# The optimisation of protocols for the extraction of nuclear DNA from animal bone for PCR.

**By**

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Submitted in partial fulfilment of the requirements for the degree of Masters by Research awarded by the Department of Forensic and Investigative Science, University of Central Lancashire.

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**DECLARATION**

I declare that while registered as a candidate for the degree for which submission is made. I have not been registered as a candidate for another award of the CNAA or of the University. No material contained in this thesis has been used for any other submission for an academic award.

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**ABSTRACT**

The general aims of this research were to find an effective method for extracting DNA from bone and to use this methodology to create Randomly Amplified Polymorphic DNA- Polymerase Chain Reaction (RAPD-PCR) profiles of different species. A modified Chelex method was selected as the preferred extraction procedure and RAPD-PCR profiles were created for several species using a number of different primers. Individuality between closely related species was also achieved. This research could go on to help in the archaeological field with identifying unknown bone fragments.

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## TABLE OF ABBREVIATIONS

| | | |
|---|---|---|
| (bp) | - | Base pairs |
| A | - | Adenine |
| aDNA | - | Ancient DNA |
| C | - | Cytosine |
| c. | - | Circa |
| DNA | - | Deoxyribonucleic acid |
| dNTP's | - | deoxynucleotide triphosphate precursors |
| EDTA | - | Diaminetetra-acetic acid |
| G | - | Guanine |
| Gly | - | Glycine |
| h | - | hours |
| HPLC | - | High performance liquid chromatography |
| kb | - | kilobase pairs |
| LCN | - | Low copy number |
| mtDNA | - | Mitochondrial DNA |
| MW | - | Molecular weight |
| PCR | - | Polmerase chain reaction |
| Pro | - | Proline |
| PTH | - | Parathyroid hormone |
| RAPD | - | Random amplified polymorphic DNA |
| RFLP | - | Restriction fragment length polymorphism |
| RNA | - | Ribonucleic acid |
| rpm | - | Revolutions per minute |
| SDS | - | Soduim dodecyl sulphate |
| SLS | - | Soduim lauryl sulphate |
| T | - | Thymine |
| UV | - | Ultra violet |
| w/v | - | weight/volume |

# 1. INTRODUCTION

Bone is one of the most durable components of the body and is often all that is left to study of ancient remains. As with other tissues, bone contains significant quantities of DNA and, given its durability and the sensitivity of current techniques for genetic analysis, it is now commonly used in the genetic investigation of human remains in the fields of archaeology and forensic science. Although this thesis is concerned primarily with the extraction and analysis of DNA from animal bones, it is worthwhile beginning this introduction by describing the properties and structure of mammalian bones, since this information underpins an understanding of how bone can be preserved, and of the experimental strategies employed in extracting DNA from these sources.

All vertebrates have similar skeletal structures and body plans. Mammals and birds and, to a lesser degree, reptiles and amphibians, have similar overall skeletal plans, which stem from a common evolutionary ancestry (O'Connor, 2000). The differences in their skeletons and bones provide clear examples of adaptations to particular life styles and selective pressures. Rather than attempt to describe a wide range of skeletal forms of little direct relevance to this thesis, I will discuss general aspects of mammalian bones with reference to the more familiar human skeletal system.

The human adult skeleton contains 206 named bones, most of which are paired on the left and right sides of the body. Unpaired bones include the 22 bones present in the skull, of which 8 form the cranium and 14 bones the face. The bones of our skeleton are grouped into two principal divisions; 80 bones comprise the axial skeleton and 126 bones the appendicular skeleton. The axial skeleton forms the longitudinal part of the skeleton, which contains the skull, vertebral column and the bony thorax. The 126 bones that make up the appendicular skeleton include those of the limbs and the pectoral and pelvic girdles, which attach the limbs to the axial skeleton (White, 2000).

The skeleton contributes to the general body shape and form as well as performing several other important functions. Its framework provides support for the body and protection for the brain by means of the skull; the vertebrae surround the spinal column and the rib cage, which protects the organs of the thoracic cavity. Skeletal muscles are attached to the bones by tendons, which use the bones as levers

to facilitate mechanical movement. Bones also serve as a reservoir for minerals such as calcium and phosphate. The bulk of blood cell formation, or haematopoiesis, takes place in the red bone marrow of certain bones, such as the ribs and long limb bones (Schultz, 1997).

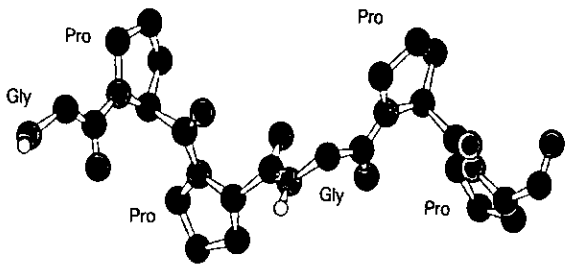
The skeleton is thus a multifunctional system, and the above functions are reflected in the properties of the bones from which it is constructed (Steele and Bramblett, 1997).

## 1.1 BONE COMPOSITION AND STRUCTURE

Living bone is made up of three main components: a complex protein matrix that forms a structural scaffold, a mineral phase that stiffens and supports the scaffolding and a ground substance made up of other organic compounds (Schultz, 1997).

Half of the dry weight of normal fresh bone is made up of the mineral phase and the remainder of the weight is made up of the organic fraction (ground substance and protein matrix) and water. These proportions can vary slightly in different hard tissues. Up to 95 % of the organic fraction of bone is made up of the protein collagen, which is also found in tendons and connective tissue. Collagen is unusual in that it contains high proportions of the amino acid glycine (Gly) and hydroxyproline, which is a hydroxylated derivative of proline (Pro) due to a repeating tripeptide sequence, Gly-X-Pro or Gly-X-hydroxyproline, where X represents any amino acid. These elements are crucial to its three-dimensional structure and characteristic properties. The high proportions of these amino acids means that collagen is incapable of forming α-helices and β-pleated sheets, and instead the polypeptides intertwine to form a unique left-handed triple helix. The molecule also has very few side chains so collagen polypeptides can be tightly packed together and can bond with each other by hydrogen bonds at regular intervals (O'Connor, 2000). This is represented in figure 1.

a)

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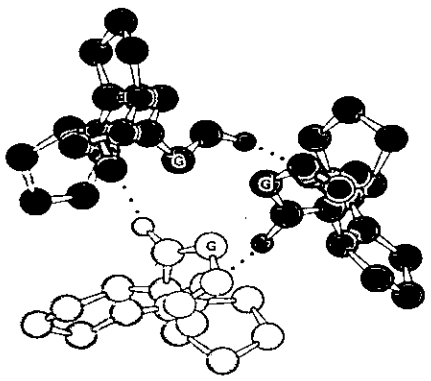



c)

**Fig 1:** a) This depicts the sequence of a representative portion of a single strand of the collagen triple helix.

b) This figure is a cross section of c) which in turn shows a model of the collagen triple helix, with each colour representing a different strand. The α carbon atom of glycine residue is represented by G. (Reproduced from Stryer, 1995).

The mineral phase is mainly hydroxyapatite, which has the general formula $Ca_{10}(PO_4)_6.(OH)_2$. This formula is a generalised representation of the mineral phase as some of its elements can be replaced by others. For instance, $Ca^{2+}$ may be replaced by $Sr^{2+}$, $Ra^{2+}$ or $Pb^{2+}$. $CO_3^{2-}$ can replace $PO_4^{3-}$ and $F^-$ can be replaced by $OH^-$. Other ions, in particular metal ions, can attach themselves to the surface of the hydroxyapatite crystals by absorption. This process can occur in living tissue as well as in dead material, it is, therefore, hard to tell whether these chemical substitutions reflect life chemistry or burial conditions (O'Connor, 2000).

There are two main types of bone tissue: compact and cancellous. Compact bone is the solid, dense material that forms the surface layers of mature bones. At joint ends this compact bone is covered by cartilage, to produce a smooth surface with no blood vessel canals: this is subchondral bone. The second main type of bone tissue is cancellous bone, which is a spongy porous material that is mainly found at the ends of long bones, in short bones and sandwiched within flat bones where it forms an internal support system. The main difference between these two types of bone tissue is their porosity (White, 2000).

The general structure of long bones can be divided into the tubular diaphysis, the epiphysis and the metaphysis (Figure 2). The tubular diaphysis or shaft constitutes the long axis of the bone. It is constructed of a relatively thick collar of compact bone that surrounds a central medullary or marrow cavity. In adults, the medullary cavity contains fat (yellow marrow) and is termed the yellow bone marrow cavity. Bone marrow is found in the medullary cavity of the bone and the pore spaces of cancellous bone. At birth this is a red bone marrow that produces red and white blood cells but as an adult active red marrow is only found in certain bones and the rest becomes inactive yellow marrow (Schultz, 1997).

The epiphyses form the ends of the bone; in most bones they are more rounded and expansive than the diaphysis. Compact bone forms the exterior of the epiphyses whereas the interior is made of cancellous bone. The joint surface is covered with a thin layer of articular cartilage that cushions the opposing bone ends during joint movement and absorbs stress. Between the diaphysis and each epiphysis in adult long bones lies the metaphysis. Here there is a structure called the epiphyseal line, which is what remains of the ephiphyseal plate. The plate is a disc like region of hyaline cartilage that grows during childhood to lengthen the bone.

Figure 2 is representative of an adult human long bone in cross section, showing the major components and features of bones described on the previous page (White, 2000).

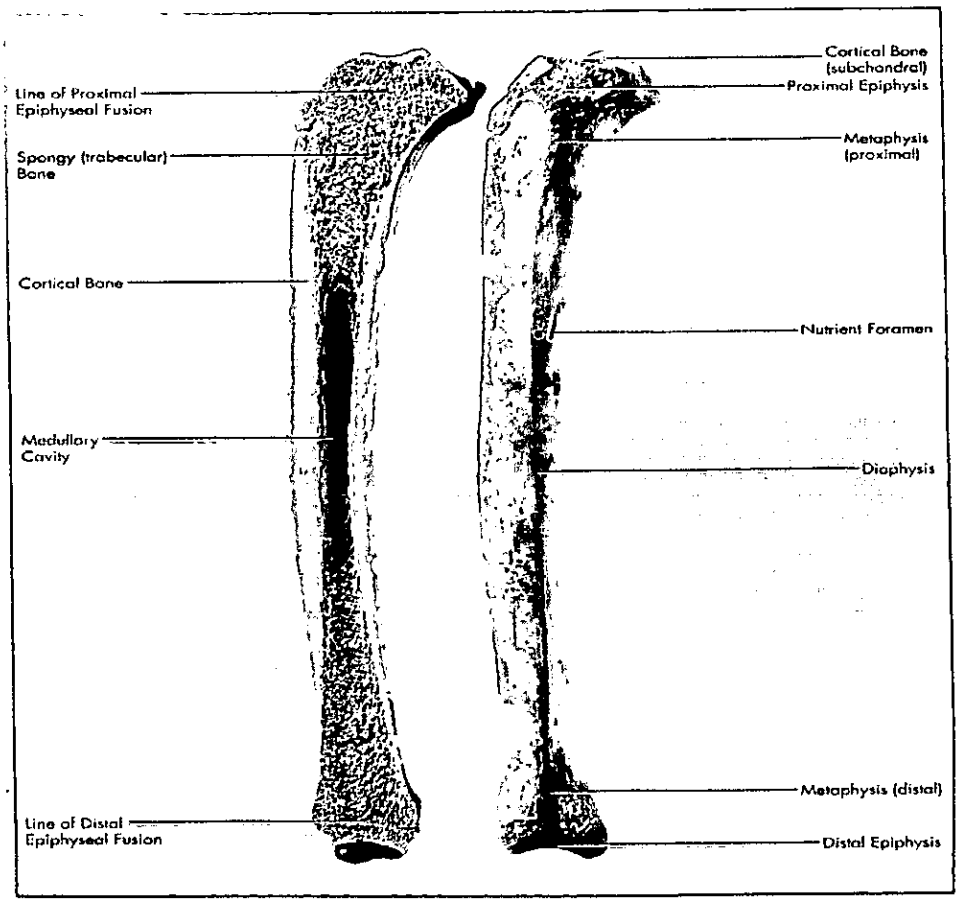



**Fig 2:** A left tibia (shin bone) sectioned to show the key elements of the gross anatomy of a human long bone (White, 2000).

Both the internal and external bone surfaces are associated with membranes. The outer surface of the diaphysis is covered and protected by a white, double-layered membrane called the periosteum. The inner surface layer of the membrane is made up of osteoblasts and the outer is composed of blood and lymphatic vessels and nerves. The inner surface of the bone is covered by the endosteum, which is similar in structure to the periosteum, and is also made up of osteoblasts (Schultz, 1997).

Short, irregular and flat bones share the same basic structure. These types of bone are not cylindrical and so they have no shaft or ephiphysis. They contain small amounts of bone marrow, but no clear medullary cavity is present (Schultz, 1997).

## 1.2 TYPES OF BONE

The bones of a mammalian skeleton can be grouped into a few basic shapes. Long bones, as described previously, are tubular in shape with expanded joint ends and have a greater length than they have width. These bones are usually slightly curved; this curvature absorbs the stress of mechanical forces, such as the body's weight, and evenly distributes it and to prevent bones snapping. These bones are predominantly limb bones and those contained in the hands and feet. Despite the obvious differences in shape of these different types of bone, their histology is remarkably similar (White, 2000).

Short bones are cuboid in shape, i.e. they are nearly equal in length and width. They are made up of cancellous bone that is covered in a thin layer of compact bone. This kind of bone is found in the wrists and ankles (Schultz, 1997).

Flat bones are generally thin and are composed of two nearly parallel plates of compact bone which sandwich a layer of cancellous bone. These bones give considerable protection and include the cranial bones, breastbone and ribs (White, 2000).

Irregular bones are complex in shape and therefore cannot be grouped into any of the other categories. They vary in the amount of compact and cancellous bone they contain and include some of the facial bones and the vertebrae of the back (Schultz, 1997).

Finally, the sesamoid bones develop in certain tendons usually near joints where there is considerable friction, tension and physical stress, such as palms and soles. The bones are typically only a few millimetres in diameter with the one

exception of the kneecaps (patellae). Their basic function is to protect tendons from excessive wear and tear as they move over the bony surface (Gunn, 1992).

## 1.3 THE HISTOLOGY OF BONE

Mineralised bone is formed by secretion of hydroxyapatite crystals upon and within the framework of collagen fibrils. This framework of collagen fibrils and hydroxyapatite is an amorphous substance described as osteoid, which is secreted by specialised bone cells called osteoblasts (Schultz, 1997). Once the hydroxyapatite crystals have been deposited, the osteoid becomes calcified and is converted to solid bone. Some of the osteoblasts may become incorporated into the matrix during this process and functionally become osteocytes. Figure 3 illustrates the Haversian system of compact bone and its composition. The Harversian system provides nutrients for osteocytes, and avenues for intercellular communication and repair. Cancellous bone lacks these Haversian systems as it gets nutrients from the blood vessels in the surrounding marrow spaces.

In the Haversian system osteocytes are enclosed in voids called lacunae. The lacunae are interconnected by a branching and interdigitating network of fine channels (canaliculi). The canaliculi are also connected at intervals to blood vessels, and this, as a whole comprises the Haversian system. The Haversian system transmits the blood vessels and nerves that support the cells within the bone, since compact bone is too dense to be nourished by surface blood vessels (Steele and Bramblett, 1997).

There are three different types of bone cells (Table 1). Osteoblasts synthesise and secrete osteoid, which then becomes mineralised to produce bone. Also found in bone are osteocytes, of which there are two types, osteoblastic osteocytes that are involved with conservation of bone tissue and osteolytic osteocytes that deal with disintegration of hard tissues (bone dissolution). These cells are essential in order to maintain the bone tissue. Finally, giant multi-nucleated cells called osteoclasts are responsible for the reabsorption of bone tissue. This places them into a group of cells which practice phagocytosis. Their primary functions are the remodelling of bone during growth and repair but are also active in destructive processes (Schultz, 1997). Table 1 contains a summary of each bone cell type and their functions.

