa Teresa di Gallura (Table 1), that were added to the results obtained by Caramelli et al. (2007). As the fragment amplified encompassed positions 16210 to 16400, haplogroup J definitory substitutions 16,069C and 16,126C were not included so the assignment of a sample to this haplogroup was always corroborated by RFLPs analysis (-13,704 Bst0I). Moreover, partial sequences

were obtained from 39 individuals that were compatible with the haplogroup subsequently obtained with the RFLPs, with 26 of them belonging to IIP, which showed the highest degree of DNA degradation (Díaz, 2009). A detailed summary of the obtained results per site can be found at Supplementary table S4.

Regarding clonation, five to ten clones from eight different individuals from three different sites were obtained (Supplementary Table S5, Supporting Information), and the sense of the original strand from each of them was determined (Supplementary Table S6, Supporting Information).

TABLE 1. Populations used from Balearic Islands (Spain) and Sardinia (Italy) and number of haplogroups and sequences in the statistical analysis.

Population	Key	N haplog	*N seq*	Period	Region	References
Ancient						
Cova des Pas	ICP	20	*17*	Neolithic	Minorca Island, Balearic Islands	Simón and Malgosa, submitted
Son Olivaret	ISOV	22	*15*	Iron Age	Minorca Island, Balearic Islands	Simón and Malgosa, 2014
Son Real	ISR	37	*22*	Iron Age	Majorca Island, Balearic Islands	Díaz, 2009
S'Illot des Porros	IIP	31		Iron Age	Majorca Island, Balearic Islands	Díaz, 2009
Can Reiners	MCR	28	*15*	Late Antiquity	Majorca Island, Balearic Islands	Díaz, 2009
Sardinia Bronze Age	ANCSARD	30	*30*	Iron Age	Sardinia	Caramelli et al., 2007; Present Study
Current						
Majorcans	MAJ	112	*112*	Current	Majorca Island, Balearic Islands	Picornell et al., 2005; Falchi et al., 2006
Minorcans	MIN	46	*46*	Current	Minorca Island, Balearic	Picornell et al., 2005

					Islands	
Chuetas	CHU	44	*48*	Current	Majorca Island, Balearic Islands	Picornell et al., 2005
Ibiza	IBI	49	*50*	Current	Ibiza Island, Balearic Islands	Picornell et al., 2005
Sardinia	SARD	115	*115*	Current	Sardinia	Richards et al., 2000

Haplogroup analysis

The haplogroup composition of the whole Ancient Balearic population shows a high percentage of haplogroup H (40.57%) and U (31.15%), being clearly their major components (71.72%). In a distant third position there is haplogroup J (7.97%) followed by haplogroups K and W (4.34%). The distribution among the series is not homogenous as can be seen in Table 2, which shows the absolute and relative frequencies in each necropolis.

TABLE 2. Haplogroup frequencies of ancient populations from Mediterranean Bronze-Iron Age and current Balearic Islands

Haplogroups	H	V	U(U5)	K	J	T	X	W	I	R0	M	L	Total
ISR	9(24.32)[1]		10(27.02)	3(8.1)	8(21.62)	1(2.7)	3(8.1)	2 (5.4)		1(2.7)			37
IIP	14(45.16)	2(6.45)	13(41.93)				1(3.22)	1(3.22)					31
MCR	12(42.85)	1(3.57)	8(28.57)	1(3.57)	1(3.57)	1(3.57)		2(7.14)	1(3.57)			1(3.57)	28
ICP	12(60)		5(25)	2(10)				1(5)					20
ISOV	9(40.9)		7(31.81)		2(9.09)	2(9.09)				2(9.09)			22
ANCMAJ[2]	35(36.45)	3(3.12)	31(32.29)	4(4.16)	9(9.37)	2(2.08)	4(4.16)	5(5.2)	1(1.04)	1(1.04)		1(1.04)	96
ANCMIN[3]	21(50)		12(28.57)	2(4.76)	2(4.76)	2(4.76)		1(2.38)		2(4.76)			42
ANCBAL[4]	56(40.57)	3(2.17)	43(31.15)	6(4.34)	11(7.97)	4(2.89)	4(2.89)	6(4.34)	1(0.72)	3(2.17)		1(0.72)	138

[1]Relative frequencies are shown between brackets (%); [2]Sum of the 3 necropolises from Majorca; [3]Sum of the 2 necropolises from Minorca; [4]Sum of the 5 necropolises from both islands.

In order to know the relevance of the haplogroups in each population, a Correspondence Analysis (CA) was carried out for all the current and ancient Majorcan and Minorcan populations (Fig. 2).

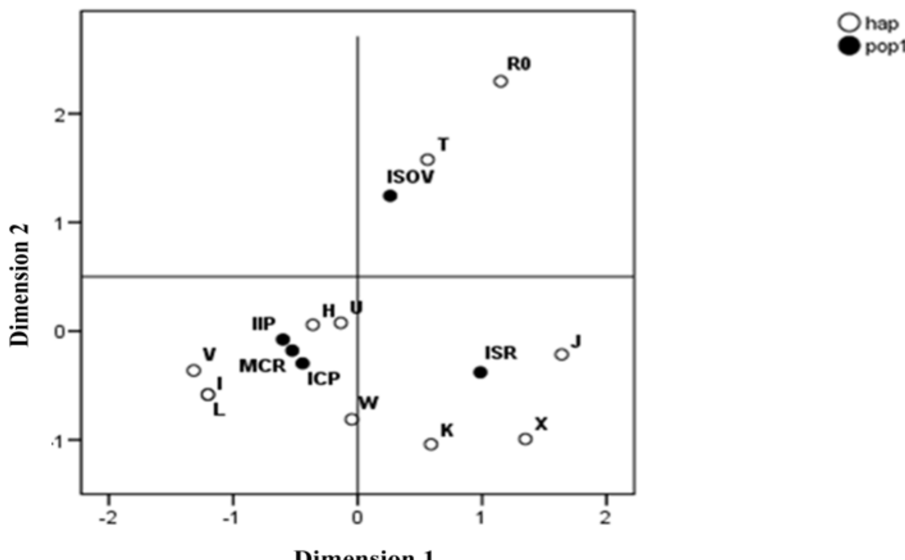

Fig. 2. Correspondence analysis from ancient Majorcan and Minorcan populations.

The placement of the Iron Age necropolises of ICP and IIP and the late Antique population of Can Reiners (MCR) is strongly determined by their high percentage of both haplogroups H and U, while ISR and ISOV stay as outsiders. ISR is characterized by a relatively high frequency of haplogroups K, J, and X (being the only ancient Balearic population showing the last one, with a high percentage of 8%) together with a relatively low frequency of H, pulling it away from the other 4 ancient populations. As a result, ISR is the only one that shows significant differences with some of its contemporaneous Balearic populations as shown with the F_{ST} distances in Table 3, including IIP, the geographically nearest population. ISOV has also a different haplogroup composition, showing a strong influence from its notorious H and U presence but modulated by relatively high frequencies of haplogroups J, R0 and T (9.09% each).

Finally, the presence of haplogroup W in ICP places it nearer the Majorcan Talaiotic necropolis of ISR and from MCR than to its contemporaneous Minorcan population of ISOV. Relative frequencies of all the populations can be found in the Supplementary Table S7.

When considering the five necropolises separately (interpopulational analysis) no significant differences were found (Table 3). However in the intra-island comparison, the Talaiotic populations of Majorca, ISR and IIP show significant distances from each other ($p = 0.028$), but they disappear in the ancient Majorcan ensemble (ISR-IIP-MCR). With regard to Minorca, the two necropolises do not

significantly differ from each other. Moreover, the ensembles from both islands, called Ancient Majorcans and Ancient Minorcans do not show significant differences between them ($F_{ST} = 0.001$; $p = 0.308$). Owing to this lack of differences in intra and inter-island comparisons when the ensemble populations of the two islands are considered, their haplogroup genetic pool has been assembled into Ancient Balearic populations (ANCBAL) in subsequent global population analyses.

TABLE 3. Distance Between populations (FST)

	ISOV	ICP	ISR	MCR	IIP
ISOV		0.010	0.007	-0.023	-0.007
ICP			0.077	-0.012	0.012
ISR				0.019	0.053
MCR					-0.013

significant p value after Bonferroni correction: p < 0,005

A CA with all the current and ancient Balearic populations shows that in spite of their differences, the haplogroup composition from ancient and current Majorca and Minorca Islands are more similar between them than with the Majorcan Jews or Chuetas and the population from the Pitiuses Islands (Ibiza) (Fig. 3); mostly due to the higher presence of R0, M and L haplogroups in the last two groups. The analysis of haplogroups using the one-tailed Z test shows that the frequency of haplogroup H (34.9%) does not hold a significant difference between the diachronic populations ($p > 0.05$), contrarily to what happens with the frequency of haplogroup U (10.58%) ($p < 0.01$) which has considerably decreased over time.

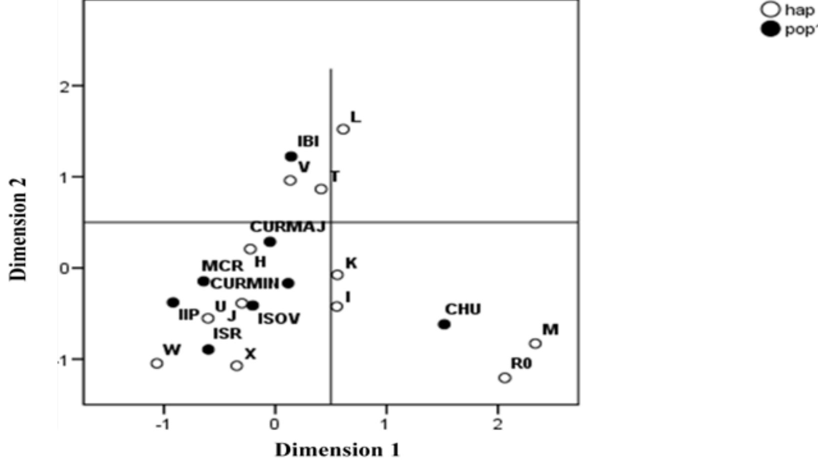

Fig. 3. Correspondence Analysis from all the Current and Ancient Balearic Populations.

The small differences between the current populations of Majorca and Minorca, are mainly due to the presence of haplogroup W in Minorca that is absent in Majorca, and a sensibly higher J frequency in Minorca (17.39% to 8.32%) (Picornell et al., 2005; Falchi et al., 2006). Moreover, while haplogroup R0 strongly influences the Chueta community, haplogroups V and L do so with Ibiza. Finally, in the four modern populations the influence of T and K has increased with respect to the ancient ones.

Genetic difference between the ancient and the current populations of Majorca was not statistically significant (two-tailed t test; $p > 0.05$). On the contrary, there was a significant difference between ancient and modern population of Minorca (two-tailed t test; $p < 0.05$). Finally the AMOVA analysis shows that there are not significant differences between the diachronic populations from the two islands ($F_{CT} = 0.026$; $p > 0.05$; $F_{SC} = 0.0075$; $p > 0.05$).

Haplogroup diversity is lower in the ancient Balearic populations than in the modern ones (Table 4), except in the Majorcan necropolis ISR (0.825). Minorcan ancient populations contain less diversity than Majorcan ones (for instance, 0.594 in ICP, versus 0.825 in ISR). Consequently, the whole set of ancient Majorcan populations show a significantly higher variability.

TABLE 4. Nei Genetic diversity in the Balearic Islands (from higher to lower)

Population	Nei genetic diversity
CHU	0.863±0.02
CURMIN[1]	0.861±0.02
ISR	0.825±0.03
CURMAJ[2]	0.776±0.02
IBI	0.760±0.04
ANCMAJ[3]	0.753±0.02
MCR	0.748±0.06
ISOV	0.740±0.06
ANCMIN[4]	0.674±0.05
IIP	0.634±0.05
ICP	0.594±0.09

[1]Current Minorca; [2]Current Majorca; [3]Ancient Majorca; [4]Ancient Minorca

In order to place the ancient necropolises from both islands inside the frame of their contemporaneous populations from the Mediterranean basin, a PC based on their haplogroup frequencies was carried out (Fig.4). The results showed that the ancient Balearic populations were very close to the ancient Catalan (ANCCAT) population in the first component, which explained 82.13% of the variability (Fig. 4). The Ancient Basque (ANCBA) and Sardinian (ANCSARD) populations were also nearby to one another.

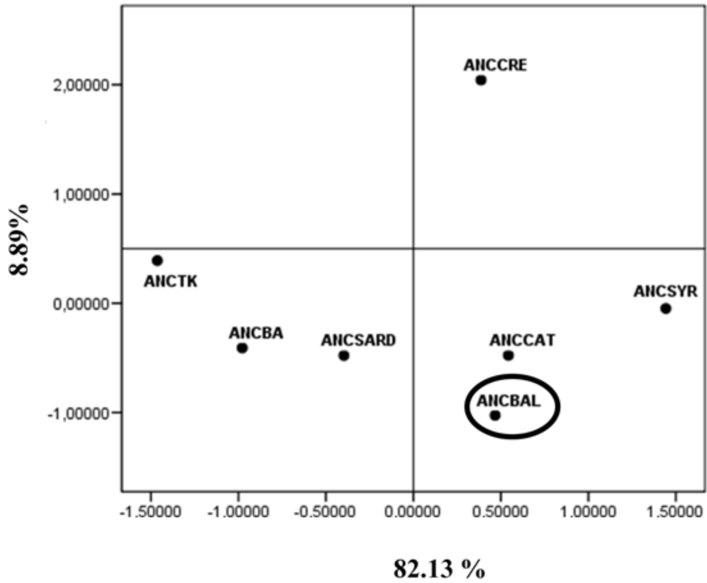

Fig. 4. Principal Components analysis from all the ancient populations

In this analysis, it is important to note that the classification of the individuals from the Ancient Sardinians taken from Caramelli et al. 2007 has been done using the following criteria: rCRS sequences have been classified as belonging to haplogroup H, and sequences with no definitory mutations for a specific haplogroup and not determined by RFLP analysis have been assigned to its most likely haplogroup according to the probabilities obtained by using Haplogrep program (Kloss-Brandstätter et al., 2011); so this result has to be taken with caution. Moreover, there were two Sardinian individuals holding the transition 16223T in their HVRI, belonging with an equal probability to haplogroups M, N and L and that have been classified as M because it made the most phylogenetic sense. The ancient populations from the Balearic Islands show significant distances to the ancient population of Sardinia (ANCSARD) in terms

of haplogroups ($F_{ST} = 0.048$; $p = 0.015$). This is explained by a significantly lower haplogroup diversity (0.551 ± 0.09 vs 0.730 ± 0.02) ($p < 0.05$) in the ANCSARD population, which also has a significantly higher frequency of haplogroup H, the lack of haplogroup T, and the possible presence of the very ancient cluster M that is lacking in ANCBAL.

Haplogroups which do not usually show high frequency in European populations as I, W, X and L are present in ANCBAL and were not found in ANCCAT (Supplementary Table 7, Supplementary Information), even though haplogroup L is present only in the Late Antiquity Majorcan population (MCR). Concerning the other haplogroups, only haplogroup J shows a significantly higher frequency in ANCCAT than in ANCBAL (23.08% vs 7.91%) ($p < 0.05$). The main features distinguishing the three ancient populations can be seen in Figure 5.

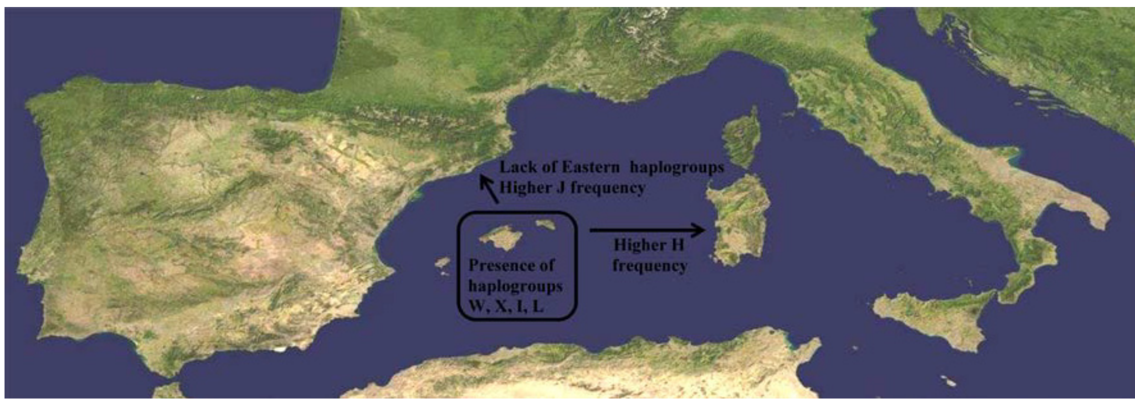

Fig. 5. Main distinctive patterns of the Balearic Islands, Sardinia and Catalunya.

Focusing in the values of genetic diversity (Table 5), ANCBAL are in the middle range considering all the values obtained in the ancient populations. As expected, both ANCSARD and ANCBA show a very low diversity. The value obtained in ancient Turkey (ANCTK) could be related to the possible existence of considerable endogamy within the region studied (Matney et al., 2012).

TABLE 5. Nei Genetic Diversity in Ancient populations

Population	Nei genetic diversity
Ancient Syria (ANCSYR)	0.944±0.07
Ancient Catalonia(ANCCAT)	0.801±0.04
Ancient Crete(ANCCRE)	0.782±0.05

Ancient Balearic Islands(ANCBAL)	0.730±0.02
Ancient Sardinia (ANCSARD)	0.551±0.09
Ancient Basque Country (ANCBA)	0.285±0.19
Ancient Turkey(ANCTK)	0

In order to assess the relationships of ancient Balearic populations with respect to other Mediterranean current and ancient populations regarding their haplogroup distribution, a PC was carried out using 32 populations representative of both the different periods and the geographic region (Table S3, Supporting information) (Fig.6).

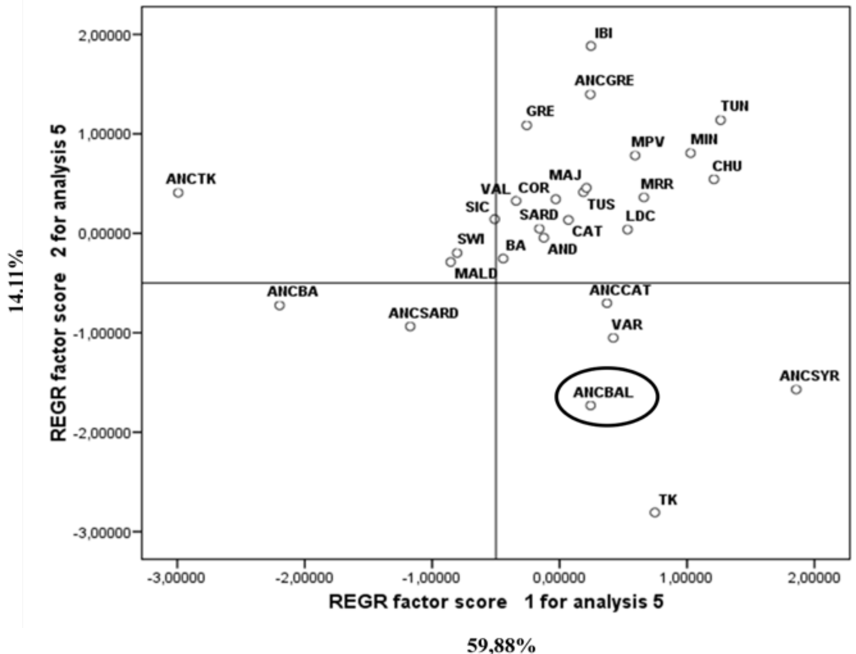

Fig. 6. Principal Components Analysis of all the populations used in the study.

