

15. Amplification of ancient DNA and determination of sex in medieval human skeletal material from Ballyhanna, Co. Donegal

Sheila Tierney

The excavation of a 'lost' medieval cemetery on the route of the N15 Bundoran–Ballyshannon Bypass has created the opportunity for an innovative research project based at the Institute of Technology, Sligo. The aim of this research study is to extract and amplify ancient DNA (aDNA) from archaeological bones excavated from the medieval cemetery discovered at Ballyhanna, Co. Donegal, in 2003 (Ó Donnchadha 2007; MacDonagh, this volume). Our hope is that by using a biomolecular approach (using molecules that naturally occur in our bodies, such as DNA) we will be able to establish the sex of individuals in skeletal assemblages, with specific interest in sub-adult, or juvenile, remains that may not be reliably sexed by conventional osteological methods.

Determining the sex of human remains relies on recording the differences between male and female skeletons, the pelvis and the skull being the main sex indicators. Sexing juvenile and fragmentary skeletal human remains using existing osteological methods, however, can provide ambiguous and unreliable results (Mays & Cox 2000). At the age of puberty males and females begin to develop differences in the pelvis, and it is these differences that can later be used by osteologists to identify the sex of an adult individual. Before this age, however, juveniles exhibit little sexual dimorphism, which means that the skeleton of a young male will not differ substantially from that of a young female. Fragmentary adult remains, on the other hand, may lack the crucial sex indicators that are needed to determine their sex (*ibid.*). Developments in molecular biology in recent years have provided methods for extracting and analysing aDNA from archaeological remains and thus have presented archaeologists with the opportunity to establish the sex of such assemblages using aDNA analysis (Hagelberg et al. 1991).

In living organisms almost all the cells of the body contain the genetic material DNA (Hermann & Hummel 1994c). While we are alive our DNA is generally protected or is unaltered by damaging forces (e.g. oxidation, hydrolysis or chemical damage). If any of these degradation processes occur, they can be corrected by enzymes and other mechanisms within the cell itself (Kaestle 2000; Lindahl 1993). Upon death, however, these protective and repair mechanisms break down resulting in the degradation of DNA molecules into smaller fragments with numerous sites of molecular damage (Kaestle 2000).

Although this ancient genetic material is severely degraded, the analysis of degraded DNA has been made possible by amplification of DNA by the polymerase chain reaction, or PCR (Hermann & Hummel 1994c). PCR is a technique that copies a specific fragment of DNA numerous times, resulting in millions of copies of the specific fragment of interest. PCR mimics the natural DNA replication process in the body. The PCR amplification process works by subjecting the extracted DNA to a number of cycles (30–50) of heating and cooling. Heating the sample causes the double-stranded DNA fragment to separate into two single strands, which are then used as templates to create new DNA fragments. By altering the temperature and using specific enzymes and DNA building-block chemicals, each of these single strands is then rebuilt, giving two new identical copies of the target

fragment. This process is then repeated in each cycle, multiplying the DNA fragment until there are millions of copies of the target DNA.

The term ancient DNA describes DNA fragments that are found in preserved biological specimens—such as animal and human remains, and also plant and microbe materials (Brown 2000). Hard tissues such as bones and teeth are generally the longest-lasting physical indication of human or animal existence at an archaeological site (Thomas et al. 2005), as they survive better than other biological tissues. This is because hard tissues lack water and enzymes and offer mechanical protection (Hermann & Hummel 1994b). The DNA within these ancient bones can still undergo decay processes over time, however, causing the DNA to gradually break down (*ibid.*).

Ancient DNA can be preserved in prehistoric specimens under a wide range of environments (O'Rourke et al. 2000). As stated earlier, however, the physical and chemical degradation that occurs over time is responsible for destroying a large proportion of the DNA molecules contained within the ancient remains. The quality and degree of DNA that survives in an ancient specimen is very much reliant on the conditions of the archaeological site, more so than on the age of the sample (Kaestle & Horsburgh 2002). It is thought that cold and dry depositional conditions are best for ancient DNA survival, as well as environmental stability (MacHugh et al. 2000).