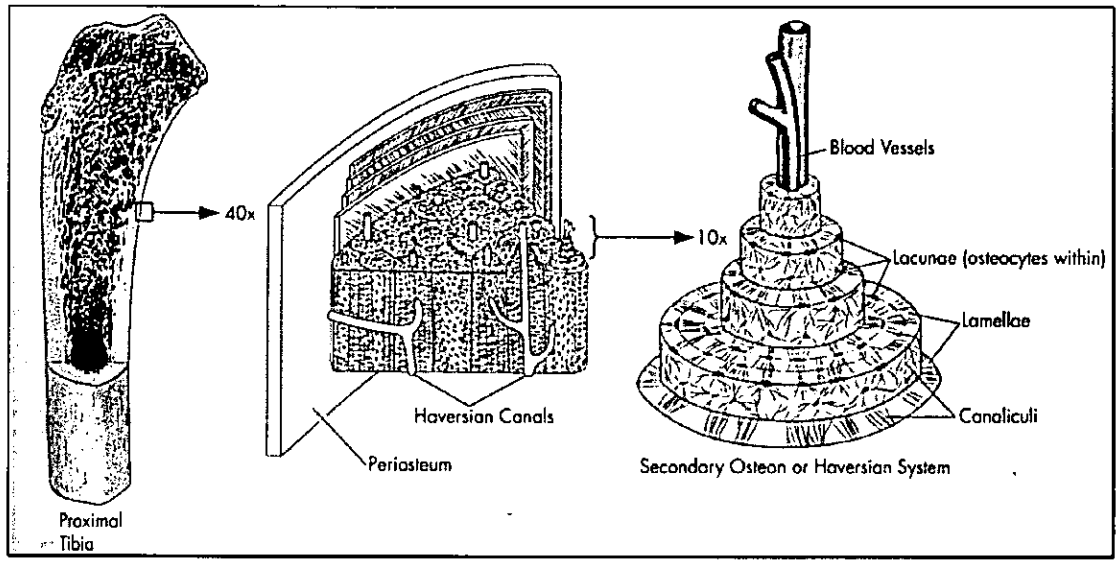



**Fig 3:** Gross and microscopic structure of bone (White, 2000).

| Cell type | Location and origin | Function |
|---|---|---|
| **Osteoblasts** | Stem from connective tissue cells. Mainly concentrated just beneath the periosteum. | Responsible for synthesising and depositing the organic substances of bone. |
| **Osteocytes** — **i Osteoblastic** / **ii Osteolytic** | Principally an osteoblast caught in the mineralised matrix. Lie in flat holes called lacunae. | i Conservation of bone tissue if they die surrounding matrix disintegrates. ii Bone dissolution. |
| **Osteoclasts** | Stem from stromal cells. Found in Howship's lacunae. | Reabsorption of bone tissue. |

**Table 1:** Summary of bone cell type, location and function. (Information taken from Schultz, 1997).

## 1.4 BONE ENDOCRINOLOGY AND METABOLISM

Approximately 1.5% of the body's dry weight is calcium. Calcium metabolism in the plasma plays a vital role in blood coagulation, cardiac and skeletal muscle contraction and nerve function. This mineral's concentration is very important to its function and is maintained in equilibrium in different parts of the body. Despite the fact that the level of calcium is balanced, it is constantly replaced through turnover in the skeleton. There is a complete turnover each year in infants and approximately an 18% yearly turnover in adults, due to constant resorption and reformation of bone tissues (Steele and Bramblett, 1997).

Vitamin D promotes the active transport of calcium and phosphate in the body as well as the absorption of calcium by the gastrointestinal tract. It is a fat-soluble vitamin and its production in the skin involves the reaction of ultraviolet light with certain pro-vitamins. If there is a deficiency of vitamin D then this can lead to rickets in the young and osteomalacia in adults. Calcium ion concentration in body fluids is partly controlled by the parathyroid. Parathyroid hormone (PTH) acts directly on bones to mobilise calcium ions, so if the parathyroid glands are not active then this may halt osteoclast activity and therefore cause calcium levels in the body to drop. On the other hand, if the parathyroid glands are overactive, osteoclastic absorption may outweigh osteoblastic deposition thus causing the bones to weaken. This can result in multiple fractures, decalcification, giant-cell tumours and cysts (Chaney, 1997).

In contrast, the hormone calcitonin decreases osteoclast activity and reduces calcium and phosphate levels in the blood. This hormone may protect bones in females from excess calcium loss during pregnancy and lactation and is present at higher levels in young individuals. A 30 % decrease in calcium ions in the body produces immediate problems, as the central nervous system and peripheral nerves begin to spontaneously discharge producing tetonic contractions of skeletal muscle, which may be fatal. If the body's levels of calcium are too high, depressed reflexes and a sluggish central nervous system result. Slightly elevated levels would lead to kidney stones as the kidneys increase the concentration of calcium in the urine. Highly elevated levels could lead to more serious conditions, such as renal failure (Litwack and Schmidt, 1997).

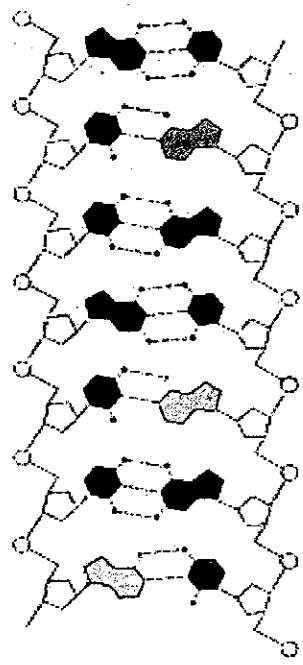


**Fig 4:** Schematic diagram of the structure of DNA. The sugar phosphate backbone is represented in black where circles represent phosphate groups, and pentangles the five carbon deoxyribose sugar residues. Each base has a different colour, yellow for A, blue for T, red for C and green for G (Stryer, 1995).

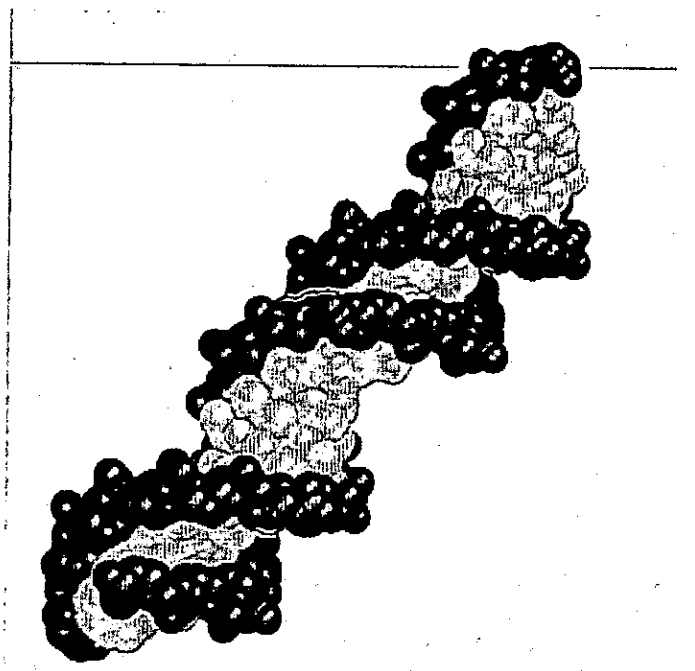


**Fig 5:** This is a model representing a 3-dimensional structure of DNA. The sugar-phosphate backbone is shown in dark colours and the bases in light colours (Stryer, 1995).

Nucleases are enzymes that act specifically on DNA and they break the 5'-3' phosphodiester bonds that link nucleotides together. In living tissue these nucleases are used in nucleotide metabolism in degradative pathways, but in dead tissue these destructive enzymes can hinder retrieval and analysis of ancient DNA samples (Hoss *et al.*, 1996 [2]).

DNA will start to be degraded by endogenous nucleases soon after death. Under some circumstances, such as rapid desiccation, low temperature or high salt concentrations, these nucleases are inactivated or their action impaired, thus halting or slowing the degradation. Nucleases are enzymes and so are susceptible to certain conditions; low temperatures decrease their activity as they work at an optimum temperature around body temperature. High salt concentrations cause proteins to precipitate out causing inactivation; a lack of moisture also causes inactivation because an aqueous environment is essential to their function (Hofreiter *et al.*, 2001). If nuclease inactivation occurs before all the nucleic acids have been converted to mononucleotides then slower chemical processes may then begin to take effect and degrade the DNA (Hofreiter *et al.*, 2001).

Oxidation and direct or indirect background radiation will affect and modify the nitrogenous bases and the sugar phosphate backbone. Deamination, depurination and other hydrolytic processes lead to destabilisation of DNA structure and single and double strand breaks in DNA (Richards and Sykes, 1995).

There is evidence that DNA is preserved longer in bones than in soft tissues. This indication comes from research carried out using DNA taken from moa soft tissues and bones around 3350 years old (Cooper *et al.*, 1992). The bone samples yielded DNA, from which was amplified a 438 base pair (bp) fragment, but the DNA from the soft tissue gave poor amplification and only fragments of around 150 bp were produced. This suggests that under similar conditions DNA may survive better in bone than in soft tissues (Cooper *et al.*, 1992). Ancient DNA (aDNA) from humans was first recovered in 1985 from a human femur carbon dated at 5,450 years old (Hagelberg and Sykes, 1989). Since then it has been extracted from skins of giant ground sloths up to 13,000 years old (Paabo *et al.*, 1989), 7000 year old human brain tissue (Paabo *et al.*, 1988), bone fragments from Pompeii up to 1,940 years old (Cipollaro *et al.*, 1999) and bone fragments from the extinct large flightless bird the moa up to 3,750 years old (Cooper *et al.*, 1992). There are many more examples from which DNA has been successfully extracted, and these are featured in Table 2.

| DNA source | Maximum age | Reference |
|---|---|---|
| Mummified human tissue | ~ 600 years | Handt *et al.*, 1996 |
| Human skeletons from the Terp of Wijnaldum | 1,800 years | Colson *et al.*, 1996 |
| Bone fragments from Pompeii | 1,940 years | Cipollaro *et al.*, 1999 |
| Bone fragments from the extinct moa | 3,750 years | Cooper *et al.*, 1992 |
| Human femur | 5,450 years | Hagelberg and Sykes, 1989 |
| Human brain tissue | 7,000 years | Paabo *et al.*, 1988 |
| Extinct wild aurochs (*B. primigenuis*) | 12,000 years | Bailey *et al.*, 1996 |
| Skins of the Giant ground sloth | 13,000 years | Hoss *et al.*, 1996 [1] |
| Saber toothed cat fossils | 14,000 years | Janczewski *et al.*, 1992 |
| Alaskan member of the horse family (*Equus hermionus*) | 25,000 years | Hoss and Paabo, 1993 |
| Ice age brown bear | 42,000 years | Leonard *et al.*, 1999 |
| Insects entombed in amber | 120-135 million years | Cano *et al.*, 1993 |

**Table 2:** Examples of specimens from which DNA has been successfully extracted and amplified. The approximate ages of samples is also listed.

Ancient DNA of this age is invariably highly damaged and degraded (Paabo *et al.*, 1989) due to post-mortem degradative processes.

Burial conditions of bodies are of great significance for the recovery of amplifiable DNA. Buried corpses are subjected to a range of chemical and physical agents, which bring about chemical changes in the tissues of the body, which in turn greatly influence the degree of preservation of tissues (O'Connor, 2000). Decomposition starts to take place soon after death has occurred (Vass, 2001). The first step of this process is autolysis or self-digestion, whereby the cells of the body become deprived of oxygen. Carbon dioxide builds up and toxic waste accumulates, which begins poisoning the cells. Cellular enzymes then begin to break down the cell from the inside out, which leads to cellular lysis. This process starts in enzyme-rich organs such as the liver and also in high water content tissues like the brain, and continues to spread throughout the body.

Once enough cells have burst open and released their contents, putrefaction begins to take effect. This process is the destruction of tissues by biotic agents such as bacteria and fungi. Their action results in the catabolism of tissues into gases, liquids and simple molecules. One of the common species of bacteria identified in putrefaction is *Clostridium* but there are many other species, depending on local and individual conditions.

Vass (2001) initiated research to determine whether microbes could be used to estimate more accurately post-mortem interval. He isolated many species, even from the very early stages of decomposition, including *Staphylococcus, Candida, Bacillus* and *Streptococcus sp.* The previously mentioned species do not include the putrefactive bacteria, such as *Clostridium.* Pathogenic organisms such as *Serratia spp, Klebsiella spp, Proteus spp* and *Salmonella spp* were also isolated. There were also environmental microorganisms such as *Agrobacterium* and varieties of fungi. With all these different species he found it impossible, due to the sheer weight in numbers and species diversity, to identify clear trends in succession.

When the corpse has no nutritional value and the remaining tissue, usually skin, has become dehydrated or desiccated, the stage of mummification is reached. This usually occurs in dry heat areas such as deserts, or areas of low humidity such as the Artic circle. As well as all these processes, bone goes through yet another complex process called diagenesis. Diagenesis alters the proportions of collagen and hydroxyapatite. Collagen is particularly susceptible in well oxygenated, moist and

slightly alkaline burial environments. Solubility of bone minerals will depend on the amount of ions already in solution in the surrounding environment (Hagelberg and Clegg, 1991).

It may well be that the age of the samples reflects the amount of damage but research has shown that age is not such an issue as the burial conditions influencing the state of preservation (Hagelberg and Clegg, 1991 and Burger *et al.*, 1999). DNA extracted from skeletal remains recovered from Abingdon abbey showed that the older medieval samples produced longer amplified products than the much younger civil war specimen (Hagelberg and Clegg, 1991). These bodies were buried in different parts of the graveyard where the amount of acid in the soil and drainage of the soil was different. Temperature is also an important factor, and specimens found in cold climates such as artic and subantartic regions, seem to yield DNA that has less damage to its bases and is therefore better preserved (Hoss *et al.*, 1996, Lindahl, 1993 and Hofreiter *et al.*, 2001). This information is consistent with the fact that a decrease in temperature of 20°C is expected to result in a 10 to 25 fold reduction in the rate of enzymatic reactions such as exonuclease digestion. Freezing, as in the case of permafrost, would further reduce the decay rate, and may halt it completely (Hoss *et al.*, 1996). Theoretical estimates from biochemical research predict that fully hydrated DNA could survive for tens of thousands of years, particularly if preserved at low temperatures (Lindahl, 1993). The factors, and their effects on the preservation of DNA are summarised in Table 3.

| FACTOR | COMMENTS |
|---|---|
| Absence of microorganisms | Microbial degradation of DNA is prevented. |
| Absence of UV radiation and isotopes | Only the surface of sample is affected by UV irradiation. |
| Aridity | Hydrolytic and oxidative damage is reduced under dry conditions. |
| DNA adsorption to mineral surfaces such as hydroxyapatite | DNA molecules are stabilised by binding to mineral surfaces. |
| Rapid inhumation after death | Infestation by microorganisms is accelerated by the formation of gas and the breakdown of soft tissue structures. |
| Hard and dry tissue samples | Hard and dry tissues prevent physical and chemical reactions. Bones and teeth protect organic residues against chemical reactions and microbial infestation. |
| Low temperatures | Low temperatures decrease the rate of most chemical reactions and inhibit the growth of microorganisms. |
| Neutral or slightly alkaline pH value | As environmental pH decreases, both DNA and its surrounding material (bone or teeth) will be degraded or destroyed. |
| Presence of chelating humic and fulvic acids | Phenolic geopolymers prevent aerobic activity of microbes. |
| Storage of samples at low temperatures | Samples should be stored at least as cold as ambient temperature where they were excavated. For longer-term storage, the use of a -20°C freezer is highly recommended. |

**Table 3:** Favourable conditions leading to aDNA preservation in archaeological material. Machugh *et al.* (2000), which was modified from Burger *et al.* (1999).

## 1.6 DNA EXTRACTION METHODS

The extraction of DNA from bone has been achieved using a number of different methods. The favoured method of the early part of the last decade seems to have been the phenol/chloroform method (Hagelberg and Clegg, 1991; Handt *et al.*, 1996; Bailey, 1996 and Colson, 1997). This method usually involves incubation of the material in a lysis buffer followed by extraction with phenol and chloroform. The lysis buffer can contain a number of different components including ethylene diaminetetra-acetic acid (EDTA), sodium dodecyl sulphate (SDS) and proteinase K. These substances are used to break open the cells and cleave proteins bound to the DNA contained within the chromosomes (Butler, 2001). Proteinase K may also inactivate nucleases present in the biological sample and EDTA, as a chelating agent binds cations required for nuclease action. The phenol precipitates the protein material and cellular debris, leaving DNA in solution. Chloroform prevents trace amounts of phenol remaining in the aqueous phase, where it would result in chemical damage to the DNA. Some of the chemicals, like SDS, phenol and proteinase K, used in the lysis buffer are potent inhibitors of the polymerase chain reaction (PCR) and so will cause major problems in further analysis (PCR techniques and applications workshop), if not subsequently removed.

To combat this, some researchers have taken to using further purification steps such as ethanol precipitation (Fisher, 1993). DNA is insoluble in ethanol, and precipitates out, facilitating its recovery by centrifugation (usually in the presence of a carrier such as glycogen or a salting agent such as sodium acetate). Another method is by the use of silica columns (Richards and Sykes, 1995 and Greenwood *et al.*, 2001). Silica-based methods have been used quite successfully (Hoss and Paabo, 1993; Cipollaro *et al.*, 1999 and Handt *et al.*, 1994), and recent comparisons by Yang and co-workers (1998) question the relative effectiveness of the phenol/chloroform stage of the extraction process. They combined elements from the methods of Hagelberg and Clegg, (1991) and Hoss and Paabo, (1993) to produce a method eliminating the phenol/chloroform stage for a more effective extraction. Although organic extraction works well for high molecular weight DNA retrieval, it is time consuming, as it requires numerous sample transfers, and is less effective with small amounts of DNA.