The Ancient Balearics, as the majority of ancient populations (ANCCAT, ANCSARD, ANCBA, ANCTK, ANCSYR), were not totally integrated inside the nucleus of European populations. However, they were in the same level as the main European nucleus with regard to the first component, which explained almost 60% of the variability observed. However, the haplogroup frequencies found in ANCBAL's current counterparts made Minorca and especially Majorca to be placed in the main nucleus of populations, where the two populations of the Middle Ages used in this comparison (MALD and MPV) were already well established.

ANCBAL did not show significant differences with Near Eastern current (TK) and ancient (ANCSYR) populations, because of the Eastern component of ANCBAL given by the high frequency of haplogroups W (4.35%), X (2.90%) and the presence of haplogroup I.

Sequences analysis

The ancient Majorcan population shows significant distances with the ancient Minorcan population ($F_{ST} = 0.025$; $p = 0.035$). The distance between the ancient and the current Majorcan populations is also slightly significant ($F_{ST} = 0.0149$; $p = 0.043$). However, the 5 necropolises do not show significant distances when a multiple comparison is made. When they are taken together, i.e. treated as a single population, and compared with the ensemble of current Majorcan and Minorcan populations (which show a somehow homogeneous genetic pool according to Picornell et al. 2005), no significant differences are found. Regarding haplotipic diversity (shown in Table 6), the AMOVA analysis (Table 7) shows no substructure in these islands populations as seen in the previous AMOVA analysis regarding the haplogroup composition. Aiming to see the overall genetic pool of this geographic area, the ancient series have been pooled again into Ancient Balearics (ANCBAL) for the following analyses in basis of geographic criteria.

TABLE 6. Molecular diversity of all Balearic current and ancient populations

Population	Haplotipic diversity	Number of haplotypes (%)
CURMIN*	0.962±0.01	28 (60.8%)
CURBAL	0.947±0	82 (32.15%)
MCR	0.933±0.05	11 (73.3%)
CURMAJ*	0.930±0.01	48 (42.85%)
IBI	0.922±0.01	16 (32%)
CHU	0,918±0.02	23 (47,91%)

ANCMAJ*	0.916±0.03	20 (54.05%)
ISR	0.909±0.04	12 (54.5%)
ISOV	0.714±0.11	6 (40%)
ANCMIN*	0.601±0.09	8 (25%)
ICP	0.500±0.13	4 (23.52%)

*See footnotes in Table 4

The current population of Majorca does not show significant differences of diversity with the ancient Majorcan necropolises (one-tailed U Mann-Whitney test; $p > 0.05$). On the contrary, the difference of diversity between ancient and current Minorcan series is statistically significant (one-tailed U Mann-Whitney test; $p < 0.05$). The reason can be found in the very low variability of the ancient Minorcan populations of ICP, which has the lowest haplotipic diversity of the analyzed populations (0.500) and that owing to its particular characteristics (Armentano et al. 2010) could quite possibly be holding kin relationships. For its part ISOV reaches a value of 0.714, also much lower than the ones found in ancient Majorcan populations (0.909 in ISR and 0.933 in MCR), but just slightly below the value obtained in the population of Ancient Catalonia (0.752).

In relation to unique haplotypes, ISR held 5 haplotypes that could not be found in the rest of ancient Balearic populations (Díaz, 2009). In turn, ISOV had also two haplotypes (294T, 296T, 304C and 301T, 343G, 356C) not found in the other Balearic populations (a detailed list of unique and shared haplotypes between ancient and current Balearic populations is shown in Supplementary Table S9, Supporting Information). Assembling the necropolises by their originary islands, a 63.6% of the haplotypes found in ancient Majorca were not found in current Majorca representing a 65.96% of the individuals, notably 9 haplotypes from ISR. The 85.71% of the haplotypes in ancient Minorca were not found in current Minorca, representing however a 46.88% of the individuals; it is due to the high percentage of individuals holding rCRS in ICP which happens to be the most common haplotype in Europe (Hervella et al., 2012). For non-shared haplotypes, additional comparisons were performed with the available databases. There were only 5 haplotypes (7.24%) that were not found in other current or ancient population: one from ISOV (U5) and four from ISR (2H, 1K and 1T) (See Supplementary Tables S8 and S9, Supporting Information, for consulted databases).

TABLE 7. AMOVA analysis

SEQUENCE ANALYSIS	FCT (among groups)	FSC (among populations within groups)
ISOV,ICP/ISR,MCR	0.025	-0.001
ANCMAJ,ANCMIN/CURMAJ,CURMIN	-0.004	0.007

The position of Balearic series in the Mediterranean frame was analyzed by a multidimensional scaling analysis. The genetic distances between the ancient populations (Fig.7) show an evident relationship between ANCBAL and ANCCAT series as it was also observed in the haplogroup analysis. In relation to other ancient series, ANCSARD, shows the logical similarity related with their geographic proximity. Basque (ANCBA), Cretan (ANCCRE) and Turkish (ANCTK) series are more distant from ancient series from occidental Mediterranean reflecting different characteristics: a high (ANCSYR, ANCBA, ANCCRE) or low degree of diversity (ANCTK). The latter necropolis has a possibly biased value due to the existence of endogamy as argued in the haplogroup analysis (Matney et al., 2012). ANCBAL are placed in the middle range of values of the ancient populations and in the lower range considering all the populations used in this study (0.803) (haplotipic diversity of all the populations is shown in Supplementary Table S10, Supporting Information).

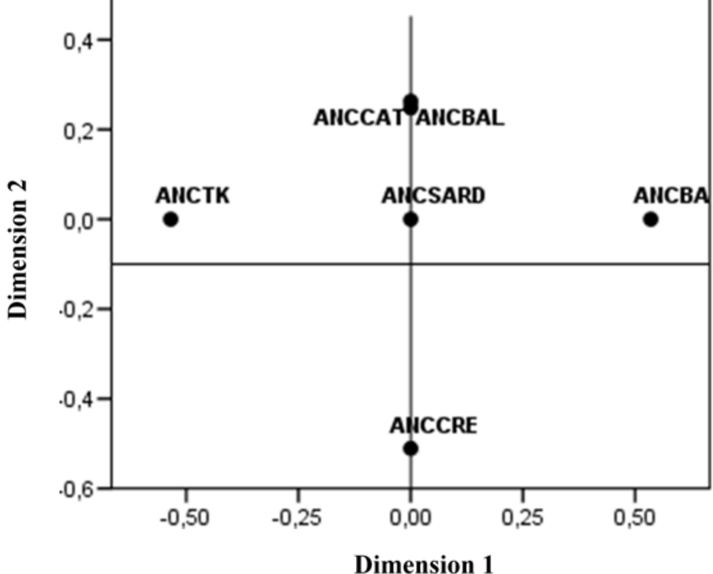

Fig. 7. Multidimensional scaling of ancient populations. ANCSYR was removed as it was so different that made all the other populations be next to one another.

In order to see how the ancient Balearic populations fit within current and ancient populations, a total number of 32 populations were used in a multidimensional scaling analysis (Fig.8). ANCBAL were very well integrated in the nucleus of European populations while ANCSARD were very far away from the rest, even very far from North African populations such as Marrakech (MRR) and Tunisia (TUN).

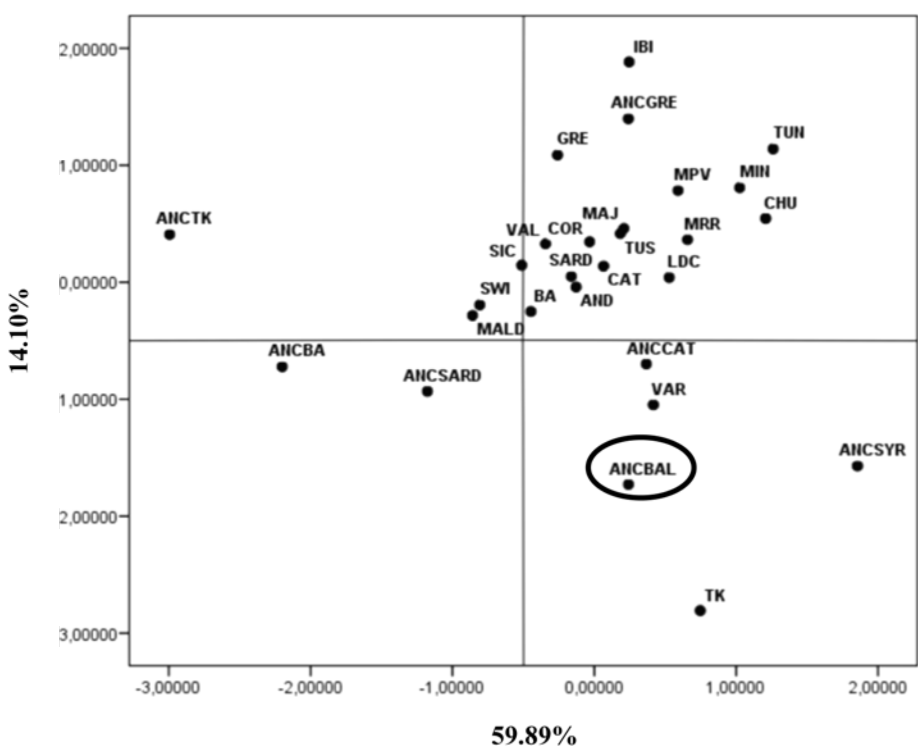

Fig. 8. Multidimensional scaling of all the populations used in this study.

A phylogenetic network including all the haplotypes from the ancient populations used in the present study was carried out (Fig. 9), which shows that the haplogroups pertaining to the macrohaplogroup JT were more frequent in the individuals from the Talaiotic culture (ancient Balearics) than in the individuals of the Nuragic culture (ancient Sardinians). However, they did not show significant differences regarding haplogroup J. Moreover, ancient Balearics and ancient Catalans have a very similar genetic pool, where the main European haplogroups (H, U, K, J) are already well represented. Most of the representatives of the minoritary haplogroups in current Western Mediterranean are represented in individuals of Ancient Eastern Mediterranean, as I (8.1% in ANCCRE and 11.11% in ANCSYR) and M (22.22% in ANCSYR), thus reflecting an ancient migration towards the former area, at least from the female lineage.

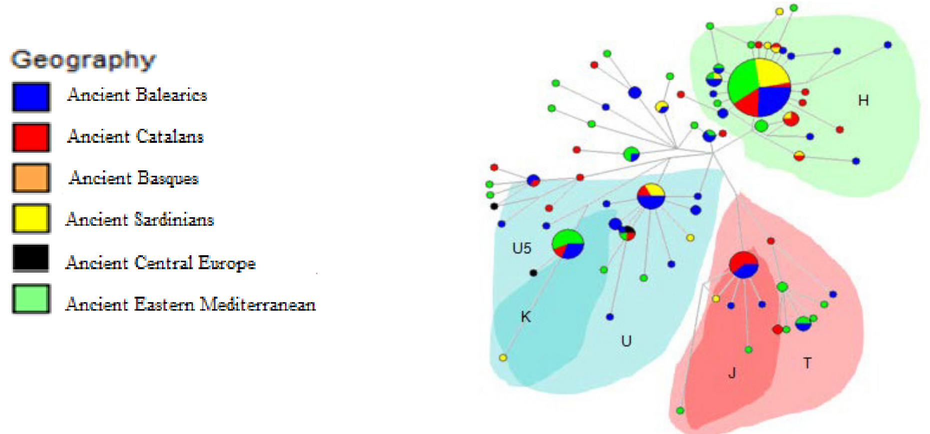

Fig. 9. Phylogenetic Network of the Ancient haplotypes from populations used in this study.

It is interesting to point out that most of the ancient individuals from Sardinia are at the central nodes of the network, constituting a lot of basal haplotypes, as expected by the high number of rCRS representatives that had been already noted (Caramelli et al., 2007).

DISCUSSION

This study is based on the concept that living populations on the major islands of the western Mediterranean basin retain a recoverable genetic record reflective of various episodes of human movements and settlements (Francalacci et al., 2003). Concerning the Balearic Islands, they are known to have received different cultural and demographic influences throughout its history that have not been homogeneous neither in time nor in the source of origin (Casasnovas, 1998), being the focus of this paper in order to shed some light to this subject.

Referring to the authenticity of the results, we have followed all the criteria recommended given each site particular circumstances. The individuals that gave positive results in independent extractions and could therefore be replicated (four of them totally and seven of them partially), giving identical results in all cases (Supplementary Table S4). Moreover, seven of the individuals were cloned showing that the consensus sequence was the one taken in consideration for any given individual (Supplementary Table S5) and the originary strand was determined in each case (Supplementary Table S6) corroborating that we were not detecting jumping-PCR artifacts.

In relation to the Talaiotic necropolises from Majorca, ISR and IIP, the archeological hypothesis suggested a distinction between them considered that the two necropolises, very close to each other, could represent a single population in two successive moments, both in their architectonic constructions (Fernández-Miranda, 1978) and in their burial rituals (Malgosa, 1992). Moreover, differences were found at a morphological level, based in a more gracile typology found in IIP site, which have been used to hypothesize that these individuals might pertain to an elite inside the Majorcan population from the Alcudia bay (Malgosa, 1992), and at a craniometric level (Jordana, 2007). As we have shown that ISR and IIP have significant differences in their haplogroup composition, our results fit with the explanation of a differential use for both necropolises.

Actually, these two cemeteries are unique in the context of the Talaiotic populations of Majorca. They were large constructive funerary spaces, while the rest of islanders used caves to make more reduced burials. These cemeteries could be a reflection of highly populated urban settlements (currently unknown), a center of attraction for people in that period (as shows the high diversity in ISR), but also demonstrated that in large groups the ruling classes, more endogamous and

economically powerful, could use other necropolises (IIP), adopting more costly funerary rites (transportation to the island and incineration). Therefore, these apparent cultural differences seem to have their reflection at the genetic level.

With respect to post-Roman population in Majorca, our results indicate that it was completely integrated in the European genetic pool, both at the haplogroup and at the haplotype level. The presence of an individual holding the haplogroup L3 demonstrates that some people from the African continent had already been present in the island during the Late Roman Age, opening the genetic Mediterranean spectrum of Majorca at a time when the native population would have received many more influences as the Roman world gathered inputs from around the Mediterranean. In addition, the late arrival of peoples from Central Europe (Vandals) increased the variability. As regard to the Minorcan necropolises, ISOV and ICP were placed in the lower range of diversity values commonly found in ancient populations, which might be indicative of consanguinity. However, in ISOV its high diversity with regard to the individuals holding haplogroup U does not favor an endogamic hypothesis, but points to a somehow diverse population. The five individuals belonging to it that could be fully sequenced showed four different haplotypes. In spite of its reduced sample size, this necropolis shows U3, J and T2b, that have been proposed as markers for the spread of farmers from Near East during the Neolithic (Richards et al., 2000), a period during which certain members of these subclusters would have expanded (Fernández, 2005). This fact points towards a high influence of Neolithic populations in this necropolis. On the other hand, ICP show the lowest haplotipic diversity value (0.5) in comparison to the other Balearic necropolises (Table 8), that points to the existence of a very close community in ICP with a high degree of endogamy, as did the common burial pattern and the low lapse of time between all of them (Armentano et al., 2010). In the comparison between contemporaneous populations, the high difference between the values of diversity of ISR and MCR on the one side and ICP on the other side causes the ancient Majorcan population to show a significantly higher variability than the ancient Minorcan one.

Regarding ISOV, the fact that 50% of the represented haplotypes are not found in any of the diachronic Balearic populations makes it the most differentiated necropolis in haplotipic terms. It is possible that the small sample size has an influence in this situation too. ISR also shows a high value of non-shared haplotypes (22.72%, shown in

Supplementary Table S8), but almost all of the other polymorphisms are represented in the other two necropolises of the island (ICP and MCR).

In a global level, the high contrast in the values of variability in the populations from both islands, together with the existence of non-shared haplotypes, make some of the Ancient Minorcan and Ancient Majorcan populations to show significant differences with one another at the haplotipic level. This argues in favor of the existence of intrapopulational variability which is also seen, although not so clearly, in their modern counterparts. Currently, just the two biggest islands show a relatively homogeneous population, although Minorca shows haplogroup W, that is absent in Majorca, and a sensibly higher frequency of haplogroup J (17.39%) with respect to Majorca (8.32%) explained by the influence of the English occupation of Minorca during the XVIIIth Century (Terrón JL, 1998).This last haplogroup is the second most frequent haplogroup in England, with a frequency of 14% (Pellecchia et al., 2007). With regard to Ibiza, the occupation of the Carthaginians in 654 BC and during at least five centuries can still be seen at the molecular level, through the presence of haplogroup L2 at a frequency of 6% (Picornell et al., 2005).

Globally, it can be stated that all of the haplogroups represented in the ancient necropolises are of European origin (with the exception of haplogroup L in MCR). In general, at the haplogroup level the differences among them could be motivated by the own social and archeological peculiarities of each site, such as the small sample size obtained owing to the poor preservation state of the remains (ISOV and IIP), endogamy (ICP) or extended population including rare haplotypes (ISR). However, the particularities of each necropolis case are eclipsed when the ancient sample is considered as a whole. In the global comparison between the five different necropolises no significant differences are found.

Meanwhile, the existence of non-shared haplotypes between ancient and current populations may be due to the higher importance of factors such as the small sample size and the genetic drift in the ancient ones, and recent historical migrations in the modern ones, but in both cases the haplotipic pool fits into the European genetic variability. Moreover, the influence of a Near Eastern component and the similarity to the genetic pool from ANCCAT cannot be disregarded and reinforces their already documented historic interactions (Pericot, 1975). Finally, the ancient Balearic population genetically differs from Ancient Sardinian population, so a hypothetic

relationship between the members of the Nuragic and the Talaiotic cultures seems unlikely at least concerning the female lineages.

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Supporting Information

Further details on the necropolises

Son Real

The necropolis of Son Real is placed in the centre of the Alcudia Bay, in a prominence known as the "cemetery of the Phoenicians" (Hernández-Gasch et al. 1998) but nothing is known about the settlement that would give rise to this big necropolis (Figure S1). It belongs to the Late Talaiotic period that chronologically encompasses from the VI to the II centuries BC, although it continued being used later on (Guerrero et al. 2006). It was characterized by different funerary architectonic structures (Tarradell and Woods, 1959; Guerrero et al. 2006). Most types of burials were multiple (Font, 1974), being the most frequent the "*micronaveta"* (Pericot et al. 1975). Owing to the changes in the burial rituals, it is believed that in the beginning it was a cemetery exclusive for dominant classes owing to the kind of monuments found, but that it evolved little by little to a certain widespread growth (Font, 1974). A total number of 165 individuals in shrunken position were found (Font, 1974). In spite of Gracile Mediterranean being the predominant morphological type, the study shows also the presence of eastern Mediterranean cranial types (brachycranic plano-occipital skull).

Fig. S1. View of the navetiform tombs of the necropolis of Son Real, in the shoreline of the Northern coast of Majorca. The little islet of Porros is located a few meters from the coastline.

Illot des Porros

This cemetery belongs to the Late Talaiotic period,dating back to the VI-II centuries BC (Malgosa 1985, 1992), It is located in a flat ellipsoidal small island, approximately 70m away from the coast in front of the aforementioned necropolis of Son Real (Tarradell, 1964) (Figure S2). There are three big burial structures (Tarradell, 1964; Hernández et al. 1998), described by Taradell as circles A, B and C, holding the majority of the 285 individuals that are found (Malgosa 1985, 1992; Alesán, 1990). Circle C was used for inhumation, while circles A and B were mainly used for cremation and incineration rites (Piga et al. 2010). The western zone of the small island was occupied by individual burials directly on the ground or with small constructions. This zone of external burials, but near to the three circles, suggests that it was used by a social group from lower status, taking into consideration the lack of wealth of the found materials (Hernández et al. 1988). The recovered remains have been deeply studied at the descriptive level (Subirà and Malgosa, 1990, 1991, 1992a, 1992b, Malgosa, 1992; Alesán et al. 1999; Hernández-Gasch et al. 2005; Piga et al. 2010 among others).