The degraded nature of the DNA within the archaeological material is a serious disadvantage to aDNA studies, although sample contamination seems to have worse implications (Yang & Speller 2006). The probability of an archaeological specimen being contaminated with modern DNA from other sources is high and is sometimes an inevitable reality. Contaminant DNA can be introduced in many ways: by archaeologists during excavation, during preparation for extraction (from DNA in the surrounding area, e.g. from the researcher's own shed skin cells), or from contaminated reagents used during the extraction or PCR amplification (Hermann & Hummel 1994c). Another major source of contaminant DNA is from molecules that have been amplified in previous PCR reactions, known as PCR carryover (Wayne et al. 1999). The sources of contamination are therefore abundant and unpredictable, hence numerous precautionary measures are enforced along with decontamination procedures that aim to minimise or eliminate contamination (Illus. 1).

Methods

Our first aim is to determine whether there are aDNA sequences within the Ballyhanna adult assemblages that can be successfully amplified through PCR, specifically amplifying the amelogenin gene, which is used in sex determination (the amelogenin gene is the sex marker in humans). As there are currently no existing osteological methods that can accurately sex juvenile human remains, morphological sex determination can only be conducted with an acceptable degree of accuracy on adult skeletons (Mays & Cox 2000). Developing a PCR assay based on the amelogenin gene will therefore be a great advantage in determining the sex of the juvenile assemblages. Depending on the successful amplification of this aDNA, the method can then be validated. Validating this method involves correlation of the PCR results with the osteological data. The sex of selected sample adult remains will have been previously determined using conventional osteological methods, and aDNA will have been extracted from the adult pelvis. Once the method is



Illus. 1—Biomolecular scientist Sheila Tierney chemically extracting DNA at the Institute of Technology, Sligo (Sheila Tierney).

validated against adult remains, the method can then be applied to determine the sex of a number of the juvenile skeletons.

Extraction of aDNA from human bones

Removing soil, dirt and contaminant DNA which has adhered to the bone is a crucial step that must be conducted before the DNA is extracted. It is most likely that the majority of the contaminant modern DNA lies on the surface of the bone, although the possibility of contaminants penetrating deep into the bone does exist. The top layer of bone (1–2 mm) is therefore removed with a sterile scalpel and discarded (Bouwman & Brown 2002). The inner layer of bone material is then removed with a dremel drill or sterile scalpel (Illus. 2) and the bone powder collected. The ultimate aim when extracting aDNA is to obtain material that is free from or has minimal modern DNA contamination; bench, equipment surfaces and drilling equipment should therefore be thoroughly decontaminated with sodium hypochlorite and UV-irradiated to aid in destroying any modern DNA contaminants (Yang & Watt 2004).

Once the bone powder has been physically extracted, the ancient nucleic acids are then chemically extracted and purified from the other cellular components (e.g. proteins and other organic components of bone) that could inhibit DNA amplification (MacHugh et al. 2000). A range of extraction methods exists for the purification of aDNA from archaeological bones. Based on a comparative study conducted by Bouwman & Brown



Illus. 2—Removal of inner layer of bone material using sterile scalpel for subsequent DNA extraction (Deirdre McCarthy).

(2002), we opted to use a silica-based kit—the Qiagen Rapid QIAquick Kit, which purifies DNA based on the binding properties of a silica membrane.

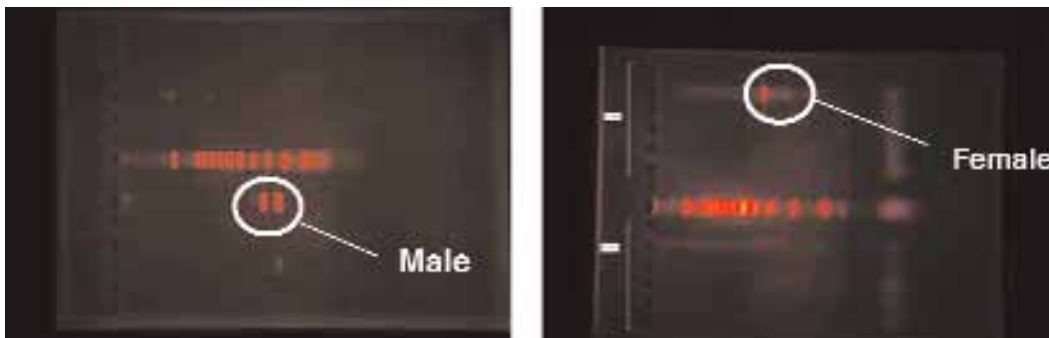
Amplifying aDNA

Successful amplification of aDNA is complicated because it is usually degraded and found in minute amounts. Furthermore, since the PCR technique can amplify as little as a single molecule of DNA, strict precautions should be put in place to prevent contamination from trace amounts of modern DNA that could ultimately serve as templates for PCR amplification (Sambrook et al. 1989). Thus it is advisable that protocols for aDNA amplification be optimised accordingly.