There are a number of other methods that have also been used which steer away from the more hazardous reagents; for example, sodium acetate precipitation

(Cattaneo *et al.*, 1995), Chelex (Walsh *et al.*, 1991), water elution (Petrishchev *et al.*, 1993) and various manufactured kits. Sodium acetate precipitation works along the same vein as ethanol precipitation. This method also requires a lysis buffer; the sodium acetate then separates the DNA from the proteins, which is then precipitated out using isopropanol. Chelex® is an ion-exchange resin, which is added in suspension to the sample. It is composed of styrene divinylbenzene co-polymers, which contain paired iminoacetate ions (Walsh *et al.*, 1991). These act as chelating groups that bind polyvalent metal ions such as magnesium and by doing this the nucleases that break down DNA are inactivated, as they require divalent cations as cofactors for their activity. Boiling with chelex, as well as denaturing DNA, disrupts the cell membrane and denatures the cell's proteins. Centrifugation pellets the beads and then the supernatant is ready for extraction (Butler, 2001). This method is very quick and can be carried out in a single microfuge tube, which reduces the risk of cross contamination. Some researchers have insisted on decalcifying bone material before use (Greenwood *et al.*, 2001) but ongoing research has indicated that decalcification is not required and actually reduces the DNA yield by half (Hagelberg and Clegg, 1991 and Fisher *et al.*, 1993).

Even the most efficient methods of extraction can fail if a poor sample is used, so careful choices have to be made with respect to the procedures that are chosen for the samples. There are various methods that can be applied to assess the suitability of a bone sample, but the most obvious one is examine its condition. If, after excavation, the bones are dense rather than brittle and the surface is undamaged then they are generally well preserved. If, on the other hand, they are damp and the surface is pitted and rubs off when handled, then the preservation is regarded as poor (Hagelberg *et al.*, 1991).

Well-preserved bone always shows the presence of osteocytes under microscopic examination (Richards and Sykes, 1995). This method involves using a portion of a transverse slice of bone that has been embedded in epoxy resin, mounted and polished on one side. An optical microscope using reflected light and around 400x magnification is then used to examine the polished side for the presence of osteocytes. Another method that could be used is analysis of the racemization of certain amino acids, such as aspartic acid, alanine and leucine, by high performance liquid chromatography (HPLC) (Poinar *et al.*, 1996). During the active metabolic

process L-amino acids undergo racemization to D-amino acids, which are optical isomers, to a point where they are present in equal amounts. The rate at which this process occurs differs depending on the amino acid, the presence of water, temperature and chelation of metal ions, all things that affect the depurination of DNA. Poinar and co-workers (1996) used this correlation to determine the amount of DNA degradation and found that for aspartic acid a D/L ratio exceeding 0.08, the sample did not produce any amplifiable DNA.

The prevention of contamination is an integral part of retrieving viable results. Ancient human material presents more of a problem than animal bones, as modern DNA could contaminate the samples and give false readings whereas with ancient animal material if they are distinct from, but related to the present species, they are considered authentic (Hofreiter *et al.*, 2001). Precautions need to be taken with every stage from excavation to laboratory to preparation. Gloves need to be used to handle the samples at all times, and protective clothing should also be used.

## 1.7 POLYMERASE CHAIN REACTION

The Polymerase Chain Reaction (PCR) has made a huge impact on molecular biology. Most of the studies mentioned in the previous sections have used this revolutionary technique to analyse DNA. Since Kary Mullis devised this method in the mid 1980's, the process of analysing particular gene sequences has become simpler and quicker. In order to study a target gene before PCR was developed, one had to resort to lengthy techniques such as cloning and hybridisation. Cloning would require taking a DNA fragment and inserting it into a vector. It would then be introduced into a host cell in order to replicate the target sequence. PCR has enabled scientists to produce an enormous number of copies of a specific sequence in just a few hours. Since only a small amount of DNA, less than a microgram, is needed, PCR has revolutionised the work on ancient material (Hofreiter *et al.*, 2001).

The reaction requires several components; firstly, a small amount of template DNA, denatured to single stranded form, is needed. Two flanking oligonucleotide primers, which are small sequences of DNA that will bind to the separated single strands of the template DNA, are also required. These primers are usually between 15-30 nucleotides in length and are designed in pairs. The G + C content of these primers must be known, and there must be similar G + C ratios between pairs. The

pairs must also lack homology in order to prevent self-annealing or the possibility of dimerisation. The actual sequence of the primers dictates the temperature of annealing ($T_M$) which can be estimated using the equation $\mathbf{T_M = 2(A+T) + 4(G+C)}$ (Stryer, 1995).

The enzyme DNA polymerase that is responsible for DNA synthesis requires the primers in order to initiate synthesis. DNA polymerase can only add deoxyribonucleotides to the 3' terminus of a DNA strand, and cannot initiate DNA synthesis *de novo*. The primers hybridise to the template DNA strand to provide these starting points for the enzyme. It also needs the four deoxynucleotide triphosphate precursors (dNTP's) to synthesise the new DNA strand. The first DNA polymerase used in this protocol was DNA polymerase I (DNA pol I) from *Escherichia coli,* as this was a well-characterised enzyme and its gene (*polA*) had been cloned, thus facilitating the large-scale production of the enzyme. However, this enzyme is sensitive to heat, and the denaturation step of the PCR procedure inactivated the enzyme, requiring the replenishment of the DNA polymerase at each round. Scientists discovered that the bacterium *Thermus aquaticus,* which lives in hot springs at temperatures above 75 °C, has a DNA polymerase (Taq polymerase) with an optimal temperature of 72 °C and which is reasonably stable at 94 °C (Watson *et al.,* 1992). Manufacturers have modified and marketed various forms of this enzyme. Taq polymerase has 5'-3' exonuclease activity and an optimum temperature of 72 °C. Amplitaq® is a recombinant form of Taq polymerase which has a temperature range of 70-80 °C, and both are patented by the Cetus corporation. The derivative Stoffel fragment, unlike Taq polymerase, has no 5'-3' exonuclease activity but a higher thermostability.

As well as these above components, the reaction also requires $MgCl_2$ at a concentration of between 1-2 mM to act as a buffer and as a co-factor for most DNA polymerases. The precise concentration of $MgCl_2$ is essential for an efficient reaction. If the concentration is too low, it will produce a low PCR yield but, on the other hand, if it is too high, it can lead to non-specific products. The final volume of the reaction can vary between 25-100 µl and may be overlaid with 50 µl of mineral oil to stop the contents from evaporating (Watson *et al.,* 1992).

The reaction itself is a cycle; the first stage is the denaturation step where the reaction mixture is heated to a temperature around 94 °C for 5 minutes, which

separates the double stranded template DNA into two strands. The temperature then drops to between 30-65 °C so that the primers can anneal to the complementary target sequence. The temperature then rises again to about 72 °C, which is the optimal temperature for Taq DNA polymerase so DNA synthesis can proceed. This stage takes about 5 minutes and then the temperature is increased back up to 94 °C again so the newly synthesised strands denature for the next cycle. This cycle is represented in Figure 6, and its effects in Figure 7.

The reaction takes place in a thermocycler, which is pre-programmed to carry out as many cycles as required at the specified temperatures. The cycle can be repeated as many as 30 to 60 times and produces an optimal number of a theoretical maximum of $2^n$ double stranded DNA molecules, where n is the number of the cycles.

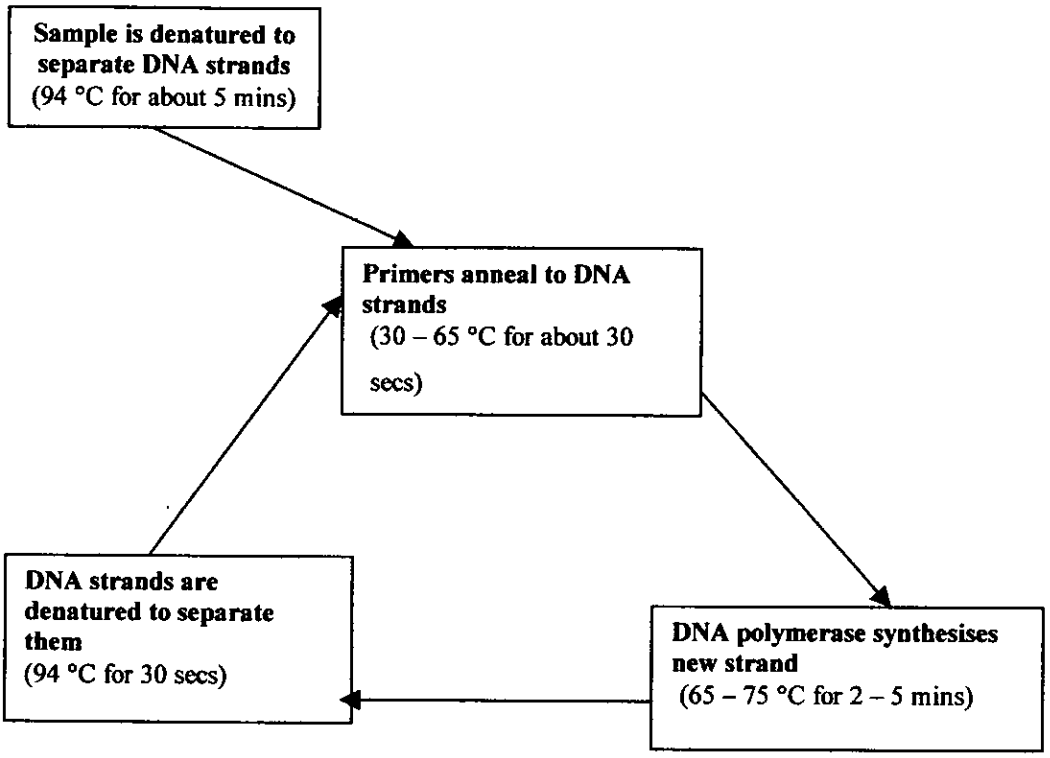



**Fig 6:** The PCR cycle (adapted from Watson *et al.*, 1992)

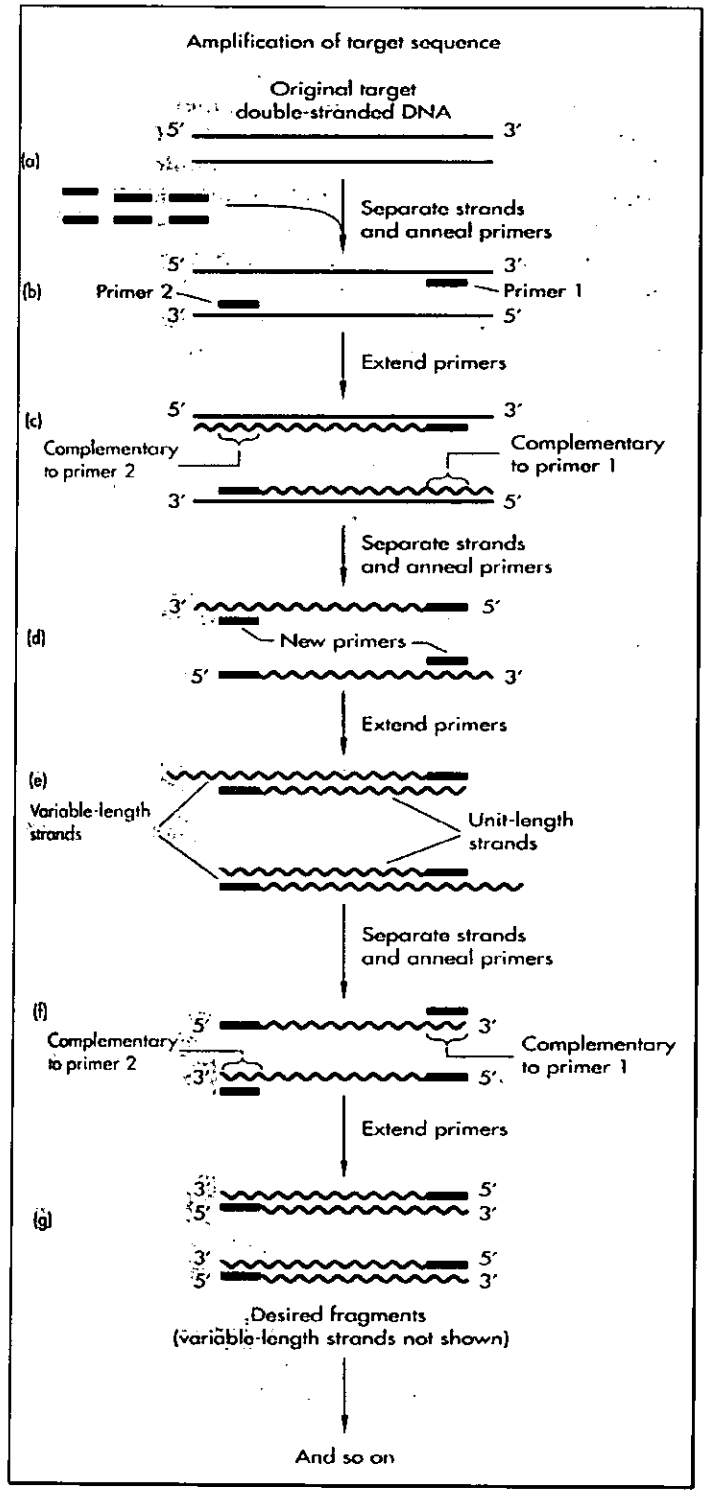



**Fig 7:** The Polymerase Chain Reaction (Reproduced from Watson *et al.*, 1992)

Randomly amplified polymorphic DNA – PCR (RAPD-PCR) is an alternative PCR technique which, as it suggests, amplifies DNA sequences using random-binding primers. With conventional PCR some knowledge of a particular sequence to be amplified, as well as that of the primers required is needed. RAPD-PCR requires no prior knowledge to carry out the reaction. The primers are typically 7-15 bases in length and are non-specific in that they bind to complementary sequences, which are effectively located at random on the template DNA. If the primers form matched pairs, as shown in Figure 8, then an amplicon is produced. These amplicons are of various sizes and therefore generate an informative fingerprint pattern, which is indicative of the species being analysed (Lee and Chang, 1994). This is due to different regions in the genome that are complementary to the primers and will differ in location and relative positive in the genomes of different species.

These fragments, when examined on an agarose gel using electrophoresis, will produce a fingerprint that is species-specific. There are a number of different primers that can be used and, although they will all produce a different fingerprint, they will still be indicative of the DNA of the species being tested.

Other methods have been used which produce species fingerprints do not use RAPD-PCR. A method based on antigen-antibody reactions has been used but it could not distinguish between closely related species like wild boar and pig, cattle and buffalo or sheep and goat (Koh *et al.*, 1998). Another method used for species fingerprinting is polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). This technique uses primers specific for the cytochrome b gene, amplifies the products using PCR and then uses 2 restriction enzymes to digest the amplified fragments. Research using this method has been carried out on fragments of bone taken from the site Head-smashed-in buffalo jump, located in southwest Alberta, Canada (Newman *et al.*, 2000).

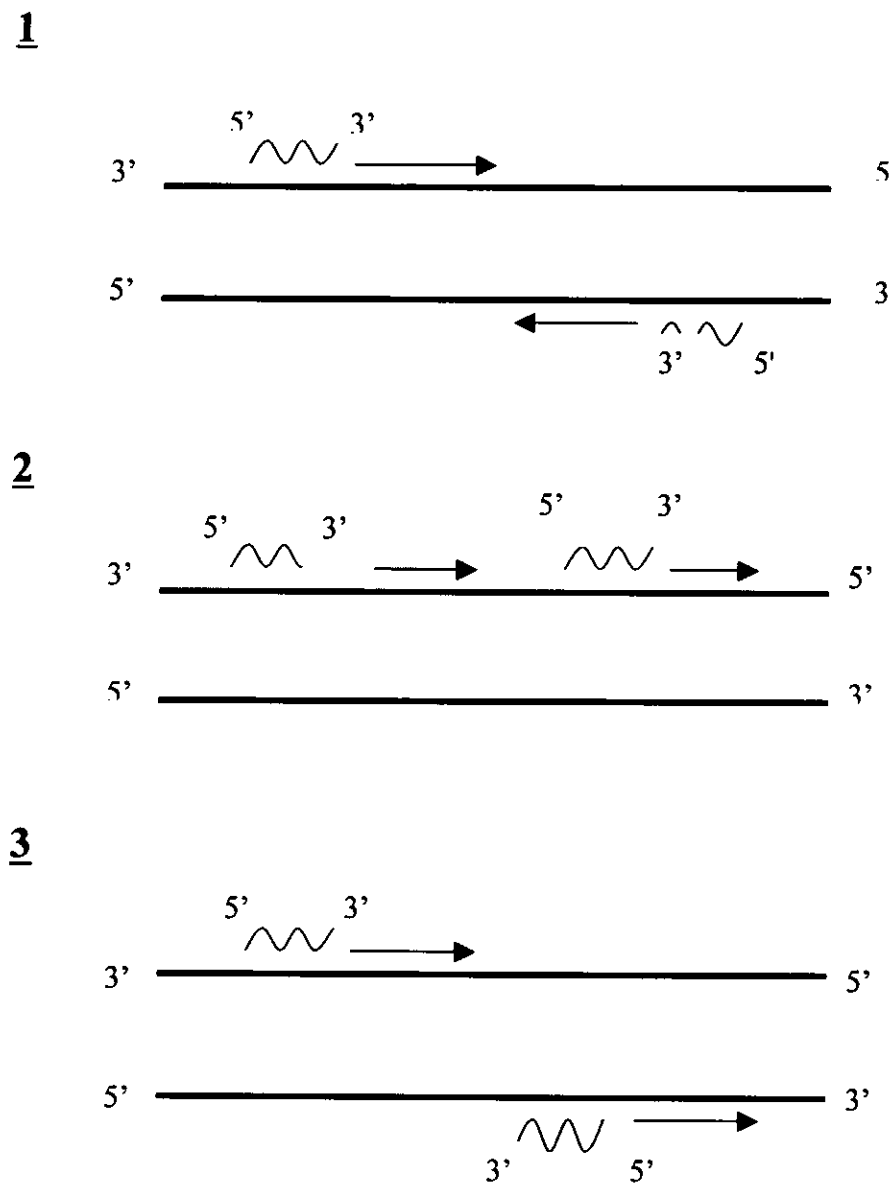



**Fig 8:** Representation of the formation of amplicons in RAPD-PCR

The diagram above shows that amplicons can only be produced if the primers anneal to the 3' end of the DNA strand and are facing each other on opposite strands with a space between them that is 5 kilobases (kb) long [1]. If they anneal on the same strand [2] or on separate strands but facing away from each other [3] then they will not produce an amplicon.