The comparative morphological data indicate a high relationship with the populations of the north of Africa and of the northeast of the Peninsula, even though it stands out a possible relationship with the populations of the east of the Mediterranean (Malgosa, 1985). The human remains found in this small island were preserved under bad conservation conditions for more than 2000 years, like extreme humidity, contributing to the deterioration of the biological structure and a higher rate of DNA degradation. (Díaz et al. 2009).

Recent craniometric and dental morphologic analysis show a close relationship of both necropolis, Son Real and S'Illot des Porros, with the Catalan populations (Jordana and Malgosa 2004; García Sívoli 2009).

Fig. S2. Overview of the little islet of Porros

Can Reiners

This necropolis, also placed in the Alcudia bay, belongs to the Late Antiquity between the fall of the Roman Empire in Western Europe and Medieval Age. The necropolis is found on the forum of the Roman city of Pollentia after it was abandoned in the IIIrd century AD (Orfila et al. 1999; Orfila 2000) (Figure S3). It is one of the most important archaeological sites and urban phenomena of Majorca (Cau MA and Chávez ME, 2003). It is in a 14m elevation over the sea level, in the isthmus that separates Alcudia and Pollença bays, initially used with maritime surveillance purposes. A total number of 138 inhumations are housed for their study at the Biological Anthropology laboratory of the Universitat Autònoma de Barcelona. Molecular, biochemical, dental, morphological and dietary studies have been applied (Ortega et al. 2003; Ortega 2005; Díaz 2009).

Fig. S3. Overview of the Pollentia forum, where the Late Antiquity necropolis of Can Reiners is located.

Son Olivaret

This burial site was a collective mortuary tomb used between 2300 and 900 BC in the southwest of Minorca, in Ciutadella. We only know the remains of a big structure formed by a double wall with oval form that has an entry oriented towards the southwest, a corridor and an inner chamber where the majority of human and ceramic remains are found (Figure S4). The absolute dating using thermoluminescence and C^{14} allow the suggestion of a first occupation phase during the third millennium BC, even though the burial continued being used up to the Late Talaiotic. The human remains were in a bad state as a result, in part, of the acid nature of the soil, which had damaged them, and also because of the fragmentation after the collapse of the architectural structure (Plantalamor et al. 2008). A high amount of human remains were recovered both from the Pretalaiotic (stratigraphic units (SU) 15 and 16) and the Talaiotic (SU 6 to 11) periods, but most of them were fragmented and poorly conserved. In this settlement, 5 different periods of use can be distinguished separated by abandonment phases. The most ancient skeletal material found dates back to 3640±40BP (KIA-27133) (Van Strydonck and Boudin, 2008).

The bone tissue was generally bad conserved as indicated by the bad preservation of the collagen in many cases (Van Strydonck and Boudin, 2008), but nevertheless the proportion C/N was good. This may indicate that the decomposition of the collagen inside the bone has been produced without the absorption of the humic acids present in the environment and without the remaining collagen present in the zone being completely degraded.

160 individuals were determined by dental analysis; 30 of them belonged to the Pretalaiotic period, and the remaining 130 to the Talaiotic period (Carrascal et al. 2008), but that no complete maxillas could be recovered, much less skeletons. The scarce preservation of the material limited the molecular study.

Fig. S4. Overview of the Son Olivaret site. Human remains were found inside this oval structure.

La Cova des Pas

This collective burial cave is placed at the southern part of Minorca, in the cliff of Trebaluger (39 ° 57 ‘50’’E, 80 msnm). It is a small cavity of 6,5 m width and 4,5m length placed 15m above the floor (Fullola et al. 2008) that acts as shelter, provoking that the remains found were preserved without major alterations. Different organic materials, such as ropes, wood of stretchers, human tissue and skin to wrap were exceptionally well preserved (Prats-Muñoz et al. 2013). The individuals inside the cave were from almost every type of age and both sexes, which suggested that they probably reflected the situation of the living population that used this small cavity 3000 years ago to bury their dead (Armentano et al. 2010, 2012). The remains occupied the whole cavity (Figure S5) and showed a good state of preservation and consistency. The anatomic connections persisted in most cases. They were all intentionally put in extremely flexed position, common in the prehistoric funerary sites. The results of C^{14} confirmed the use of the Cave as a cemetery during at least 100 years (Van Strydonck et al. 2010). The morphological study showed that there were 66 individuals inside the cave. The first burials were deposited around 1100 BC, at the end of the Bronze Age, although the largest number of inhumations took place between 900 and 800 BC (Van Strydonck et al. 2010), during the Talaiotic period (Cabanes and Albert, 2011).

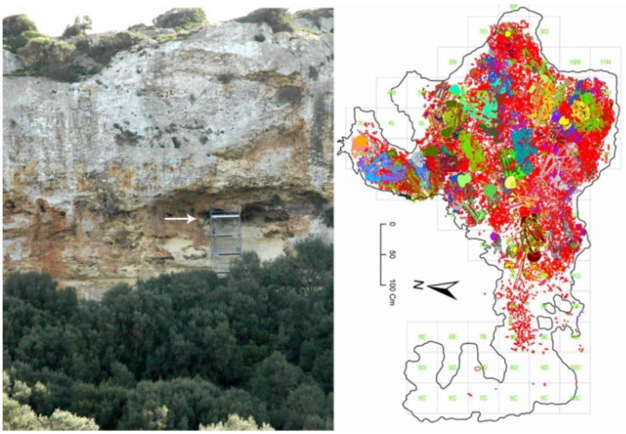

Fig. S5. Overview of La Cova des Pas. The ubication in the cliff is signalled by an arrow (left) and the schematic deposition of the 66 individuals is shown (right).

Supplementary Tables

TABLE 1. Primers used to amplify and sequence the Hypervariable region I of mtDNA (HVR-I).

Region		Sequence	References
HVR I	16190 –	5’-CCCCATGCTTTACAAGCAAGT -3’	(Montiel et al. 2001)

16420	5’-TGATTTCACGGAGGATGGTG -3’	(Vigilant et al. 1991)
16190 – 16339	5'-CCCCATGCTTTACAAGCAAGT -3'	(Montiel et al. 2001)
	5'-GTGCTATGTACGGTAAATGG-3'	(Díaz 2009)
16292 – 16420	5'- CACCCTTAACAGTACATAGTAC-3'	(Montiel et al. 2001)
	5'- TGATTTCACGGAGGATGGTG -3'	(Vigilant et al. 1991)
16030 – 16230	5'- CATGGGGAAGCAGATTTGGG-3'	(Simón et al. 2011)
	5'- GATAGTTGAGGGTTGATTGCTG-3'	(Simón et al. 2011)

TABLE 2. Primers used to amplify and sequence the fragments of the mtDNA coding region

Restr.Site	Primers	Coordinates	Primer sequences
-7025 Alu I	L6968/H7052	6949-6968	5’-CCGTAGGTGGCCTGACTGGC-3’
	(Francalacci, p.c.[1])	7071-7052	5’-TGATGGCAAATACAGCTCCT-3’
-1715 Dde I	L1698/H1776	1678-1698	5’-TAGCCCCAAACCCACTCCAC-3’
	(Montiel, 2001)	1797-1776	5’-CTTTCCCTTTGCGGTACTATATC-3’
-13704 Bst0I	L13640/H13720	13621-13640	5’-TCACCCTAACAGGTCAACC-3’
	(Montiel, 2001)	13739-13720	5’-ATGAGAAATCCTGCGAATAG-3’
-9052 Hae II	L9020/H9082	9000-9020	5’-ACGCCTAACCGCTAACATTAC-3
	(Montiel, 2001)	9103-9082	5’-AGATGATAAGTGTAGAGGGAAG-3’
+11718 Hae III	L11650/H11771	11669-11650	5’-AGTAACAGCCATTCTCATCC-3’
	(Díaz, 2009)	11791-11771	5’-GAGTGCGTTCGTAGTTTGAG-3’
+15606 Alu I	L15561/H15625	15543-15561	5’-CCCACATCAAGCCCGAATG-3’
	(Montiel, 2001)	15646-15625	5’-GATGAGGATGGATAGTAATAGG-3’
+12308 Hinf I	L12237/H12309	12216-12237	5’-CACAAGAACTGCTAACTCATGC-3’
	(Izagirre, 1998)	12279-12308	5’-ATTACTTTTATTTGGAGTTGCACCAAGATT-3’
-4577NlaIII	L4538/H4621	4519-4538	5’-CACTCATCACACAGCGCTAAGC-3’
	(Izagirre, 1998)	4638-4621	5’-TGGCAGCTTCTGTGGAAC-3’
-8994 HaeIII	L8927/H9014	8908-8927	5’-TTCTTACCACAAGGCACACC-3’
	(Montiel, 2001)	8995-9014	5’-AGGTGGCCTGCAGTAATGT-3’
+14465 AccI	L14399/H14533	14417-14398	5’-ACACTCACCAAGACCTCAA-3’
	(Díaz, 2004)	14513-14533	5’-GGGAGGTTATATGGGTTTAA-3’

[1]personal communication

TABLE 3. Populations used for haplogroup analysis and sequence analysis.

Population	Shortcut	N haplog	N seq	Period	Region	Reference
S'Illot des Porros	IIP	31	19	Iron Age	Majorca Island, Northeast Spain	Díaz, 2009
Son Olivaret	ISOV	22	15	Iron Age	Majorca Island, Northeast Spain	Simón and Malgosa 2014
Cova des Pas	ICP	20	17	Iron Age	Majorca Island, Northeast Spain	Simón and Malgosa, 2015, in press
Son Real	ISR	37	22	Iron Age	Majorca Island, Northeast Spain	Díaz, 2009
Can Reiners	MCR	28	15	Late Roman Age	Majorca Island, Northeast Spain	Díaz, 2009
Ancient Catalans	ANCCAT	26	26	Iron Age	Catalonia, northeast Spain	Sampietro et al. 2005, 2007
Ancient Sardinians	ANCSARD	30	30	Bronze-Iron Age	Sardinia, west Italy	Caramelli et al. 2007, present study
Ancient Cretians	ANCCRE	37	37	Bronze-Iron Age	Crete island, east Europe	Hughey et al. 2013
Ancient Basques	ANCBA	7	7	Bronze-Iron Age	Basque Country, north Spain	Fernández 2005; Hervella et al. 2012
Ancient Turkey	ANCTK	12	12	Iron Age	Turkey, east Europe	Matney et al. 2012
Ancient Syria	ANCSYR	9	9	Bronze-Iron Age	Syria, Near East	Fernández 2005; Witas et al. 2013

Aldaieta	MALD	56	56	Middle Ages	Basque Country, North Spain	Alzualde et al. 2006
Plaça Vella	MPV	24	24	Middle Ages	Catalonia, Northeast Spain	Montiel, 2001
Majorcans	MAJ	112	112	Current	Balearic Islands, northeast Spain	Picornell et al. 2005; Falchi et al. 2006
Minorcans	MIN	46	46	Current	Balearic Islands, northeast Spain	Picornell et al. 2005
Chuetas	CHU	47	48	Current	Balearic Islands, northeast Spain	Picornell et al. 2005
Ibiza	IBI	50	50	Current	Balearic Islands, northeast Spain	Picornell et al. 2005
Marrakech	MRR	50	45	Current	North Africa	Brakez et al. 2001
Languedoc	LDC	85	85	Current	Southwest France	Richard et al. 2007
Valencia	VAL	39	42	Current	Northeast Spain	Picornell et al. 2005
Tuscany	TUS	49	49	Current	West Italy	Torroni et al. 1996
Greece	GRE	25	25	Current	East Europe	Bosch et al. 2006
Switzerland	SWI	74	74	Current	Center Europe	Pult et al. 1994
Turkey	TK	75	75	Current	East Europe	Mergen et al. 2004
Tunisia	TUS	155	154	Current	North Africa	Fadhlaoui-Zid et al. 2004
Corsica	COR	53	47	Current	Corsica Island, West Italy	Varesi et al. 2000
Sardinia	SARD	115	115	Current	Sardinia Island, West Italy	Richards et al. 2000
Sicilia	SIC	105	49	Current	South Italy	Vona et al. 2001
Basques	BA	491	106	Current	North Spain	Bertranpetit et al. 1995, Côrte-Real et al. 1996, Izagirre 1998, Richards et al. 2000, Alfonso-Sánchez et al. 2008, García et al. 2011
Andalusia	AND	157	99	Current	South Spain	Plaza et al. 2003; Casas et al.

						2006
Catalonia	CAT	141	62	Current	Northeast Spain	Bertranpetit et al. 1995; Côrte-Real et al. 1996; Álvarez et al. 2007; García et al. 2011
Var	VAR	37	37	Current	Southeast France	Dubut et al. 2004

TABLE 4. Detailed results from all the individuals that gave positive results in the study.

Son Real	Sample	HVR-I sequence (np 16210-16400), minus np 16000	7025 Alu I	12308 HinfI	11718 HaeIII	8994 HaeIII	9052 Hae II	13704 Bst0I	15606 AluI	1715 DdeI	4577 NlaIII	14465 AccI	10871 MnlI	Hg HVR-I	RFLPs
	54.2;71.1[2];0001	rCRS	-											H,U,R0,J	H
	1-67-A(1)	221T	-											Unk.	H
	22.D	291T,293G	-											Unk.	H
	2.3	278T	-											H,U	H
	1-67 A;5303;56.1	n.d.	-											Unk.	H
	49.1[2]	rCRS		+										H,U,R0,J	U
	54.1	270T,290T,292T,294T		+										¿U5?	U
	13	270T		+										U5	U
	29.6[2]	355T		+										Unk.	U
	70.1	270T		+										U5	U
	Sep 74[4]	270T		+										U5	U
	75.2;1.3;34;4402	n.d.		+										Unk.	U
	4-67(2)	223T,278T										+		X	X

	43,48.1	n.d.										+		Unk.	X
	3-67-A;43.1[4];1-67-A	rCRS						-						H, U, R0, J	J
	1-67-A(2)	278T						-						H,U,J	J
	43.5	242T						-						Unk.	J
	72.2;6.12(4407);20.1	n.d.						-						Unk.	J
	47.3	292T,294T,344T							+					T	T
	1-67-A(5)	rCRS			11719A									H, U, R0, J	R0
	75.1,53.4	224T,311C		+			-							U,K	K
	29.05	311C,327T		+			-							U,K	K
	Sep68	223T,292T				-								W	W
	Sep72	223T,292T				-								W	W
Illot des Porros	**Sample**	HVR-I sequence (np 16210-16400), minus np 16000	7025 Alu I	12308 HinfI	11718 HaeIII	8994 HaeIII	9052 Hae II	13704 Bst0I	15606 AluI	1715 DdeI	4577 NlaIII	14465 AccI	10871 MnlI	Hg HVR-I	RFLPs
	C-18;N15	rCRS(314-358;236-285)	-											H, U, R0, J	H
	C-N14	223T, 262T, 293G	-											Unk.	H
	C-49	250A, 256T(236-270)	-											Unk.	H

Can Reiners	Sample	HVR-I sequence (np 16210-16400), minus np 16000	7025 Alu I	12308 HinfI	11718 HaeIII	8994 HaeIII	9052 Hae II	13704 Bst0I	15606 AluI	1715 DdeI	4577 NlaIII	14465 AccI	10871 MnlI	Hg HVR-I	RFLPs
	SW 97-3	378T(237-387)	-											Unk.	H
	B-331962,B-21,B-33,B-36,B-1,B-24,B-25,B-31,H5a	n.d.	-											Unk.	H
	B-7;SW97-9;98-1;B-3;N 5b	rCRS (239-285 and 313-356;325-387;314-387;314-351;325-378)		+										H,U,R0,J	U
	A 1961	270T, 378T		+										U5	U
	B-5	270T, 304C (239-285 and 304-378)		+										U5	U
	B 1961-1;C1964-42’	270T (256 -285; 261-285 and 325-348)		+										U5	U
	B-2	257Y,270T(250-285 and 313-387)		+										U5	U
	C-56’	261C,270T(243-285 and 313-344)		+										U5	U
	BC-16	325C		+										Unk.	U
	C-20	325C,355C,356T (210-357)		+										Unk./U4	U
	C1964-42’	270T(261-285 and 325-348)		+										U5	U
	B-23	223T(223-285)									-			¿¿V??	
	B-11	n.d.									-			V	

	1[2], 147[2],93.1,41[1],31.1,77.1[1],G-9	rCRS	-											H, U, R0, J	H
	32.1	274A	-											Unk.	H
	17.2	290	-											Unk.	H
	E-89	266T, 294T, 311C	-											Unk.	H
	32.1	291T	-											Unk.	H
	15.2,16.1	n.d.	-											Unk.	H
	17.1, 107.1,164.1[2]	rCRS		+										H, U, R0, J	U
	43.1[1]	278T		+										H,U	U
	131.1	256T, 270T		+										U5	U
	47.57	260T, 278T		+										Unk.	U
	23.2, 10.1	n.d.		+										n.d.	U
	70.1	223T											-	Many	L3
	23.1	223T,292T				-								W	W
	163.1	n.d.				-								n.d.	W
	39-1(29)	n.d.						-						n.d.	J
	39-1(50)	n.d.							+					n.d.	T

Son Olivaret	Sample	HVR-I sequence (np 16210-16400), minus np 16000	7025 Alu I	12308 HinfI	11718 HaeIII	8994 HaeIII	9052 Hae II	13704 Bst0I	15606 AluI	1715 DdeI	4577 NlaIII	14465 AccI	10871 MnlI	Hg HVR-I	RFLPs
	67.1	223T								-				Many	I
	83.1	261T,298C									-			H,V	V
	169.1	224T,311C					-							U,K	K
	Ind 23,32,34	rCRS	-											H, U, R0, J	H
	Ind 13	278T	-											H,U	H
	Ind 44	256T, 257T, 290T, 294T[4]	-											Unk.	H
	Ind41	rCRS[1]	-											Many	H
	Ind 20,21,42	n.d.	-											-	H
	Ind 1,46	rCRS	+	+										H, U, R0, J	U
	Ind 10	rCRS[1]	+	+										Many	U
	Ind 8,26	362C	+	+										U2e	U
	Ind 9	304C	+	+										H, U	U
	Ind 25	301T, 343G, 356C	+	+										U3	U

	Ind 3, 4	rCRS	+		11719A									R0[7]	-
	Ind 16, 27	294T, 296T, 304C	-						+					T2	T
	Ind 43,48	rCRS						-						H, U, R0, J	J
Cova des Pas	**Sample**	HVR-I sequence (np 16210-16400), minus np 16000	7025 Alu I	12308 HinfI	11718 HaeIII	8994 HaeIII	9052 Hae II	13704 Bst0I	15606 AluI	1715 DdeI	4577 NlaIII	14465 AccI	10871 MnlI	Hg HVR-I	RFLPs
	Ind 25[6],26[6],29,30,31, 47[6], 57[3], 62[3,5], 69	rCRS	-											H	H
	Ind 2[7]	362C	-											H	H
	Ind 9	362C, 390A	-											H	H
	Ind 4[6,7],11[7],35	rCRS	-	+										H,U,R0,J	U
	Ind 22[2]	399G	-	+										Many	U
	Ind 37[5]	256T, 270T, 389A, 399G												U5	U
	Ind 17[1,5]	223T, 301C	+	-		-								Many	W
	Ind 41[6,7],50	224C, 311C	-	+			-							U,K	K
Santa Teresa di Gallura	**Sample**	HVR-I sequence (np 16210-16400), minus np 16000	7025 Alu I	12308 HinfI	11718 HaeIII	8994 HaeIII	9052 Hae II	13704 Bst0I	15606 AluI	1715 DdeI	4577 NlaIII	14465 AccI	10871 MnlI	Hg HVR-I	RFLPs
	Ind. 22	rCRS	-											H	H

	Ind. 35[1]	rCRS	-			Many	H
	Ind.19	256T	-			Many	H
	Ind. 38[1]	304C	-			Many	H
	Ind. 40	270T					
	Ind. 43	294T, 304C					
	Ind.18	224C, 246T		+		Many	U
	Ind. 25	224C, 242T, 290A, 303A, 311C		+		Many	U
	Ind. 34[1]	256T, 270T, 311C				Many	U
	Ind. 27[1]	224C, 246T, 311C		+	-	U, K	K

[1]Segment amplified from positions 16210 to 16319. [2] Segment amplified from positions 16314 to 16400. [3]Fully duplicated individuals.[4] Duplicated in two different laboratories of the same building. [5]Cloned individuals. [6]RFLP study fully replicated in two independent extractions. [7]Sequernce partially replicated in two independent extractions.