Identifying extracted aDNA fragments

Agarose gel electrophoresis is the standard method used to separate and identify DNA fragments. By applying an electric field across an agarose gel, negatively charged DNA is forced to migrate or sieve through the gel towards the positive anode. DNA fragments that are the same size migrate the same distance and collect together to form a band. A special dye is incorporated into the gel which binds to the DNA fragments. When looked at under UV light the DNA fragments (or bands) fluoresce and thus can be seen and identified.

To determine the sex of humans using these biological techniques of PCR and agarose gel electrophoresis, DNA is amplified from the amelogenin gene, which is found on both X and Y chromosomes. Once the DNA fragment of interest is amplified, it is then separated on an agarose gel. The sizes of the separated fragments are estimated by comparison with fragments of known size. Male DNA shows two bands on an agarose gel (Illus. 3)—one fragment from the X chromosome, one from the Y chromosome. Female DNA shows one band on a gel—two fragments of the same size from the X chromosome (XX) (Mays & Cox 2000).



Illus. 3—Agarose gels showing amplified male and female DNA extracted from modern sources (Sheila Tierney).

Results

Before any DNA samples could be extracted from our ancient skeletal material, all methods had to be optimised and validated using modern DNA. Once satisfied that our methods were optimised, we began DNA extractions from ancient skeletal material. Extractions were conducted on disarticulated (fragmentary) adult material—specifically disarticulated pelvis and femurs. None of the first extracted samples successfully yielded any amplifiable aDNA when amplified using PCR. After each unsuccessful attempt, all of our techniques and methods were re-evaluated and some troubleshooting was conducted to try and determine why our samples yielded no DNA. It was thought that either the DNA fragments present in the samples were too degraded or that the DNA was not being amplified owing to the presence of PCR inhibitors that had co-extracted with the DNA. To determine whether this was the case, our extraction samples were tested for PCR inhibitors by spiking the aDNA samples with modern DNA. All spiked samples amplified, proving that there were no PCR inhibitors present. As a result it was determined that the problem must be associated with the size of the DNA fragment we were trying to amplify (235-bp and 329-bp). Accordingly the target DNA fragment was decreased to 112-bp and 106-bp—a fragment size that should amplify more easily if the extraction samples contained highly degraded DNA fragments. We are currently optimising this PCR amplification method.

Although we have not been able to sex any skeletal material to date using aDNA analysis, we have, however, been successful at amplifying mitochondrial DNA from a number of disarticulated bones. Mitochondrial DNA is another type of DNA found in small structures outside the nucleus called the mitochondria (the function of the mitochondria is to convert energy from food into a form that can be used by the body) (Brown 2000). Although the majority of our DNA (called nuclear DNA) is packaged into chromosomes within each cell's nucleus, a small amount of an individual's DNA can be found within the mitochondria. It is inherited only from the mother and is useful for tracing ancestry through the female line. There are up to 1,000 separate copies of mitochondrial DNA in every cell of the body compared to restricted copy numbers of nuclear DNA in each cell (*ibid*). The chances of finding mitochondrial aDNA are therefore much higher.

It is unclear, as of yet, whether the results we obtained reflect aDNA sequences or are the result of modern contaminants that have penetrated the bones and have subsequently

been extracted and amplified. All negative controls included in the analysis yielded no amplifiable DNA, which proves that contamination more than likely did not occur during extraction or PCR amplification. It is only by conducting the necessary authenticity criteria, such as independent replication, repeat extractions and quantitation, that we can authenticate our results and therefore confirm if there are aDNA sequences preserved within the archaeological remains from Ballyhanna.

Conclusion

There are still many challenges that must be overcome when conducting aDNA studies and much scepticism still surrounds this aspect of archaeological science. No one can dispute, however, that aDNA research provides a unique opportunity to both molecular scientists and archaeologists to recover genetic information about past populations unavailable through any other approach. Regardless of the serious obstacles that are associated with aDNA analysis it still has the potential to become a revolutionary research tool in molecular evolution and archaeology (Hagelberg 2005).

Acknowledgements

The author would like to thank the National Roads Authority for funding this project through Donegal County Council, Dr Jeremy Bird for his guidance and help, Michael MacDonagh, NRA Senior Archaeologist, and all of the members of the Ballyhanna Research Project. Thanks also to the excavation director and Irish Archaeological Consultancy Ltd.