RAPD-PCR could be used to identify the species from which bone fragments originate in archaeological investigations where the bone that cannot be

morphologically identified. This type of PCR can also be useful when testing food samples. For example when expensive meats are fraudulently replaced with cheaper ones and visual differences are not evident, then this method has been used to identify the species (Koh *et al.*, 1997). Another use could be detecting the illegal trafficking of endangered species products like powdered rhino horn for instance (Wetton *et al.*, 2002, Koh *et al.*, 1997 and Lee and Chang, 1994).

The PCR reaction is very sensitive and if there is any contamination with recent DNA material that is not damaged, then it is more likely to amplify the recent material rather than the ancient. At every stage of the procedure protective clothing must be worn. A dedicated PCR workstation is also useful and this must be kept separate from areas for clean up and extraction procedures to avoid cross contamination. DNAse® is also a good tool for removing trace amounts of DNA as well as UV irradiation to cross-link DNA remnants. The PCR reaction has also got a number of inhibitors as well as the ionic detergents mentioned earlier, silica particles from extraction methods can affect the reaction, so extreme care must be taken when transferring DNA samples into the reaction. Organic inhibitors could also be co-extracted for example tannins, humic and fulvic acid and maillard products which not be removed during the process. All these things can be used for troubleshooting and possible clean-up methods (Machugh, 2000).

## 1.8 ANALYSIS OF DNA EXTRACTED FROM BONE

Extraction of DNA from ancient bone samples has been put to use in numerous different ways. DNA analysis has been used for species-level identification when bone remains cannot be morphologically distinguished (Barnes *et al.*, 2000 and Loreille *et al.*, 1997). Bone fragments have also been analysed and using various methods species-specific fingerprints have been produced for use in identification (Lee and Chang, 1994 and Newman *et al.*, 2002). This application is central to the subject of this thesis and will be discussed in more detail later.

Researchers have analysed the phylogeny of extinct species and studied the relationship with extant species (Cooper *et al.*, 1992, Greenwood *et al.*, 2001, Hoss *et*

*al.*, 1996 [1] and Janczewski *et al.*, 1992). This also leads to the analysis of genetic variation within and between populations of the same species (Cooper, 2000).

The identification of victims found in mass graves (Boles *et al.*, 1995; Primorac *et al.*, 1996), correlates with work in gender identification of infants and small children's remains when morphology cannot be used (Colson *et al.*, 1997; Faerman *et al.*, 1995). The identification of skeletons found to be those of the Romanov family has used these techniques and mitochondrial DNA (mtDNA) (Gill *et al.*, 1994).

DNA analysis of bones has major forensic implications and has been used in criminal cases to aid the identification of murder victims (Lee *et al.*, 1991).

The retrieval of DNA from ancient human samples (Handt *et al.*, 1996, Hoss *et al.*, 1996 [2] and Meyer *et al.*, 2000) has been successfully achieved and this knowledge can then contribute to research into the ancestry of different races (Stoneking, 1995).

DNA has been extracted from ancient wheat seeds and has been used in taxonomic identification to asses quality of possible bread making of wheat grown at an Early Bronze Age site in Greece (Brown, 1999).

There has been some investigation into how environmental factors affect the preservation of DNA (Burger *et al.*, 1999) and bone diagenesis studies have been carried out on skeletons found in a house in Pompeii (Cipollaro *et al.*, 1999).

Researchers have also spent time trying to improve the methods used by comparing extraction methods to determine their effectiveness (Cattaneo *et al.*, 1997), and comparing extractions taken from bone and soft tissues (Lassen *et al.*, 1994). All this research into different areas will aid further studies and improve techniques.

## 1.9 AIMS OF THE RESEARCH

There were a number of different aims to this research project. Given the lack of expertise within the laboratory regarding analysis of DNA from archaeological samples, an initial aim was to find a reliable method of extracting DNA from bone. Subsequently, this DNA would be used in the RAPD-PCR reactions to produce RAPD profiles of a range of species to create a library of profiles so that unknown samples could be compared and identified.

The applications of this research would mainly of value in the field of archaeology, where unknown bone fragments are often recovered. Although anthropologists are experienced in identifying human bones, even in fragmentary states, small fragments often present problems in objective identification, and if suspected to be non-human in origin, then further difficulties are presented in determining the animal species, given the wide range of potential species. For example, this University recently acquired the contents of some votary urns, which are used to carry cremated remains. These urns can invariably contain animal remains as well as human, and so with this test we might be able to determine the origin of the contents, which could lead to an insight into diet, general communal practices and lifestyles of these people.

A molecular approach, based on RAPD-PCR methodology, could be used as a quick and convenient initial test to determine their species origin. Further methods could then be used to confirm these initial results like PCR-RFLP (Newman *et al.*, 2000) or antigen-antibody reactions (Koh *et al.*, 1997). One challenge of the approach would be to see if it could distinguish between species that are closely related, such as pig and wild boar.

If successful in this regard, the method might also be used to possibly identify different strains or breed of species, along the lines of related work carried out by Cooper, 2000 in analysing genetic variations between populations of the southern brown bandicoot (*Isoodon obesulus).*

## 2. MATERIALS AND METHODS

### 2.1 BONE SAMPLES

Bone samples were collected from a number of different sources (See Table 2)

| Species | Origin | Ages of samples |
|---|---|---|
| Pig (*Sus scrofa)* | Preston abattoir, Preston | hours after slaughter |
| Bovine (*Bos bubalis*) | Brindle brothers butchers, Bamber Bridge | hours after slaughter |
| Lamb (*Ovis aries*) | Brindle brothers butchers, Bamber Bridge | hours after slaughter |
| Rabbit (*Oryctolagus cuniculus)* | Courtesy of David Pepper, N. Wales | c. 20 years |
| Dog (*Canis familiaris*) | The Veterinary centre, Blackburn | 2 months |
| Roe Deer (*Capreolus capreolus*) | The Farm centre, Blackburn | hours after slaughter |
| Wild boar (*Sus scrofa*) | The Farm centre, Blackburn | hours after slaughter |
| Unknown long bone | Garden, Penwortham, Preston | c. 20 years |
| Unknown scapula | Garden, Penwortham, Preston | c. 20 years |

**Table 2.1:** Bone samples used for analysis

All fresh samples were stored at –80 °C, after being ground to a fine powder using a freezer mill (Glen Creston Ltd, model 6750). The bone samples were then stored in sterile vessels at –20 °C. Old samples were stored in plastic bags until cleaning. After grinding they were stored at –20 °C. The dog samples were extracted teeth, which after grinding were stored in the same manner as the bone samples.

#### 2.1.1 CLEANING OF BONE

Old bone material was cleaned first using a soft brush and a scalpel to remove any dirt or soil debris. A large metal file was then used to remove to top layer of the bone; rough sandpaper was also used for the bits the larger file was too big for. Throughout this

whole procedure lab coat, glasses and a dust mask were worn to prevent inhalation of dust. The file was sterilised using isopropanol after each use. The bone was wrapped in paper and then fragmented using a hammer. The pieces were then placed on foil to provide a reflective surface and exposed to UV light for 20 minutes using a UV hood (Scie plas GLE-UVSC). The dog teeth were cleaned by using a scalpel to remove plaque and dirt and were then soaked overnight in 5% Chloros.

### 2.1.2 BONE GRINDING

Fresh bone samples were cut into small pieces using a tenon saw. They were then weighed and placed into sterile tubes with impacters. The bone material was ground into a fine powder using a freezer mill (Glen Creston Ltd 6750) under liquid nitrogen on a setting of 1 cycle for 2 minutes at the rate of 10 impacts per second. Old bone samples were treated in the same way except they were wrapped in clean, sterile paper and then fragmented using a hammer rather than by sawing.

## 2.2 DNA EXTRACTION FROM FRESH BONE

### 2.2.1 CHELEX METHOD

This method was adapted from the DNA extraction protocols handbook published by Perkin Elmer Biosystems. 0.1 g of the bone powder was placed into an autoclaved microcentrifuge tube (1.5 ml) with 200 µl of 5% Chelex 100®. The sample was incubated in a water bath at 56 °C for two hours and then removed and vortexed thoroughly at high speed for 10 seconds. The microcentrifuge tube lid was then pierced, to prevent pressure build up, with a sterilised needle before placing the tube in a boiling water bath for an incubation period of 8 minutes. The sample was vortexed again for 10 seconds and centrifuged in a microcentrifuge (MSE Micro centaur) for 2 minutes at 13,000 rpm. The supernatant was removed and placed in a fresh 1.5 ml microfuge tube; the sample was now ready for DNA quantification and PCR amplification. The remainder of the sample was stored at either 2 to 6 °C or –15 to –25 °C. Frozen samples were thawed at room

temperature, vortexed and centrifuged for 2 minutes using a microcentrifuge before undertaking subsequent analysis.

### 2.2.2 DNAce KIT (Bioline, London)

0.1 g of bone powder was placed into an autoclaved microcentrifuge tube and to it was added 1 ml of Lysis buffer (manufacturer's recipe) and 200 µl of Bone lysis enhancer (manufacturer's recipe). If the Lysis buffer contained any precipitate, it was incubated at 50 °C until it had completely dissolved. The tube and contents were then incubated in a water bath with occasional agitation at 50 – 60 °C for 15 – 20 hours. The sample was then placed in the microcentrifuge and centrifuged at full speed for 2 minutes after which the supernatant was transferred to a fresh 1.5 ml microfuge tube. After thoroughly vortexing the Carrier suspension (manufacturer's recipe), 15 µl was added to the microcentrifuge tube and vortexed again. The sample was then left to incubate at room temperature for 5 minutes before centrifuging at 10,000 rpm briefly to form a pellet. The supernatant was carefully discarded to leave a pellet, which was then resuspended in 1 ml of Wash buffer. The sample was centrifuged again briefly at 10,000 rpm briefly and the supernatant discarded; the Wash buffer (made up according to manufacturer's instructions) was then added and the process was repeated twice. Finally, it was centrifuged and the residual Wash buffer removed. The pellet was dried using an incubator, then resuspended in 80 µl of Elution buffer D (manufacturer's recipe) and incubated at 60 °C for 5 minutes in a water bath in order to elute the DNA. It was then centrifuged for 2 minutes at 13,000 rpm and the supernatant was removed. Care was taken to avoid transferring any carrier particles when using a Gilson pipette.

### 2.2.3 GENOMESTAR KIT (Hybaid,)

Using a 1.5 ml microcentrifuge tube, 600 µl of Buffer 1 (manufacturer's recipe) was added to 0.1 g of bone powder in the tube and then incubated in a water bath at 68 °C

for 5-10 minutes. The suspension was deproteinised by adding 450 µl of chloroform whilst mixing to form an emulsion, and was then centrifuged at 10,000 rpm for 2 minutes. The upper phase was transferred to a sterile 1.5 ml microcentrifuge tube and 450 µl of distilled water and 50 µl of Buffer 2 (manufacturer's recipe) were added. After gently mixing by inversion, the sample was then centrifuged at 10,000 rpm for 2 minutes to pellet the precipitated DNA. This was then resuspended using 0.3 ml of Buffer 3 (manufacturer's recipe) and 375 µl of 96 % ethanol, and mixed by inversion. The sample was centrifuged at 10,000 rpm for 10 minutes and the supernatant was discarded. The pellet was then rinsed with 70 % ethanol, and the DNA dissolved in 100 µl of TE buffer.

## 2.3 SPECTROPHOTOMETRIC ANALYSIS OF DNA SOLUTIONS

Absorbance readings were taken of the different samples at 230 nm, 260 nm and 280 nm to determine carbohydrate, nucleic acid and protein concentrations respectively. All samples were placed in 0.5 ml quartz cuvettes and, using a water standard, the UV/Vis spectrophotometer (Ultraspec λLKB Biochrom) was set to a reference zero before each reading. The samples were prepared by taking 20 µl of DNA solution and diluting with 380 µl of water to create a 1 in 20 dilution. If the absorbance readings were above 1.0 then further dilutions were required. All results were recorded in order to calculate concentrations and yield. Results were calculated using the equations;

$A_{260}$ of 1.0 = 50 µg/ml of double stranded DNA

$A_{260}$ of 1.0 = 40 µg/ml of single stranded DNA

The Chelex method of DNA extraction produces single stranded DNA due to the denaturation step of this process and therefore the second equation was used, for samples produced by this method.

## 2.4 DNA QUANTIFICATION

A 0.8 % agarose gel was used to quantify the sample DNA by comparing brightness of bands with those containing a known quantity of DNA. Sheared Herring sperm DNA was used for the standard and a series of dilutions were produced from a stock solution of 10 mg/ml. These dilutions were loaded on to the gel with 2 µl of tracking dye (0.25 % bromophenol blue, 0.25 % xylene cyanol FF and 40 % (w/v) sucrose in water), to make a total of 20 µl, in descending order and 18 µl of sample DNA was also loaded with 2 µl of tracking dye. The gel was then placed in an electrophoresis tank in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA [pH 8]) and electrophoresed at 50 mA for approximately 3 hours. It was then placed into an ethiduim bromide solution (0.5 µg/ml) to stain for 10 minutes before placing in the UV image analyser (UVP Bio-docit image analyser) to photograph. Comparison of the standard bands and the sample band were made in order to assess the amount of DNA present, so the amount included in PCR reactions could be determined. The results taken from the spectrophotometry analysis were also used to quantify the amount of DNA present. This is not an accurate method for the determination of absolute DNA content values as inevitably the supernatant will contain other components including protein, carbohydrate and also RNA.

## 2.5 PCR ANALYSIS

### 2.5.1 Ready-To-Go™ RAPD Analysis (Amersham Pharmacia Biotech, Little Charlton)

Extreme care was taken with this procedure to prevent DNA contamination but due to the lack of facilities available at the time of the research the precautions that were most suitable were put in to place. The extraction and PCR processes were separated by placing the different areas at opposing ends of the room or carrying out the procedures at different times with thorough cleaning and UV irradiation in between. Sterile filter pipette tips were always used to prevent cross contamination from stock solutions. A RAPD analysis bead (AmpliTaq® DNA polymerase and Stoffel fragment, 0.4 mM of

### 2.5.2 Ready-To-Go™ PCR Analysis (Amersham Pharmacia Biotech, Little Charlton)

Extreme care was taken with this procedure to prevent DNA contamination and fresh sterile filter pipette tips were always used to prevent cross contamination from stock solutions. A Ready-to-go PCR analysis bead ((1.5 units of Taq DNA polymerase, 200 μM of each dNTP in a 25 μl reaction, Bovine Serum Albumin (2.5 μg), and buffer [1.5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl, (pH 9.0) in a 25 μl volume]) was placed in a 0.2 ml PCR tube making sure the bead sits at the bottom. 25 ppmol of a single pair of primers (5 μl) was then added to the tube, followed by a quantified amount of template DNA between 5 and 50 ng and then distilled water to a final volume of 25 μl. The primers used were the same as those for RAPD (Table 2.2).

The contents of the tube were then mixed by vortexing and then centrifuged, in the microcentrifuge using adapters, for 1 sec at 13,000 rpm to collect the contents at the bottom of the tube. The reaction mixture was then overlaid with 50 μl of mineral oil and the tube then placed in the thermocycler (Applied Biosystems Geneamp™ 2700). The thermocycler was set to cycle at 95 °C for 5 minutes followed by 45 cycles at 95 °C for 1 minute, 36 °C for l minute and 72 °C for 2 minutes then to hold the samples at 4 °C. All reactions had a positive and negative control. The positive control used was 2 μl of *E. coli* C1a DNA and the negative control replaced DNA with double distilled and sterile water.

Reactions were prepared in a UV hood (Scie plas GLE-UVSC) and equipment was irradiated for 20 minutes.

### 2.5.3 Agarose Gel Electrophoresis

The amplicons produced were then analysed using a 2% agarose gel in TAE buffer. A 100 base pair ladder was used as a marker, loading 3 μl of ladder and 2 μl of tracking dye. For each of the amplified samples 10 μl were loaded with 2 μl of tracking dye. The gel was then electrophoresed at 50 mA for about 3 hours or until the tracking dye was around 3 cm away from the bottom of the gel. The gel was then removed from

the tank and then placed in a separate tank containing ethiduim bromide (0.5 μg/ml) and left for about 10 minutes. The gel was placed in a UV image analyser to visualise the bands and photograph the gel.

| Fragment | Size (kb) |
|---|---|
| A | 23.13 |
| B | 9.42 |
| C | 6.56 |
| D | 4.36 |
| E | 2.32 |
| F | 2.02 |
| G | 0.56 |

**Table 2.3:** Represents the sizes of λ *Hin*dIII molecular weight marker DNA fragments. This table was used to size the fragments of RAPD fragments of the different species using a standard curve using tan $^{-1}$ molecular weight (in kb) versus distance (mm) (Sanger *et al.* 1982).