TABLE 5. Clones obtained from the sequences.

CLONES

SOV26

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      11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
      66666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666
      22222222222222222222222222222222222222222222222222222222222222222222222222222222222222222233333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333334
      11111111112222222222333333333344444444445555555555666666666677777777778888888888999999999900000000001111111111222222222233333333334444444444555555555566666666667777777777888888888899999999990
      01234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
      ACAGCAATCAACCCTCAACTATCACACATCAACTGCAACTCCAAAGCCACCCCTCACCCACTAGGATACCAACAAACCTACCCACCCTTAACAGTACATAGTACATAAAGCCATTTACCGTACATAGCACATTACAGTCAAATCCCTTCTCGTCCCCATGGATGACCCCCCTCAGATAGGGGTCCCTTGAC
26.1                 ....................................................................................................................G...................................C......................................
26.2                 .......................................C...................................................................................................C....C......................................
26.3                                                                                                                                             ................................C......................................
26.4                 ........................................................................................................................................................C......................................
26.5                 ................G...................................................................................................................................C........G.............................
26.6                 ........................................................................................................................................................C........G.............................
26.7                 ........................................................................................................................................................C......................................
26.8                 .....................C.......................................C..........................................................................................C......................................
```

SOV27

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      11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
      66666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666
      22222222222222222222222222222222222222222222222222222222222222222222222222222222222222222233333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333334
      11111111112222222222333333333344444444445555555555666666666677777777778888888888999999999900000000001111111111222222222233333333334444444444555555555566666666667777777777888888888899999999990
      01234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
      ACAGCAATCAACCCTCAACTATCACACATCAACTGCAACTCCAAAGCCACCCCTCACCCACTAGGATACCAACAAACCTACCCACCCTTAACAGTACATAGTACATAAAGCCATTTACCGTACATAGCACATTACAGTCAAATCCCTTCTCGTCCCCATGGATGACCCCCCTCAGATAGGGGTCCCTTGAC
27.1                 .......C.............................................................................T.T......C....................................................................................A...........
27.2                 .....................................................................................T.T......C................................................................................................
27.3                 .....................................................................................T.T......C................................................................................................
27.4                 .......C.............................................................................T.T......C...............A....................................................................A...........
27.5                 .....................................................................................T.T......C................................................................................................
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CPAS17

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      11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
      66666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666
      22222222222222222222222222222222222222222222222222222222222222222222222222222222222222222233333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333334
      11111111112222222222333333333344444444445555555555666666666677777777778888888888999999999900000000001111111111222222222233333333334444444444555555555566666666667777777777888888888899999999990
      01234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
      ACAGCAATCAACCCTCAACTATCACACATCAACTGCAACTCCAAAGCCACCCCTCACCCACTAGGATACCAACAAACCTACCCACCCTTAACAGTACATAGTACATAAAGCCATTTACCGTACATAGCACATTACAGTCAAATCCCTTCTCGTCCCCATGGATGACCCCCCTCAGATAGGGGTCCCTTGAC
                                 17.1                          .............T.............................................................................T.........
                                 17.2                          .............T.............................................................................T.........
                                 17.3                          .............T.............................................................................T.........
                                 17.4                          .............T...............................................C.............................T.........
                                 17.5                          .............T.............................................................................T.........
```
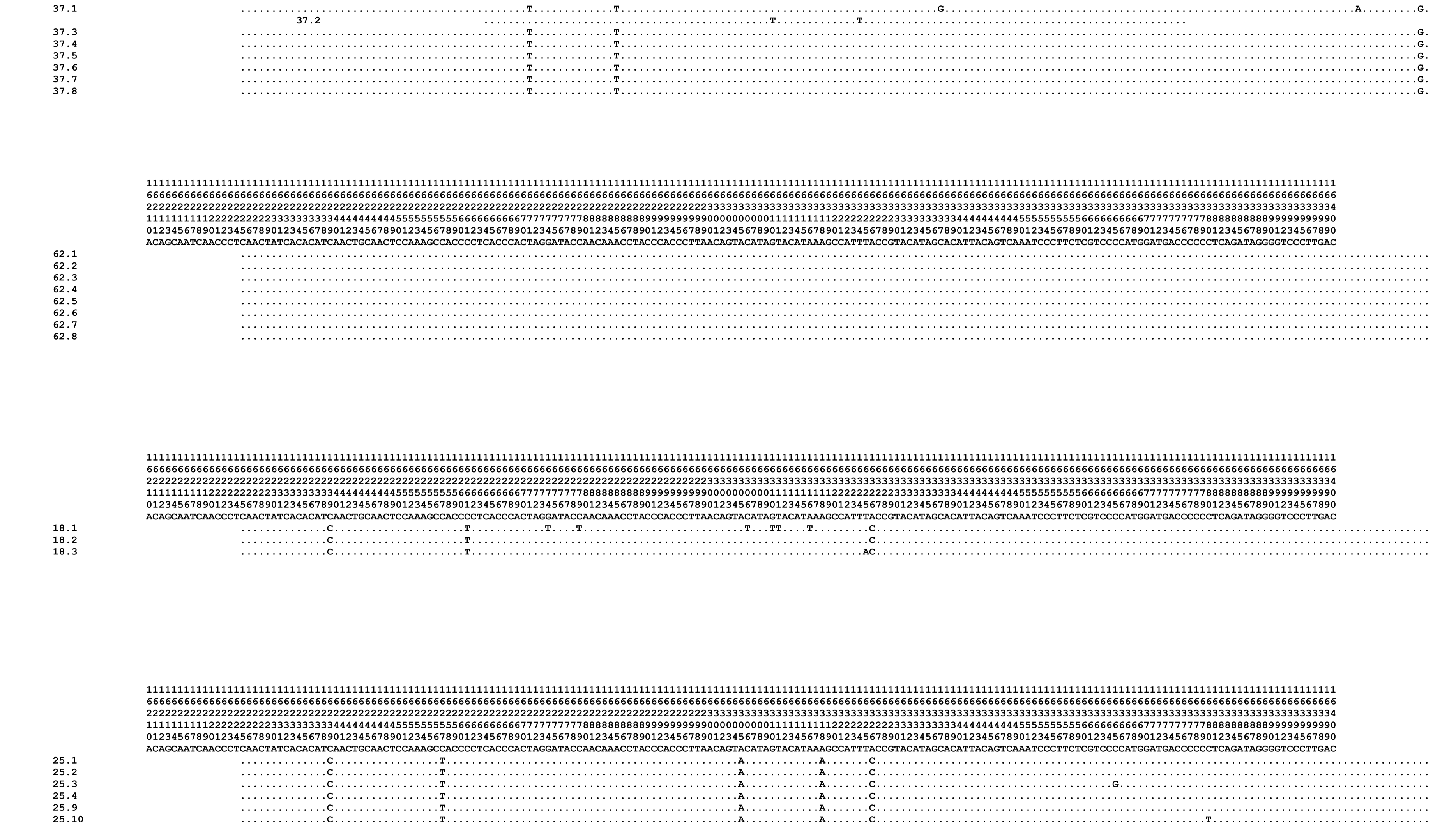

CPAS37

```
      11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
      66666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666
      22222222222222222222222222222222222222222222222222222222222222222222222222222222222222222233333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333334
      11111111112222222222333333333344444444445555555555666666666677777777778888888888999999999900000000001111111111222222222233333333334444444444555555555566666666667777777777888888888899999999990
      01234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
      ACAGCAATCAACCCTCAACTATCACACATCAACTGCAACTCCAAAGCCACCCCTCACCCACTAGGATACCAACAAACCTACCCACCCTTAACAGTACATAGTACATAAAGCCATTTACCGTACATAGCACATTACAGTCAAATCCCTTCTCGTCCCCATGGATGACCCCCCTCAGATAGGGGTCCCTTGAC
      ..............................................T.............T................................................................................................................................G.
```