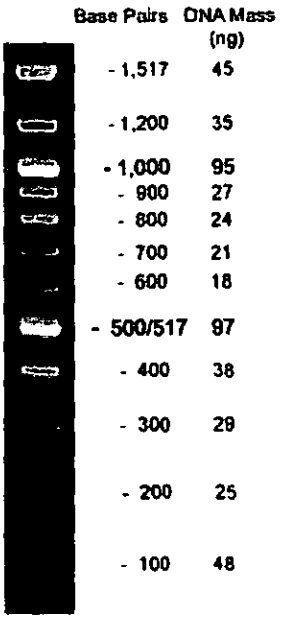



**Fig 2.1:** Represents the sizes of fragments of 100 bp ladder used in the reproduction of gels (Figures 3.9.1, 3.9.2 and 3.9.3). Fragments were sized again using a standard curve using $\tan^{-1}$ molecular weight (in kb) versus distance (mm).

# RESULTS

## 3.1 Extraction of DNA from bone

At the start of this project, there was no expertise in our laboratory in the extraction of DNA from bone, and so it was necessary to spend some time and effort in identifying a protocol suitable for this purpose. It was decided to begin by applying simple methods to fresh bone samples, and to then test the suitability of these methods for more ancient animal bones.

The Chelex extraction method, as described in the DNA extraction protocols handbook from Perkin Elmer Biosystems (materials and methods section 2.2.1), was selected for initial trials. This method was applied to small (2 mm diameter) samples of fresh animal bone, but no DNA from these sources could be detected after agarose gel electrophoresis and ethiduim bromide staining. The Chelex extraction method involved the suspension of the bone sample in an extraction buffer, and despite increasing incubation times (overnight); no DNA could be recovered using this approach. Controls using tissue samples (muscle tissue) also gave poor results, and so it was considered that the procedure could be improved by breaking up the bone sample using mechanical methods.

Use of a Potter's homogeniser to disrupt bone marrow samples removed from fresh bone material failed to produce detectable quantities of DNA and, in any case, this approach would not have been applicable to complete or ancient bone samples, where marrow could not be obtained. It was therefore decided to grind bone samples to a fine powder using a freezer mill. This apparatus, (model 6750, manufactured by Glen Creston Ltd), grinds solid bone or dental samples by impaction in sterile containers under liquid nitrogen. Samples frozen under these conditions are brittle and easily powdered by impaction. A fine bone powder was produced by this process, which was then used in subsequent extraction methods. All methods described below employed this approach.

However, initial attempts to extract DNA from the powdered bone samples using the published Chelex extraction method failed to yield detectable amounts of DNA. The extraction procedure was therefore modified to include a detergent in the buffer during the incubation of the sample at 56 °C, since it was reasoned that this should facilitate cellular lysis and release of DNA. The non-ionic detergent Triton X-

100 at a concentration of 1 % in the incubation buffer was successful in this respect, and amounts of DNA were produced that were easily observed on agarose gels, giving our first success in extracting DNA from bone.

It was then decided to refine the modified procedure further, through a comparison of Triton X-100 with the ionic detergent sodium lauryl sulphate (SLS), and by establishing the optimal incubation time with detergent for cellular lysis and DNA recovery. Extraction experiments were thus performed using multiple identical samples of fresh bone incubated with buffer containing either a 1 % Triton X-100 solution or a 1 % SLS solution. These samples were used for extraction of DNA over a range of incubation times that ranged from 1 to 18 hours (overnight). As controls, duplicates were set up without incubation. DNA was extracted from these samples as described in section 2.1, and 16 µl of the final solution was subjected to electrophoresis. In addition, the absorbances of the final solutions were determined at wavelengths of 230, 260, and 280 nm to determine whether carbohydrate or protein were present as contaminants in the samples.

This was undertaken to determine the purity of the samples that were being extracted; if the 230 nm and 280 nm values were high, then this would indicate that samples were respectively contaminated with carbohydrates and proteins. The biggest concern was that the protein fraction could include enzymes that might degrade the DNA in solution.

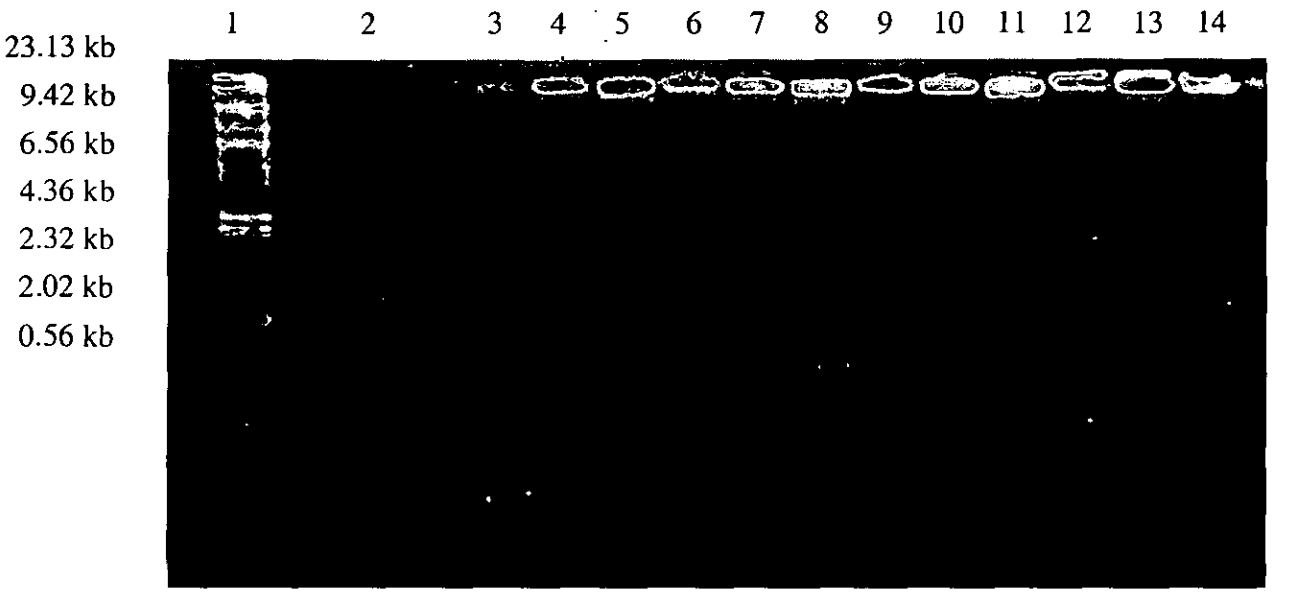
By comparing the results of agarose gel images (Figure 3.1) and the absorbance readings (Table 3.1) it was determined that an incubation time of 2 hours using the detergent SLS optimised the amount of DNA yielded by the extraction process. Incubation of samples for longer periods did not increase significantly the yield of DNA. There was also present cellular RNA, which is evident, as it appears below the 0.56 kb λ *Hin* dIII fragment. The table also shows that the samples were pure as the amount of protein present is relatively small. The protein contamination in the samples increases as the DNA yield increases. These values are typical of the results obtained throughout the project.

**Table 3.1: Effect of incubation time on yield of DNA using a modified Chelex extraction method.**

| Incubation time (hrs) | $A_{230}$ | $A_{260}$ | $A_{280}$ | Conc. of DNA (μg/ml) | Conc. of protein (μg/ml) | Yield (μg/0.1 g of bone) |
|---|---|---|---|---|---|---|
| 0 | 0.103 | 0.045 | 0.044 | 36 | 34 | 2.88 |
| 1 | 0.588 | 0.231 | 0.162 | 184.8 | 75.5 | 14.8 |
| 2 | 0.840 | 0.362 | 0.212 | 289.6 | 53.5 | 23.17 |
| 4 | 0.664 | 0.304 | 0.257 | 243.2 | 167.4 | 19.46 |
| 18+ | 0.896 | 0.342 | 0.306 | 273.6 | 214.4 | 21.89 |

The figures presented are an average of three experiments. The method incorporated SLS in the incubation buffer. Calculations for the DNA and protein concentrations were taken from Boyer (2000).

**Figure 3.1: Agarose gel electrophoresis of DNA samples prepared by the modified Chelex extraction method.**




DNA extracted from pig bone using the SLS modified Chelex method.

Samples visualised in the above agarose gel (lanes 2 – 14) were incubated for various times in the presence of SLS. Their incubation times, and the amounts of the DNA sample loaded on to the gel are indicated in the table below.

Lane 1 contains MW marker DNA (λ *Hin* dIII)

**Table 3.2: Represents incubation times of samples and amount loaded on to gel.**

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Incubation time (h) | 0 | 0 | 1 | 1 | 1 | 2 | 2 | 2 | 4 | 4 | 4 | 18+ | 18+ | 18+ |
| Volume of DNA solution loaded on to gel (μl) | 20 | 20 | 2 | 10 | 20 | 2 | 10 | 20 | 2 | 10 | 20 | 2 | 10 | 20 |

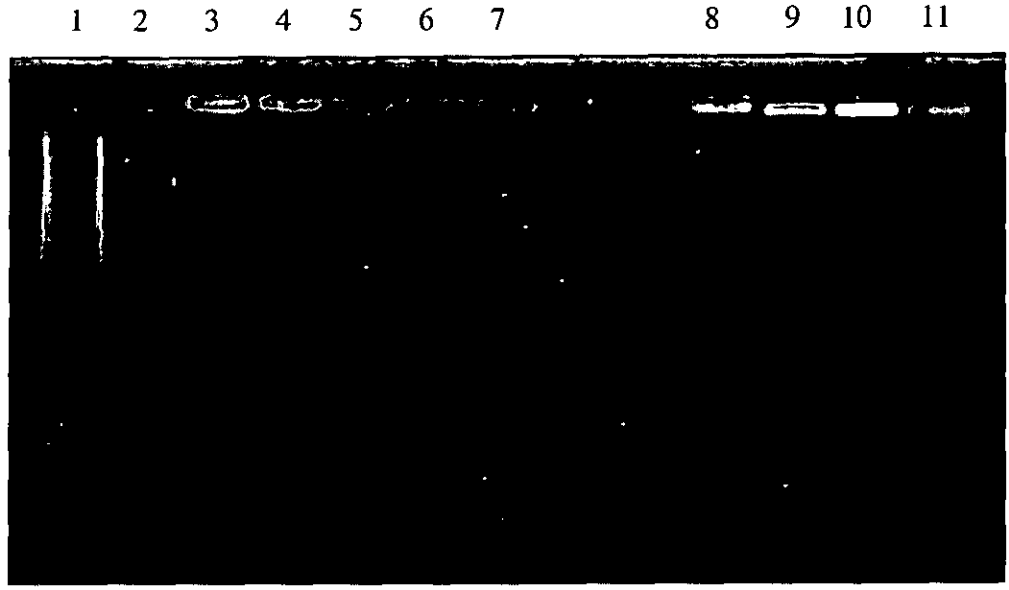
The next step taken was to investigate other extraction methods to compare their efficiency and yield with the modified Chelex method. Two manufactured kits were chosen; DNAce (Bioline, London) and Genomestar (Hybaid). Both methods, described in sections 2.2.2 and 2.2.3, were followed according to the manufacturers' instruction using 0.1 g of bone powder produced using the freezer mill.

The DNAce kit used a lysis buffer to split open the cells to release the DNA, which then attached itself to the carrier suspension. This then was centrifuged to produce a pellet, which was washed to remove any impurities, and an elution buffer was added to the pellet to remove it from the carrier suspension. The supernatant (60 μl) was placed in a fresh 1.5 ml microfuge tube and was then ready for analysis.

The Genomestar kit uses a different lysis buffer and the lysate is de-proteinised using chloroform. The manufacturer does not describe the composition of these buffers. DNA was precipitated out using a second buffer and then re-suspended using a third buffer. Ethanol was then used to precipitate the DNA to remove any impurities from the mixture and centrifuged to produce a DNA pellet, which was finally resuspended in TE buffer.

Samples from each of the different methods were quantified using known standards and electrophoresed on an agarose gel and visualised using ethiduim bromide. The results are shown in Figure 3.2.

**Figure 3.2: Visualisation of DNA obtained by three different extraction methods, by agarose gel electrophoresis.**




Lanes 1 – 7 include the following amounts of Herring sperm DNA for quantification:

Lane 1. 2 μg
Lane 2. 1 μg
Lane 3. 0.5 μg
Lane 4. 0.2 μg
Lane 5. 0.1 μg
Lane 6. 0.05 μg
Lane 7. 0.01 μg

DNA extracted from fresh pig bone is shown in the remaining lanes:

Lane 8. DNA extracted by modified Chelex, using SLS.
Lane 9. DNA extracted by DNAce kit.
Lane 10. DNA extracted by Genomestar kit.
Lane 11. DNA extracted by Genomestar kit.

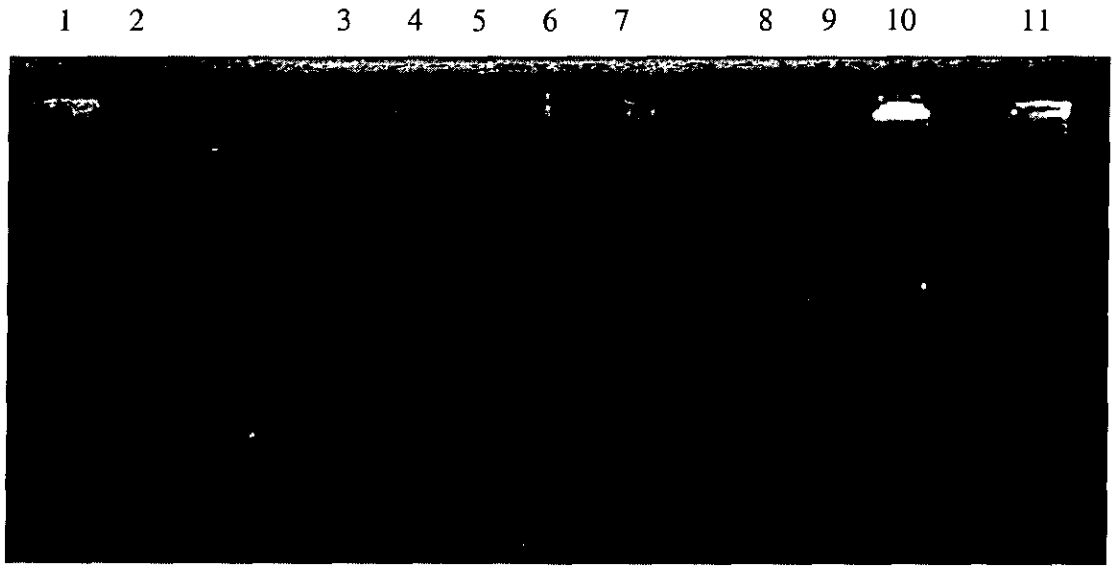
From Figure 3.2 it was concluded that both the DNAce kit, represented by lane 9, and the Genomestar kit, represented by lanes 10 and 11, extracted around 0.5 μg DNA from 0.1g of bone. The original method for the Genomestar kit produced a final volume of over 1.5 ml; in order to overcome this problem the amounts stated in the original method were split between two tubes. The contents from each extraction were electrophoresed. It was also evident from the results that the Genomestar kit also extracted significant amounts of RNA, as shown by the bright band at the bottom of the DNA band in lane numbers 10 and 11.

Although both of the manufactured kits were efficient in the extraction of DNA, the Genomestar kit extracted RNA with DNA. Given our preliminary experiences with the three methods, it was decided to proceed with the Chelex method on the grounds of cost, convenience and the ability to modify the procedure.

### 3.2 PCR amplification of DNA samples

Quantification gels (Figure 3.3) were used to estimate the amount of DNA in samples for use in PCR reactions. For the Ready-to-go® RAPD-PCR kit, the recommended amounts of DNA used for the reaction were between 5 and 50 ng. Following the method described in Section 2.5.1 for RAPD-PCR, samples from all three extraction methods were used in the reaction and in this experiment primer set 1 was used. The RAPD kit provided a choice of short random primers for use. Nucleotide sequences of these primers are detailed in Table 2.2. The DNA used for these experiments was taken from extracted samples using the Chelex method and the samples extracted with the DNAce and Genomestar kits.

**Figure 3.3: Visualisation of RAPD-PCR amplicons from DNA extracted using three different extraction methods.**




Primer 2 was used for these experiments and DNA was extracted from fresh pig bone. Lane 1 is the positive control (*E. coli)* supplied with the RAPD-PCR analysis kit and Lane 2 contains the negative control.

Lanes 3 – 7 were samples extracted using the modified Chelex method using SLS. Lane 8 was amplified from DNA extracted using the DNAce kit and Lane 9 used extracted DNA using the Genomestar kit.

Herring sperm DNA in lane 10 was used as a second standard.