CPAS62
SD18
SD25

SD27*

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      1111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
      6666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666666
      2222222222222222222222222222222222222222222222222222222222222222222222222222222222222222223333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333333334
      1111111111222222222233333333334444444444555555555566666666667777777777888888888899999999990000000000111111111122222222223333333333444444444455555555556666666666777777777788888888889999999999
      0123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789
      ACAGCAATCAACCCTCAACTATCACACATCAACTGCAACTCCAAAGCCACCCCTCACCCACTAGGATACCAACAAACCTACCCACCCTTAACAGTACATAGTACATAAAGCCATTTACCGTACATAGCACATTACAGTCAAATCCCTTCTCGTCCCCATGGATGACCCCCCTCAGATAGGGGTCCCTTGAC
27.1   ..............C.....................T.............T..................................................C..........................................TT..T.T...................T.T..................
27.2   ..............C.....................T.............T.........................................G........C..........................................TT..T.T...................T.T..................
27.3   ..............C.....................T.............T..................................................C..........................................TT..T.T...................T.T..................
27.4   ..............C.....................T.............T..................................................C..........................................TT..T.T...................T.T..................
27.5   ..............C.....................T.............T..................................................C..........................................TT..T.T...................T.T..................
27.6   ..............C.....................T.............T..................................................C..........................................TT..T.T...................T.T..................
27.7   ..............C.....................T.............T..................................................C..........................................TT..T.T...................T.T..................
27.8   ..............C.....................T.............T..................................................C..........................................TT..T.T...................T.T..................
27.9   ..............C.....................T.............T..................................................C..........................................TT..T.T...................T.T..................
27.10  ..............C.....................T.............T..................................................C..........................................TT..T.T...................T.T..................
```

*Second half of the sequence quite possibly affected by postmortem damage

TABLE 6. Origin of postmortem damage.

INDIVIDUAL	mtDNA positions (+16000)																																						
	210	217	223	224	226	231	246	249	256	259	260	264	270	271	291	294	295	296	301	302	304	310	311	319	322	326	354	355	357	358	360	362	371	380	382	388	389	399	400	Original template strange damaged[3]
rCRS	A	T	C	T	A	T	A	T	C	C	C	C	C	T	C	C	C	C	C	A	T	G	T	G	A	A	C	C	T	C	C	T	A	C	C	G	G	A	C	
SOV 26.1																										G[2]						C								L
SOV 26.2								C[1]																					C[1]			C								H
SOV 26.5					G[2]																											C	G[2]							L
SOV 26.6																																C	G[2]							L
SOV 26.8						C[1]								C[1]																		C								H
SOV 27.1		C[1]														T		T			C															A[2]				H
SOV 27.4		C[1]														T		T			C			A[2]												A[2]				H
CPAS 17.4	.		T						.				.	C[1]					T						.	.														H
CPAS 37.1	.		.						T				T	.					.						.	.											.[2]	G		L
CPAS 37.2	.		.						T				T	.					.						G[2]	.											A	G	.	L

B129.1				C			T			T^2		T^2			T^2		T^2	T^2	T^2				C																	L
B129.3				C			T															A^2	C																	H
T237.2				C			T				T									G^2			C				T^2	T^2		T^2	T^2			T^2	T^2					L
T237.1, T237.3-.10				C			T				T												C				T^2	T^2		T^2	T^2			T^2	T^2					L

[1]Type 1 transitions; [2]Type 2 transitions; [3]Following the criterion established in Gilbert et al. 2003

TABLE 7. Relative frequencies of all the populations

	Haplogroups												
Population	H	I	J	K	T	X	U	V	W	OT	M	RO	L
IIP	,4516	,0000	,0000	,0000	,0000	,0322	,4193	,0645	,0322	,0000	,0000	,0000	,0000
ISOV	,4090	,0000	,0909	,0000	,0909	,0000	,3181	,0000	,0000	,0000	,0000	,0909	,0000
ICP	,6000	,0000	,0000	,1000	,0000	,0000	,2500	,0000	,0500	,0000	,0000	,0000	,0000
ISR	,2432	,0000	,2162	,0810	,0270	,0810	,2702	,0000	,0540	,0000	,0000	,0270	,0000
MCR	,4285	,0357	,0357	,0357	,0357	,0000	,2857	,0357	,0714	,0000	,0000	,0000	,0357
MALD	,6071	,0000	,1428	,0178	,0535	,0000	,1250	,0178	,0000	,0000	,0357	,0000	,0000
MPV	,3333	,0416	,1250	,1666	,0833	,0000	,0833	,1250	,0000	,0416	,0000	,0000	,0000
MRR	,3200	,0000	,1000	,0200	,0400	,0000	,1400	,1000	,0000	,0200	,0000	,0000	,2600
LDC	,3529	,0117	,0823	,0588	,1529	,0117	,1882	,0235	,0235	,0117	,0000	,0588	,0235
TUS	,4081	,0408	,1428	,0612	,1020	,0816	,1020	,0000	,0204	,0000	,0000	,0204	,0204
GRE	,4800	,0000	,0800	,0000	,1600	,0000	,0400	,0000	,0000	,1600	,0800	,0000	,0000
SWI	,5945	,0135	,1081	,0540	,0270	,0000	,1216	,0540	,0135	,0135	,0000	,0000	,0000

TK	,3200	,0000	,0800	,0533	,0133	,0000	,4000	,0000	,0133	,0000	,1066	,0133	,0000
TUN	,2129	,0258	,0387	,0645	,1419	,0000	,0967	,0516	,0000	,0387	,0451	,0709	,2129
COR	,4528	,0000	,1132	,1320	,0943	,0943	,1132	,0000	,0000	,0000	,0000	,0000	,0000
SARD	,4782	,0086	,0608	,0521	,1130	,0086	,1565	,0521	,0086	,0086	,0086	,0260	,0173
SIC	,5428	,0000	,0476	,0571	,0761	,0285	,1142	,0000	,0000	,0476	,0000	,0857	,0000
BA	,5254	,0020	,0407	,0488	,0610	,0244	,1649	,1059	,0061	,0122	,0020	,0061	,0000
AND	,4649	,0191	,0700	,0636	,0445	,0318	,1210	,0573	,0127	,0318	,0382	,0254	,0191
VAL	,5128	,0000	,1025	,0256	,1282	,0000	,1282	,0512	,0000	,0000	,0000	,0256	,0256
CHU	,2340	,0212	,0212	,1276	,1063	,0212	,1063	,0425	,0000	,0000	,0425	,2340	,0425
MAJ	,4107	,0089	,0892	,0982	,1428	,0178	,1339	,0714	,0000	,0000	,0089	,0178	,0000
IBI	,4000	,0000	,0400	,0600	,2600	,0000	,0600	,1200	,0000	,0000	,0000	,0000	,0600
MIN	,2608	,0217	,1739	,1521	,1304	,0217	,0869	,0217	,0434	,0000	,0000	,0869	,0000
CAT	,4326	,0070	,0283	,0921	,1063	,0283	,1560	,0425	,0425	,0141	,0070	,0070	,0354
VAR	,3783	,0540	,0000	,0540	,0810	,0000	,2702	,0540	,0000	,0000	,0000	,1081	,0000
ANCCAT	,3846	,0000	,2307	,0769	,0384	,0000	,1923	,0384	,0000	,0000	,0000	,0384	,0000
ANCGRE	,4054	,0810	,0270	,1621	,1891	,0270	,0540	,0000	,0270	,0000	,0000	,0270	,0000
ANCSARD	,6666	,0000	,0333	,0333	,0000	,0000	,1666	,0333	,0000	,0000	,0666	,0000	,0000
ANCBA	,8571	,0000	,0000	,0000	,0000	,0000	,1428	,0000	,0000	,0000	,0000	,0000	,0000
ANCTK	1,0000	,0000	,0000	,0000	,0000	,0000	,0000	,0000	,0000	,0000	,0000	,0000	,0000
ANCSYR	,1111	,1111	,1111	,1111	,0000	,0000	,2222	,0000	,0000	,0000	,2222	,1111	,0000

TABLE 8. Shared and unique haplotypes in ancient and current Balearic populations

Populat (number of sequen)	H	rCRS-U	rCRS-R	rCRS-J	362C-U	304-U	H	278-J	294C, 296T, 304C-T2	301T, 343G, 356C-U	362C-H	390A-H	224C, 311C-K	256T, 270T, 389A, 399G	223T, 278T-X	221T-H	292T,294T, 344T	270T, 290T, 292T, 294T-U5	242T-J	291T,293G-H	311C,327T-K	270T-U5	223T,292T-W	274A-H	290T-H	266T, 294T, 311C-H	291T-H	223T-I	223T-L3	260T, 278T-U	256T,270T-U5	261C,298T-V
ICP (17)	9	3									1	1	2	1																		
ISOV (15)	3	2	1	2	2	1	1		2	1																						
ISR (22)	2	0	1	3			1	1					2		1	1	1	1	1	1	1	3	2									
MCR (15)	2	2											1										1	1	1	1	1	1	1	1	1	1
Current Bal Pop																																
MAJ (112)	Yes	1??	1??	2				1	2		2	1	3		2							1		1								
MIN (46)	Yes			3							1		5			1						2										
IBI (50)	Yes						4+1 ?	2+1 ?	2													1									1	
CHU (48)	Yes					1?							3																			
Shared	Yes	?	?	?	No	?	Yes	Yes	Yes	No	Ye	Ye	Ye	No	Ye	Ye	N	No	No	No	No	Ye	No	Ye	No	No	No	No	No	No	Ye	No

Populations used are those appearing in Picornell et al. 2005 and Falchi et al. 2006; ?In Picornell et al. just their not pertinence to haplogroup H was checked by RFLP analysis, their sequence was not unequivocal to determine an haplogroup; ??They show rCRS from 16048 to 16400, their pertinence to haplogroup H has been discarded by RFLPs, so they can belong to haplogroups U and R.

TABLE 9. Search for shared and unique populations in the databases

Haplotype	Richards et al. 2000* (4246 haplotypes)	GenBank (Benson et al. 2012)	Human Genome Mitochondrial Database (Ingman and Gyllenstein 2006) (MITOMAP 2013) (23966 haplotypes)	EMPOP CR Database (Parson and Dür 2007) (34617 haplotypes)	Van Oven et al. 2009 (20666 haplotypes)
301T, 343G, 356C-U3	1 (Norwegian, Opdal et al. 1998)**	3 in FBI-Caucasian Forensic Sci Comm (2002) 4 Online: (USA.CAU.0302, USA.CAU.0719 and USA.CAU.0853)	0	0	0
256T, 270T, 389A, 399G-U5	0	0	0	0	0
270T, 290T, 292T, 294T-T	0	0	0	0	0
242T-J		2 Scottish, one unpublished, one in Helgason et al. 2001			2 (1 italian and 1 Turkish, Pala et al. 2012)
291T, 293G-H	0	0	0	0	0
311C, 327T-K	0	0	0	0	0
266T, 294T, 311C-H	0	0	0	0	0
260T, 278T-U	0	0	0	0	0

261T, 298C-V	1 (Europe North Central), 4 (North Caucasus)***	1 Ghelli et al. 2009	0	1 Admixed American	0

*all the databases except Richards et al. 2000 were consulted in 25-1-15 **291T-H, 223T-L3, 223T-I: widely represented in the database, not rare. ***they cover from position 16100 to 16365.

TABLE 10. Diversity Data From all the populations used in the study from highest to lowest.

Population	**Haplotipic diversity**
ANCSYR	1±0.05
VAL	0,962±0,02
MIN	0,962±0,01
CAT	0,953±0,01
ANCBA	0.952±0.09
SIC	0,944±0,02
GRE	0,940±0,03
MAJ	0,930±0,01
IBI	0,922±0,01
TUS	0,921±0,02
MPV	0,920±0,03
AND	0,919±0,01
CHU	0,918±0,02
SWI	0,914±0,01
LDC	0,912±0,02
ANCCRE	0.911±0.03
COR	0,903±0,02
SARD	0,899±0,01
VAR	0,898±0,03

MRR	0,865±0,02
BA	0,806±0,02
ANCBAL[1]	**0.803±0.04**
TK	0,764±0,01
ANCCAT	0.752±0.07
MALD	0,666±0,03
ANCSARD	0.576±0.10
ANCTK	0.318±0.16

[1]Population under study

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4.6 Molecular analysis of ancient caries

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Molecular analysis of ancient caries

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An 84 base pair sequence of the *Streptococcus mutans* virulence factor, known as dextranase, has been obtained from 10 individuals from the Bronze Age to the Modern Era in Europe and from before and after the colonization in America. Modern samples show four polymorphic sites that have not been found in the ancient samples studied so far. The nucleotide and haplotype diversity of this region have increased over time, which could be reflecting the footprint of a population expansion. While this segment has apparently evolved according to neutral evolution, we have been able to detect one site that is under positive selection pressure both in present and past populations. This study is a first step to study the evolution of this microorganism, analysed using direct evidence obtained from ancient remains.

1. Introduction

In the past few decades, genetic tools have made it possible to confirm the presence of the bacteria responsible for some of the diseases observed in ancient human remains. The aims of these studies have been numerous: to confirm the early diagnosis based on skeletal evidence, to identify the causative agent of a disease that has notably influenced some periods of human history, or to improve the general knowledge on the interaction between the human beings and the bacteria. Moreover, it has been used to reconstruct historical migrations by characterizing the diseases of the past, their morbidity, their expansion/diffusion and their evolution over time.

The diseases that have been molecularly studied so far are those believed to have caused the majority of the documented human epidemics, such as tuberculosis (*Mycobacterium tuberculosis*) [1,2], plague (*Yersinia pestis*) [3,4], leprosy (*Mycobacterium leprae*) [5,6] and syphilis (*Treponema pallidum pallidum*) [7,8], among others [9–11]. However, there are other microorganisms, such as bacteria from the dental plaque, that have accompanied humans since remote times for which their history is still not well established, and that could help us to understand prehistoric populations in depth. Thus, besides assessing issues such as the kind of diet and whether the meals were correctly handled and prepared [12], the general hygienic conditions [13], the gathering of people in populations [14], the routine contact of humans with animals, especially during the process of domestication [15], the recent discovery that some ignored illnesses can be traced back to far more ancient times than previously thought [16] implies that palaeogeneticists should be able to study human-bacterial interactions in dental plaque since their emergence.

In fact, oral infections have generally been overlooked when studying infections from the past even considering that caries has been the most persistent infection in the history of humanity. Palaeopathological studies have shown that their incidence go back to at least 1.5 Myr ago, being already found in a *Paranthropus robustus* specimen [17]. However, the most dramatic increase in caries frequency occurred in the transition towards agriculture owing to a change in diet. This change can be seen in both Europe and America [18–20],

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although there are places where such a relationship is not so clear, as in mainland southeast Asia, where the relative non-cariogenicity of rice, at that time an increasingly important subsistence mode in the region, and the retention of broad-spectrum livelihood strategies put the global application of this theory into question [21]. But this looks like the exception rather than the rule. Thus, study of Mesolithic–Neolithic transition has become a matter of great interest for learning about the infectivity of the bacteria involved in the formation of this lesion. Moreover, in recent times, the evidence that dental problems like the periodontal diseases and caries can cause other serious health problems have increased, so their development has been a matter of growing interest. New research suggests that periodontal diseases may contribute to the development of heart diseases [22], increase the risk of stroke [23] and may be a serious threat for people whose health is already harmed by diabetes, respiratory diseases or osteoporosis [24]. Thus, characterizing the microorganisms involved in these processes would be a step forward to gain a better knowledge of their interaction with human beings and the consequences that result from it. Following this reasoning, this study focuses on DNA characterization of the main agents responsible for the carious lesions from ancient remains.

Even though caries is the consequence of the loss of enamel as a result of the acidification of the oral plaque by a quite heterogeneous group of bacteria [25], *Streptococcus mutans* has been consistently associated with its presence [26]. The current advances in biomolecular technology offer the possibility of genetically characterizing these bacteria in human ancient remains, as well as determining the characteristics of the virulence factors that they need to carry out a successful infection. Preceding this study, in 2007 our group showed that it was possible to recover *S. mutans* DNA from human ancient remains [27]. On that basis, it has been possible to begin an evolutionary study of *S. mutans* in relation to the development of caries in humans [28], which has been extended in this work.

Caries lesions could be used to obtain information about the health of the individuals and their way of life in ancient times. Two combined factors increase the feasibility of carrying out the genetic analysis of this disease in ancient times. First, the resistance of teeth, which makes them particularly suitable for the study of the evolution of an organism that has one of its niches there. Second, the results of the activity of the cariogenic agents are evident in the teeth and make caries easy to detect.

Good markers of the *S. mutans* adaptation to its human host can be found in its virulence factors, thus their study may clarify some aspects of the evolution of this widely extended infection that has accompanied humankind during millennia, and therefore give us a more complete image of our evolutionary history. One of the most relevant points would be to know whether this microorganism has evolved according to the changes in the way of life of the host.

Moreover, the direction and strength with which natural selection has acted in different genomic regions of this bacterium could provide some clues over how good the adaptation to its host has been, and open the possibility to predict its evolution. Some things must be taken into account: (i) in many proteins, a high proportion of amino acids may be largely invariable owing to strong functional constraints [29], and (ii) adaptive evolution most probably occurs at a few time points and affects a few amino acids [30]. Thus, it is necessary to detect the ratio of non-synonymous to synonymous rates (ω), which measures the selective pressure at the protein level and can take values of $\omega < 1$, $=1$ or >1 indicating negative, neutral or adaptive evolution, respectively. In addition, a method that can measure it in a whole nucleotidic fragment, on the one hand, and at each single codon on the other hand, will be especially important for this matter, because in cases of very local action of positive selection, the overall ω ratio will not be significantly greater than 1. The aim of this study is to recover genetic material from the caries of individuals from the Bronze Age up to the twentieth century in Europe and America, and to characterize a fragment of the gene that encodes the virulence factor known as dextranase. This will permit us to carry out comparisons with current strains in order to determine whether the genetic diversity in ancient times was as high as it is nowadays, and whether there was any other relevant difference in relation to geographical or chronological differentiation. Moreover, we want to assess whether any specific site has been submitted to positive selection pressure in past and present populations, and whether its intensity has changed over time.

2. Material and methods

(a) Study species and sampling

Samples of different antiquity and geographical origin, European and American, were chosen from different archaeological sites and from a skeletal collection housed at Universitat Autònoma de Barcelona (UAB) (see table 1 for Genbank accession numbers, the electronic supplementary material for technical details and electronic supplementary material, table S1 for further description). The selection was made visually, as caries is macroscopically detectable.

The samples were stored and analysed in the Palaeogenetics Laboratory of the UAB. The conditions of sterility and the precautionary measures taken are previously described [11]: sample preparation (see the electronic supplementary material), DNA extraction and PCR reactions were performed in a laboratory dedicated specifically to work with ancient DNA (aDNA), positively pressurized and physically isolated from the laboratories used to carry out post-PCR processes. Laboratory overalls covering the whole body of the investigator, masks and protective lenses were also used. All the samples were amplified twice at the UAB, and the majority of them (7 out of 10) were cloned. In addition, samples T1, U1 and LO1 were analysed in the Laboratorio Nacional de Genómica para la Biodiversidad (Langebio, CINVESTAV-IPN, Mexico). In the Mexico laboratory, DNA extractions were performed essentially with the same protocol and procedures as in the UAB (see below) in an especially dedicated facility for aDNA analysis. Also, PCR amplifications of a dextranase gene fragment were conducted as described below, although in Langebio an end-point PCR apparatus (Veriti, Applied Biosystems) was used. Two of the samples (U1 and LO1) yielded positive amplification and were cloned and sequenced, and from the U1 sample two independent extractions were obtained.

The genome region of *S. mutans* chosen to be amplified was an exonic fragment of 84 base pairs (bp) in length of the first variable region of the dextranase gene. This gene is one of the so-called virulence factors of the pathogen. It codes for an enzyme which cleaves α-1,6-linkages of glucans and is thought to be responsible both for the control of the amount and content of extracellular glucans and for the metabolic utilization of extracellular glucans ([31] and references therein). The primers used to carry out the PCR process were L-344 (forward primer) and R-467 (reverse primer) [27].

Table 1. Alignment of the obtained sequences. (Sample references can be found in the electronic supplementary material, table S1. En dashes (–) indicate the same nucleotide as the reference sequence D4930.1 in that position, while polymorphisms are shown in bold. Samples U1 and L01 were duplicated at the Laboratorio Nacional de Genómica para la Biodiversidad from Mexico.)

	strain	accession no.	site	period	364	367	368	380	385	390	400	410	420	429	430	432	437	440	447
	D4930.1	D4930.1	England	Modern	T	A[a]	A[a]	A	G[a]	T	C	A	G	A[b]	G	G[b]	C[a]	C	A
modern samples	NN 2025	APO 10655	Japan	Modern	–	G	C	–	A	–	–	–	–	G	–	–	–	–	–
	LJ23	AP012336.1	Japan	Modern	–	–	C	–	A	–	–	–	–	G	–	–	–	–	–
	U159	AEO 14133	North America	Modern	–	G	C	–	A	–	–	–	–	G	–	–	T	–	–
	GS5	CP003686.1	North America	Modern	–	G	C	–	A	–	–	–	–	G	–	–	–	–	–
	ATCC 25175	HQ711852.1	England	Modern	–	–	–	–		–	–	–	–	G	–	–	–	–	–
	5DC8	AOBX00000000	England	Modern	–	–	C	–	A	–	–	–	–	G	–	–	–	–	–
	KK21	AOBY00000000	Germany	Modern	–	G	C	–	A	–	–	–	–	G	–	–	T	–	–
	KK23	AOBZ00000000	Germany	Modern	–	G	C	–	A	–	–	–	–	G	–	A	–	–	–
	AC4446	AOCA00000000	Germany	Modern	–	G	C	–	A	–	–	–	–	G	–	–	–	–	–
	NCTC11060	AOCC00000000	Denmark	Modern	–	G	C	–	A	–	–	–	–	G	–	–	–	–	–
ancient samples	U1	KJ950640	Granollers, Catalonia	twentieth century	–	–	C	–	A	–	–	–	–	G	–	–	–	–	–
	M1	KJ950631	Montanissell, Lleida	Bronze Age	–	G	C	–	A	–	–	–	–	G	–	–	–	–	–
	CR1	KJ950636	Can Reiners, Majorca	seventh century	–	–	C	–	A	–	–	–	–	G	–	–	–	–	–
	V1	KJ950637	Vilomara, Catalonia	ninth to tenth centuries	–	–	C	–	A	–	–	–	–	G	–	–	–	–	–
	SP1	KJ950634	Sant Pere Churches, Catalonia	fifth to twelfth centuries	–	G	C	–	A	–	–	–	–	G	–	–	–	–	–
	SP2	KJ950635	Sant Pere Churches, Catalonia	fifth to twelfth centuries	–	G	C	–	A	–	–	–	–	G	–	–	–	–	–
	T1	KJ950638	Tlatelolco, Mexico	precontact twenty-fifth to twenty-sixth centuries	–	–	C	–	A	–	–	–	–	G	–	–	–	–	–

(*Continued*.)

Table 1. (*Continued*.)

strain	accession no.	site	period	364	367	368	380	385	390	400	410	420	429	430	432	437	440	447
D4930.1	D4930.1	England	Modern	T	A[a]	A[a]	A	G[a]	T	C	A	G	A[b]	G	G[b]	C[a]	C	A
T2	KJ950639	Tlatelolco, Mexico	precontact twenty-fifth to twenty-sixth centuries	–	–	T	–	A	–	–	–	–	G	–	–	–	–	–
LO1	KJ950632	Los Olmos, Mexico	postcontact twenty-sixth century	–	G	C	–	A	–	–	–	–	G	–	–	–	–	–
LO2	KJ950633	Los Olmos, Mexico	postcontact twenty-fifth century	–	G	C	–	A	–	–	–	–	G	–	–	–	–	–

[a]Positions where the polymorhism results in a non-synonymous change.
[b]Positions where the polymorphism results in a synonymous change.

(b) Experimental protocol

For DNA extraction, 0.5 g of powder were collected from the teeth cavities of each individual. Samples were divided into groups of three to five for the DNA extraction process, to keep a low sample-to-blank control ratio. A real-time PCR reaction using the Qiagen Rotor-Gene Q (QIAgen, Turnberry Lane, USA) and the Type-it HRM PCR kit(400) (QIAgen, Turnberry Lane, USA) was then carried out in a final volume of 25 µl. The PCR process consisted of the following steps: an initial denaturation step at 94°C for 5 min, followed by 45 cycles of PCR including 10 s at 94°C and 30 s at 55°C. The obtained product was purified using the PCRapace kit (Invitrogen, Carslbad, CA, USA) following supplier instructions.

In the cloned samples, after purification the amplified product was cloned into the TOPO TA cloning kit (Invitrogen). Cloned fragments were amplified by colony-PCR using pM13 forward and reverse primers with the following profile: an initial denaturation step at 94°C for 5 min, followed by 35 cycles of PCR including 1 min at 94°C, 1 min 30 s at 55°C, 1 min at 72°C and a final extension step of 7 min at 72°C. The amplified product was purified again as described above and then sequenced.

Sequence reactions were carried out using the sequencing kit BigDye Terminator v. 3.1 (Applied Biosystems, Carslbad, USA) according to the manufacturer's specifications, and run in an ABI 3130XL sequencer (Applied Biosystems, Foster City, USA). The BLAST program [32] was used to search for similar sequences in the GenBank database (NCBI). The consensus sequence for each gene fragment was determined by alignment of the forward and reverse sequences using BioEdit v. 7.0.5.3 (Ibis Biosciences, Carslbad, USA).

Finally, two teeth were purposely chosen to be of different geographical origins for parallel DNA extraction from dentine. MtDNA haplogroup identification was carried out in order to have a general overview of the degree of conservation of the samples, and to check whether the results were consistent with the population of origin. The samples were amplified in the second half of the mitochondrial Hypervariable Region I sequence and the obtained haplogroup was corroborated by means of restriction fragment length polymorphisms.

The data sequence assembly is available at the electronic supplementary material.

(c) Statistical analyses

The nucleotide diversity per site (π) and haplotype diversity (H) were calculated using SPSS 15.0.1 software (IBM, New York, NY, USA). The best-fit model of nucleotide evolution was selected using the Bayesian Information Criterion implemented in the best-fit model test included in MEGA 5.05 software [33,34]. The F_{ST} distance between the two groups was calculated with Arlequin v. 3.11 [35] and finally, a median-joining network was constructed using the Network 4.1.5.6 software (Fluxus Technology Ltd, Suffolk, UK).

Maximum-likelihood estimations (MLEs) of the dN/dS ratio (ω) were obtained by using the Codeml program from the PAML package [36], and normalized values of dN–dS on a codon-by-codon analysis were obtained using the HyPhy software [37]. The ML phylogenetic tree was reconstructed with the PHYML package [38] (see the electronic supplementary material for technical details).

A branch-site test of positive selection was applied to both groups of samples to check whether they were evolving according to neutral evolution. Moreover, three of the tests supported by PAML package, M0 versus M3 to test for variable ω among sites, and M1a versus M2a and M7 versus M8 to test for possible positive selection at specific sites, were carried out [39]. To calculate the posterior probabilities that each site belongs to a particular site class, a Bayes Empirical Bayes approach [29] was

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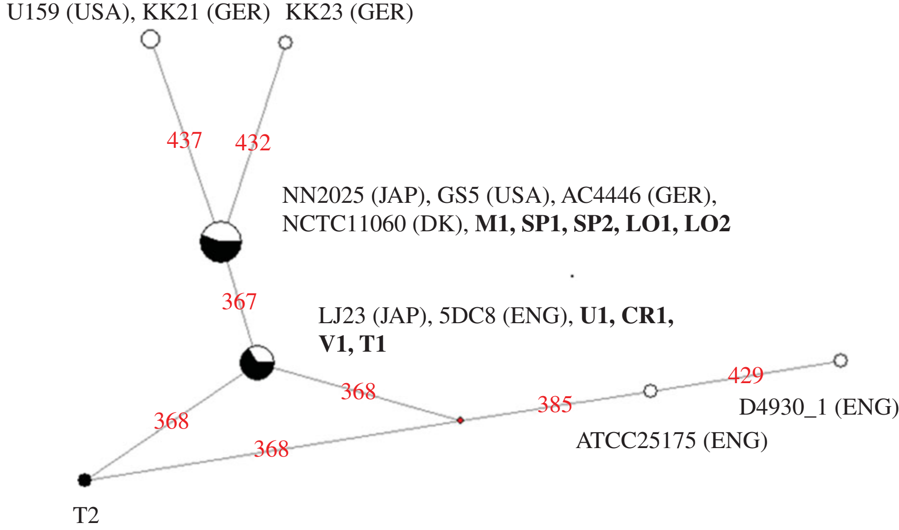

Figure 1. Median-joining network of the sequences presented in this study. Modern sequences (11 modern strains from Genbank obtained in this study) are: U159 and GS5 (USA), NN2025 and LJ23 (Japan), D4930.1, ATCC 25175 and 5DC8 (England), KK21, KK23 and AC4446 (Germany) and NCTC11060 (Denmark). Ancient samples frequency is in black inside the circles and their names are highlighted in bold, and modern samples frequency is represented in white. (Online version in colour.)

applied, and sites coming from the class with $\omega > 1$ with a high posterior probability ($p > 0.95$) were inferred to be under positive selection. Branch lengths were fixed at their MLEs under M0 (one-ratio). In addition, the ω rate ratios, estimated using the method of Nei & Gojobori [40] for pairwise sequence comparison, were compared between the total amount of sequences and those from current populations to check whether any change in their values could be detected over time (see the electronic supplementary material for technical details).

3. Results

A 84 bp DNA fragment of the dextranase gene of *S. mutans* was obtained from caries samples from 10 individuals (see table 1 for Genbank accession numbers). Six samples belonged to ancient European populations and four belonged to ancient American ones. The results are summarized in table 1, with the sequences obtained in this study aligned with the 11 modern sequences of this segment of the dextranase gene currently available in the Genbank database (see the electronic supplementary material, table S1). Seven of the samples (M1, CR1, LO1, LO2, SP1, U1 and T2) were cloned (see the electronic supplementary material, table S2) and the consensus sequences obtained from each one always matched the one obtained by direct PCR. The sequences from the samples that belong to the caries of the ancient individuals were identical to those observed in some modern populations, with the exception of the American sample T2, a sample prior to European contact. In addition, two of the ancient samples (U1 and LO1) were independently replicated in Langebio, Mexico, giving coincident results.

The results showed that five of the nine ancient samples (M1, SP1, SP2, LO1 and LO2) matched up exactly with one of the two modern Japanese strains (NN2025) and also with modern North American (GS5), Danish (NCTC11060) and German (AC4446) ones. The remaining five showed a G to A transition in the nucleotide position 367 (U1, CR1, V1, T1 and T2), which is also currently extended worldwide as observed in current Japanese (LJ23) and English (5DC8) strains. One sample (T2) also harboured a C to T transition in the position 368 that was not seen in any other sample, as the other two samples that showed a change in that position were two modern English strains harbouring a C to A transversion (ATCC25175 and D49430.1).

Amplifications of human mtDNA, as quality controls, were successful and yielded the expected results. One sample originally coming from Mexico (LO1, accession no. KJ950642) harboured an haplogroup of American origin (A), and one coming from Catalonia (M1, accession no. KJ950641) harboured one of European origin (K) (see the electronic supplementary material, table S3).

The nucleotidic (π) and haplotypic (H) diversity indexes were higher in the modern than in the ancient sequences (0.019 ± 0.004 versus 0.009 ± 0.002 for the π values, and 0.848 ± 0.074 versus 0.639 ± 0.126 for the H values). Both the increase in nucleotide and haplotype diversity was statistically significant (one-tailed Mann–Whitney test [41], $p < 0.01$ and $p < 0.05$, respectively) (see the electronic supplementary material, table S4).

The best-fit model of nucleotide evolution was the Kimura two-parameter model [42], with a discrete Gamma distribution of rate variation among sites (+G), with five gamma rate categories and alpha shape parameter of 0.06 and a transition to transversion ratio of 6.31. Pairwise F_{ST} distances between the ancient and the current population were calculated, showing that there were no significant differences ($p > 0.05$; see the electronic supplementary material, table S5). A phylogenetic network was constructed under the assumptions of this model (figure 1).

The maximum-likelihood phylogenetic tree was calculated with PHYML [38] using the best-fit model of nucleotide evolution obtained, and used as the basis to estimate the mean number of changes per codon per branch. The test statistics M0 versus M3, M1a versus M2a and M7 versus M8 gave significant results in both the current and the ancient populations, and also whether all the samples were considered as a single population ($p < 0.01$ in all cases) (see the electronic supplementary material, table S6). We considered the signal of positive selection to be strong when both M1a versus M2a and M7 versus M8 were significant at the 5% level. Only codon site 2 showed

positive selection with a high posterior probability ($p > 0.99$) in both sets of sequences, once the Bayes Empirical Bayes approach was applied [15]. Site 25 fell near the threshold value to reject neutral evolution in the M7 versus M8 test (see the electronic supplementary material, table S7).

At site 2, the selective strength seemed stronger in ancient populations than in modern ones, using all the models of amino acid evolution available at the HyPhy package (see the electronic supplementary material, table S8).

Among the ancient sequences, only non-synonymous substitutions were observed. As no orthologous sequences from a closely related species could be found performing a BLAST search (see the electronic supplementary material, table S9), two samples from this study, M1 and NN2025, were used as background branches to carry out the branch-site test for positive selection. These tests showed that ω was not significantly greater than 1, neither in the ancient nor in the modern population (see the electronic supplementary material, table S10).

4. Discussion

The physical presence of *S. mutans* in ancient samples was first detected by our team in 2007 [27], and some of the preliminary sequences of this study were published in 2011 [28]. In addition, this bacterium was recently detected by other groups using the gold-labelled antibody transmission electron microscopy [43], and in 2012 an aDNA segment from *S. mutans* DNA was amplified from an ancient dental calculus of an individual dating from approximately 500 years BP [44]. Ten sequences from the Bronze Age to the beginning of the twentieth century from ancient dental carious lesions are presented in this study, allowing our team to carry out a phylogenetic analysis of the ancient strains of this bacterium.

This sample size is related to the difficulties inherent to the analysis of aDNA. The work on aDNA is subjected to important difficulties: (i) the biochemical damage it can suffer [45,46], (ii) the risk of amplifying exogenous (contaminant) DNA that may outcompete aDNA in downstream analyses [47], and (iii) the possible inhibitors that the samples may carry [48]. Owing to this, all the samples suspicious of bearing postmortem damage, as an excess of type II transitions [45,46], and those that did not amplify after three amplifications were discarded to avoid obtaining sequences affected by miscoding lesions or products resulting from carryover contamination.

Regarding other authenticity criteria, no positive controls had been used, and no DNA from *S. mutans* had been previously amplified in our laboratories. In addition, some of the obtained sequences were cloned in order to obtain the consensus sequences, which always matched up with the one obtained by direct sequencing of PCR products. Moreover, two samples from different origins were amplified with primers for human mtDNA, and the results coincided with the expectations, as each sample harboured a haplogroup that made phylogenetic sense, considering their different geographical sources. The diversity of the results further guaranteed that they were not the product of a general process of contamination. Finally, two of the samples were successfully replicated in the aDNA laboratory of Langebio, CINVESTAV-IPN, in Mexico, showing that the products obtained were not affected by intra-laboratory contamination. Thus, the authenticity of these results can be verified [47], and it can be stated that it is possible to isolate DNA from this bacterium in archaeological remains from periods as ancient as the Bronze Age (Montanissell sample, M1).

Focusing on the sequences, six polymorphic positions can be seen (367, 368, 385, 429, 432 and 437) in present-day populations, while in ancient samples just the first two of the cited polymorphisms have been observed. In the near future, more ancient samples sharing a similar origin with the samples used in this study, such as Asia, shall be analysed to warrant a minimum representation of geographical variability. The African continent, not represented here, must also be a focus of interest in future studies regarding past and present populations. Therefore, it will be necessary to increase the sample size of both modern and ancient groups. Nevertheless, and although the small sample size recommends taking these results with caution, it seems that both the nucleotide and haplotype diversities of this region of the dextranase gene are increasing over time (both values are significantly higher in the modern than in the ancient populations).

Alternatively, the increase in the genetic diversity we found might be attributed to a bias in the choice of the modern strains of *S. mutans*. However, this seems unlikely because in any of the original papers reporting the modern sequences it is specified that the strains were chosen for any particular reason other than characterizing partially or totally the genome from different strains of this organism [49–54], or distinguishing the different functional regions of the dextranase gene sequence in relation to its enzymatic activity [55]. Therefore, no sampling bias is evident.

This increase in diversity is not translated into an increase of the ω value, as the new observed substitutions bring a negative value in the overall dN – dS difference (see the electronic supplementary material, table S8), pointing to a slight decrease of ω over time, and suggesting a constraint of the selective pressure in this dextranase segment over *S. mutans* recent history. In fact, empirical data have demonstrated that in closely related microbial sequences, a relative preponderance of non-synonymous changes is seen, leading to a high value of ω [56]. The interpretations of this fact have been numerous, from statistical artefact [57], to relaxed [58] or positive [59] selection, recent ancestry [60,61] or a lag in the removal of slightly deleterious non-synonymous mutations that have survived via hitch-hiking to a nearby strong adaptive mutation [56]. Whatever the reason, the advantage of our study is that obtaining samples from archaeological remains allows comparison of the ω of this population at different points in time, separated by hundreds and thousands of years not by inferred phylogenetic reconstruction, but by directly observed data. As a fall in the ω value is detected, we can rule out the possibilities that neither relaxed nor positive selection have been the driving forces in the full segment over time. The fact that no positive selection is observed when considering the segment as a whole, in spite of being a known factor of virulence, agrees with the fact that dextranase has not been included among the genes that were under Darwinian positive selection in previous studies [62,63]. Nevertheless, using likelihood-ratio tests of codon evolution one specific site (site 2) appears to have been subjected to positive selection throughout the evolutionary history of the segment and continue to do so, although to a lesser extent, and a second one (site 25) could be departing from neutral evolution.

The majority of the samples, both modern and ancient, belong to the two central nodes, reflecting that the full segment

is still evolving neutrally while, with the exception of sample T2, the extremes of the phylogenetic network are represented by modern-day sequences, thus reflecting the increase in genetic variability over time. This is supported by the previously mentioned rise in the genetic diversity of the segment and a recent study showing that the *S. mutans* population started expanding exponentially around 10 000 years ago, approximately coinciding with the onset of human agriculture [63].

Finally, as observed in previous studies focusing in *S. mutans* comparative genomics of current strains [50,63,64], no characteristic differences in relation to geographical distribution were seen either in the modern or ancient population.

Nevertheless, more samples of different periods and longer sequences will be needed in future studies in order to fully certify the constriction of selective pressure in this segment inferred from our results. Tracing back *S. mutans* history demands the review of the history of caries. As stated previously, adaptive evolution most probably occurs at a few time points [17], where the frequency of caries has significantly changed, so those periods in time are the ones most likely to show a process of non-neutral selection, marking interesting moments to check the evolution of the bacteria involved in the process.

Six such moments in the history of this illness stand out and it will be worth studying them in depth in future works: the first stage of farming in the American continent (8000–5000 BP) when caries has been recorded with relatively high indices as a consequence of the consumption of endemic fruits rich in maltodextrines and sugar [65–67]; the introduction and spread of cereals in the Old World, reaching a 75% in caries frequency around 4500 BC [68]; its increment in America since 2300 BC, clearly related to maize consumption (*Zea mays*) [69,70] and more specifically to a gradual replacement of popcorn for a more cariogenic and amylaceous maize [66,71]; the contact between the people of the Old and the New World, especially from before and after AD 1550 when sugar and sugar cane started being imported in large scales from America to Europe [72,73]; the advent of Industrial Revolution that gave rise to cooking technologies that broadened the dietary breath and implied a major dietary shift to increased animal fat, sugar and processed foods [74]; and finally, the decrease since the 1970s throughout industrialized countries related to dental treatment, the introduction of fluoride water and toothpaste [75,76] on the one hand, and a range of changing social factors linked to improvements in general health indicators [77–79] on the other. Caries has such a well-documented story that once this technology can be routinely applied the palaeogeneticists will know which populations warrant this kind of study.

The analysis of *S. mutans* in individuals from these historical periods could start an interesting field that aims to understand more issues about the way of life of ancient people. The information obtained could also be used to reconstruct past population movements. In this sense, this work reports molecular genetic evidence obtained from ancient bacteria associated with archaeological caries lesions that will help in this aim. Analysing such an ancient genetic material also brings about the possibility of determining whether its evolution can be somehow correlated with changes in the way of life of its hosts. Owing to *S. mutans* being, to some extent, maternally transmitted [80], it could consequently reflect the migration pattern of women, which may be inferred by mtDNA analysis. A first paper reporting this kind of study was published by Moodley *et al*. [81] by using another human pathogen, *Helicobacter pylori*, demonstrating that two different strains of this bacterium accompanied the two human prehistoric migrations along the Pacific.

Also, it would be important to check the adaptation of this bacterium at molecular level to the different niches that it can find inside a human host.

The answer to some of these questions may be related to the changes that the virulence factors of the bacterium have experienced over time; so this field needs to continue expanding in order to be able to respond to them.

Data accessibility. DNA sequences: Genbank accessions KJ950631–KJ950642.

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4.6.1 Supporting information

Supplementary Text

Pretreatment of the samples and DNA extraction method

All the reagents were prepared in a laboratory specifically dedicated to work with ancient DNA under restrictive sterility conditions. Before starting the extraction process the samples were briefly cleaned with a 5% hypochlorite solution trying to manipulate them as little as possible. All the process was conducted inside a laminar flux hood. Each sample was obtained using a different set of dental material that included forceps and a high speed dental diamond bur inserted in a 220 V micromotor. The powder was directly extracted from the lesion itself and put in a 15ml polypropylene tube, where it was incubated overnight at 37ºC in 5ml of extraction buffer (250ul Tris HCl 1M (pH 8.0-8.5), 250ul SDS 10%, 250ul sterile deionised water and 4,25ml EDTA 0,5M) and 50ul of 0.01 g/ml proteinase K. After incubation, the DNA was subsequently extracted with a standard phenol-chloroform extraction protocol and the aqueous phase was concentrated using a Centricon-30 filter column (Millipore) up to a 30 ml volume [13]. An extraction blank was prepared in each extraction process to check for contamination.

Sequence used as reference and other modern sequences

The *Streptococcus mutans* (*S. mutans*) D4930.1 strain [1] was used as reference. The numbering was done starting from the first nucleotide that appears in the *S. mutans* dexA gene for dextranase, complete cds, Accesion number D49430.1. The other modern sequences used are U159 [2] and GS5 (USA) [3], NN2025 and LJ23 (Japan) [4,5], ATCC 25175 (England) [6], 5DC8 (England), KK21, KK23 and AC4446 (Germany) and NCTC11060 (Denmark) [7].

Ancient sequences obtained

The full-length caries ancient sequences retrieved were all originally obtained in this work. Some aspects of the ancient human populations from those sites were described in: M1 (Catalonia) [8], CR1 (Majorca) [9], V1 (Universitat Autònoma de Barcelona (UAB) archaeological collection) (Catalonia, present study), SP1 and SP2 (Catalonia) [10], T1, T2, LO1 and LO2 (México) [11] and U1 (UAB archaeological collection) (Catalonia, present study).

Determination of human mitochondrial haplogroups

The mitochondrial haplogroups of two individuals, one of European and one of American origin, were obtained combining the information obtained by the sequenciation of the second half of the Hypervariable Region I (nucleotide positions 16210 to 16400) and generating restriction-length polymorphisms

Detailed data

Branch-site test of positive selection

The branch-site test of positive selection [14,15] was applied. This compares the modified model A with the corresponding null model with $\omega = 1$ fixed (fix_omega = 1 and omega = 1). χ_1^2 with critical values 3.84 and 5.99 was used to guide against violations of model assumptions, as recommended in [16]. To calculate the *p* value based on this mixture distribution, *p* was calculated using 2Δl, and then the obtained value was divided by 2. As no orthologous sequences from a closely related species could be found performing a Blast search [17] (see the electronic supplementary material, table S8), the most ancient sample studied, M1, was taken as the background branch when applying the branch-site test of positive selection to the ancient sequence data set only, or to the whole sequence data set. When applying the test to the modern sequence data only, strain NN2025 (Japan) was used as the background branch because

it was in the principal node, and Japan was the only country with representatives in the two principal nodes of the network.

Likelihood-ratio tests using the site models

Three of the tests supported by PAML package were carried out. The first test compared the one-ratio model (M0) with the discrete model (M3), which tests whether ω can vary among sites. The two likelihood-ratio tests (LRTs) used to check for positive selection were M1a vs M2a and M7 vs M8 [18]. In these tests, a null model that does not allow $\omega>1$ in the class distribution of this value (M1a and M7) is compared with an alternative model that does (M2a and M8, respectively). These are the two best LRTs used so far to test for positive selection [19]. Twice the log likelihood difference (2Δl) between the values obtained under these models can be compared to a χ^2 distribution with 4 degrees of freedom (df) in M0 vs M3, and with 2 df in M1a vs M2a and M7 vs M8 [20]. The F3x4 model of codon frequencies (the equilibrium codon frequencies are calculated from the average nucleotide frequencies at the three codon positions) was used to accommodate biased codon usage. Under the conditions set by positive selection models M2a and M8a, the most likely site category (with the associated *d*N/*d*S ratio) at each codon (amino acid) site was inferred. In all cases, branch lengths were fixed at their Maximum-Likelihood Estimation (MLE) under M0 (one-ratio) to save computation, as several previous studies have shown that tests of positive selection and detection of specific sites under its action are insensitive to minor errors in the tree topology or to different estimates of branch lengths [21-23]. After detecting that sites under positive selection were present using LRTs, we applied a procedure known as Bayes Empirical Bayes (BEB) [24] to calculate the posterior probabilities that each site belonged to the class $\omega>1$. BEB appears to avoid the high false-positive rates of the

naïve empirical Bayes (NEB) approach in small non-informative data sets, as it better accommodates uncertainties in the MLE of parameters in the ω distribution [15].

Different tests of codon selection (Twice the log-likelihood was calculated and compared with the corresponding χ^2 test).

For ancient sequences

M0 vs M3: 2x (-109.230-(-117.484)) = 16.632, p<0,05 for χ^2_4

M1a vs M2a: 2x (-109.230-(-118.530)) = 18.6, p<0,01 for χ^2_2

M7 vs M8: 2x (-109.230-(-118.757)) = 19.054, p<0,01 for χ^2_2

For modern sequences

M0 vs M3: 2x (-129.751-(-134.917)) = 10.332, p<0,05 for χ^2_4

M1a vs M2a: 2x (-129.752 -(-135.555)) = 11.506, p<0,01 for χ^2_2

M7 vs M8: 2x (-130.253-(-135.816)) = 10.586, p<0,01 for χ^2_2

For all the sequences

M0 vs M3: 2x (-153.352-(-174.916)) = 43.128, p<0,05 for χ^2_4

M1a vs M2a: 2x (-153.352 -(-172.471)) = 38.238, p<0,01 for χ^2_2

M7 vs M8: 2x (-154.681-(-172.478)) = 35.594, p<0,01 for χ^2_2

Codon-by-codon analysis of natural selection

For each codon, the numbers of sites that are estimated to be synonymous (S) and non-synonymous (N) were calculated. These estimates were produced using the joint Maximum Likelihood reconstructions of ancestral states under a Muse-Gaut model [25] of codon substitution and all the models of nucleotide substitution provided by the HyPhy software package [26]. To estimate MLE values, a tree topology was automatically computed. The test statistic dN - dS was calculated, where dS is the number of synonymous substitutions per site (s/S) and dN is the number of non-synonymous substitutions per site (n/N). A positive value for the test statistic indicates

an over-abundance of non-synonymous substitutions. Normalized dN - dS for the test statistic was obtained using the total number of substitutions in the tree (measured in expected substitutions per site), in order to make comparisons between the two data sets. Maximum Likelihood computations of dN and dS were performed using HyPhy software package as done in [27].

BLAST search

A search for short, nearly exact matches was carried out using the BLAST program [24] from 7 to 20bp in length in the Bacillus/ Lactobacillus/Streptococcus group (taxid: 91061) and with somewhat similar sequences (blastn) that includes 20bp or longer with just *S. mutans* dextranase giving significant results (E value lower than 0.1) (http://blast.ncbi.nlm.nih.gov/Blast.cgi, last accessed 15/4/13).

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Supplementary Tables

Table S1. Used samples and references. The references from the sites where some other aspects of the ancient human populations were described are: M1[8], CR1[9], SP1and SP2 [10], T1, T2, LO1 and LO2 [11].

	Strain/Accession number (modern samples)[1] Strain/Used Tooth (ancient samples)[2]	Site	Period	Reference
Modern samples	D49430.1[3]	England	Modern	1
	NN 2025/APO 10655	Japan	Modern	2
	LJ23/AP012336.1	Japan	Modern	3
	U159/AEO 14133	North America	Modern	4
	GS-5/CP003686.1	North America	Modern	5
	ATCC 25175/HQ711852.1	England	Modern	6
	5DC8/AOBX00000000	England	Modern	7
	KK21/AOBY00000000	Germany	Modern	7
	KK23/AOBZ00000000	Germany	Modern	7
	AC4446/AOCA00000000	Germany	Modern	7
	NCTC11060/AOCC00000000	Denmark	Modern	7
Ancient a samples	U1/(24)[4]	Granollers, Catalonia	XXth century	This study
	M1/(16)	Montanissell, Lleida	Bronze Age	This study
	CR1/(47)	Can Reiners, Majorca	VIIth century	This study
	V1/(47)	Vilomara, Catalonia	IX-Xth centuries	This study
	SP1/(46)	Sant Pere Churches, Catalonia	V-XIIth centuries	This study
	SP2/(31)	Sant Pere Churches, Catalonia	V-XIIth centuries	This study
	T1/(46)	Tlatelolco, Mexico	Precontact XV-XVIth centuries	This study
	T2/(26)	Tlatelolco, Mexico	Precontact XV-XVIth centuries	This study
	LO1/(41)	Los Olmos, Mexico	Postcontact XVIth century	This study
	LO2/(32)	Los Olmos, Mexico	Postcontact XVth century	This study

[1]: for current samples; [2]: for ancient samples; [3]: sequence used as reference; [4]following the nomenclature set by the FDI.World Dental Federation.

Table S2. Alignment of clones from the amplified sequences. The English sequence D4930.1 was used as reference. Clones highlighted in blue have been obtained at the Laboratorio Nacional de Genómica para la Biodiversidad, Mexico. Polymorphic positions found in this study are highlited in bold.

D4930.1[1] (Modern)	TCA**AA**TGAAACGGTGGATTCG**G**CCATTAACTCTTTTCAAGAGACAGACCTTAAGGTGCAAGAGAAAGAGGATGCTGCGGCTGCA
M1 (Bronze Age)	TCA**GC**TGAAACGGTGGATTCG**A**CCATTAACTCTTTTCAAGAGACAGACCTTAAGGTGCAAGAGAA**G**GAGGATGCTGCGGCTGCA
M1.1	....
M1.2	....
M1.3	....
M1.4	....
M1.5	....
M1.6	....
M1.7	....
M1.8	....
M1.9	....
M1.10	..........T...
M1.11	....
M1.12	..........T...

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LO1 (XVIst cent)    TCAGCTGAAACGGTGGATTCGACCATTAACTCTTTTCAAGAGACAGACCTTAAGGTGCAAGAGAAGGAGGATGCTGCGGCTGCA
LO1.1               ....................................................................................
LO1.2               ................G............T.T.........................................T..T.......
LO1.3               ....................................................................................
LO1.4               ....................................................................................
LO1.5               .........................................A..............................A...........
LO1.1               ....................................................................................
LO1.2               ....................................................................................
LO1.3               ....................................................................................
LO1.4               ....................................................................................
LO1.5               ....................................................................................

LO2  (XVIst cent)   TCAGCTGAAACGGTGGATTCGACCATTAACTCTTTTCAAGAGACAGACCTTAAGGTGCAAGAGAAGGAGGATGCTGCGGCTGCA
LO2.1               ....................................................................................
LO2.2               ...A................................................................................
LO2.3               ....................................T..........TT......C...........................G
LO2.4               ....................................................................................
LO2.5               ................................C...................................................
LO2.6               ....................................................................................
LO2.7               ....................................................................................

SP1 (V-XIIst cent)  TCAGCTGAAACGGTGGATTCGACCATTAACTCTTTTCAAGAGACAGACCTTAAGGTGCAAGAGAAGGAGGATGCTGCGGCTGCA
SP1.1               ....................................................................................
SP1.2               ....................................................................................
SP1.3               ..........T.........................................................................
SP1.4               ............................................................A.A.....................
SP1.5               ....................................................................................
SP1.6               .........................................A..........................................
SP1.7               ....................................................................................
SP1.8               ....................................................................................
SP1.9               ....................................................................................

CR1 (VIIst cent)    TCAACTGAAACGGTGGATTCGACCATTAACTCTTTTCAAGAGACAGACCTTAAGGTGCAAGAGAAGGAGGATGCTGCGGCTGCA
CR1.1               ....................................................................................
CR1.2               ....................................................................................
CR1.3               ....................................................................................
CR1.4               ....................................................................................
CR1.5               ....................................................................................
CR1.6               ....................................................................................
CR1.7               ....................................................................................
CR1.8               ....................................................................................
CR1.9               ....................................................................................
CR1.10              ....................................................................................
CR1.11              ................G.....T.........................T........T..........................

T2 (XV-XVIth cent)  TCAATTGAAACGGTGGATTCGACCATTAACTCTTTTCAAGAGACAGACCTTAAGGTGCAAGAGAAGGAGGATGCTGCGGCTGCA
T2.1                ....................................................................................
T2.2                ....................................................................................
T2.3                ........G...........................................................................
T2.4                .......G............................................................................
T2.5                .............................................................T......................
T2.6                ....................................................................................
T2.7                ....................................................................................

U1 (XXth cent)      TCAACTGAAACGGTGGATTCGACCATTAACTCTTTTCAAGAGACAGACCTTAAGGTGCAAGAGAAGGAGGATGCTGCGGCTGCA
U1.1                ............A.......................................................................
U1.2                ....................................................................................
U1.3                ............................................................A.......................
U1.4                .....................................................................A....C.........
U1.5                ....................................................................................
U1.6                ....................................................................................
U1.7                ....................................................................................
U1.1                ...........................G........................................................
U1.2                ...........................G........................................................
U1.3                ....................................................................................
U1.4                ....................................................................................
U1.5                ....................................................................................
U1.6                ....................................................................................
U1.7                ....................................................................................
U1.8                ....................................................................................
U1.9                ...................................................................................G
U1.10               ...........................G........................................................
```