Lane 11 contains MW marker λ *Hin* dIII.

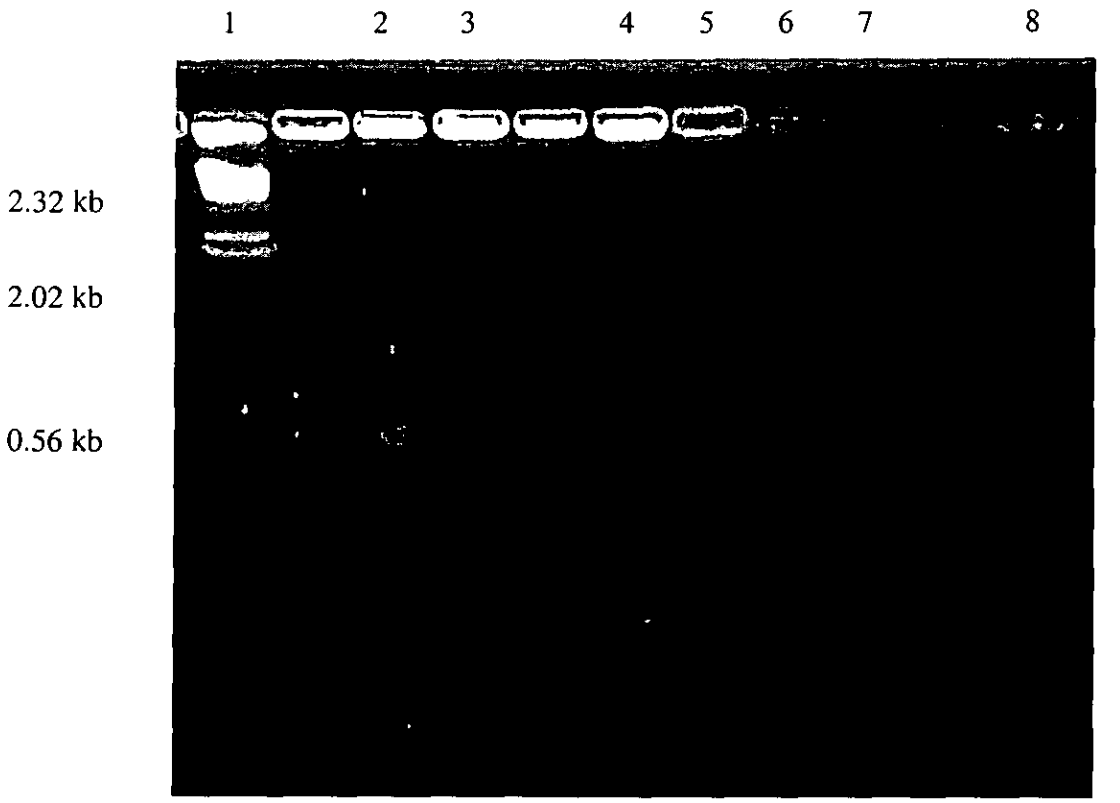
Lanes 6 and 9 show very clear amplicons at approximately 1.02 kb and 0.79 kb. These amplicons are also evident in lane 8. In all three of these lanes there is also evidence of a number of amplicons, which range between 0.36 kb and 0.51 kb in size but because they are so close together it is impossible to determine how many amplicons there are. There also evidence of a further band present in lane 9, of about 0.18 kb.

The Herring sperm DNA (lane 10) did not produce a banding pattern at first. This was found to be a result of the sample not being adequately sheared to smaller fragments. The reason for its use as a second standard was so that we had a sample of a larger eukaryotic genome, as this was what we would be mainly dealing with, as opposed to a prokaryotic genome such as that of *E. coli* which was provided with the kit as a reference DNA sample.

DNA extracted from bone by the modified Chelex procedure (lanes 3 – 7) failed to yield reproducible results. Only lane 6 revealed any amplicons after PCR amplification, even though DNA was present in each sample. This result led to the conclusion that the PCR reaction was being in some way inhibited.

The first step to find the cause was to take different elements of the method and change them to see if the results of the PCR improved. The first change was to remake the 5 % Chelex solution; this was done in order to eliminate it as a problem. The next modification that was tried was the use of the extraction method with and without detergent. Figure 3.4 below shows results obtained using samples extracted with and without the use of detergent. Those samples with detergent produced no amplicons whereas the samples produced without the detergent removed produced a banding pattern, even though the image was slightly blurred. Blurring resulted from electrophoresing samples at a high voltage: lower voltages and longer times for electrophoresis subsequently produced tighter, more distinct amplicons.

**Figure 3.4: Effects of detergent during extraction on the PCR reaction.**




The experiment shows the effect of detergent (SLS) during extraction from fresh pig bone on the PCR reaction.

Lane 2 is the positive control (*E. coli*) supplied with RAPD-PCR analysis kit and lane 3 is the negative control.

Lanes 4 and 6 contain samples extracted without using SLS detergent during incubation.

Lanes 5 and 7 contain samples that were extracted including SLS in the incubation period.

Lane 8 contained Herring sperm DNA to use as a second standard.

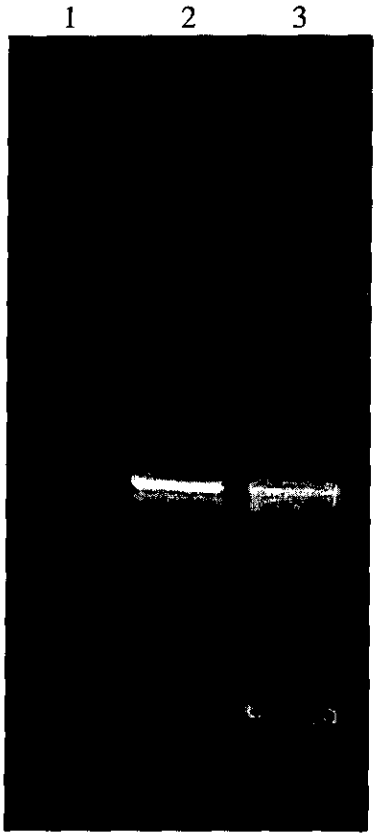
The fragment sizes found in the negative control sample (lane 3) are 1.04 kb, 0.74 kb and 0.49 kb. All these fragments sizes are very similar to those from Figure 3.3 representing pig DNA using primer 2, and are interpreted as being characteristic of amplicons produced from pig DNA using these primers. Although lanes 4 and 6 are blurred, it is still evident from Figure 3.4 that the banding pattern is the same as that of the negative sample. Therefore it was believed that the negative sample was contaminated with the DNA under analysis.

The conclusion from this experiment was that the detergent included during the incubation period of the extraction process was inhibiting the PCR reaction. Therefore because of this result it was decided that the Chelex method should omit the detergent from the process (c.f. pg 39).

### 3.3 Comparison of Ready-to-go® RAPD-PCR with Ready-to-go PCR®.

Amersham Pharmacia produce two PCR kits: the Ready-to-go RAPD kit and the simpler Ready-to-go PCR product. Since the latter was cheaper, it was decided to compare the two kits for use in these experiments; using the *E. coli* positive control supplied with the kits we used both PCR reactions in order to compare them. Figure 3.5 shows results from both kits with the *E. coli* control DNA.

**Figure 3.5: The differences between Ready-to-go PCR beads and Ready-to-go RAPD-PCR.**




This experiment was to compare the two different PCR beads in an attempt to decide which was best for use in this research. Lane 1 contains a negative control. Lane 2 contains *E. coli* DNA amplified using the Ready-to-go® PCR beads and lane 3 contains *E. coli* DNA amplified using the Ready-to-go® RAPD-PCR beads.

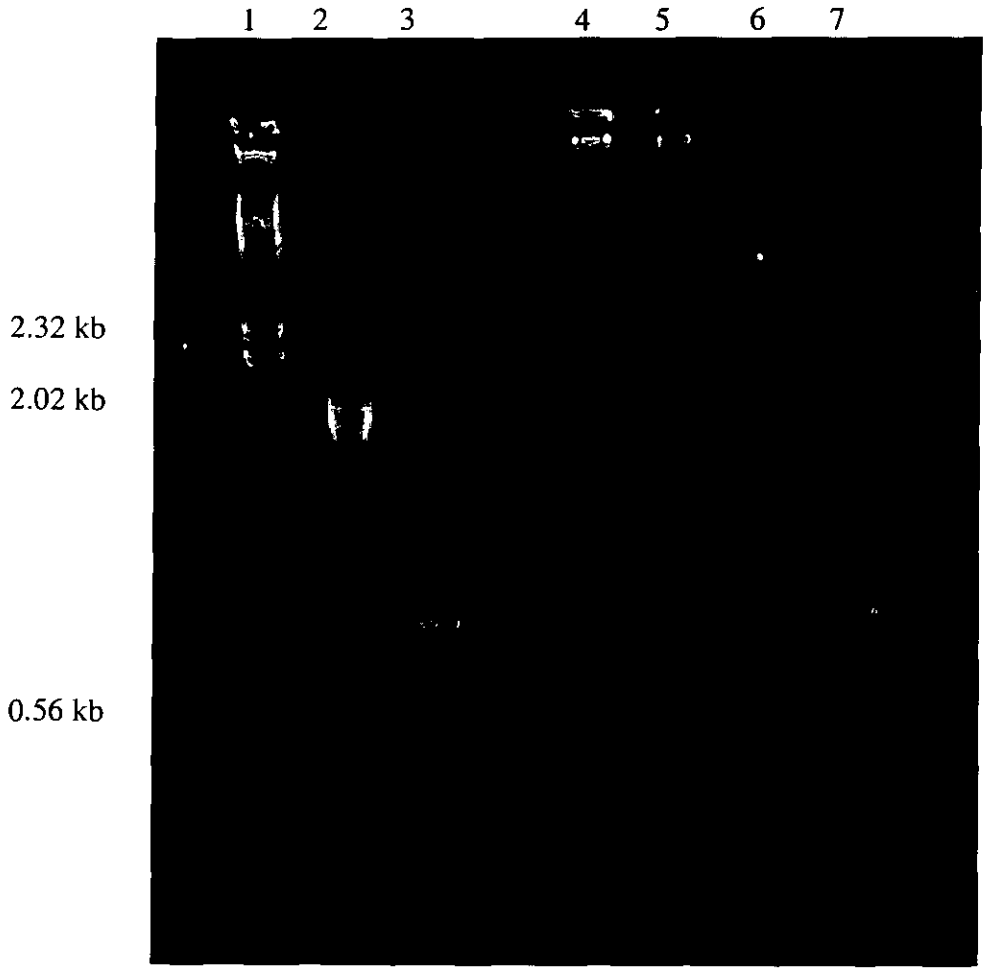
Contamination was again present in the negative sample shown by lane 1. It was also apparent that the number of amplicons produced using the Ready-to-go® PCR beads (lane 2) was less than the number of amplicons produced by the Ready-to-go® RAPD-PCR beads (lane 3). The reason attributed to extra amplicons was an ingredient contained in the RAPD bead that was missing from the PCR bead, which was Stoffel fragment. It was preferable to have more amplicons present for species identification as it makes the process more precise.

The possibility of multiplexing with the slightly cheaper kit was then explored. Multiplexing is a technique whereby a number of different primers are used in the reaction in order that more regions can be copied simultaneously. This was done to increase the number of amplicons produced, which would then increase the discrimination of the test. In order for this reaction to work successfully the primers involved need to be compatible. To be compatible the primers need to have similar annealing temperatures and also excessive regions of complementarities to avoid primer-dimers, where they bind to each other and not the template DNA (Butler, 2001).

Unfortunately this attempt failed to show any viable results as there was no clear distinction between bands they all blurred together. Therefore the decision was made to stick with the Ready-to-go RAPD-PCR beads as it produced a much wide-ranging profile pattern.

A dilution experiment was then set up to determine which concentration of Herring sperm DNA would produce the best profile pattern so it could be used as a control after being cut with the restriction enzyme. Figure 3.6 shows the different dilutions used and which is best in the PCR reaction.

**Figure 3.6: Agarose gel electrophoresis of PCR amplicons produced using different dilutions of Herring sperm DNA.**




The experiment was to determine what dilution of 10mg/ml solution of Herring sperm DNA is required to produce viable amplicons during RAPD-PCR. Lane 2 is the positive control (*E. coli*) supplied with RAPD-PCR analysis kit and lane 3 is the negative control. Lane 1 contains MW marker DNA (λ *Hin dIII*)

Lanes 4-7 contains different concentrations of Herring sperm DNA:

Lane 4: 1 μg/μl solution.

Lane 5: 100 ng/μl solution.

Lane 6: 10 ng/μl solution.

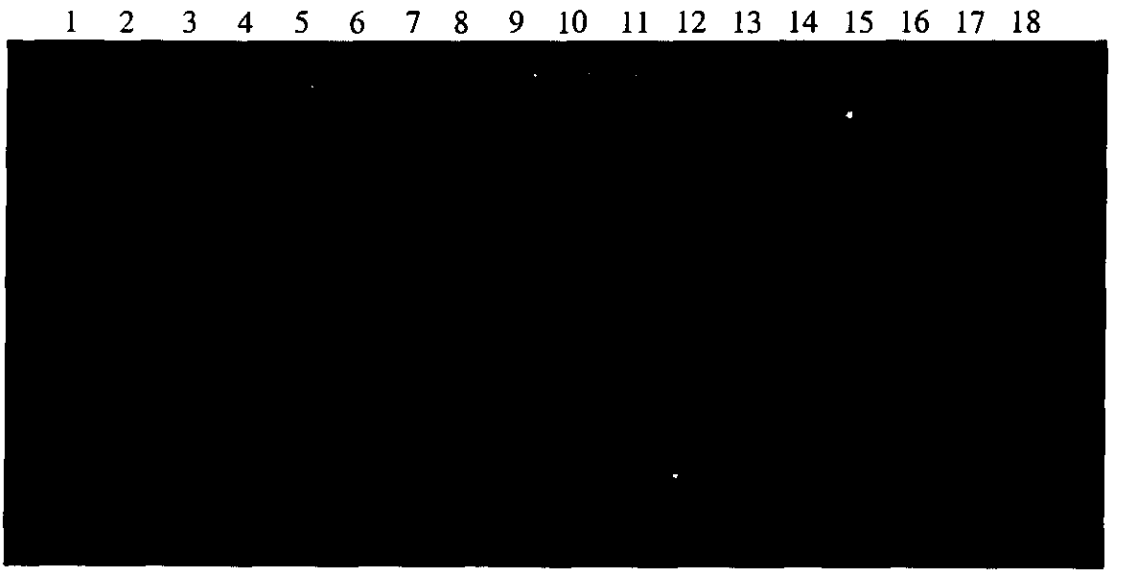
Lane 7: 1 ng/μl solution.

The dilution that produced the best profile pattern was between 10 ng/μl and 1 ng/μl of DNA this was shown in lanes 5 and 6. Contaminating DNA is present in the negative sample with a fragment size of 0.77 kb.

### 3.4 Comparisons of DNA samples from different species

After the control dilution concentration was determined we went on to grind more samples of bone from different species to compare profile patterns for individual species. The first sample used for comparison with original pig bone was bovine. Each different bone sample was extracted using the chelex method and then some of the samples were treated with ethanol in order to precipitate the DNA in an attempt to clean up the samples by removing any impurities. This approach was used to see if this made a difference in the amplicons and their resolution. Each of the differently treated samples were put in the PCR reaction using both 2 μl of template DNA and 5 μl of template DNA and also using each of the four different primers. This experiment is represented by the figure 3.7; it was an attempt to study several different factors at the same time.

**Figure 3.7: Visualisation of PCR products amplified using several different factors including different primers, species, amounts of DNA and treatments.**




In this experiment a number of different factors were used in PCR amplification in order to compare results. These different factors included two different species, different primers, different amounts of template DNA and whether the template DNA was treated with or without ethanol precipitation. Table 3.3 explains what each sample is in the different lanes.

**Table 3.3: Represents the positions of samples visualised in Figure 3.7.**

| | Species | Amount of DNA | Primer | Ethanol precipitation |
|---|---|---|---|---|
| 1 | *E. coli* | 1 µl | 1 | - |
| 2 | Negative control | 0 | 1 | - |
| 3 | Pig (*sus scrofa*) | 2 µl | 1 | - |
| 4 | Pig (*sus scrofa*) | 2 µl | 1 | + |
| 5 | Pig (*sus scrofa*) | 5 µl | 2 | - |
| 6 | Pig (*sus scrofa*) | 5 µl | 2 | + |
| 7 | Pig (*sus scrofa*) | 2 µl | 3 | - |
| 8 | Pig (*sus scrofa*) | 2 µl | 3 | + |
| 9 | Pig (*sus scrofa*) | 5 µl | 4 | - |
| 10 | Pig (*sus scrofa*) | 5 µl | 4 | + |
| 11 | Bovine (*Bos bubalis*) | 2 µl | 1 | - |
| 12 | Bovine (*Bos bubalis*) | 2 µl | 1 | + |
| 13 | Bovine (*Bos bubalis*) | 5 µl | 2 | - |
| 14 | Bovine (*Bos bubalis*) | 5 µl | 2 | + |
| 15 | Bovine (*Bos bubalis*) | 2 µl | 3 | - |
| 16 | Bovine (*Bos bubalis*) | 2 µl | 3 | + |
| 17 | Bovine (*Bos bubalis*) | 5 µl | 4 | - |
| 18 | Bovine (*Bos bubalis*) | 5 µl | 4 | + |

The bovine DNA showed the best results but the use of ethanol precipitation was inconsistent in this case. All the different primers were used and it was concluded that primers 3 & 4 seemed the best candidates for the experiments using all the different species, as they were the ones that showed viable results.

All the different species that had been gathered were ground using the freezer mill and extracted again using the modified Chelex method. Each sample was then quantified using the UV- Vis spectrophotometer because it seemed a more convenient way of quantifying the DNA rather than an estimation using the agarose gel method. Table 3.4 shows the results from spectrophotometric analysis, from which we then calculated the yield as in previous experiments.

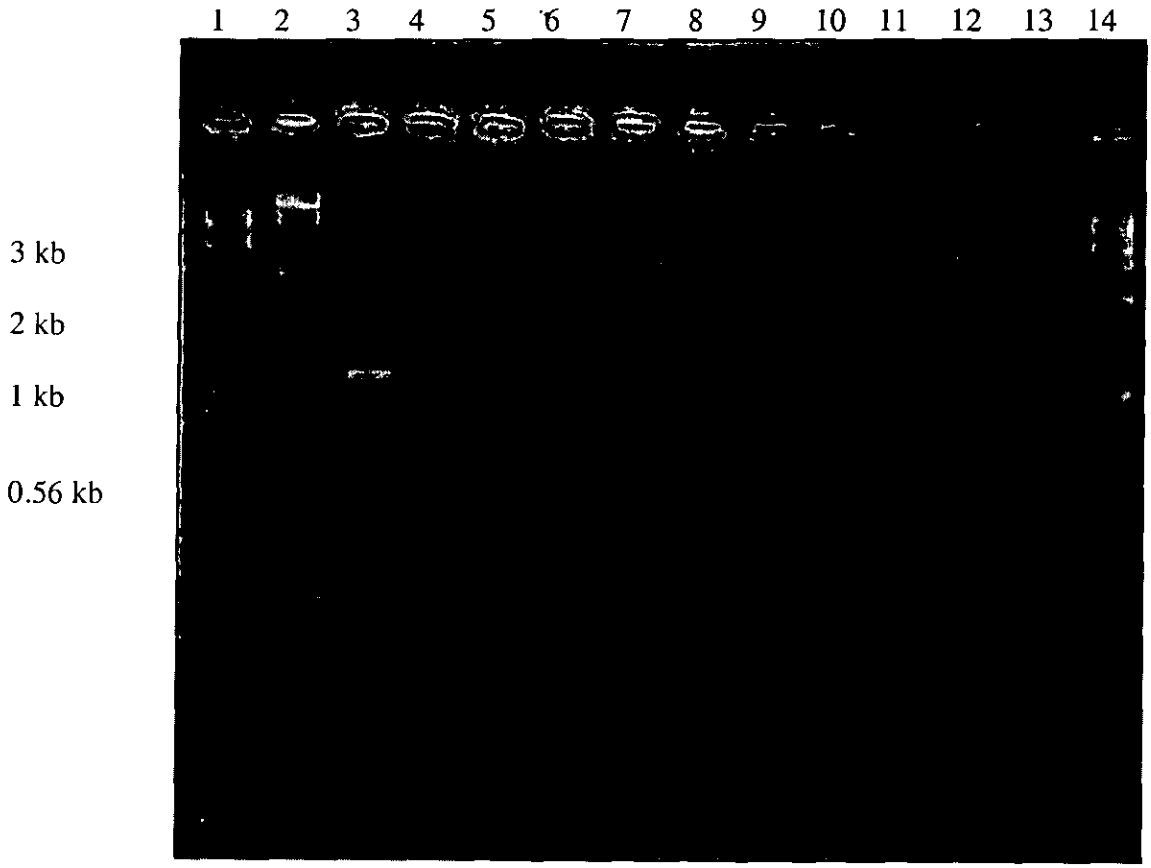
These were results were used to calculate amount of DNA needed to put into the PCR reaction to produce viable results. Primers 3 & 4 were used for comparison. Figures 3.8 and 3.9 represent the different species used and the two different primers used for comparison.

**Table 3.4: Yields of DNA obtained from different species extracted using the modified Chelex method.**

| Species | Dilution factor | $A_{230}$ | $A_{260}$ | $A_{280}$ | Conc$^{n}$ (μg/ml) | Yield (μg/0.1g of bone) |
|---|---|---|---|---|---|---|
| Pig | 100x | 0.442 | 0.224 | 0.161 | 896 | 358.4 |
| Bovine | 50x | 0.525 | 0.286 | 0.209 | 572 | 205.9 |
| Lamb | 20x | 0.642 | 0.242 | 0.169 | 193.6 | 69.7 |
| Rabbit | 20x | 0.400 | 0.070 | 0.043 | 56 | 17.9 |
| Roe deer | 50x | 0.611 | 0.188 | 0.136 | 376 | 142.9 |
| Wild boar | 50x | 0.557 | 0.258 | 0.179 | 516 | 206.4 |
| Dog | 20x | 0.403 | 0.072 | 0.046 | 57.6 | 23 |
| Unknown long bone | 100x | 0.503 | 0.241 | 0.192 | 964 | 173.5 |
| Unknown scapula | 50x | 0.137 | 0.022 | 0.018 | 44 | 13.2 |

This table lists the yields of DNA obtained from the different species being analysed extracted using the modified chelex method.

**Figure 3.8.1: Visualisation of PCR products amplified using primer 3.**




The figure represents a RAPD-PCR profile of a number of different species using primer 3. Lane 3 is the positive control (*E. coli*) supplied with RAPD-PCR analysis kit and lane 4 is the negative control.

Lanes 5-13 were the different species used:

Lane 5: Pig

Lane 6: Cow

Lane 7: Lamb

Lane 8: Rabbit

Lane 9: Roe deer

Lane 10: Wild boar

Lane 11: Dog

Lane 12: Unknown long bone

Lane 13: Unknown scapula

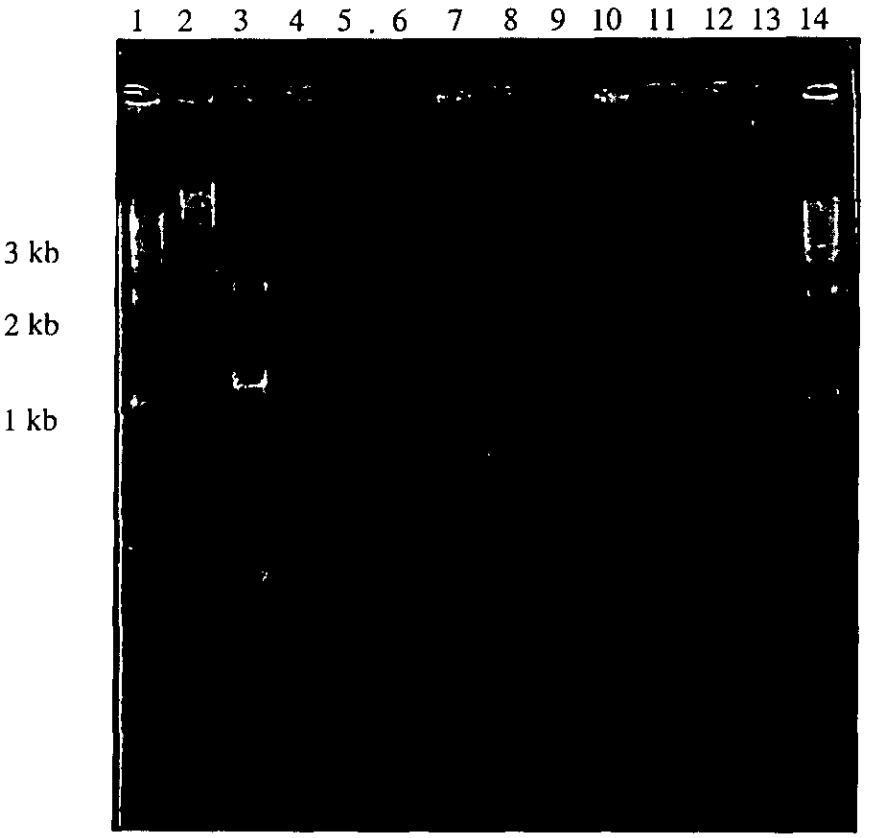
Lanes 1 & 14 contain 1kb ladders and lane 2 contains MW marker DNA (λ *Hin dIII*).

**Table 3.5: Different fragment sizes of PCR amplicons of different species visualised in Figure 3.8.1.**

| Species | Fragment size (kb) |
|---|---|
| Pig | 0.48, 0.81 |
| Cow | 0.25, 0.62 |
| Lamb | No amplicons present |
| Rabbit | 0.69 |
| Roe deer | No amplicons present |
| Wild boar | 0.58 |
| Dog | No amplicons present |
| Unknown long bone | No amplicons present |
| Unknown scapula | 0.25, 0.51 |

Represents the amplicons present on the gel for each species and their sizes. Contamination was present in the negative sample and the band was 0.53 kb in size. This size does not correspond with any of the other species fragment sizes present on the gel.

**Figure 3.8.2: Visualisation of PCR products amplified using primer 4.**




The figure represents a RAPD-PCR profile of a number of different species using primer 4. Lane 3 is the positive control (*E. coli*) supplied with RAPD-PCR analysis kit and lane 4 is the negative control.

Lanes 5-13 were the different species used:

Lane 5: Pig

Lane 6: Cow

Lane 7: Lamb

Lane 8: Rabbit

Lane 9: Roe deer

Lane 10: Wild boar

Lane 11: Dog

Lane 12: Unknown long bone

Lane 13: Unknown scapula

Lanes 1 & 14 contain 1kb ladders and lane 2 contains MW marker DNA (λ *Hin dIII*).

**Table 3.6: Different fragment sizes of PCR amplicons of different species visualised in Figure 3.8.2.**

| Species | Fragment size (kb) |
|---|---|
| Pig | 0.45 |
| Cow | 0.16 |
| Lamb | No amplicons present |
| Rabbit | 0.51, 1.26, 1.38 |
| Roe deer | No amplicons present |
| Wild boar | No amplicons present |
| Dog | No amplicons present |
| Unknown long bone | No amplicons present |
| Unknown scapula | 0.14, 0.20, 0.92 |

Represents the amplicons present for each species and their sizes on the gel image (Figure 3.8.2).

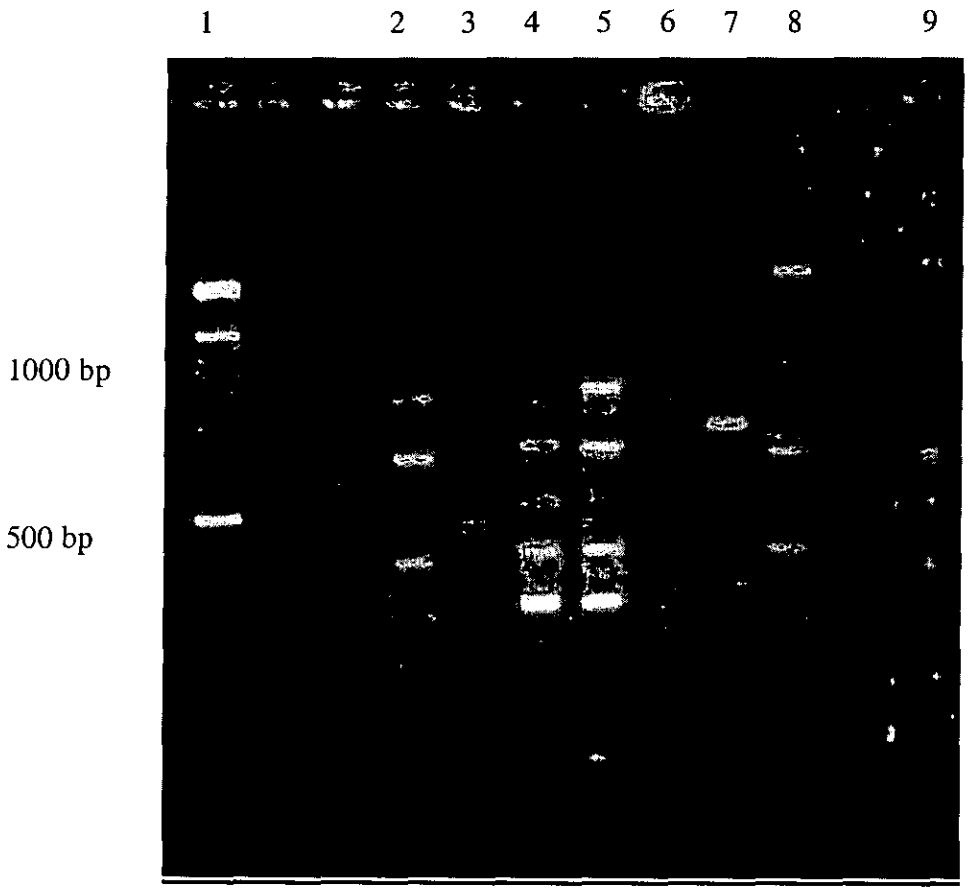
This gel does not have any contaminating DNA present, as well as this there was no presence of a profile for roe deer and the unknown long bone DNA. There is slight smearing and the possibility of DNA but no profiles for lamb, wild boar and dog. This smearing was not evident in the primer 3 experiment.

From these results it is evident from the profiles that they are different, unfortunately we were unable to verify what animal the unknown samples were from our results, only that they were not any of the samples that produced profiles.

### 3.5 Further RAPD-PCR analysis.

The results obtained in this section were obtained through collaboration with my colleague Ashley Matchett. It was decided to repeat and expand the RAPD-PCR profiles of the different species using primers 2,3 and 4. The objective was to obtain a wider range of RAPD-PCR profiles with a view to comparing these with previous results. The amplified samples were examined as before on 2 % agarose gels by electrophoresis, but with a 100 bp ladder as molecular weight markers for more accurate estimation of amplicon size. The use of these molecular weight markers facilitated the measurement of the sizes of small fragments. The results are depicted in figures 3.9.1, 3.9.2 and 3.9.3. The control used was a negative extraction control, where at the extraction stage no bone powder was added in order to trace contamination.

**Figure 3.9.1: Reproduction of RAPD-PCR species profiles using primer 2.**




The figure represents a RAPD-PCR profile of a number of different species using primer 2. Lane 9 is a negative extraction control.

Lanes 2 – 8 were the different species used:

Lane 2: Unknown long bone

Lane 3: Unknown scapula

Lane 4: Wild boar

Lane 5: Pig

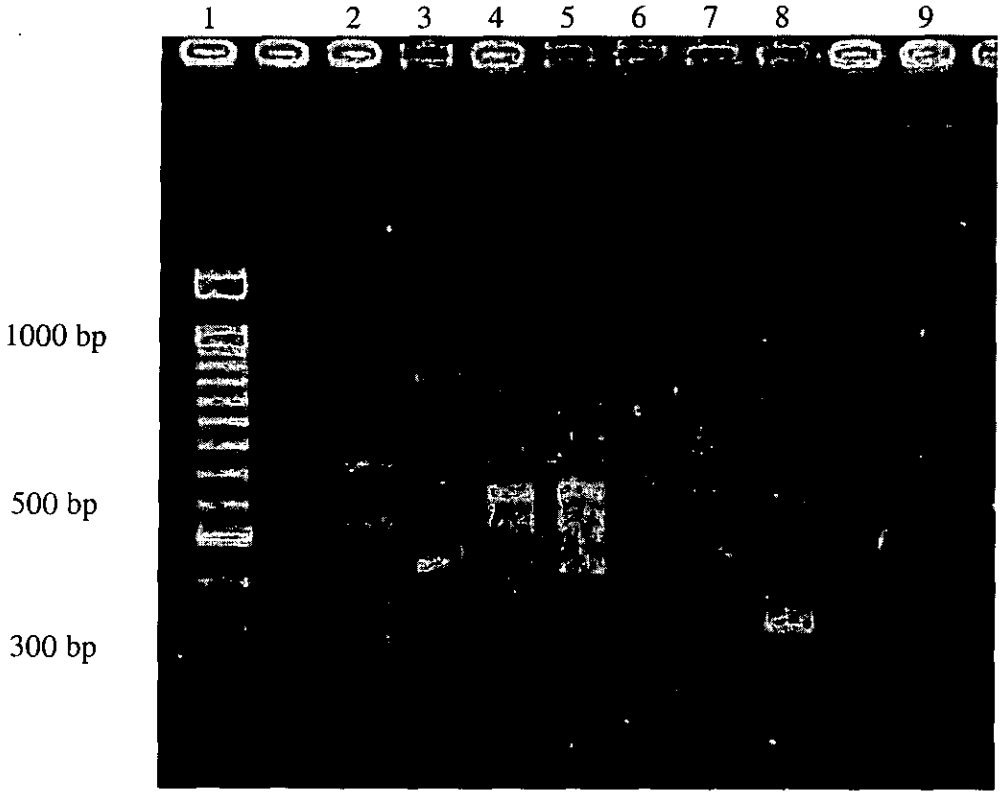
Lane 6: Roe deer

Lane 7: Dog

Lane 8: Lamb

Lane 1 contains 100 bp ladder as a molecular weight marker.