[1]Clones obtained at the Laboratorio General para la Biodiversidad from Mexico are marked in blue.

Table S3. Primers used to amplify and sequence the Hypervariable region I of mtDNA (HVR-I) and enzymatic restriction for the RFLP study.

Sample	Nucleotide position 16210-16400	Haplogroup by sequence	Primers L-16209 [12] and H-16401 [13]	+HaeIII 663 Primers L-635 and H-708 [15]	-HaeII 9052 Primers L-9020 and H-9082 [12]	+HinfI 12308 Primers L-12215 and H-12309 [14]	Haplogroup by RTFLPs	Consensus haplogroup [1]

M1	224C, 311C	U, K		-	+	K	**K**
LO1	223T, 290T, 319A, 362C	A	+			A	**A**

[1]Haplogroup classification following [16].

Table S4: Nucleotide data

Variables	Ancient populations	Current populations
Haplotype diversity	0.639	0.848*
Nucleotide diversity	0.009	0.019**

*p<0,05, **p<0.01

Table S5: Maximum Likelihood Estimates of nucleotide evolution and associated F_{ST} distances between both populations. The model with the lowest BIC score (Bayesian Information Criterion) is considered to describe the substitution pattern the best. AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (*lnL*), and the number of parameters (including branch lengths) are also presented [17]. Non-uniformity of evolutionary rates among sites is shown using a discrete Gamma distribution (+*G*) with 5 rate categories. Estimates of gamma shape parameter are shown. Assumed or estimated values of transition/transversion bias (*R*) are also shown.

Model	Parameters	BIC	AICc	*lnL*	(+*I*)	(+*G*)	*R*	F_{ST}
K2+G	41	620.646	398.158	-157.079	n/a	0.06	6.31	0,0018

Table S6. Log-likelihood values for the region studied to estimate positive selection. In these tests used, twice the log likelihood difference (2Δl) between the values obtained under these models can be compared to a χ^2 distribution with 4 degrees of freedom (df) in M0 vs M3 and with 2 df in M1a vs M2a and M7 vs M8 [18]. Last column indicates the site under positive selection after the BEB [19] analysis is applied.

Population	n	M0vsM3	M1avsM2a	M7vsM8	Positively selected sites BEB
Ancient Strains	9	16,508**	18,6**	19,054**	2A
Modern Strains	12	30,012**	24,436**	31,05**	2A
All Strains	21	43,128**	38,238**	35,594**	2A

**p<0,01

Table S7: Detailed Bayes Empirical Bayes inference of relevant codons posterior probabilities

Ancient sequences

M2a

Site	Pr(ω >1)	post mean ± SE for ω
2 A	0.999**	8.988 ± 1.623

M8

Site	Pr(ω >1)	post mean ± SE for ω
2 A	1**	8.943 ± 1.642

Modern sequences

M2a

Site	Pr(ω >1)	post mean ± SE for ω
2 A	1**	9.399 ± 1.305
8 T	0.638	6.291 ± 4.245
25 A	0.886	8.435 ± 2.964

M8

Site	Pr(ω >1)	post mean ± SE for ω
2 A	1**	9.380 ± 1.322
8 T	0.747	7.164 ± 3.961
25 A	0.942	8.884 ± 2.383

All sequences

M2a

Site	Pr(ω >1)	post mean ± SE for ω
2 A	1**	9.940 ± 0.856
8 T	0.644	9.552 ± 2.020

25 A	0.885	9.252 ± 2.537

M8

Site	Pr(ω >1)	post mean ± SE for ω
2 A	1**	9.805 ± 0.975
8 T	0.750	7.515 ± 4.049
25 A	0.941	9.271 ± 2.331

**p>0.99

Table S8: Maximum-likelihood analysis of natural selection codon by codon

Site		Ancient population	Modern population
2	Normalized dN-dS[1]	39.901	12.0165
8	Normalized dN-dS	0	6.925
25	Normalized dN-dS	0	6.925

[1]Values shown are those obtained by applying the Felstentein model of codon evolution [20].

Table S9: Alignment of the obtained segment with similar sequences. Only the matches with the 8 lowest E values are included.

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Streptococcus mutans dexA gene for dextranase, complete cds	152	152	100%	8e-36	100%	D49430.1
Streptococcus mutans strain ATCC 25175 dextranase gene, complete cds	147	147	100%	3e-34	99%	HQ711852.1
Streptococcus mutans LJ23 DNA, complete genome	138	138	100%	2e-31	96%	AP012336.1
Streptococcus mutans GS-5, complete genome	134	134	94%	2e-30	97%	CP003686.1
Streptococcus mutans NN2025 DNA, complete genome	134	134	94%	2e-30	97%	AP010655.1
Streptococcus mutans UA159, complete genome	129	129	94%	9e-29	96%	AE014133.2
Streptococcus parauberis KCTC 11537, complete genome	35.6	35.6	53%	1.5	80%	CP002471.1
	33.7	33.7	21%	5.4	100%	CP003017.1

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Bacillus megaterium WSH-002, complete genome						

Table S10: Log-likelihood values for the branch-site test of positive selection.

	n	Null model (fix ω=1) 2Δl	Alternative model 2Δl
Ancient Strains	9	0	0
Modern Strains	12	0	0
All Strains	21	0	0

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5. DISCUSSION

Owing to the amount of information that can be obtained in studies involving aDNA, paleogenetics has become a useful tool to provide answers to a high amount of questions: from the genetic characterization of very ancient remains to the analysis of the relations inside and between groups or populations in the past, as well as the reconstruction of species' migrations over time and the evolution of pathologies. The present thesis intends to help in some of these purposes, and to offer new data about ancient populations, mainly from our natural location, the Western Mediterranean.

In order to do so it is unavoidable to try to improve all processes carrried out in our lab to maximize the effort and the results of our projects. In this sense, in view of the lack of eficiency in the recovery of ancient genetic material in one of the studied sites, we have developed a comparison test to check whether a silica-based method could outperform the phenol-clorophorm method usually employed in our laboratory.

As for the genetic studies, we have first focused on the kinds of relations inside close groups and what this meant to the concept of family, using a common burial ground from Bronze Age in northeast Catalunya. In order to do so, we have checked for the possibilities of a feasible genetic familiar relationship, using frequencies fom ancient and current populations from the same region. In the second place, we have addressed the genetic characteristics of Late Bronze/Early Iron Age necropolises from Minorca, and in a subsequent population analysis we have added them to two other populations from the same period in Majorca to test whether Balearic Islands constituted an homogeneous genetic pool and whether they behaved as a genetic isolate with respect to their surrounding regions.

Finally, taking into account that all the populations studied showed oral pathologies, we have tried to know more about the characteristics of the oral health in these populations over time, using a virulence factor of one of the bacteria highly responible for the appearance of caries, *Streptococcus mutans*.

5.1 IMPROVEMENT OF THE METHODOLOGY

Up to the current days, no extraction method to recover ancient genetic material has undoubtedly proven to be the best. The first study of this PhD work came as a result of the need to improve the poor output in the obtained results in La Cova des Pas using our routinary protocol (Malgosa et al. 2005). We tried an alternate method consisting of a new kit based on the silica membranes selective binding to DNA named QIAamp DNA Investigator, and compared the results. Two fragments of different sizes of mitochondrial DNA were amplified in order to evaluate the success rates of both methods, checking whether any of them worked differentially better in global terms and also with respect to the skeletal piece used, tooth or bone. Apart from adding this work to the ones carried out to date for a possible future metanalysis, we wanted to improve the results for this

site and see if we could draw some conclusions that trascended our study.

Our results showed that the silica-based method significantly improved the results with respect to our modified phenol-clorophorm method independently of the hard tissue used, pointing towards an efficient inhibitor removal by this alternative extraction method. Probably the elimination of inhibitors permitted to compensate the decrease in the total amount of DNA recovery known to occur when not using the phenol-clorophorm methodology (Rohland and Hofreiter 2007) and to overcome previous results. Although not knowing the exact nature of the inhibitor(s) present, we could reduce the scope due to some observed results: as the use of both methods did not diminish the overall availability of the genetic material but significantly changed the length of the obtained fragments, it could be stated that the inhibitor(s) did not generically affect DNA, because in that case the damage would equally affect the results whatever the employed method was. In this sense we could discard some inhibitors: first, EDTA and calcium ions, as the modified phenol-clorophorm procedure carried out in our laboratory (Malgosa et al. 2005) had already proven to be suitable for ancient DNA studies, with both substances being correctly removed during the extraction procedure as testified by previous works (Solórzano 2006 and Díaz 2009); also, tannic acid, an agent found in leather as well as in some types of plant material (Wilson 1997), because additional Taq and additional magnesium did not relieve inhibition, contrarily to what happens when this substance is its causing agent (Opel et al. 2010). On the other hand, some facts pointed to the humic acids such as the color of the samples extracted using the phenol-clorophorm method ranging from light to dark yellow and PCR results fitting with their ability to inhibit the reaction through sequence specific binding to DNA, limiting the length of the amplified product. Finally, and coincidentally with the work by Opal et al. in 2010, additional Taq or magnesium did not relieve inhibition. This work demonstrated that in spite of a previous work proving that the QIAgen kit can bring about a degree of inhibition as high as the phenol-clorophorm (Rohland and Hofreiter 2007), this statement cannot be applied as a general rule. Thus, we concluded that testing the best extraction procedure for each site seems the most reasonable way of proceeding, as there is not a universal method which can be carried out to obtain the highest degree of success for all the samples and archeological sites. This is because DNA preservation, as well as the type of inhibitory substances, may vary in samples which have suffered different taphonomical processes such as those ocurred on permafrost, open sites (rather than caves), or warmer climates; thus, other methods may perform better on such samples (Rohland and Hofreiter 2007).

5.2 RELATIONSHIPS INSIDE CLOSE GROUPS

The kind of relations that could be present inside a small ancient group buried in close association has longly been a matter of discussion and it is fair to say that genetic tools can help to analyze the interactions within them. The individuals found inside a cave located in Sant Joan de Montanissell in Lleida constituted a good opportunity to furtherly explore this question, considering

that the age and gender distribution of the individuals present pointed to a nuclear family (López et al. 2005). Taking into account that there was an adult male and woman, a juvenile woman and five children and the grave goods encountered, the finding seemed to be in accordance with the statement made by Heyd in 2007, who proposed that burials closely related both spatially and by their regular equipment with similar pottery vessels most likely implied close community and/or familial links (Weinig 1991, 1993, Rieder 1997, Meixner and Weinig 2002). We tried to solve questions regarding these individuals, checking a possible parenthood between some of the members inside the cave and whether it fitted the hypothesis given for this and similar type of burials found in prehistoric contexts, where the concept of family is often associated with the nuclear family in terms of an occidental model of society, showing very close blood ties. Thus far few studies have been devoted to the presence of genetic kinship in ancient populations (Alt et al. 1995; Hummel and Herrmann 1997; Schultes et al. 2000; Scholz et al. 2001; Keyser-Tracqui et al. 2003; Gilbert et al. 2007a; Bouwman et al. 2008; Haak et al. 2008; Vanek et al. 2009; Lalueza et al. 2011) and even less have proven a direct child-parent relationship, being the work by Haak et al. in 2008 the oldest molecular genetic evidence of a nuclear family in one Neolithic burial from Germany.

Our results show that Montanissell collective burial presents high mtDNA variability inside a small group, with four different haplogroups and at least five different haplotypes along the eight individuals in the cave. In spite of this, the high J frequency (in four of the eight individuals), an unusual haplogroup in Catalunya in ancient times, indicates that some individuals are probably relatives although we do not know the degree of kinship. Therefore, together with the evidence of non-simultaneous interments we can conclude that the cave was most likely used as an “attritional” cemetery of an extended family.

In relation with the composition of the group, none of the adult women was the mother of any of the individuals present in the cave and their haplogroups were not shared by any other individual, so a patrilocal group where adult women migrated to foreign groups to take care of children, quite possibly mating with the adult males, appears as a plausible explanation. This work also signals the importance of knowing the intragroupal relations before doing statistical analyses, as shown when the results obtained applying the correction for the presence of relatives (Vernesi et al. 2004) were quite different than when it was not, with the individuals showing significant differences with almost all the used populations when it was not applied while being much more in their same range when it was. So we suggest a profound analysis at the time of establishing the possible existence of kinship relations when the information available is limited, taking into account the different characteristics of the places where the samples are found.

The high diversity present in the individuals and the short time spent among the successive burials suggest two possible scenarios. In our case, while a small group with a patrilocal mating system with the integration of women to incorporate new haplotypes was the most likely explanation, we conclude that it cannot be discarded that the interment corresponds to the death of part of a more numerous and itinerant (nomadic) group. In conclusion, our study points towards a group of

individuals that shared their existence and were not just connected by kinship, but also by culture, as shown by the common funerary ritual. So while the currently predominant structure is the nuclear family strongly conditioned by genetic ties, in the Bronze Age it could have been a somewhat looser structure, where cultural issues might have played an important role too. This is in accordance with the hypothesis suggested by Hill et al. in 2011, in which our species' biological success would find its origin in cooperation with non-kin that would finally imply a higher degree of cultural transmission. In fact, the study by Haak et al. in 2008 states that, while kinship relationships were very important in that period in the treatment of the dead, there were also cases where the emphasis was put on the supragenetic social connections between the individuals buried together. As argued, this is compatible with some of the individuals inside the group sharing genetic ties, in what can be seen as an extended family structure. If we were dealing with a patrilocal mating system, as seems reasonable, this would fit with the model suggested by Chapais in 2008, upon which the affiliation of several men to the same woman, related to each other as consanguineal and affinal kin, ameliorates hostile between-group relations and allows visiting and opportunistic coresidence in human meta-group social structures (multiple residential bands exchanging spouses, goods and information).

5.3 POPULATIONAL ANALYSIS OF ANCIENT BALEARIC ISLANDS

While the molecular characterization of many western Mediterranean populations since the Neolithic period had been carried out during the two previous decades, no information about past populations of the Balearic Islands was available. To solve this deficiency, in this thesis we characterized the mitochondrial genetic pool of two Late Bronze/Early Iron Age necropolises from Minorca. This period coincided with the beginning of the so-called talaiotic culture in these islands, which in spite of showing a common background in the funerary practices in Minorca and Majorca, had particular characteristics that were different enough to study them individually. The first necropolis studied, Son Olivaret, was used as well to carry out a study of the evolution and importance of haplogroup H, the most frequent in current European populations, to trace its presence over the history of the Balearic Islands and to check how it related to the surrounding regions. Another Minorcan population set, that of Cova des Pas, was analyzed. The geographical characteristics, conservation and ritual of the burials required a thorough study about the internal relationships in this group, connecting with the first study about familial ties and being the third study of the thesis.

Joining these data from Minorca with previously obtained data from protohistoric contemporary populations of Majorca (Díaz 2009), we focused on analyzing the differences and similarities of the genetic content of both islands during the talaiotic period, and on comparing the Balearic Islands' genetic pool with the one from contemporary nearby populations to understand aspects of their origin and evolution over time. At a temporary level, the main goal was to compare their ancient genetic pools, to check if a genetic continuity up to the current days could be seen and to know if they constituted an homogeneous genetic pool at that point in time. Finally, we compared the

ancient Balearic community with other ancient populations to characterize its possible population influences. The addition of a fifth Majorcan necropolis from the Late Roman period (Díaz 2009) in the global population analysis permitted us to englobe approximately 1.000 years of history, from the VIth century BC to theVIIth century AD.