**Figure 3.9.2: Reproduction of RAPD-PCR species profiles using primer 3.**




The figure represents a RAPD-PCR profile of a number of different species using primer 3. Lane 9 is a negative extraction control.

Lanes 2 – 8 were the different species used:

Lane 2: Unknown long bone

Lane 3: Unknown scapula

Lane 4: Wild boar

Lane 5: Pig

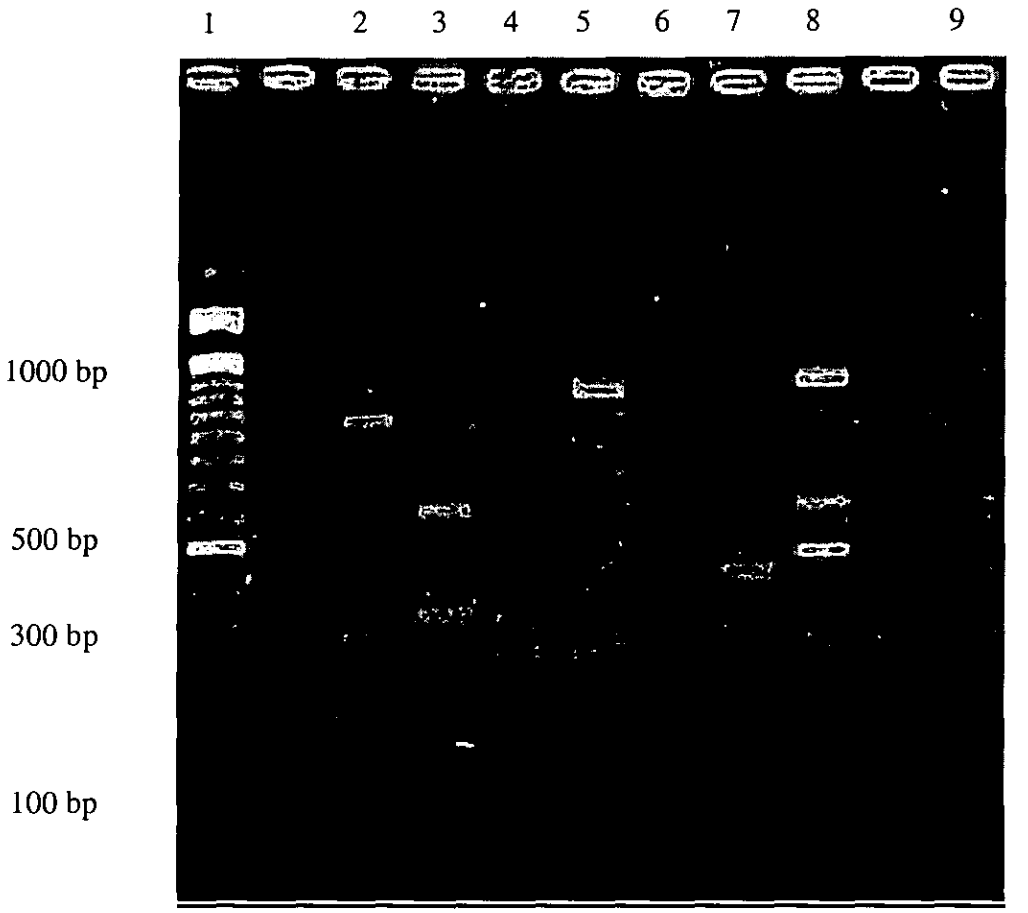
Lane 6: Roe deer

Lane 7: Dog

Lane 8: Lamb

Lane 1 contains 100 bp ladder as a molecular weight marker.

**Figure 3.9.3: Reproduction of RAPD-PCR species profiles using primer 4.**




The figure represents a RAPD-PCR profile of a number of different species using primer 3. Lane 9 is a negative extraction control.

Lanes 2 – 8 were the different species used:

Lane 2: Unknown long bone

Lane 3: Unknown scapula

Lane 4: Wild boar

Lane 5: Pig

Lane 6: Roe deer

Lane 7: Dog

Lane 8: Lamb

Lane 1 contains 100 bp ladder as a molecular weight marker.

Figures 3.9.1, 3.9.2 and 3.9.3 showed an increased number of bands compared to the previous gels shown in figures 3.8.1 and 3.8.2. The unknown samples that were analysed show no similarity to any of the other species profiles on the gel and are they themselves individual concluding that they came from different species. None of gels (Figures 3.9.1, 3.9.2 and 3.9.3) show evidence of a profile from Roe deer, which agrees with the result from the earlier gel (Figures 3.8.1 and 3.8.2). The profile created for the dog sample on each of the reproduced gels contained fewer bands than the other species. The profiles of the wild boar and pig are both similar but have enough differences to be able to distinguish between them. The extraction controls of each gel seem to be contaminated.

# 4 DISCUSSION

The general aims of this research were to find an effective method for extracting DNA from bone and to use this methodology to create RAPD-PCR profiles of different species. The initial aim was to find an appropriate extraction method, and the first technique tried involved the use of Chelex resin. At the start of the project, we were concerned primarily with establishing this method in the laboratory for bone samples. In response to the initial lack of success, the inclusion of detergent in the extraction process, and the application of a freezer mill, was tried. Although detergent increased the yield of DNA, residual amounts of detergent interfered with subsequent PCR amplification. This was evident from results of the experiments with and without detergent at the incubation stage of the extraction process (Figure 3.4).

However, the use of the freezer mill to grind the produced good results. The next step after gaining these results was to look at the incubation time for samples in extraction buffer; we reduced the amount of time given by the Perkin Elmer handbook to see if the yield was affected. The results indicated that 2 hours was sufficient time to gain a suitable yield. Different manufactured kits were used for comparisons and to check suitability but although they were effective the decision was made to stick with Chelex method, as it was more adaptable.

The next stage was to carry out PCR analysis of the DNA solutions extracted from bone samples. RAPD-PCR was used as it was a completely random reaction and no primer design was required, enabling us to complete more research in the allotted time. Researchers including Lee *et al.* (1994) and Koh *et al.* (1998) have used this method to distinguish different species. Lee *et al.* (1994) had also used bone fragments and so it was decided this was an appropriate route to take. This method of PCR was, like any other, very sensitive. Contamination of the negative was apparent. Table 4.1 represents different precautions that can be taken to avoid contamination issues.

| Contamination sources | Precautions taken |
|---|---|
| Handling sample | Gloves to be used while handling any sample. |
| Contamination of sample during extraction. | Wear protective clothing e.g. lab coat, glasses, gloves, etc.<br>Keep extraction area completely separate from PCR area.<br>Make sure reagents are changed regularly and new tips for every stage of reaction.<br>Positive and negative extraction controls. |
| Contamination of sample during PCR. | Wear protective clothing e.g. lab coat, glasses, gloves, etc.<br>Keep PCR area completely separate from extraction area.<br>Make sure reagents are changed regularly and new tips for every stage of reaction.<br>Positive and negative PCR controls.<br>Dedicated equipment for the PCR process.<br>Daily irradiation of equipment by UV light. |

**Table 4.1:** The table shows different stages of the process at which a sample can be contaminated and precaution that can be taken to avoid it happening. This is an integral part of dealing with ancient DNA samples as many contain low copy number (LCN) DNA and so will be out-competed with by recent contaminating DNA. Information taken from Handt *et al.* (1994).

The precautions described in Table 4.1 were taken, with the exception that, due to lack of space, we were unable to put a lot of distance between each working area, although all PCR reactions took place in a UV hood, which was irradiated after each use. With the experience I have gained through this research more stringent controls would be put in place especially if dealing with ancient remains. In order to control the process properly a human RAPD-PCR profile would need to be done for extraction controls but also to help distinguish between bone fragments.

However, research into Ready-to-go™ RAPD analysis beads has revealed that bands and/or smears in negative control are normal with RAPD analysis (Williams *et al.* 1990). These bands are thought to arise from small amounts of DNA contamination purified with the polymerases, and these are not apparent if there is sufficient template DNA from the sample under test to out-compete the contaminating DNA that may be present. This observation fits with our experience in these experiments, as bands equivalent to those seen in the negative controls are not observed when template DNA of good quality is added to the PCR reaction.

The results in Figure 3.6 support the idea of the negative controls showing bands due small amounts of DNA contamination in the polymerases. Lane 4 contained a 1 μg/μl solution of herring sperm DNA and showed no bands, leading to the belief that at this concentration there was too much template DNA which overloaded the DNA polymerase and therefore was unable to amplify. Lanes 5 and 6 produced identical bands therefore a concentration of between 100 ng/μl and 10 ng/μl was sufficient to produce a species profile. Lane 7 had only a 1 ng/μl of DNA solution and although there are bands, they do match any of the bands in lanes 5 and 6. This lead to the belief that there is insufficient template DNA to amplify so any extraneous contaminating DNA has been amplified in its place.

The experiment to compare the Ready-To-Go™ RAPD Analysis kit and the Ready-To-Go™ PCR Analysis kit confirmed that the action of both Stoffel fragment and Amplitaq® DNA polymerase contained in the RAPD analysis kit, as opposed to just Taq DNA polymerase alone, produced smaller amplicons, thus permitting greater distinction between species. The Stoffel fragment itself is more specific and efficient during the PCR reaction (Varadaraj and Skinner, 1994) than Amplitaq® DNA polymerase but the combination of the two works better than simply Taq DNA polymerase. It was therefore decided to forego the possibility of multiplexing using

the Ready-to-go system and multiple primer pairs, and to use the RAPD products solely.

When choosing the species to be included in this project for analysis, choices were primarily governed by the availability of the different animals. However, given the potential application of this approach to archaeological excavations in the UK, it was important to select species indigenous to these islands. 4 out of the 9 samples of bone were fresh and so there was no need to check the suitability of the bones and the condition they were in. The rabbit samples supplied from an excavation in North Wales were in good condition and had no pitting and the surface was not soft and brittle. Researchers have shown that bones have to be in a suitable condition or the likeliness of extracting good quality DNA is poor (Hagelberg *et al.* 1991 and Haynes *et al.* 2002). With the two unknown bone samples, the scapula was in good condition whereas the long bone was pitted near the diaphysis and was slightly softer. The condition of these two bones were thought to have played an integral part in whether the DNA extracted from the samples was viable for PCR amplification as initially the unknown long bone produced no results (Figures 3.8.1 and 3.8.2). Further research showed that in fact profiles could be produced from DNA extracted from this sample (Figures 3.9.1, 3.9.2 and 3.9.3). Careful choice of sample is still needed in order to reduce the probability of failure due to poor quality DNA.

The unknown long bone as described previously was not in excellent condition, also when the sample was extracted the supernatant was discoloured, being orange/brown in appearance. This colouration could be due to humic and fulvic acids in the soil leaching into the sample and these substances are known PCR inhibitors, which could have contributed to the initial lack of results (Burger *et al.* 1999, Paabo *et al.* 1989, Paabo, 1989 and Hagelberg *et al.* 1991).

The final amplified products of each species obtained using the two different primers pairs 3 and 4 were electrophoresed on a 2 % agarose gel but not all samples produced a RAPD profile. The four bone samples that consistently showed no results after electrophoresis were lamb, roe deer, dog and the unknown long bone (Figures 3.8.1 and 3.8.2). The dog sample was the only sample that was teeth not bone; there were very few numbers of teeth and they were very small in size so it was thought that there was possibly that not enough DNA extracted or it was highly fragmented and unable to amplify. The further RAPD-PCR analysis showed that a profile was achievable from both dog, lamb and as mentioned previously the unknown long bone

but roe deer sample still produced no results (Figures 3.9.1, 3.9.2 and 3.9.3). It is unclear why the roe deer samples did not produce any viable results.

Another problem could have been that, because this method makes use of random primers, the annealing temperature is slightly lower at 35-39 °C compared to 50-55 °C during amplification (Bowditch *et al.*, 1994). This lower temperature then allows for non-specific binding to the DNA, which then may result in amplified products not being reproducible (Hadrys *et al.*, 1992 and Ellsworth *et al.*, 1993). Despite this several researchers have shown that if special care is taken and a standardised protocol is followed then reproducible bands are achievable (Hadrys *et al.*, 1992, Bowditch *et al.*, 1994, and Rothuizen and Van Wolferen, 1994). Any non-reproducible bands tend to be faint and can be dismissed as genetic markers (Hadrys *et al.*, 1992).

In collaboration with my colleague Ashley Matchett the final species profiles were repeated using primers pairs 2, 3 and 4. These repeated results (Figures 3.9.1, 3.9.2 and 3.9.3) showed profiles with higher numbers of bands, which made them easily distinguishable from the others. Although the previous results (Figures 3.8 and 3.9) showed differences in the number and sizes of amplicons for each species, the greater number of bands in the later sets of results provided far greater discrimination. The Roe deer sample again produced no results but the dog and unknown scapula that had previously shown no results now produced profiles. Wild boar and pig profiles, although different, still shared at least two bands of the same size, so it could be seen they are closely related but are still different. This test then is better than the method chosen by Koh *et al.*, 1997, as it cannot distinguish between these closely related species. It also provides an indication that the method may be suitable for distinguishing between different breeds of domestic animals, and possibility that is worth investigating given the prevalence of remains from domestic breeds at archaeological sites.

The samples of unknown origin produced RAPD profiles (Figures 3.9.1, 3.9.2 and 3.9.3). These profiles differed from each other, showing that the bones were from two different species, but since these two profiles did not match those of the known, the species origin of these bones remains unknown. However, it can be concluded that

they do not belong to a pig, wild boar, dog or lamb. The fragment sizes of each profile using the different primers are represented in Table 4.2.

The extraction controls were contaminated but as this phenomenon is well documented (Williams *et al.* 1990) and the bands were not apparent in sample lanes on gels, this is not considered a major problem in interpreting results. Results, in the form of the sizes of amplicons produced for each species and bone are presented in Table 4.2 as a summary.

| Species | Primer 2 (fragment sizes in kb) | Primer 3 (fragment sizes in kb) | Primer 4 (fragment sizes in kb) |
|---|---|---|---|
| Wild boar | 0.92, 0.85, 0.74, 0.55, 0.50, 0.44, 0.38, 0.32 | 0.95, 0.89, 0.62, 0.56, 0.42, 0.37, 0.26 | 0.50, 0.44, 0.37, 0.31, 0.24, 0.16 |
| Pig | 1.30, 1.21, 0.95, 0.82, 0.74, 0.59, 0.54, 0.44, 0.40, 0.32 | 0.89, 0.80, 0.62, 0.43, 0.38, 0.26 | 0.98, 0.95, 0.82, 0.62, 0.50, 0.49, 0.44, 0.37, 0.31 |
| Roe deer | No profile | No profile | No profile |
| Dog | 0.78, 0.70, 0.36 | 1.17, 0.47 | 0.49, 0.44, 0.29 |
| Lamb | 1.41, 0.97, 0.79, 0.70, 0.67, 0.43, 0.36, 0.28 | 0.60, 0.52, 0.30 | 0.99, 0.65, 0.62, 0.50, 0.48, 0.44, 0.40 |
| Unknown long bone | 0.82, 0.69, 0.40, 0.29, 0.19 | 0.70, 0.54, 0.24 | 0.88, 0.67, 0.59, 0.51, 0.30, 0.16 |
| Unknown scapula | 0.60, 0.50 | 1.02, 0.43 | 1.00, 0.96, 0.86, 0.72, 0.62, 0.52, 0.49, 0.43, 0.38, 0.05 |
| Extraction control | 1.15, 0.92, 0.67, 0.54 | 0.73, 0.24, 0.08 | 1.52, 0.99, 0.65, 0.48, 0.37, 0.16 |

**Table 4.2**: The table shows the different species and primers used to create the RAPD-PCR profiles.

This research indicates that this is a possible method for the initial species identification of bone fragments found at archaeological sites. It would be essential to create as wide a library of profiles as possible, covering all relevant indigenous species, in order that the method could be used a presumptive test to help identify most unknown samples. It has shown that the method can distinguish between closely related species where some methods have failed (Koh *et al.*, 1997).

Other methods could be used to confirm the results of the RAPD-PCR procedure and to verify species identification. For instance, PCR-RFLP using primers specific for the cytochrome b gene (Newman *et al.*, 2000 and Wetton *et al.*, 2002) have been used in this respect and mitochondrial DNA (mt DNA) sequencing has been used by a number of researchers where a primer is designed to copy a specified region for a particular species (Barnes *et al.*, 2000, Colgan *et al.*, 2001, Cooper *et al.*, 1992, Green wood *et al.*, 2001, Hoss *et al.*, 1996 [1], Janczewski *et al.*, 1992 and Loreille *et al.*, 1997).

Not only could RAPD-PCR identify unknown species, but it may also be used to identify different genetic variations within species (Cooper, 2000) or possibly even different breeds.

RAPD-PCR could also be used not only for bone fragments but possibly ancient plant remains, in order to identify species and study their distribution across different continents and civilisations. Work carried out in this area, but using different PCR methods, has been conducted by researchers like Brown, (1999). Again, RAPD-PCR could be used as a quick initial that would lead to further in depth analysis.

There is a lot of scope for this research to be taken further, and a database of different species using the different primers should be set up as a reference tool for other researchers. This should include samples from breeds of domestic species as well as wild and domestic species indigenous to the UK.

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