5.3.1 Analysis of the necropolis of Son Olivaret

The prehistoric site of Son Olivaret is a collective sepulchre that extended from the pretalaiotic (second half of the IIIrd millennium BC) to the talaiotic (1.500-500 AD) period. The human remains showed a high state of degradation (Carrascal et al. 2008) that prevented their characterization and the comparisons with other contemporary populations from Minorca as La Cova des Pas (Armentano et al. 2010, 2012) and Majorca as Son Real (Font 1974) or Illot des Porros (Malgosa 1992). In order to overcome this problem we used genetic tools to study the individuals from this necropolis, analysing their mitochondrial DNA and focusing on the haplogroup H, the most common haplogroup in Europe and the Mediterranian basin (Torroni et al. 2006; Roostalu et al. 2007), while a more general population study would be later carried out. Although it was not possible to follow two of the standard authenticity criteria (the duplication of the samples and the replication in another laboratory) because the state of the material forced us to use the same dental piece for all the individuals, all the other criteria were applied (Montiel et al. 2007). Regarding all the precautions taken and in view of the results, we believe we can guarantee the veracity of the data.

Our results show that haplogroup H, which has been found in low frequency in ancient European remains since the Neolithic and has been incrementing since then (Brotherton et al. 2013), was present in Son Olivaret in a 40.9%, almost coinciding with the frequency found in the current European population (40%, Roostalu et al. 2007). To analyze the importance of this haplogroup in this necropolis and in the ancient Minorcan population, we compared this value with the ones found in other series related to it both spatially and temporally. The first comparison was made with another talaiotic Minorcan series, constituted by the individuals of La Cova des Pas (Armentano et al. 2012, Simón et al. 2012), where the frequency reached a 60%. This difference could be due to the presence of a higher endogamy in la Cova des Pas, but in any case we showed that haplogroup H had a high frequency in talaiotic Minorca. As the individuals studied at Son Olivaret might be separated by centuries of difference and the individuals of La Cova des Pas were almost contemporary, the hypothesis that the latter were affected by endogamic relations becomes even more plausible. The frequency in ancient Minorca was similar to the ancient series of S'Illot de Porros (Majorca), but significantly higher than that of Son Real (23.7%, Díaz 2009). This last necropolis has very unique characteristics at both constructive (Font 1974) and genetic (Díaz 2009) levels, with haplogroups that are uncommon in Western Mediterranium and unique haplotypes which have not been found in

current populations. In the comparison with the European populations, Son Olivaret shows a very similar H percentage to the current European mean and that does not differ from the whole Balearic, Valencian, Catalan and Greek ancient populations, but much higher than the ancient series representing the Middle East (Syria). Just the European groups considered among the most ancient ones as Basques and Sardinians had a sensibly higher H frequency, which is not strange as haplogrup H is as much as 30.000 years old and owing to its frequency may have spread into Europe more than once (Metspalu et al. 1999; Richards et al. 2000).

Diachronical analysis shows that the ancient Minorcan population has a higher H frequency than the current one (24%, Picornell et al. 2005). This could be an artefact due to the low sample size of the modern sample (46 individuals, Picornell et al. 2005), but we must take into account the importance of the historical events which have taken place in the island during the last centuries, as the formation of an important English colony which is reflected in the high frequency of haplogroup J. We concluded that the frequencies of the ancient Minorcan populations reflected a pool with a high percentage of haplogroup H, as in the majority of European and Peninsular populations both current and ancient, but that it did not show a clear continuity with current Minorca possibly due to the most recent events in Minorca's history.

5.3.2 Analysis of the necropolis of La Cova des Pas

La Cova des Pas is a natural cave considered one of the most important Minorcan sites among the ancient funerary necropolises originary from Late Bronze owing to the unusually well conserved organic remains (Fullola et al. 2008). The 66 individuals found there belonged to the same population as the common funerary pattern evidenced. They were deposited there between 1.100 cal. aC and 800 cal. aC (Armentano et al. 2010, 2012). We pretended to genetically characterize this inhumated population to know its origin and the intra and interpopulational relations, as well as determining their sex to see if some kind of bias existed in this special burial. The analysis of mitochondrial DNA shows that at the population level, as in the case of Son Olivaret, La Cova des Pas haplogroup composition did not differ from any of the other ancient Balearic necropolises with the exception of Son Real, a very particular necropolis (Díaz 2009). The most frequent haplogroups in the site are the most common ones in almost all of Europe since the Calcolithic (Ricaut et al. 2012), but the presence of only 4 haplogroups (H, U, K and W) reflects a low genetic diversity (0.594). This fact points to a high level of endogamy, as does the high number of basal haplotypes found (rCRS in haplogroups H and U, and 224T, 311C in haplogroup K) and the much lower haplotipic diversity in comparison with the other ancient Balearic populations (0.5); conversely it is in accordance with the common funerary patterns observed. The differences seen between La Cova des Pas and its contemporaruy Minorcan sample of Son Olivaret are possibly related to the low diversity in the former, which seem to be a reflection of a different way of life: Son Olivaret in the plain central zone of the island and La Cova des Pas in a less accessible zone, possibly representing a more isolated population that carried out

different funerary rituals. Moreover, our data corroborated the presence of individuals from both genders, discarding a sex bias as had already been suggested by Armentano et al. in 2010. With respect to our previous work, we could add two more guarantees of authenticity: we could replicate partially or totally ten of the obtained individuals, with the results being coincident in each case, and the correspondence between the morphological and genetic sex in the adult individuals supported the veracity of the results in subadult individuals.

5.3.3 Population analysis of the Balearic Islands: from the Talaiotic to the current era

Using the data obtained by the mitochondrial DNA from the previous two populations and adding them to three previously studied ancient Majorcan populations (Díaz 2009), we wanted to know the evolution of the Balearic population from a genetic point of view during the boundary between the Pretalaiotic and the Talaiotic. Also it is interesting to verify whether there has been an eventual continuity from then to the current days or if some kind of replacement has happened. In this sense, a Late Medieval Majorcan necropolis named Can Reiners (Díaz 2009) permitted our study to encompass approximately 1.000 years of history, from the VIth century BC to theVIIth century AD. In a subsequent analysis, our purpose was placing the ancient Balearic populations in the mitochondrial genetic context of both ancient and current populations from the Mediterranean basin, while also seeing if the hypothesized relationship between the members of the Talaiotic and Nuragic cultures had genetic support. The published Sardinian sample is scarce and comes from diferent series. For this reason, we tried to extend the sample with the analysis of bones coming from Santa Teresa de Gallura.

Our results showed that concerning the talaiotic necropolises from Majorca, Son Real and Illot des Porros, they presented significant differences in their haplogroup composition. Both necropolises probably represented two successive moments of its burial rituals (Malgosa 1992); therefore our results fit with the explanation of a differential use for both of them. This was previously suggested by differences found at a morphological level (Malgosa 1992), craniometric analysis (Jordana 2007) and dental shape (García Sivoli 2008). As these two necropolises were totally different at both structural and group level, we concluded that these results were likely the genetic reflection of the apparent cultural differences between both of them (Díaz 2009). With respect to the Late Roman Age population of Can Reiners, our results indicated that it was totally integrated in the European genetic pool and the presence of an individual holding the haplogroup L demonstrated that some people from the African continent had already been present in the island during that period. As regards the Minorcan necropolises, Son Olivaret had an overall moderate diversity value most likely as a result of its low sample size as argued in 5.2.1, because its number of lineages with respect to the individuals holding haplogroup U pointed to a diversified population. Moreover, showing U3, J and T2b amongst its haplotypes, proposed as markers for the spread of farmers from Near East during the Neolithic (Richards et al. 2000, Fernández 2005), the indication of a high influence from Neolithic population dispersal was palpable. Concerning La Cova des Pas, its particularities in the haplogroup and

haplotype dotation that strongly pointed to a close community have just been discussed in 5.2.2. Each of these populations has shown its own particular characteristics which permit to identify them, a fact which also shows that despite their common origin and the ineludible Balearic connections, they maintain their own uniqueness.

Concerning the variability in ancient and modern populations, we observed that Son Real's high level of Nei's gene diversity caused that the necropolises of Majorca and its current population did not show significant differences in their genetic diversity, contrarily to what happened in Minorca owing to the low value in La Cova de Pas. The contrast between Son Real and La Cova des Pas values provoked that the ancient Majorcan population showed a significantly higher variability than the ancient Minorcan one.

Focusing on the genetic differences, Son Olivaret having 50% of the represented haplotypes not found in any of the diachronic Balearic populations and Son Real holding 5 exclusive haplotypes (Díaz 2009) show the loss of some lineages. Moreover, the high contrast in the values of variability from both islands and the lack of shared haplotypes between them made the ancient Minorcan and Majorcan populations show significant differences at the haplotipic level. The genetic particularities identified in the Balearic necropolises could complement previous analyses, with signals from the Neolithic wave in Son Olivaret, a high number of unique haplotypes in Son Real pointing to a very particular situation, or the existence of a high degree of endogamy in La Cova des Pas (Armentano et al. 2010) being the main examples. This fact argued in favor of the existence of intrapopulational variability which is also seen, although not so clearly, in their current counterparts: Minorca's genetic pool shows haplogroup W, lacking in Majorca, and a sensibly higher frequency of haplogroup J, the second most frequent in England (Pellecchia et al. 2007), as a result of Minorca's English occupation during the XVIIIth century (Terrón JL 1998). On the other hand, Ibiza shows haplogroup L2 possibly as a footprint of the Carthaginian occupation in 654 BC (Picornell et al. 2005).

Therefore we proved the existence of a heterogeneous European genetic pool in the Balearic Islands from the Iron Age to the current days at both the haplogroup and the haplotype level and demonstrated how different population influences, at a cultural and migratory level, can modify it over history. However, with our results it seems possible to talk about a certain degree of diachronic continuity at the haplogroup level because while facts such as small sample size (ISOV, MCR), endogamy (ICP) or rare haplotypes (ISR) might be affecting particular results, almost all of the haplogroups represented are of European origin and the influence of each necropolis seems to decrease when the ancient sample is considered as a whole, reducing the incidence of each single case. The haplotipic analysis shows that the ancient Balearic population already belongs to the European genetic variability, without discounting the influence of a Near Eastern component, and that it shows a very similar genetic pool to the ancient Catalan population, thus reinforcing their already documented historic interactions (Pericot 1975). In summary, we confirmed genetically the argument stated by Casasnovas in 1988 upon which the Balearic Islands have received different cultural and demographic

influences throughout their history that have not been homogeneous neither in time nor in the source of origin. Finally, we gave genetic support to the documented historic interactions between the Balearic Islands and Catalunya (Pericot 1975) as their ancient populations showed a very homogeneous genetic pool, while the ancient Sardinian population showed differences with both of them. This dissimilarity rules out a direct relationship between the members of the Nuragic and the Talaiotic cultures at least concerning the feminine lineages, although relationships between these neighboring islands are undeniable and their influence should be felt in some way.

5.4 ORAL PALEOPATHOLOGY

During all the studies which have been described, we faced the problem of dental diseases, having rejected many teeth for this reason. We realized that all the studied populations suffered from caries (Carrasco and Malgosa 1990, Ortega et al. 2002 among others), the most persistent infection in the history of humanity. However, from a genetic point of view there were no studies concerning the evolution from the bacteria from the dental plaque that cause caries, despite having accompanied humans since remote times. Thus, in the last work of this thesis we focused on the characterization of the microorganisms involved in this process in order to gain a better knowledge of their interaction with humans. Following this reasoning, the study focuses on DNA characterization of one of the main agents responsible for the carious lesions from ancient remains, *Streptococcus mutans*. Even though caries is a polymicrobial disease (Belda-Ferre et al. 2012), *S.mutans* has been consistently associated with its presence (Forssten et al. 2010). The proof that the study of this bacterium is gaining interest is that it has recently been detected by other groups using the gold-labeled antibody TEM (Preus et al. 2011), and in 2012 a DNA segment from *S. mutans* DNA was amplified from ancient dental calculus of an individual dating from approximately 500 YBP (De la Fuente et al. 2013). On our side, continuing the study started in 2007 by our group showing that it was possible to recover *S. mutans* DNA from human ancient remains (Smerling et al. 2007) and continued by Solórzano et al. in 2011, we incremented the sample size and intended to carry out an evolutionary study of this bacterium in relation to the development of caries in human beings. For this study, we took advantage from the resistance of teeth, which provided a suitable tissue to work with as *S. mutans* has one of its niches there, and from the macroscopic proofs, because the activity of the cariogenic agents are evident in dental pieces. As a marker, we chose the gene codifying for one of its virulence factors, named dextranase, as its evolution over time would give information about the bacterium's adaptation to its human host. We pretended to decipher not only its genomic but also its amino acid sequence, making statistical tests to check whether this factor had evolved according to neutrality and/or had deviated from it at some particular points in history. These tests were done at two stages, first checking the ratio of non-synonymous to synonymous rates (ω) for the whole obtained fragment, and second on a codon by codon basis, as in cases of very local action for natural selection the overall ω

value would not significantly differ from neutrality and the non neutral evolution at a very specific region might go unnoticed.

We extracted material from caries of individuals from the Bronze Age up to the XXth century in Europe and America and characterized a fragment of the dextranase gene, comparing it with current strains in order to determine whether there had been a change in the genetic diversity and if there was any other relevant difference in relation to geography or chronology.

Contrarily to what happened with human samples, no DNA from *S. mutans* had been previously amplified in our laboratory. In addition, two samples, one from each of the two different continents represented in the study, were amplified with primers specific for human mtDNA with results making phylogenetic sense, and two of the samples were successfully replicated in an independent laboratory showing that the products obtained were not affected by intra-laboratory contamination. Thus, the authenticity of these results could be verified (Montiel et al. 2007).

Our results show that it is possible to isolate DNA from this bacterium in archaeological remains from periods as ancient as the Bronze Age (sample from Montanissell, site described in 5.1.1). We demonstrated that while in present-day populations there were six polymorphic positions in the amplified segment in ancient samples just two of them were present which, with the caution needed because of the small sample size, seemed to point towards an increase in both nucleotide and haplotype diversities of this dextranase gene region over time. Nevertheless, we showed that this increase was not translated into an increase of the ω value, because the new observed substitutions pointed to a slight decrease of ω over time and suggested a constraint of the selective pressure in this segment over *S. mutans* recent history, highlighting the importance of also knowing the amino acid sequence. This fact higlighted the main advantage of our study, which is that it allowed comparing the ω of this population at different points in time separated by hundreds and thousands of years not with inferred phylogenetic reconstructions, but with directly observed data. It permitted us to rule out relaxed or positive selection as the driving forces in the full segment over time because of the decrease detected in the ω value, and the codon-by-codon analysis allowed us to refine the analysis to the extent of suggesting a constraint of selective pressure over time as the most likely hypothesis. The fact that no positive selection was observed when considering the segment as a whole is in accordance with dextranase not being included among the genes that have been shown to be under Darwinian positive selection in previous studies (Maruyama et al. 2009; Do et al. 2010). In spite of that, we were able to detect one specific codon that has been subjected to positive selection throughout the evolutionary history of the segment and continues to do so, although to a lesser extent, but the relevance of this finding will just be known in future analyses.

We also proved with a phylogenetic network that the majority of modern and ancient samples belonged to the two central nodes reflecting that the full segment is still evolving neutrally, while the increase in genetic variability over time was reflected by the extremes of the phylogenetic network being represented by modern-day sequences. Based on the decrease in Tajima's D values over time, we suggested the existence of an excess of low frequency polymorphisms in modern sequences and a

possible continuous population expansion. To support this statement we based on the previously mentioned raise in the genetic diversity of the segment and a recent study showing that *S. mutans* population started expanding exponentially around 10.000 years ago, approximately coinciding with the onset of human agriculture (Cornejo et al. 2012).

Another observation that we made which agreed with data obtained in previous studies focusing in *S. mutans* comparative genomics of current strains (Maruyama et al. 2009, Do et al. 2010, Cornejo et al. 2012) was that no characteristic differences in relation to geographical distribution were seen either in the modern or the ancient population. We concluded that the molecular genetic evidences obtained from fossil bacteria associated with archaeological caries lesions reported in this work will set the stage to understand more issues about the way of life of ancient people and bring about the possibility of determining whether caries evolution can be somehow correlated with changes in the lifestyle of their hosts, so dextranase and more virulence factors' evolution will have to continue being studied in order to be able to respond to them.

5.5. GENERAL COMMENTS

The works carried out in this thesis are the product of an effort carried out on ancient samples to comprehend the origin and diversity of the talaiotic populations in Minorca in particular and at the Balearic Islands in general. This purpose has been achieved by analyzing different genetic aspects: on a population by population basis first, studying a close group from the same period in Catalunya to see what it could tell us about the relationships inside this kind of social organization, similar to the one which would be later analyzed in La Cova des Pas. Secondly, a comparison between populations from Minorca and Majorca belonging to that period has been done, their relationships with the surrounding Mediterranean regions have been studied, and finally a widely disseminated infection which was found in all the populations used has been analyzed. Obviously, all these analyses have been possible with constant methodological updates that have also been published. However the study of a specific population, such as the one from the Balearic Islands, needs to be supplemented with the analysis of some cultural and health features. Cultural rules are reflected in the work about family relationships performed on reduced groups such as in the Montanissell case. The results show that the nuclear family concept from a current Western point of view which has frequently been assumed for people of the antiquity is not always applicable. The issue of health has also been studied using a very specific but widespread disease, caries, also in the Balearics.

All the works are different, but all of them coincide on the attempt to interpret new information coming from old skeletal remains for a more comprehensive understanding of the Past.

6. CONCLUSIONS

1. In relation to the methodological problem motivated by the low extraction rate of success in the DNA retrieval

1.1. We can conclude that a silica-based method significantly improved the obtained results with respect to our modified phenol-clorophorm method in the Early Iron Age site of la Cova des Pas affected by the presence of inhibitory substances, showing an efficient inhibitor removal which could have compensated for the decrease in the total amount of DNA recovered.

1.2. There is not a method which invariably provides the highest degree of success for all the kinds of samples and archaeological sites, because DNA preservation, the type of molecular damage and the possible inhibitory substances may require different strategies to recover it with the highest possible quality.

2. In the second question focused on ancient familial ties, our study revealed that what seemed a nuclear family in a Late Bronze Age cave from Catalunya was really a group of individuals from the same clan or extended family but were not just connected by kinship. While the predominant structure nowadays is the nuclear family strongly conditioned by genetic ties, our study shows that the concept of nuclear family cannot be extrapolated to all the different periods and cultures.

3. About the Balearic populations,

3.1. The analysis of the ancient Minorcan populations reflected a genetic pool with a high percentage of haplogroup H as in most European and Peninsular populations both current and ancient, but a temporal discontinuity can be observed with current Minorca possibly due to the most recent events in this island's history.

3.2. The low genetic diversity and the high number of basal haplotypes in La Cova des Pas site points to a high level of endogamy, in accordance with the common funerary patterns observed.

3.3. At the population level, La Cova des Pas and Son Olivaret haplogroup composition did not differ from any other ancient Balearic necropolis with the exception of Son Real which has very particular characteristics that allow for its differentiation. The most frequent haplogroups in the ancient Minorcan populations are the most common ones in almost all of Europe since the Calcolithic. Their differences are possibly related to the low diversity in the first one, which seems to be a reflection of a different way of life regarding their different geographic location and accessibility, with possibly La Cova des Pas representing a more isolated population.

3.4. A genetic continuity in the Balearic Islands from the Iron Age to the current days exists, but particular situations and different population influences must be considered in both past and present populations when making a detailed analysis.

3.5. The documented historic interactions between the Balearic Islands and Catalunya have been confirmed with regard to their feminine lineages by the homogeneity in their ancient mitochondrial genetic pool, while the opposite happens with ancient Sardinia, discarding a hypothetic relationship between the members of the Nuragic and the Talaiotic cultures as new archaeological hypotheses suggest.

4. In relation to the analysis of caries regarding changes on the impact on ancient people health, the diachronic study of a segment of *Streptococcus mutans* dextranase showed that it probably evolved under neutral evolution since at least the Late Bronze Age, while its increase in genetic variability indicated a possible continuous population expansion. Evolutionary changes which could motivate the differences in caries frequency or its severity have not been found in the studied segment.

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